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Clinical Research Article

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Nonalcoholic Fatty Liver Disease and Estimated Insulin Resistance in Obese Youth: A Mendelian Randomization Analysis

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Abstract

Context: Nonalcoholic fatty liver disease (NAFLD) is associated with insulin resistance (IR) and predicts type 2 diabetes. Currently, it is uncertain whether NAFLD may directly cause IR or vice versa.
Objective: To test the hypothesis that NAFLD is causally related to IR.
Design and Methods: We performed a Mendelian randomization (MR) in 904 obese children/adolescents using an NAFLD-related genetic risk score (GRS) as an instrumental variable. We assessed NAFLD by ultrasonography and IR by homeostasis model assessment (HOMA-IR). We also interrogated the MAGIC Consortium dataset of 46 186 adults to assess the association between *PNPLA3* rs738409 (ie, the most robust NAFLD-related polymorphism) and HOMA-IR, and we performed a 2-sample MR with 2 large datasets to test reverse causation (HOMA-IR increasing the risk of NAFLD).
Results: Nonalcoholic fatty liver disease prevalence increased by 20% for every increase in the GRS (β-coefficient = 0.20, $P < 0.001$), and NAFLD was associated with ln-HOMA-IR (β-coefficient = 0.28, $P < 0.001$). Thus, the expected increase in ln-HOMA-IR for every increase in the GRS (expected β-coefficient) was 0.056 (0.28*0.20) in the case of complete NAFLD-HOMA-IR causal association, and 0.042 in the case of 75% causality. In our cohort, the GRS did not predict ln-HOMA-IR (β-coefficient = 0.007, $P = 0.75$). In the MAGIC cohort, the *PNPLA3* rs738409 did not associate with ln-HOMA-IR. The 2-sample MR failed to show a causal association between ln-HOMA-IR and NAFLD.
Conclusions: Our study shows that genetically-influenced NAFLD does not increase HOMA-IR, and genetically-influenced HOMA-IR does not increase the risk of NAFLD. Shared pathogenic pathways or NAFLD subtypes not "captured" by our MR design might underpin the association between NAFLD and HOMA-IR.

Freeform/Key Words: NAFLD, insulin resistance, childhood obesity, causal association

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Nonalcoholic fatty liver disease (NAFLD) is a metabolic liver disease that encompasses a spectrum of progressive pathologic conditions, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis (1, 2). Nonalcoholic fatty liver disease is the most common cause of chronic liver disease in industrialized countries (1, 2). The global epidemic of NAFLD has paralleled that of the so-called "diabesity," because NAFLD is associated with Western diets, obesity, and greater insulin resistance (IR). Insulin resistance increases hepatic de novo lipogenesis and impairs insulin-mediated suppression of adipose tissue lipolysis with consequent increased fluxes of free fatty acids into the liver (1, 3). Both of these factors may contribute to hepatic fat accumulation and increased lipids in circulation. To date, the worldwide prevalence of NAFLD is estimated to be around 5% to 10% in children and adolescents, reaching the rate of nearly 50% among children and adolescents with obesity (4–6). Thus, clarifying which are the main complications of NAFLD in childhood is crucial in order to guide prevention, treatment, and prognosis.

Among adults, elevated serum transaminase concentrations and/or NAFLD diagnosed by ultrasonography are 2 well-recognized predictors of incident type 2 diabetes (2, 7–11). The intimate association between NAFLD and increased long-term risk of incident type 2 diabetes has raised the hypothesis that NAFLD is not only induced by IR but that it may also directly aggravate IR and, subsequently, increase the risk of developing type 2 diabetes (12). This intimate and bidirectional relationship between NAFLD and type 2 diabetes would justify searching for treatments aimed to improve NAFLD in order to prevent the development of diabetes and would justify a worse metabolic prognosis in individuals with NAFLD, regardless of their degree of IR and metabolic impairment. However, this master hypothesis has not yet been definitely proved and, currently, it remains uncertain whether NAFLD itself may directly induce IR.

