# Mendelian randomisation suggests no beneficial effect of moderate alcohol consumption on the severity of nonalcoholic fatty liver disease

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**Publication data**
Submitted 30 July 2016
First decision 21 August 2016
Resubmitted 25 August 2016
Resubmitted 23 September 2016
Accepted 24 September 2016

*The Handling Editor for this article was Professor Stephen Harrison, and it was accepted for publication after full peer-review.*

## SUMMARY

### Background
Previous epidemiological studies suggest that patients diagnosed with non-alcoholic fatty liver disease (NAFLD) who drink light to moderate amounts of alcohol (up to ~30 g per day) have less severe histological lesions compared with nondrinkers. However, while the cross-sectional nature of current evidence precludes assessment of causality, cumulative lifetime-exposure of moderate alcohol consumption on histological outcomes has never been evaluated.

### Aim
To overcome these limitations, a Mendelian randomisation study was performed using a validated genetic variant (rs1229984 A;G) in the alcohol dehydrogenase (*ADH1B*) gene as a proxy of long-term alcohol exposure.

### Methods
We first assessed whether the instrumental variant (rs1229984) was associated with the amount of alcohol consumption in our cohort. We further explored the association between the variant and histological outcomes; a sample of 466 individuals, including 266 patients with NAFLD confirmed by liver biopsy, was studied.

### Results
We found that carriers of the A-allele consumed significantly lower amounts of alcohol compared with noncarriers (2.3 ± 5.3 vs. 8.18 ± 21 g per day, mean ± s.d., $P = 0.03$). The analysis of association with the disease severity showed that carriers of the A-allele had lower degree of histological steatosis (1.76 ± 0.83 vs. 2.19 ± 0.78, $P = 0.03$) and lower scores of lobular inflammation (0.54 ± 0.65 vs. 0.95 ± 0.92, $P = 0.02$) and NAFLD-Activity Score (2.9 ± 1.4 vs. 3.7 ± 1.4, $P = 0.015$) compared with noncarriers.

### Conclusion
Mendelian randomisation analysis suggests no beneficial effect of moderate alcohol consumption on NAFLD disease severity.

*Aliment Pharmacol Ther*

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doi:10.1111/apt.13828

## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease that has reached global epidemic proportions.[1] The disease is defined as abnormal accumulation of fat in the liver in the absence of significant alcohol consumption (less than 30 g per day for men and 20 g per day for women) and other causes of secondary hepatic steatosis.[2] The natural history of NAFLD is characterised by relatively benign prognosis; however, the disease may progress to severe histological stages, including nonalcoholic steatohepatitis (NASH), cirrhosis and eventually hepatocellular carcinoma.[3] Many factors influence the disease progression, including the coexistence of metabolic syndrome-associated diseases (type 2 diabetes, obesity and cardiovascular disease – CVD), genetic predisposition and environmental factors, such as diet, lifestyle and alcohol.[3]

Findings of several earlier epidemiological studies suggest that individuals that drink light to moderate amounts of alcohol (up to ~30 g per day) not only have low prevalence of NAFLD but also less severe histological disease.[4–9] Quantitative summarised evidence yielded by cross-sectional observational studies indicates a protective effect of about 31% on the risk of having NAFLD associated with low to moderate amounts of alcohol consumption.[8] A recently published meta-analysis suggests an ethnically related protective effect of light to moderate alcohol consumption on the prevalence of NAFLD as the beneficial effect was only observed in Japanese.[10] Accordingly, a longitudinal study on NAFLD prevalence in Japan suggests a protective effect of moderate alcohol consumption[11]; however, the longitudinal assessment included a very short follow-up. In addition, moderate alcohol consumption showed an average protective effect of about 50% on the risk of developing an advanced disease stage (NASH).[8] Nevertheless, these results must be interpreted with caution because the cross-sectional nature of the available evidence prevents any assessment of causality or reverse causation. Bias introduced by either interviewers or subjects' responses regarding the exact amount of alcohol consumed is likely to jeopardise the final interpretation of the results. More importantly, there are no available results on the cumulative lifetime effect/s of light to moderate alcohol consumption on NAFLD-histological outcomes. The definitive answer as to whether moderate alcohol consumption has any beneficial effect on the natural history of NAFLD should be provided by prospective-cohorts studies or, ideally, by randomised clinical trials (RCT). Although an RCT is the most rigorous way to assess whether a cause–effect relation exists between moderate alcohol consumption and NAFLD, an intervention based on this design applied to humans would be virtually unfeasible because of the need to operate on the randomisation of a variable such as 'alcohol consumption', which is at least ethically questionable. An alternative approach that can overcome these difficulties is the implementation of a Mendelian randomisation study in which a genetic variant (or instrument) is used as the proxy of alcohol exposure. The rationale of this strategy is based on the premise that genetic variants are determined at conception by the random allocation of chromosomes and are unlinked to potential confounders.[12] The goal of Mendelian randomisation analysis is to implement a genetic testing that has a strong and robust biological impact on the instrumental variable (alcohol) while not being related to the phenotype itself.

