



HHS Public Access

Author manuscript

Gastroenterology. Author manuscript; available in PMC 2024 May 01.

Published in final edited form as:

Gastroenterology. 2023 May ; 164(6): 966–977.e17. doi:10.1053/j.gastro.2023.01.040.

PNPLA3 genotype and diabetes identify patients with non-alcoholic fatty liver disease at high risk of incident cirrhosis

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Abstract

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) can progress to cirrhosis and hepatic decompensation, but whether genetic variants influence the rate of progression to cirrhosis or are useful in risk stratification among patients with NAFLD is uncertain.

Methods: We included participants from two independent cohorts, Michigan Genomics Initiative (MGI) and UK Biobank (UKBB), who had NAFLD defined by elevated alanine aminotransferase (ALT) levels in the absence of alternative chronic liver disease. The primary predictors were genetic variants and metabolic comorbidities associated with cirrhosis. We conducted time-to-event analyses using Fine-Gray competing risk models.

Results: We included 7,893 and 46,880 participants from MGI and UKBB, respectively. In univariable analysis, *PNPLA3*-rs738409-GG genotype, diabetes, obesity, and ALT >2x upper limit of normal were associated with higher incidence rate of cirrhosis in both MGI and UKBB. *PNPLA3*-rs738409-GG had additive effects with clinical risk factors including diabetes, obesity, and ALT elevations. Among patients with indeterminate Fibrosis-4 (FIB4) scores (1.3–2.67), those

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Disclosures: Vincent Chen, Antonino Oliveri, Matthew Miller, Karn Wijarnpreecha, Xiaomeng Du, Yanhua Chen, Kelly Cushing, Anna Lok, and Elizabeth Speliotes: nothing to disclose

Writing assistance: none

Data transparency: Individual-level data will not be made publicly available due to privacy restrictions for Michigan Genomics Initiative and UK Biobank. Full UK Biobank data are available to the public upon application.

with diabetes, and *PNPLA3*-rs738409-GG genotype had incidence rate of cirrhosis comparable to that of patients with high-risk FIB4 scores (>2.67) and 2.9 to 4.8-times that of patients with diabetes but CC/CG genotypes. In contrast, FIB4<1.3 was associated with incidence rate of cirrhosis significantly lower than that of FIB4>2.67, even in the presence of clinical risk factors and high-risk *PNPLA3* genotype.

Conclusions: *PNPLA3*-rs738409 genotype and diabetes identified patients with NAFLD currently considered indeterminate risk (FIB4 1.3–2.67) who had similar risk of cirrhosis as those considered high-risk (FIB4 2.67). *PNPLA3* genotyping may improve prognostication and allow for prioritization of intensive intervention.

Keywords

genetics; predictive modeling; risk stratification; TRIB1; polygenic risk score

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a leading cause of cirrhosis and end-stage liver disease ¹ and is implicated in 36% of liver-related deaths and over one billion dollars in annual healthcare costs in the United States ^{2, 3}. However, while NAFLD is a leading cause of liver-related mortality, most patients with NAFLD do not die from liver disease or even develop cirrhosis ⁴. NAFLD pathophysiology is heterogeneous with variable rates of disease progression and spontaneous regression, and we have limited ability to predict which patients with NAFLD are most likely to progress to cirrhosis ⁵. The most consistent clinical predictor of incident cirrhosis and liver-related death is fibrosis stage ^{6, 7}. However, determining fibrosis stage requires testing such as liver elastography or liver biopsy which are infrequently available outside of a subspecialist setting ⁸. Other factors such as diabetes and obesity are less strongly predictive ^{9–11}. Improving risk stratification is critical to prioritizing the highest-risk patients to intensive interventions.

Incorporating genetic variants may help predict risk of disease progression. Liver cirrhosis and fibrosis are largely genetically mediated with estimated heritability around 50% ^{12–14}. In addition, genetic and environmental risk factors may interact to further exacerbate risk of hepatic steatosis or cirrhosis. We previously found that genetic susceptibility to hepatic steatosis interacted with insulin resistance to increase risk of hepatic steatosis and that *PNPLA3* genotype, insulin resistance, and their interaction accounted for 8% of variability in hepatic steatosis ¹⁵. In addition, a recent genome-wide multi-trait association study for cirrhosis and alanine aminotransferase (ALT) found that a polygenic risk score (PRS) for cirrhosis interacted with alcohol intake and body mass index to increase lifetime risk of cirrhosis ¹⁶.