Up to now, preliminary evidence has been produced by studies using a Mendelian randomization (MR) approach (13–16). These studies have assessed the association between NAFLD-predisposing genetic variants and type 2 diabetes or markers of IR in adult individuals. These genetic variants are attributed randomly at conception and are consequently free from confounders, and they are suitable to replace random treatments typical of randomized clinical trials in order to establish a causal relationship between NAFLD and either type 2 diabetes or IR in large population-based cohorts. However, the results of these studies have not been conclusive. The carriers of the patatin-like phospholipase domain-containing protein 3 (*PNPLA3)* rs738409 GG gene variant, who have ~75% more liver fat content than noncarriers (17), have shown a small but significant increase in risk of incident type 2 diabetes among 100 323 adult individuals from a publicly available diabetes genome-wide association studies (GWAS) database (odds ratio [OR] 1.04 [95% confidence interval (CI): 1.01–1.07]) (13). Another NAFLD-related genetic variant, the trans-membrane 6 superfamily member 2 (*TM6SF2)* rs58542926 C > T polymorphism, was found to be associated with a 20% to 40% significant increase in risk of incident type 2 diabetes in about 9000 adults overall, from the METSIM and FINRISK studies, as well as with a small increase in risk of type 2 diabetes in 452 244 adult individuals from 54 studies (pooled OR 1.07 [95% CI: 1.05–1.10]) (14, 15).

To date, however, data about a causal relationship between NAFLD and IR are very limited. Based on the literature, only 1 MR study has been conducted in adults, suggesting that NAFLD may increase IR in patients with severe obesity only when NAFLD is associated with liver inflammation, while it does not increase IR in overweight or obese individuals from the general population (16). To our knowledge, no study up to now has attempted to assess the possible causal relationship between NAFLD and IR in children, which is an ideal population in which to investigate early, unbiased, pathogenic pathways. Moreover, no study has investigated, by MR approach, the causal relationship between NAFLD and markers of IR in large pediatric cohorts in order to assess the existence of an even small causal association between NAFLD and IR.

Therefore, the main aim of the present study was to test the hypothesis of a causal relationship between NAFLD and IR as estimated by the homeostasis model assessment (HOMA-IR score) in a large cohort of Italian obese children and adolescents, by using an MR approach.

In addition, we tested the same hypothesis by MR approach in a large cohort of adults, and we also tested the hypothesis of reverse causation, that is, IR causing NAFLD, by MR analysis employing 2 large adult datasets.

Research Design and Methods

Participants

A total of 904 unrelated obese children and adolescents were recruited at their first visit at the obesity outpatient clinic of the Pediatrics Section of the "Luigi Vanvitelli" University of Naples (Italy). Inclusion criteria were as follows: aged between 2–20 years, European ancestry, Italian family origin, and a body mass index (BMI) greater than the age- and sex-specific BMI cutoffs for obesity (according to the World Health Organization BMI cutoffs). Exclusion criteria were as follows: secondary obesity (eg, Prader–Willi syndrome, Cohen syndrome, mutation in

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the MC4R gene), known liver diseases (eg, viral hepatitis, drug-induced hepatitis, autoimmune hepatitis, hemochromatosis, Wilson's disease), alcohol abuse (≥140 g/week), and chronic use of any drugs. Written, informed consent was obtained from the children and their parents. The protocol of this study was approved by the local Ethics Committee of the "Luigi Vanvitelli" University of Naples.

Clinical and laboratory data

A team of trained pediatricians examined the study participants. They performed the examinations in accordance with standard protocols and used calibrated instruments. Weight was measured to the nearest 0.1 kg on a scale placed on a level floor while the subjects were wearing light clothes, and height was measured to the nearest 0.1 cm, without shoes. A trained pediatrician assessed the pubertal stage according to Tanner staging. A fasting blood drawing served to obtain participants' DNA and to measure several biochemical parameters, of which plasma glucose and insulin levels are those of major interest for the present study and were centrally measured at the hospital's chemistry laboratory. The HOMA-IR score [plasma glucose (mmol/L)*insulin (mU/L) / 22.5] was calculated as a proxy measure for IR. Serum alanine aminotransferase (ALT) levels were measured enzymatically and classified as elevated if ALT ≥ 44 IU/L in boys and ≥ 52 IU/L in girls, respectively, according to the North American Society of Pediatric Gastroenterology, Hepatology, and Nutrition guidelines (18).

Instrumental variable

As the instrumental variable for our MR analysis, we used a weighted genetic risk score (GRS) built using the genotypes from 3 established NAFLD-associated single-nucleotide polymorphisms (SNPs) previously validated in a cohort of obese Italian children (19): rs738409 C>G at *PNPLA3*, rs58542926 C>T at *TM6SF2* E167K, and rs2236212 at *ELOVL2*. Rs1260326 at GCKR was also previously validated in the same pediatric cohort, but it was excluded from this analysis because this genetic variant suffers from pleiotropy, for its known direct effect on glucose metabolism (20). In fact, in accordance with the assumptions for instrumental variables in MR (21), the NAFLD-related weighted GRS was supposed to have no pleiotropic effects potentially influencing IR in a NAFLD-independent way, based on the literature.