A genetic polymorphism (rs1229984 G>A, G being the ancestral allele) in the gene that encodes a form of the alcohol dehydrogenase ADH1B has been robustly associated with the level of alcohol consumption and the risk of alcoholism.[13] The polypeptide encoded by this gene is the beta subunit of a complex enzyme that catalyses the rate-limiting step for ethanol metabolism; the rs1229984 is a missense variant that involves a residue change from Histidine to Arginine at position 48 (p.His48Arg). Carriers of the A (His-48)-allele are at a low risk of alcohol dependence and consume low levels of alcohol because the variant is associated with an increased activity (indicating more rapid oxidation of ethanol to acetaldehyde) making them more likely to find drinking unpleasant.[13] The rs1229984 variant accounts for a two to threefold variation in alcohol elimination rate[14] and the A (His-48)-allele increases the ADH1B enzymatic activity by 32% compared with the frequent allele (G-Arg48 allele).[15]

The purpose of this study was to evaluate the effect of light to moderate alcohol consumption in NAFLD severity by Mendelian randomisation analysis that uses the rs1229984 as a surrogate of the cumulative lifetime effect of alcohol on the histological outcomes.

We first assessed whether the instrumental variant (rs1229984) was associated with the amount of alcohol consumption in our cohort. We subsequently explored the association between the variant and NAFLD-histological outcomes, which may be inferred as a 'causal' estimate of the effect of moderate alcohol consumption in the natural history of the disease.


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## PATIENTS AND METHODS

### Patients and control subjects: selection criteria

For validating the instrumental variable (the rs1229984 variant as a proxy of alcohol consumption), we included 466 unrelated individuals, 135 of whom were healthy subjects (Table S1) and 331 were patients with NAFLD (Table S2). For testing the hypothesis of a relation between moderate alcohol consumption and the histological disease severity, we included in the analysis patients who had histopathological evidence of NAFLD (either simple steatosis or NASH) on liver biopsy performed within this study ($n$ = 266) (Table S3).

Secondary causes of steatosis, including alcohol abuse, total parenteral nutrition, hepatitis B and hepatitis C virus infection, and the use of drugs known to precipitate steatosis were excluded. In addition, patients diagnosed with any of the following diseases were precluded from participation: autoimmune liver disease, metabolic liver disease, Wilson's disease and α-1-antitrypsin deficiency. When defining NAFLD, alcohol consumption of 30 g alcohol daily for men and 20 g for women was used as the threshold. Patients who consumed higher daily amounts of alcohol than the explained cut-off were excluded from the study; then there were no heavy drinkers in this study.

The screening criterion was liver ultrasonographic (US) examination indicative of fatty infiltration, which was carried out by the same operator and was performed in all the participants.

Healthy subjects were selected from the patient population attending our hospital for check-up purposes whose sex matched the NAFLD patients. In addition to the standard heath examination, all control individuals were subjected to a liver US; they were included in the study if no evidence of fatty change or biochemical abnormalities was found. Furthermore, healthy subjects were confirmed not to have any of the features of the metabolic syndrome as defined by the National Cholesterol Education Program Adult Treatment Panel III and did not abuse alcohol.

The case participants and the controls were selected during the same study period from the same population of patients attending our institution, and all shared the same demographic characteristics (occupation, educational level, place of residence and ethnicity).

Recruitment of participants and collection of biospecimens was initiated in January 2006 and ended in December 2014.

All investigations performed were conducted in accordance with the guidelines of the 1975 Declaration of Helsinki. Written consent from participating individuals was obtained in accordance with the procedures approved by the ethical committee of our institution.

### Physical, anthropometric and biochemical evaluation

Health examinations included anthropometric measurements, a questionnaire on health-related behaviours and biochemical determinations. The body mass index (BMI) was calculated as weight/squared height (kg/m$^2$) and was used as an index for relative weight. Elevated blood pressure was defined as systolic (SABP) ≥130 mmHg and/or diastolic (DABP) ≥ 85 mmHg or anti-hypertensive treatment.

Blood was drawn from 12-h fasting subjects who had been in a supine resting position for at least 30 min. Serum insulin, total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, triglycerides, plasma glucose and liver enzymes (alanine-ALT and aspartate aminotransferase-AST, gamma glutamyl transferase-$\gamma$GT and alkaline phosphatase-AP) were measured using standard clinical laboratory techniques. All biochemical determinations were measured using a Hitachi-912 Autoanalyzer (Roche, Diagnostic, Buenos Aires, Argentina) or an Immulite 1000 (DPC, Buenos Aires, Argentina). HOMA-IR was used to evaluate an insulin resistance index, calculated as follows: Fasting serum insulin (μU/mL) × Fasting plasma glucose (mmol/L)/22.5. The leucocyte count was measured automatically using a Sysmex XE 2100 (Roche Diagnostics) (normal range: 6–10$^9$/L).

Caspase-generated CK-18 fragment (CK-18) – a non-invasive quantification of hepatocellular apoptosis – concentration was measured by the one-step *in vitro* immunoassay M30-apoptosense ELISA kit (PEVIVA AB; DiaPharma, West Chester, OH, USA).

### Alcohol consumption

Participants were asked to report the number of standard glasses of alcoholic beverages and this information was used to calculate average alcohol consumption in g of alcohol per day. Details regarding the amount, pattern and frequency of alcohol use were specifically recorded by direct interview during the clinical assessment by physicians (GOC and SS).

### Liver biopsy and histopathological evaluation

Liver biopsy was performed before any intervention with ultrasound guidance and a modified 1.4-mm-diameter Menghini needle (Hepafix, Braun, Germany) under local anaesthesia on an out-patient basis. A portion of each liver biopsy specimen was routinely fixed in 40 g/L


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formaldehyde (pH 7.4), embedded in paraffin, and stained with haematoxylin and eosin, Masson trichrome, and silver impregnation for reticular fibres. All the biopsies were at least 3 cm in length and contained a minimum of eight portal tracts.