Weighting of these genotypes was based on previously reported coefficients in cohorts of individuals of European ancestry (19, 22, 23). Moreover, the weighted GRS was also supposed to increase the risk of NAFLD in a consistent way across individuals, thus avoiding any stratification effect, according to the monotonicity assumption. In this study, we applied a linear model to test the associations between GRS and NAFLD or between NAFLD and ln-HOMA-IR, because in both cases the linear model was that with the highest F statistics among a group of other tested models, including linear, logarithmic, quadratic, cubic, composed, or exponential models.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using salting-out procedures. Genotyping was carried out by predesigned TaqMan probes (Applied Biosystems, Foster City, CA, USA.), according to the manufacturer's protocol. Single-nucleotide polymorphism genotyping was performed using 7900 HT Real Time PCR (Applied Biosystem). The genotypes were in Hardy-Weinberg equilibrium for each of the three SNPs analyzed.

Diagnosis of NAFLD

At recruitment, as part of the routine investigation of children, an experienced radiologist, who was blinded to the clinical details of participants, performed liver ultrasonography in all children. Hepatic steatosis was diagnosed according to ultrasonographic characteristics, including diffuse hyperechogenicity of the liver relative to the kidneys, ultrasonography beam attenuation, or poor visualization of the intrahepatic vessel borders and diaphragm (24, 25). Ultrasonography has proved 85% sensitivity and 94% specificity in detecting moderate to severe hepatic steatosis in adults, as well as 80% sensitivity and 86% specificity in detecting moderate to severe steatosis in children (24, 25). Moreover, this imaging method is recommended for assessing NAFLD in children and adolescents according to the European and Italian pediatric hepatology and obesity guidelines (26, 27).

Statistical analysis

Cohort description. Normal and skewed continuous variables are described by mean (standard deviation [SD]) or medians (interquartile ranges), respectively, while dichotomous variables are described as proportions. Continuous variables were compared across genders by the unpaired Student's *t*-test or the Mann-Whitney U test according to their normal or skewed distribution, respectively. The prevalence of dichotomous variables across genders was compared by the chi-squared test.

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Mendelian randomization analysis in children/adolescents with obesity. As previously mentioned, MR analysis is a statistical method that avoids confounding and reverse causation, by using genetic variation as an instrument to establish a causal role of an exposure variable (ie, NAFLD in our study) in determining an outcome of interest (in ie, HOMA-IR in our study). This statistical method relies on the assumption that as an individual's genotype is determined randomly at conception, it is not related to lifestyle variables or other potential confounding factors, and thus can serve as an unconfounded and lifelong proxy for the exposure of interest. In our cohort of children, we performed a classical 1-sample MR analysis following the steps listed below:

1. We assessed, with binary logistic regression, the association between ultrasound-detected NAFLD and the NAFLD-related GRS;
2. Once the existence of this significant association was verified, we rescaled the NAFLD-related GRS so that it had a SD of 1. This allowed us to perform a linear regression analysis between NAFLD and the GRS to obtain a β-coefficient of association, which represents the absolute NAFLD risk increase for each SD increase in GRS, despite the noncontinuous distribution of the dependent variable. We called this β-coefficient β_x.
3. We naturally log-transformed HOMA-IR (ln-HOMA-IR) to obtain the normally distributed residuals of this parameter adjusted for age, sex, z-BMI, and pubertal stage.
4. We assessed, with a general linear model, the association between NAFLD and ln-HOMA-IR adjusted for age, sex, z-BMI, and pubertal stage, so as to obtain a β-coefficient that represents the increase in ln-HOMA-IR associated with NAFLD. We called this coefficient β_y.
5. We multiplied β_x by β_y, obtaining the $\exp\beta_z$ coefficient, to estimate the expected β-coefficient of the association between NAFLD-related GRS and ln-HOMA-IR in the hypothesis of complete causality in the NAFLD-IR association. This represents the expected increase in ln-HOMA-IR for each SD increase in GRS, and in the case of NAFLD, causally underlying the 100% NAFLD-IR association.
6. To estimate the expected β-coefficient of association between GRS and ln-HOMA-IR in the hypothesis of 75% and 50% causality in the NAFLD-IR association, we multiplied β_x by β_y by 0.75 and 0.50, respectively, obtaining the $\exp\beta_{z75}$ and $\exp\beta_{z50}$ coefficients. These represent the expected increase in ln-HOMA-IR for each SD increase in GRS, and in the case of NAFLD, causally underlying the 75% or 50% of NAFLD-IR association.
7. We performed a general linear model with ln-HOMA-IR as the dependent variable and GRS, sex, age, pubertal stage, and z-BMI as covariates, obtaining an actual β-coefficient to assess the actual association between NAFLD-related GRS and ln-HOMA-IR. We called this coefficient actual β_z.
8. Finally, we compared actual β_z to $\exp\beta_z$, $\exp\beta_{z75}$, and $\exp\beta_{z50}$ to infer whether and at which extent NAFLD increases ln-HOMA-IR.

We calculated the statistical power of our study sample by using a power calculator specifically designed for MR analysis and that is freely available at https://sb452.shinyapps.io/power/.

Besides the above-described analyses, we also performed a secondary MR analysis that included all of the 4 above-mentioned NAFLD genetic variants (rs738409 at *PNPLA3*, rs58542926 at *TM6SF2*, rs2236212 at *ELOVL2*, and rs126326 at *GCKR*) using the Egger's regression strategy, which meta-analyses the separate effects of each single SNP, adjusting the effect estimates for pleiotropy.

All the statistical analyses, apart from the power calculation, were performed with SPSS0.24 statistical package (IBM, Chicago, Illinois).

Assessment of causal effect of NAFLD on HOMA-IR in nondiabetic adults. We interrogated the publicly available data issued from a meta-analysis of 21 GWAS informative for ln-HOMA-IR in 46 186 nondiabetic individuals (https://www.magicinvestigators.org/downloads/) in order to assess the association between *PNPLA3* rs738409 (ie, the SNP most robustly associated with NAFLD) and HOMA-IR. No information was available about the SNP-NAFLD or NAFLD-HOMA-IR associations. This kind of analysis cannot be considered a formal MR analysis, in that it is not suitable to quantify any causal relationship between an exposure and an outcome, because the information about the exposure is not available. However, due to the large size of the cohorts included, it is suitable to suggest the existence or the lack of existence of any exposure–outcome causal relationship, in the case of existence or lack of existence of any SNP–outcome association, respectively. Thus, this analysis is part of the MR approache (28). The dataset we interrogated displays the coefficients and *P*-values for the association between ln-HOMA-IR and the SNPs genotyped in the meta-analyzed GWAS, adjusted for age-, sex-, and study-specific covariates.

Assessment of causal effect of HOMA-IR on NAFLD (reverse causation) in nondiabetic adults. We conducted a 2-sample MR analysis using the MAGIC GWA dataset, including 46 186 nondiabetic adults, to obtain the instrumental variable for the exposure (ln-HOMA-IR), as well as the Medical Research Council-Integrative Epidemiology Unit Consortium GWA dataset (including 462 356 healthy

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adults and 654 adults with fatty liver) to assess the existence of a direct causality between ln-HOMA-IR and NAFLD (http://app.mrbase.org/). We performed these analyses by the MR-BASE platform (http://app.mrbase.org/) (29).

Results

Assessment of causal effect of NAFLD on HOMA-IR in obese children and adolescents

Participants included in our study are described in Table 1. Girls were slightly younger and had significantly lower body weights, heights, BMI, serum ALT, and fasting glucose levels than boys (all $P < 0.05$). Pubertal stage, z-BMI, fasting insulin levels, and ln-HOMA-IR did not significantly differ between sexes. Boys also had a markedly higher prevalence of ultrasound-defined NAFLD compared with girls ($P = 3.6*10^{-14}$).

As shown in Table 2, children with NAFLD were more likely to be male, older in age, and had higher z-BMI, ln-HOMA-IR, and serum ALT levels compared with their counterparts without NAFLD (all $P < 0.05$).

The weighted GRS predicted the presence of NAFLD (OR = 1.66 [95% CI: 1.50–1.84], $P = 2.2*10^{-23}$, Nagelkerke $R^2 = 0.16$). The prevalence of NAFLD increased by 20% for every 1-SD increase of the GRS rescaled to have variance = 1 ($\beta_x = 0.20$, $P = 1.2* 10^{-24}$). Nonalcoholic fatty liver disease was associated with a 0.28 increase in ln-HOMA-IR ($\beta_y = 0.28$, 95% CI: 0.14–0.39, $P = 1*10^{-4}$, $R^2 = 0.11$), independently of age, sex, z-BMI, and pubertal status (Table 3). Male sex did not significantly predict the presence of NAFLD in this adjusted regression model.