The degree of steatosis was assessed according to the system developed by Kleiner *et al.*, based on the percentage of hepatocytes containing macrovesicular fat droplets,[16] while NASH[17] and NAFLD Activity Score (NAS)[16] were defined as reported previously; a NAS threshold of 5 was used for further comparisons with variables of interest. NASH was defined as steatosis plus mixed inflammatory-cell infiltration, hepatocyte ballooning and necrosis, glycogen nuclei, Mallory's hyaline and any stage of fibrosis, including absent fibrosis.[16]

### ADH1B genotyping

The genetic analysis was conducted on genomic DNA extracted from white blood cells. Genotyping of rs1229984 was performed using a TaqMan Drug Metabolism genotyping assay (dbSNP rs1229984 assay C_2688467_20, Cat. #4362691; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. To ensure genotyping quality, we included DNA samples as internal controls, hidden samples of known genotype, and negative controls (water). To account for possible population stratification, we used a collection of 13 SNPs[18] before analysing the data with the Structure program Version 2.[19] We found no evidence of stratification in our sample because the cases and the controls showed similar $Q$ values and the Structure program assigned a similar distance to clusters with no further improvement in the fitting model resulting from adding up to four clusters (the maximum ln of likelihood was obtained for $K = 1$). Moreover, all the participants in this study self-reported Caucasian ethnicity as a surrogate of ancestry, which is consistent with the minor allele frequency (MAF) found.

For the genetic analysis, we used a dominant model of inheritance owing to the low prevalence of the A (His-48)-allele[20]; data from homozygous AA+ heterozygous AG subjects were pooled and compared with subjects homozygous for the G-allele. The GG homozygous was regarded as the reference group.[20]

Using the CaTS power calculator for genetic association studies,[21] our sample had 98% power for the dominant model.

### Statistical analysis

Quantitative data were expressed as mean ± s.d. unless otherwise indicated. As a significant difference was observed between the groups in most of the variables and/or the distribution was significantly skewed in most cases, we chose to be conservative and assessed the differences between the groups using nonparametric Mann–Whitney $U$-test. For histological outcomes, we used generalised lineal models (ANCOVA or ANOVA) for an ordinal multinomial distribution (Probit as the Link function) with histological outcomes (response as ordinal categorical variables), genotypes as grouping variables, and age and BMI as continuous predictor variables. For ordinal multinomial analysis, logistic analysis or ANCOVA, we adjusted for co-variables that were not normally distributed through log-transformation. The CSS/Statistica program package version 6.0 (StatSoft, Tulsa, OK, USA) was used in these analyses.

## RESULTS

### The rs1229984 variant is a reliable proxy of the level of alcohol consumption in our cohort

Clinical and biochemical features of subjects according to rs1229984 genotypes are shown in Table 1. Carriers of the A-allele had similar demographic (age and sex proportion), lifestyle-related (smoking habit and physical activity) and anthropometric features (BMI) to those of noncarriers (Table 1). There were no differences in either blood lipid traits, including total cholesterol, HDL, LDL-cholesterol and triglycerides, or glucose-related variables, including fasting glucose and insulin, and HOMA-IR (Table 1). Levels of liver enzymes, including ALT, AST and γGT, were similar between the two groups of genotypes (GG vs. AG+AA).

However, carriers of the A-allele had lower levels of serum AP, peripheral leucocyte count (cells/mm$^3$) and uric acid (Table 1); the variant was associated with arterial hypertension ($P = 0.04$) as a disease trait.

The rs1229984 variant was significantly associated with the amount of alcohol exposure; carriers of the A (His-48)-allele consumed significantly lower levels of alcohol relative to the noncarriers (Figure 1a). The distribution of the level of alcohol consumption between carriers and noncarriers of the A-allele is depicted in Figure 1b.

The analysis of the variant and NAFLD as dichotomised variable (disease trait) between controls and cases showed no significant association.

The Hardy–Weinberg test confirmed the independent segregation of the individual rs1229984 alleles ($P = 0.43$); the minor A-allele had a frequency of 7.22%.



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### The relation between the rs1229984 A-allele and NAFLD-histological outcomes: Genetically predicted moderate alcohol consumption does not have a protective effect on the disease severity

Among patients with NAFLD confirmed by liver biopsy, there were no differences in demographic features (age and sex proportion), lifestyle-related variables (smoking habit and physical activity) or biochemical parameters between carriers and noncarriers of the A-allele (Table 2). However, carriers of the A-allele had significantly lower BMI, and lower levels of serum AP and uric acid as compared with noncarriers (Table 2). Significant differences ($P = 0.02$) in daily alcohol intake between carriers of the A-allele and noncarriers (0.5 ± 2 vs. 10 ± 25 g per day, mean ± s.d.), were also observed. Association analysis between rs1229984 genotypes and alcohol intake in patients with NAFLD definitively confirmed the significance of the variant in predicting drinking habits (Figure 2a); abstainers were significantly over-represented in the group of carriers of the A-allele ($P = 0.018$).

The genetic association analysis of the rs1229984 variant and the disease severity showed that carriers of the A-allele had significantly lower degree of histological steatosis, as well as lower scores of necroinflammatory activity (lobular inflammation) and NAS, when compared with noncarriers (Figure 2b); no association with fibrosis was observed, though lower scores in A-allele carriers compared with noncarriers were noted.

The difference of NAS score between the A-allele carriers and noncarriers (OR 0.219, 95% CI 0.049–0.987) was explained by lower steatosis grade and lesser lobular inflammation but not the score of ballooning degeneration, which was remarkably similar between the two groups (Table 2). Levels of cytokeratin-18 fragments were also comparable between the two groups (Table 2).

To adjust for potential confounders, we used a regression analysis for an ordinal multinomial distribution with Probit function by coding the histological variable of interest, as explained in methods section using the Kleiner score.[16] Following this analysis, the associations of the rs1229984 variant with histological lesions persisted after adjusting for BMI as an independent continuous predictor variable (degree of steatosis: chi-square

**Table 1 |** Assessment of the instrumental variant: clinical and biochemical characteristics of the population according to *ADH1B*-rs1229984 genotypes

| Variables | GG | AG + AA | *P* value |
|---|---|---|---|
| Number of subjects | 401 | 65 | – |
| Female/male (%) | 64/36 | 69/31 | NS |
| Age (years) | 49.9 ± 12.6 | 52.9 ± 12.6 | NS |
| Smoking habit (cigarettes/day) | 3.58 ± 8.6 | 2.1 ± 4.9 | NS |
| Physical activity (h/week) | 1.5 ± 4.2 | 1.9 ± 4.9 | NS |
| BMI (kg/m$^2$) | 30.4 ± 6.4 | 28.8 ± 4.9 | NS |
| SABP (mmHg) | 122.2 ± 16 | 121.5 ± 15.6 | NS |
| DABP (mmHg) | 75.8 ± 11.4 | 74.6 ± 9.9 | NS |
| Fasting plasma glucose (mg/dL) | 104.3 ± 77 | 92.0 ± 18 | NS |
| Fasting plasma insulin (UI/L) | 11.6 ± 8.7 | 11.9 ± 10.6 | NS |
| HOMA-IR index | 3.2 ± 4.37 | 2.8 ± 3.1 | NS |
| Total cholesterol (mg/dL) | 207.9 ± 51 | 207.2 ± 41 | NS |
| HDL-cholesterol (mg/dL) | 51.4 ± 19 | 50.5 ± 14 | NS |
| LDL-cholesterol (mg/dL) | 125 ± 45 | 119 ± 46 | NS |
| Triglycerides (mg/dL) | 158 ± 98 | 170 ± 113 | NS |
| Uric acid (mg/dL) | 4.6 ± 5.0 | 3.9 ± 2.2 | 0.05 |
| Leucocyte count (cells/μL) | 7416 ± 2237 | 6544 ± 1880 | 0.05 |
| ALT (U/L) | 54.3 ± 55.7 | 41.6 ± 27.9 | NS |
| AST (U/L) | 38.3 ± 28 | 32.6 ± 15.9 | NS |
| γGT (U/L) | 3.9 ± 0.82 | 3.7 ± 0.6 | NS |
| AP (U/L) | 212.3 ± 112 | 185.4 ± 97.6 | 0.05 |

BMI, body mass index; SABP and DABP, systolic and diastolic arterial blood pressure; HOMA, homeostatic model assessment; ALT and AST, serum alanine and aspartate aminotransferase; γGT, gamma-glutamyl-transferase; AP, alkaline phosphatase. NS, nonsignificant.

Results are expressed as mean ± SD. *P* value stands for statistical significance using Mann–Whitney *U*-test, except for female/male proportion that *P* value stands for statistical significance using chi-squared test.


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5.59, $P = 0.019$, lobular inflammation: chi-square 4.74, $P = 0.033$ and NAS score: chi-square 5.11, $P = 0.023$). Interestingly, the effect of the rs1229984 variant also persisted after adjusting for abstainers and moderate alcohol consumption (degree of steatosis: chi-square 6.99, $P = 0.0082$, lobular inflammation: chi-square 3.74, $P = 0.05$ and NAS score: chi-square 5.80, $P = 0.016$).

## DISCUSSION

While the evidence yielded by previous cross-sectional studies suggests that moderate alcohol consumption might have a beneficial effect on the histological outcomes of NAFLD,[5, 6, 8, 9, 22, 23] protective associations are disputable because many potential uncontrolled factors could have introduced bias into the analyses. Particularly, the heterogeneous quality of alcohol exposure assessment as well as definition of drinking patterns (either self-reported or by direct interview) might have led to systematic misclassification of past drinkers and occasional drinkers to the abstainer or nondrinker categories. In addition, confounding factors, including lifestyle, socio-demographic variables, environmental influence, and the impossibility of accurately assessing the long-term pattern of exposure, definitively preclude a clear interpretation of the current evidence. Of great importance, the protective effect of moderate alcohol consumption on NAFLD severity has never been demonstrated in a RCT.