Based on the β_x and β_y coefficients, the expected increase in ln-HOMA-IR for each unitary increase in the NAFLD-related GRS, in the case of a complete NAFLD–HOMA-IR causal relationship, was 0.056 ($\exp\beta_z = 0.056$). The expected increases in ln-HOMA-IR in the hypothesis of 75% or 50% causality were 0.042 ($\exp\beta_{z75} = 0.042$) and 0.028 ($\exp\beta_{z50} = 0.028$), respectively. In our cohort, the NAFLD-related GRS did not significantly predict ln-HOMA-IR at all (actual$\beta_z = 0.007$, 95% CI: -0.038–0.052, $P = 0.75$) (Table 4).

The study design was 99%, 90%, and 62% powerful to detect, with a 1-sided 0.05 α error, an actual β_z of 0.056, 0.042, and 0.028, corresponding to 100%, 75%, and 50% of direct causality in the observed NAFLD-HOMA-IR association, respectively.

The Egger's regression analysis, including the 4 tested SNPs, also failed to confute the null hypothesis (estimated β of causal association = 0.013, 95% C.I: -0.02–0.05, $P = 0.28$, p for heterogeneity = 0.15).

Assessment of causal effect of NAFLD on HOMA-IR in nondiabetic adults

In the 46 186 adult individuals of the MAGIC Consortium, the *PNPLA3* rs738409 polymorphism was not significantly associated with ln-HOMA-IR. The association's β-coefficient was 0.007, and the p-value of 0.014 was largely below the GWAS significance threshold.

Assessment of causal effect of IR on NAFLD in nondiabetic adults

The Egger's regression analysis employing 21 independent SNPs associated with ln-HOMA-IR (total variance explained = 1.3%, $P \leq 2.5 \times 10^{-5}$) failed to infer any causative

Table 1. Characteristics of Study Participants

	Boys (n = 464)	Girls (n = 440)	*P*-value	Total (n = 904)
Age, years	11.06 (2.6)	10.21 (2.9)	$5*10^{-6}$	10.65 (2.8)
Pubertal stage (cases/total)	200/464	210/440	0.16	410/904
Weight, kg	72.9 (22.7)	62.9 (19.8)	$2.5*10^{-12}$	68.1 (21.9)
Height, m	1.50 [1.40–1.59]	1.43 [1.32–1.55]	$3.3*10^{-13}$	1.47 [1.36–1.56]
BMI, kg/m^2	30.8 [28.3–34.0]	29.2 [26.8–32.6]	$3*10^{-6}$	30.1 [27.4–33.4]
Z-BMI	2.43 [2.16–2.68]	2.39 [2.05–2.67]	0.19	2.41 [2.09–2.67]
Fasting glucose, mg/dL	82.1 (8.7)	79.2 (8.6)	$8.8*10^{-7}$	80.7 (8.7)
Fasting insulin, mU/L	18.1 [11.0–27.3]	17.5 [11.1–27.7]	0.67	17.7 [11.1–27.4]
HOMA-IR score	3.6 [2.2–5.6]	3.3 [2.1–5.3]	0.21	3.5 [2.2–5.4]
ln-HOMA-IR	1.23 (0.68)	1.18 (0.65)	0.29	1.21 (0.66)
NAFLD (cases/total)	276/464	151/440	$3.6*10^{-14}$	427/904
ALT, IU/L	31 [23–43]	25 [18–33]	$1.8*10^{-11}$	28 [19–38]
Elevated ALT (cases/total)	75/464	52/440	0.06	127/904

Data are expressed as means (SD) or medians [interquartile ranges]. Differences between the 2 groups were tested by the unpaired Student's *t*-test or the Mann-Whitney test (as appropriate).

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; HOMA-IR, homeostasis model assessment-insulin resistance, NAFLD, Non Alcoholic Fatty Liver Disease.

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Table 2. Comparison Between Children With and Without Ultrasound-defined NAFLD