In this study, we used a reliable approach (Mendelian randomisation analysis) to overcome the tremendous difficulties of performing an RCT in which not only alcohol would be the intervention-treatment variable, but the RCT should theoretically contemplate a control (unexposed) and a treated (exposed) group. The Mendelian randomisation analysis (Figure 3), in which the genetic test (rs1229984) is the instrumental variable that allows the classification of subjects into two categories, has the advantage of reflecting with substantial accuracy the lifetime pattern of alcohol exposure (lower and higher) (Figure 3). Another key advantage of the Mendelian

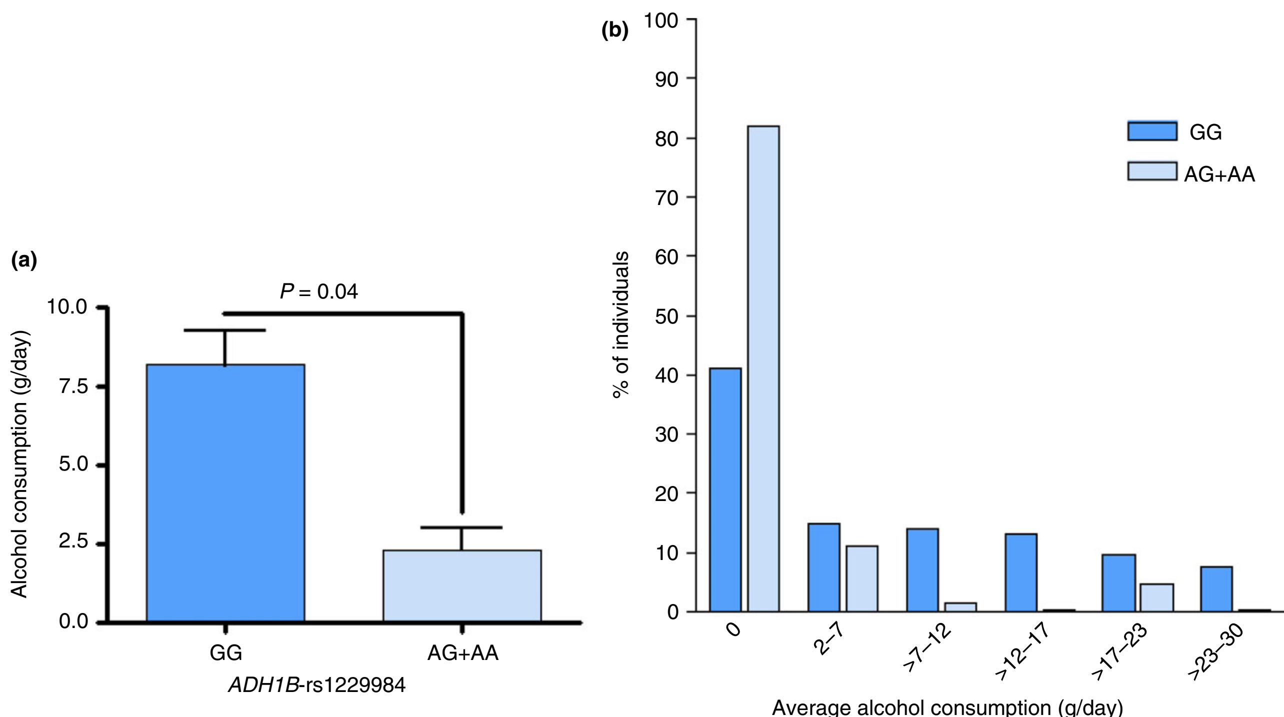


**Figure 1 |** The rs1229984 variant is a reliable proxy of alcohol consumption. (a) Assessment of the instrumental variant (the rs1229984) as a proxy of the amount of alcohol consumption in our population. Data from homozygous AA+ heterozygous AG subjects ($n = 65$) were pooled and compared with that pertaining to subjects homozygous for the G-allele ($n = 401$); the G (ancestral)-allele was regarded as the reference group. Bars represent daily alcohol intake expressed in g per day (mean ± s.d.). *P* value stands for statistical significance using Mann–Whitney *U*-test. (b) Histogram showing the distribution of alcohol consumption between carriers and noncarriers of the rs1229984 A-allele. Data on alcohol drinking were recorded by direct interview during the clinical assessment by physicians.


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randomisation analysis is that is a robust tool for appraising causality.[12]

Consistent with other reports,[13, 20, 24–26] we observed that rs1229984 is a reliable tool for classifying subjects according to the level of alcohol consumption; the average daily alcohol intake was ~5.88 g lower in carriers of the A-allele compared to noncarriers, even in the lower range of alcohol consumption imposed by the exclusion criteria of NAFLD.

The *ADH1B* locus encodes for a protein of the class I enzymes (ADH1A, ADH1B and ADH1C), which belong to a family of homo/heterodimeric complex enzymes involved in ethanol oxidation in the liver.[13] The role of the missense rs1229984 variant in alcohol metabolism and dependence has been extensively used in different populations around the world, including at genome-wide association level (GWAS),[27] although the effects could be different in Asians relative to Caucasians.[25–27] The minor A-allele significantly increases the activity of the ADH1B enzyme.[13, 27] A previous study showed that the population risk of alcoholism attributable to the rs1229984, specifically the Arg48 (G)-allele, was 62–67% in Caucasian population; however, it ranged from 9% to 24% in East Asian population.[15]

It should be highlighted that rs1229984 is not the only genetic proxy of the level of alcohol consumption. Genetic variation in other locus, including coding variants in *ADH1C* and *ALDH2* genes, is associated with altered kinetic properties of the resulting enzymes.[13] Nevertheless, many observations suggest that rs1229984 is a suitable instrument for validating Mendelian randomisation assumptions. For example, *ADH1C*-rs698

**Table 2 |** Association analysis with histological outcomes: clinical and biochemical characteristics of patients with NAFLD according to *ADH1B*-rs1229984 genotypes

| Variables | GG | AG + AA | *P* value |
|---|---|---|---|
| Number of subjects | 230 | 36 | – |
| Female/male (%) | 61/39 | 76/24 | NS |
| Age (year) | 52.3 ± 11 | 52.8 ± 10 | NS |
| Smoking habit (cigarettes/day) | 4.8 ± 10 | 1.5 ± 5 | NS |
| Physical activity (h/week) | 1.4 ± 5 | 2.7 ± 6.5 | NS |
| BMI (kg/m$^2$) | 33 ± 6 | 30 ± 4 | 0.009 |
| SABP (mmHg) | 127 ± 15 | 124 ± 16 | NS |
| DABP (mmHg) | 78.9 ± 11.6 | 76.1 ± 9.7 | NS |
| Fasting plasma glucose (mg/dL) | 117.8 ± 20.7 | 100.8 ± 19 | NS |
| Fasting plasma insulin (UI/L) | 14.7 ± 9.7 | 12.8 ± 10.8 | NS |
| HOMA-IR index | 4.3 ± 4.3 | 3.4 ± 3.8 | NS |
| Total cholesterol (mg/dL) | 210 ± 48 | 203 ± 40 | NS |
| HDL-cholesterol (mg/dL) | 52 ± 19 | 47 ± 15 | NS |
| LDL-cholesterol (mg/dL) | 127 ± 43 | 108 ± 47 | NS |
| Triglycerides (mg/dL) | 172 ± 110 | 202 ± 136 | NS |
| Uric acid (mg/dL) | 5.4 ± 6.3 | 3.8 ± 2.4 | 0.006 |
| Leucocyte count (cells/μL) | 7803 ± 2302 | 6626 ± 1486 | 0.06 |
| ALT (U/L) | 68 ± 61 | 51 ± 27 | NS |
| AST (U/L) | 45 ± 30 | 39 ± 16 | NS |
| γGT (U/L) | 78.3 ± 71 | 56 ± 42 | NS |
| AP (U/L) | 236 ± 110 | 188 ± 97 | 0.007 |
| Haemoglobin A1c (HbA1c) | 7.1 ± 2.2 | 6.4 ± 1 | NS |
| CK-18 (U/L) | 285.4 ± 241 | 280.1 ± 304 | NS |
| Histological outcomes | | | |
| Degree of steatosis | 2.2 ± 0.8 | 1.7 ± 0.8 | 0.03 |
| Ballooning degeneration | 0.47 ± 0.6 | 0.5 ± 0.7 | NS |
| Necroinflammatory activity | 0.95 ± 0.92 | 0.54 ± 0.65 | 0.02 |
| Fibrosis stage | 0.78 ± 1.2 | 0.63 ± 1.0 | NS |

BMI, body mass index; SABP and DABP, systolic and diastolic arterial blood pressure; HOMA, homeostatic model assessment; ALT and AST, serum alanine and aspartate aminotransferase; γGT, gamma-glutamyl-transferase; AP, alkaline phosphatase; CK18, caspase-generated CK-18 fragment (CK-18). NS, nonsignificant.

Results are expressed as mean ± s.d. *P* value stands for statistical significance using Mann–Whitney *U*-test, except for female/male proportion that *P* value stands for statistical significance using chi-squared test.


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(Ile349Val) showed a lesser impact on ADH activity (*ADH-1C**2 allele decreases ADH activity by 2% compared with the most frequent allele *ADH-1C**1).[14] In addition, a comprehensive analysis of variants in all seven ADH-genes, including 110 SNPs, did not find association with any SNP in or near *ADH1C* and alcohol drinking behaviour.[28] Finally, in European population and among many candidate polymorphisms in alcohol-metabolising genes, rs1229984 showed superior performance and strong association with alcohol consumption.[15, 20, 24, 26, 29]

Our results show that carriers of the rs1229984 A-allele had decreased scores of histological steatosis, lobular inflammation and NAS. Remarkably, the results pertaining to histological lesions remain significant after adjustment for BMI, the major potential confounding factor, and even if subjects were abstainers or consumers of moderate amount of alcohol. A previous study showed that being overweight or obese increase the effect of the average daily alcohol consumption on hepatic steatosis.[23]

This finding expresses the robustness of the instrumental variable, and suggests that there is no attenuation of the gene-outcome relation caused by confounders. Contrary to previous evidence,[4–8, 10] we can conclude that genetically predicted light to moderate alcohol consumption does not have a beneficial effect on the histological outcomes of NAFLD and in fact suggest the opposite.

Furthermore, in the group of A-allele carriers, we noted a lower prevalence of arterial hypertension, as well as decreased plasma levels of uric acid and peripheral leucocyte count, suggesting that the effect of alcohol on systemic inflammation is consistent. Moreover, these results denote vertical pleiotropy, implying that the variant affects multiple phenotypes in the same pathway. Together, our findings suggest that the lower the long-term level of alcohol drinking (especially abstinence) the better the final outcome; hence, moderate alcohol consumption does not seem to be protective.