	Without NAFLD	With NAFLD	*P*-value
Male/Female	188/289	276/151	$3.5*10^{-14}$
Age, years	10.34 (2.95)	10.99 (2.60)	0.001
Pubertal stage (cases/total)	202/477	208/427	0.055
Weight, kg	59.1 [49.2–75.1]	71 [57–89]	$3.4*10^{-16}$
Height, m	1.44 [1.32–1.55]	1.49 [1.40–1.59]	$3.9*10^{-7}$
BMI	29.05 [26.8–31.6]	31.7 [28.9–35.3]	$1.07*10^{-20}$
z-BMI	2.32 [2.0–2.36]	2.5 [2.2–2.8]	0.001
Fasting glucose, mg/L	80 [75–85]	81 [75–87]	0.003
Fasting insulin, mUI/L	15.4 [9.5–23.1]	21.5 [13.0–31.5]	$1.2*10^{-18}$
HOMA-IR score	3.0 [1.9–4.6]	4.3 [2.5–6.7]	$1.7*10^{-20}$
ln-HOMA-IR	1.04 (0.6)	1.39 (0.68)	$1.02*10^{-15}$
ALT, IU/L	22.15 (9.55)	44.95 (24.9)	$2.5*10^{-64}$
Elevated ALT (cases/total)	13/477	114/427	$3.9*10^{-25}$

Data are expressed as means (SD), medians [interquartile range] or proportions. Differences between the 2 groups were tested by the unpaired Student's *t*-test or the Mann-Whitney test (as appropriate).
Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; HOMA-IR, homeostasis model assessment-insulin resistance.

Table 3. Association Between NAFLD and ln-HOMA-IR, Adjusted for Age, Sex, Pubertal Status, and z-BMI

	β-coefficient	95% CI	*P*-value
Age, years	0. 08	0.06–0,10	$5.4*10^{-8}$
Pubertal status (0 = not pubertal, 1 = pubertal)	-0.22	-0.18–0.42	0.066
Sex (0 = boys, 1 = girls)	0.10	-0.03–0.22	0.131
z-BMI (for each unit)	0.21	0.14–0.29	$8*10^{-11}$
NAFLD (yes vs no)	0.28	0.14–0.39	$1*10^{-4}$

Sample size, n = 904. Data are expressed as β-coefficients (β) and 95% CI tested by multivariable linear regression analysis. Logarithmically transformed HOMA-IR was included as the dependent variable in this multivariable regression model.
Abbreviations: BMI, body mass index; CI, confidence interval; HOMA-IR, homeostasis model assessment - insulin resistance; NAFLD, non alcoholic fatty liver disease.

Table 4. Association Between NAFLD-related GRS and ln-HOMA-IR, Adjusted for Age, Sex, Pubertal Status, and z-BMI

	β-coefficient	95% CI	*P*-value
Age, years	0.034	0.017–0.052	$1.15*10^{-4}$
Pubertal status (0 = not pubertal, 1 = pubertal)	0.19	0.06–0.32	0.004
Sex (0 = boys, 1 = girls)	0.09	-0.004–0.18	0.062
z-BMI	0.26	0.18–0.33	$3.5*10^{-10}$
GRS	0.007	- 0.038–0.052	0.750

Sample size, n = 904. Data are expressed as β-coefficients (β) and 95% CI tested by multivariable linear regression analysis. Logarithmically transformed HOMA-IR was included as the dependent variable in this multivariable regression model.
Abbreviations: BMI, body mass index; CI, confidence interval; HOMA-IR, homeostasis model assessment - insulin resistance; NAFLD, non alcoholic fatty liver disease.

effect of ln-HOMA-IR on risk of NAFLD (OR associated with each SD increase in ln-HOMA-IR = 1.001, $P = 0.37$, p for heterogeneity = 0.38) (Fig. 1).

Conclusions

Using an MR approach, we did not observe any significant evidence that NAFLD is causally associated with IR as estimated by the HOMA-IR score. In both the pediatric and adult cohorts analyzed, the β-coefficient of association between the NAFLD-related GRS (ie, the instrumental variable) and HOMA-IR approached zero, suggesting no causal association between genetically-influenced NAFLD and HOMA-estimated IR.

To our knowledge, the present study includes the first MR analysis performed in a pediatric population to assess the early causal role of NAFLD in the development of IR as estimated by HOMA-IR, as well as the first large GWA dataset interrogation to assess the causal association between NAFLD and HOMA-IR in nondiabetic adults. However, it must be acknowledged that our MR analysis is not completely conclusive as regards to the pediatric age, in that a very small causal effect of NAFLD on IR cannot be definitely excluded. Indeed, our study was underpowered to detect any HOMA-IR increase caused by NAFLD lower than ~0.3 SD. In contrast, the lack of any causal association highlighted by the adult GWA dataset interrogation appears to be more robust. In fact, even in the conservative hypothesis of *PNPLA3* rs738409 explaining only 1.5% of NAFLD risk in the pooled cohorts of the interrogated meta-analysis, the dataset of more than 46 000 individuals would have a power of 100% to detect an HOMA-IR increase

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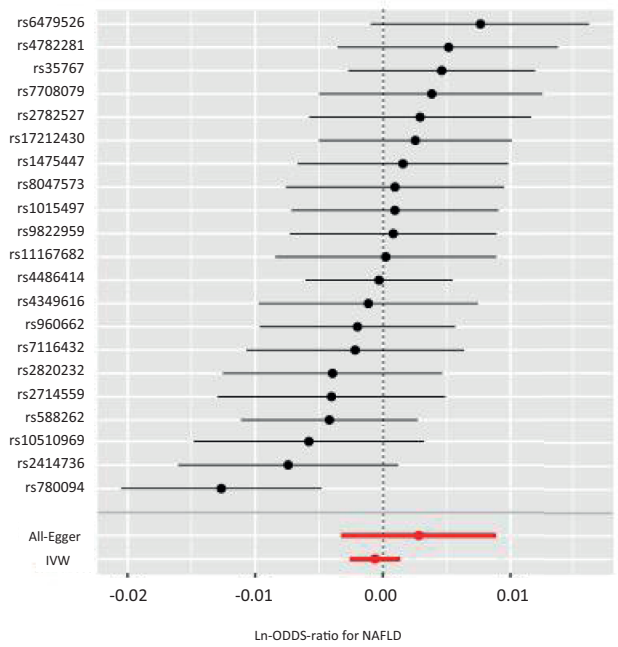

Figure 1. Association between HOMA-IR-related SNPs and NAFLD, by the Egger's regression and Inverse Variance Weighted (IVW) methods. Abbreviations: HOMA-IR, homeostasis model assessment-insulin resistance; NAFLD, non alcoholic fatty liver disease; SNP, single nucleotide polymorphism.

produced by NAFLD as low as 0.2 SD. The power would be 98% to detect a HOMA-IR increase produced by NAFLD as low as 0.15 SD.

Our results are consistent with those recently published by Dongiovanni and colleagues, who did not find any association between HOMA-IR and a robust NAFLD-related GRS in 4570 American adults from the Dallas Heart Study (16). In another study that adopted an MR approach in investigating Finnish adults, Sliz et al showed that NAFLD was significantly associated with several metabolites that were implicated in insulin-signaling pathways (such as hepatic beta-oxidation, glycolysis. and gluconeogenesis), but that the *PNPLA3* rs738409 and *TM6SF2* rs58542926 variants did not associate significantly with these metabolites in almost 25 000 Europeans from a publicly available GWAs dataset (30). Thus, this latter result further supports the lack of any direct causality between NAFLD and IR.

That said, the idea of NAFLD as an "innocent bystander" in the pathophysiology of IR is somewhat challenged by the fact that SNPs predisposing to NAFLD have been associated with a small increase in diabetes risk in some population-based cohort studies (13–15). We believe that this paradox may have more than 1 plausible explanation. First, its not the "generic" NAFLD phenotype but its more severe phenotype, NASH with increasing levels of liver fibrosis, might be the main driver of hepatic IR and the subsequent development of diabetes in NAFLD. This would explain why NAFLD-predisposing SNPs have been associated with increased diabetes risk mainly in very large cohorts that are probably characterized by a high enough prevalence of NASH to uncover even small associations between NAFLD-predisposing SNPs and the risk of type 2 diabetes. In accordance with this latter hypothesis, in 2 adult cohorts of patients with severe obesity and biopsy-confirmed NASH, Dongiovanni et al reported that NAFLD was significantly associated with HOMA-IR, but this association was largely dependent on the histologic severity of liver damage (16). In line with this, a recent large MR analysis also showed that elevated serum transaminase levels, which are thought to be a proxy of NASH, are causally associated with an increased risk of incident type 2 diabetes (31). Second, it must be acknowledged that MR analysis has the intrinsic limitation to rely on the paradigm of a well-defined, unique exposure (NAFLD, in this case), whose genetic predisposing polymorphisms are just instrumental markers employed for their freedom from confounders. However, genetic instrumental variables may underpin multiple pathophysiologic and metabolic subtypes of the exposure, unfavoring the opportunity to uncover pathogenic roles, if any, for other potential subtypes of the exposure. Thus, although we did not observe any significant causal association between NAFLD and HOMA-IR through our MR analysis, we cannot definitely exclude the existence of NAFLD subtypes increasing the risk of diabetes by contributing to IR. Finally, another plausible explanation for the aforementioned paradox is that NAFLD might contribute to the development of diabetes through various pathogenic mechanisms other than IR, for example, by decreasing the pancreatic β-cell function in still unknown ways.