Three main inferences should be discussed when interpreting our findings. First, contrary to earlier reports, long-term co-occurring NAFLD-associated metabolic risk factors and low to moderate alcohol drinking seem to act synergistically to increase the accumulation of fat in liver. In fact, in our study, carriers of the A-allele showed a reduction of ~20% in the steatosis score. Second, long-term coexistence of metabolic risk factors and light to moderate alcohol consumption exacerbates the liver inflammatory picture. As already known, inflammation is not only one of the hallmark features of NASH but plays a pivotal role in promoting the disease progression; the

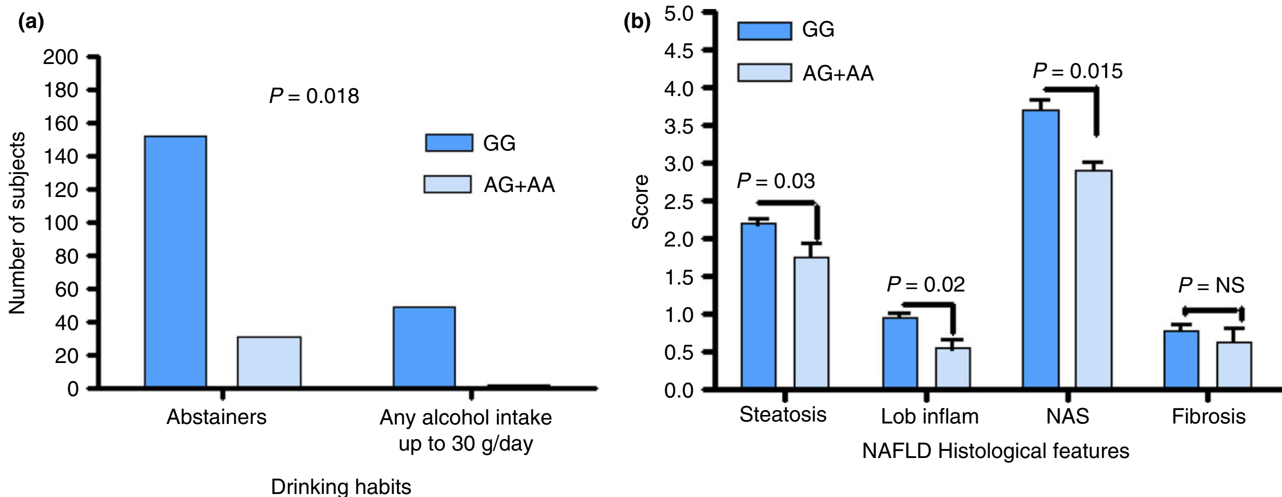


**Figure 2 |** The relation between light to moderate alcohol intake and the histological severity of nonalcoholic fatty liver disease (NAFLD). (a) Association analysis of drinking habits between carriers and noncarriers of the rs1229984 A-allele. (b) The score of histological lesions according to rs1229984 genotypes in the dominant model of inheritance. Steatosis: indicates the grade of steatosis; lob inflam: denotes the score of lobular inflammation; NAS: NAFLD activity score. Histological outcomes were assessed and scored according to Kleiner *et al.*[16] Details on number of subjects are provided in Table 2. Results are expressed as mean ± s.d.. *P* value denotes statistical significance using the Mann–Whitney *U*-test.




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detrimental effects of inflammatory cells in the liver were elegantly reviewed recently.[30] In our study, the rs1229984 A-allele was associated with a reduction of ~43% in the inflammatory score, suggesting that the joint effect of alcohol and metabolic factors amplifies the liver injury. Third, our results suggest that genetically predicted moderate alcohol consumption might not have any benefit in reducing cardiovascular risk factors, including arterial hypertension, as was suggested in earlier cross-sectional studies.[31] Accordingly, a recent Mendelian randomisation analysis of the risk of CVD showed that reduction in alcohol consumption, even for light to moderate drinkers, is beneficial to CV health.[20]

The strength of our study stems not only from the novelty of the approach employed to assess the effects of genetically predicted moderate alcohol consumption on NAFLD severity, but also the scope and implications of its findings in the clinical setting. Moreover, we used an instrument (rs1229984) that seems to be independent of the outcome (NAFLD) from the biological point of view. This aspect suggests that the effect of the variant on the explored outcome (histological severity) is mediated exclusively by the amount of alcohol consumed and not by any other direct biological effect.

Nevertheless, some limitations to our research should be noted, namely the small number of events owing to the low frequency of the variant. The MAF in our sample was 7.22%, which is similar to the MAF reported in European populations.[20] Interestingly, the last observation highlights the point that the G variant is found in more than 92% of Caucasian population alleles, implying that the alcohol degradation rate is slow in around 85% of individuals (GG homozygous subjects) of this racial origin. In addition, the study sample comprised of a small number of subjects who drink alcohol in moderate amounts, which is justified by the fact that the inclusion criteria for NAFLD impose indeed obvious restrictions onto this variable. Specifically, the last point presents a clear limitation for the assessment of the effect of moderate alcohol consumption on the incidence of fatty liver in the general population, which remains to be explored further. Finally, our study does not address exactly the portion of disease variability associated with the range

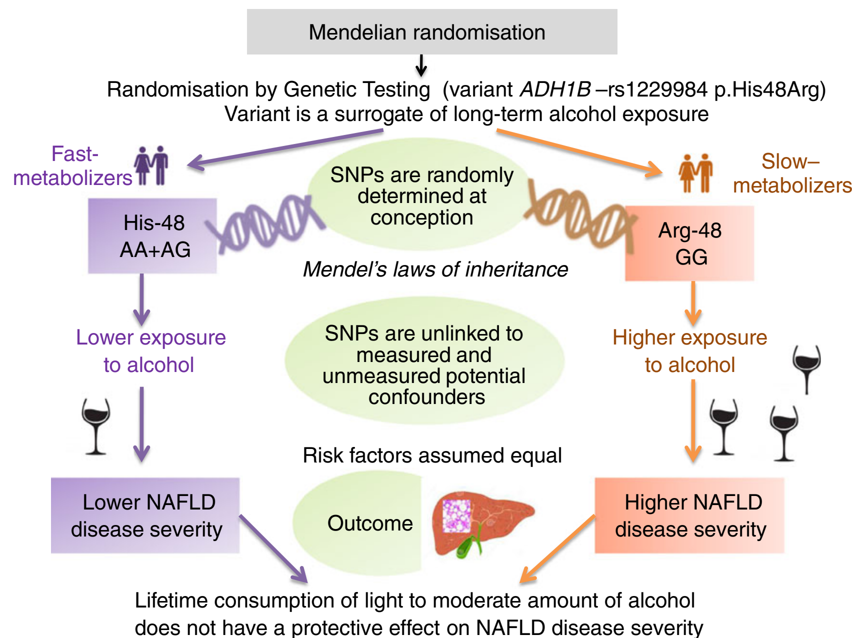


**Figure 3 |** Graphical summary of the Mendelian randomisation approach for exploring the role of light to moderate alcohol consumption in the severity of NAFLD. Images illustrate a parallelism between Mendelian randomisation and classical randomised control trials, in which participants are randomised to either intervention (treatment) or no-treatment (placebo or control) group, and the randomisation ensures that potential confounders (known or unknown) are distributed equally. By adopting this approach, the possibility of reverse causation is eliminated, indicating that if higher alcohol exposure is associated with liver histological outcomes, it can be treated as the causal factor and not the consequence. Otherwise, if increased alcohol exposure is not the cause, we would expect no increase in the severity of NAFLD.


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of absent to low/moderate amount of alcohol ingestion; indeed, carriers of the A allele were mostly abstinent. The fact that rs1229984-A allele carriers usually consume less alcohol does not necessarily mean that a person carrying this allele is a 'moderate drinker'. Furthermore, it seems unreasonable to assume that the inclusion criteria of NAFLD –20 or 30 g of alcohol per day- are absolute, and none of the patients consume alcohol exactly above these cutoffs. Rather, one might *a priori* anticipate a right skew distribution; for instance, it should be expected the majority of rs1229984-homozygous GG to be moderate drinkers (consume <30 g per day), some to be moderate–heavy drinkers, and few to be heavy drinkers. Unfortunately, our study was not focused on the pattern of alcohol consumption in the general population; hence, we could not assess the accuracy of the model in separating the whole spectrum of alcohol drinking, including heavy-drinkers. Conversely, whether there were heavy-drinkers that recognised themselves as moderate-drinkers cannot be either discriminated by our analysis but Mendelian randomisation may suggest that this is not the case. At any rate, the classification of the trait of interest as 'non-alcoholic' fatty liver disease relies on the premise that patients honestly disclose their drinking practices as well as clinicians' confidence as to what constitutes alcohol misuse.

Besides, the question remains as to whether the influence of the genetic variant is strong enough to condition the alcohol consumption among the low to moderate drinkers. At any rate, the instrumental variant is useful for validating Mendelian randomisation assumptions on the level of long-term alcohol consumption; however, the variant does not allow separate never-drinkers from non-drinkers. However, this instrument is particularly advantageous for the exploration of the outcome of interest, which was the effect of the long-term pattern of alcohol exposure in NAFLD severity. We did not find a significant association between the variant and liver fibrosis, although it was mostly lower among the A-allele carriers. Nevertheless, this assertion does not imply that the lack of association should be interpreted as evidence disputing the link between the exposure and outcome. Studies with specific focus on liver fibrosis, employing larger sample size, should thus be conducted to reach a definitive conclusion on this feature. At higher levels of alcohol consumption, there is no doubt that alcohol is detrimental to liver fibrosis.[32]

In conclusion, Mendelian randomisation analysis suggests that light to moderate alcohol intake has no beneficial effect on the histological outcomes of NAFLD. Likewise, in patients with NAFLD, the adverse effects of alcohol – even at low doses – seem to enhance the risk of CVD, potentially aggravating further the long-term morbidity and mortality associated with the disease.

On the other hand, the present Mendelian randomisation not only challenged earlier epidemiological results of the effect of moderate alcohol consumption on NAFLD, but our findings definitively support simple and classical way of thinking based on common sense suggesting that two 'joined insults' – such as metabolic deregulation plus alcohol – whatever the level of exposure, will have much greater potential to cause liver injury than each one separately. Collectively, our findings support the argument that in patients with NAFLD at high-risk for progressing to end-stage liver disease, alcohol consumption even at moderate amount might be harmful.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Clinical and biochemical characteristics of healthy subjects included in the validation of the instrumental variable.

**Table S2.** Clinical and biochemical characteristics of patients with NAFLD included in the validation of the instrumental variable.

**Table S3.** Clinical and biochemical characteristics of patients with NAFLD included in the association study between moderate alcohol consumption and the histological outcomes.

## AUTHORSHIP

*Guarantor of the article:* Silvia Sookoian.
*Author contributions:* SS was involved in the study concept and design, data acquisition, liver biopsies and collection of biological material, data analysis and interpretation, general study supervision, drafting of the manuscript and also the securing of funding; DF: performed genotyping; GOC performed liver biopsies and collected biological samples; CJP was involved in the study concept and design, data acquisition, statistical analysis and interpretation, general study supervision, drafting of the manuscript and the securing of funding. All authors reviewed the manuscript. All authors approved the final version of the manuscript.

## ACKNOWLEDGEMENT

*Declaration of personal interests:* None.
*Declaration of funding interests:* This study was partially supported by grants PICT 2014-432 (Dr Silvia Sookoian) and PICT 2014-1816 (Dr Carlos J Pirola), Agencia Nacional de Promoción Científica y Tecnológica.


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