The failure to observe any causal association between NAFLD and HOMA-IR in our pediatric cohort further feeds the debate about the complexity of the association between NAFLD and diabetes. The evidence from this and other studies suggests that we should probably move the research focus from a "generic" NAFLD concept to that of more specific and severe subphenotypes of liver disease (ie, NASH with varying amounts of liver fibrosis). This, besides exploring potential pathogenic pathways other than IR, would help to disentangle the steps leading from NAFLD to the development of type 2 diabetes, increasing the possibility to formulate correct metabolic prognosis for individuals with NAFLD and conceptualize effective preventive treatments.

From a clinical point of view, the failure to observe a causal association between NAFLD and HOMA-IR imposes some caution in formulating a worse metabolic prognosis for children with NAFLD compared with peers without NAFLD, but with similar levels of IR and other established risk factors for diabetes, such as obesity. On the other hand, it is also reasonable to hypothesize that early recognition of more advanced histologic forms of NAFLD

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that might accelerate the progression towards IR and/or diabetes would allow for the selection of individuals who will benefit from treatment of NAFLD to decrease IR and the risk of developing type 2 diabetes over time. The latter scenario may come true in the future, thanks to studies targeting metabolic consequences and potential specific treatments of such advanced NAFLD phenotypes.

Notably, our study has also included a 2-sample MR analysis assessing the possible causative role of IR on NAFLD. The estimated causal association between HOMA-IR and NAFLD was not statistically significant, though it was in the expected direction. It must be acknowledged that the analysis had about a 75% statistical power to detect quite a strong causative association, that is, a double risk of NAFLD for every SD increase in HOMA-IR, assuming consistent coefficients of association of the instrumental variables with HOMA-IR across the samples. Thus, it is possible that our study was not powerful enough to observe weaker causative associations. Moreover, the sample used to infer causality was issued from several population-based European cohorts of adult individuals, while any causative role of IR on NAFLD could be stronger and easier to detect among persons with obesity. However, a β-coefficient of estimated association as low as that issued from our analysis allows us to hypothesize that the well-known association between NAFLD and HOMA-IR is likely due to shared pathogenic pathways rather than direct or reverse causation. However, this hypothesis should be considered with some , as analyses of large cohorts of obese individuals assessing both HOMA-IR and NAFLD have not been analyzed.

Our study has some important limitations, such as the above-mentioned lack of statistical power to detect a very small causal effect between NAFLD and HOMA-IR in our pediatric cohort, as well as the lack of any liver biopsy data. The lack of liver biopsy is unavoidable among children or adolescents with primary obesity, for whom this invasive method is recommended in only a few selected cases (32). As mentioned previously, since we used liver ultrasonography for diagnosing NAFLD (hepatic steatosis) in this study, it cannot be definitely excluded that NASH with varying levels of liver fibrosis could directly induce systemic and hepatic IR. Another important limitation is that we used HOMA-IR, which is only a surrogate measure of IR, as a candidate mechanistic consequence of NAFLD. In fact, HOMA-IR does not accurately reflect hepatic IR. Thus, the design of the study does not completely allow us to confute the hypothesis of a causal relationship between NAFLD and hepatic IR. Finally, the model we adopted to assess the association between NAFLD and HOMA-IR in our pediatric cohort took into account some major confounders, that is, age, sex, z-BMI, and pubertal status, but it lacked several other covariates that are potentially useful to exhaustively explain one's variation in the risk to be affected by NAFLD or IR. These covariates may be social, lifestyle-related, genetic, perinatal, or epigenetic factors concurring to determine one's metabolic capacity and one's metabolic load, according to the so-called "capacity-load model" (33). Notwithstanding these limitations, our study has also important strengths, like the assessment of a (relatively) large pediatric cohort, which is suitable to investigate early and long-standing pathogenic pathways, and the use of a robust weighted GRS as an instrumental variable for NAFLD, explaining a remarkable percentage of NAFLD risk variance in our cohort.

In conclusion, the results of our MR study, issued from a cohort of obese children and from large adult GWA datasets, do not suggest that genetically influenced NAFLD (as detected by ultrasonography) directly increases HOMA-IR or that HOMA-IR directly increases the risk of NAFLD. This may have more than one explanation: shared pathogenic pathways might underpin the association between NAFLD and IR; some subtypes or severity categories of NAFLD "not captured" by the MR design, actually induce IR and are induced by IR. However, further research is certainly needed to corroborate these findings and test the above-mentioned hypotheses in other pediatric cohorts.

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Additional Information

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Data Availability: The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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