Despite this, there has been little uptake of genotyping for clinical management of NAFLD. The literature on effects of genetic variation on NAFLD-related cirrhosis has largely consisted of case-control studies comparing patients with vs. without cirrhosis, but there is very limited literature on how genetics impacts rate of disease progression. In addition, most genetic studies on incident cirrhosis in NAFLD have used the general population as controls, so the effects of genetic variants on disease progression in patients with established

NAFLD are not well-characterized^{17, 18}. Most importantly, whether incorporating genetics into risk stratification results in meaningful improvement over clinical predictors such as Fibrosis-4 (FIB4), diabetes, and obesity status, is unknown. Improved understanding of how genetics influences rate of disease progression and how it interacts with established risk factors for advanced liver disease is crucial for genetic testing to be applicable in clinical practice¹⁹.

We conducted an exploratory study in two independent cohorts to determine how known NAFLD-promoting genetic variants influence rate of progression to cirrhosis among patients with NAFLD and whether genetics can improve upon risk stratification.

METHODS

Ethics

The Institutional Review Board of the University of Michigan (Ann Arbor, MI) approved all research conducted in this study. All Michigan Genomics Initiative (MGI) participants provided written informed consent. UK Biobank (UKBB) protocols were approved by the National Research Ethics Service Committee and all participants provided written informed consent. UKBB analyses in this project were conducted under Resource Project 18120. This study followed STROBE guidelines.

Cohorts and genotyping

MGI is an ongoing prospective cohort that has been recruiting patients seen at Michigan Medicine (Ann Arbor, MI, USA) since 2012, with >80,000 subjects with genotyping of whole blood on an Illumina HumanCoreExome v.12.1 array, a combined GWAS and exome array consisting of >500,000 SNPs at the time of analysis²⁰. Imputation was performed to Haplotype Reference Consortium (release 1 for chromosomes 1–22 and 1.1 for X)¹³.

UKBB is a prospective cohort of >500,000 individuals living in the UK aged 40–69 years at recruitment (2006–2010)²¹. Participants were genotyped on one of two arrays: UK BiLEVE Axiom Array (n = 50,520) or UK BioBank Axiom Array (n = 438,692) with >95% overlap. SNPs were imputed to Haplotype Reference Consortium and UK10K. For quality control, we used EasyQC (version 9.2) with an imputation quality cutoff of 0.85.

Genetic predictors

We selected genetic variants previously associated with hepatic steatosis and/or cirrhosis^{22–25}, with individual variants modeled based on genotype (e.g., AA, AC, vs. CC alleles). The specific variants evaluated were: PNPLA3-rs738409, TM6SF2-rs58542926, HSD17B13-rs6834314, TRIB1-rs28601761, MBOAT7-rs641738, APOE-rs429358, GPAM-rs2792751, and GCKR-rs1260326. We also evaluated a previously reported PRS for cirrhosis, based on dosages of individual genetic variants, weighted by the beta value for each genetic variant¹⁶.

NAFLD definition

In both MGI and UKBB, we included patients with NAFLD defined based on ALT elevations in the absence of excess alcohol intake or other etiologies of chronic liver disease including chronic viral hepatitis and alcohol-related liver disease (Supp. Table 1)^{26–28}. In MGI, we required outpatient ALT levels ≥ 31 U/L in women or ≥ 40 U/L in men, on at least two occasions at least six months apart, or an ICD code for NAFLD (571.8, K76.0, or K75.81). We previously reported that in a Michigan Medicine cohort these criteria have 89–96% specificity for NAFLD²⁶. In UKBB, serial ALT values are unavailable in >90% of participants, so we required only a single ALT elevation. In both cohorts, the NAFLD index date was the first date of ALT elevation.

Outcomes

The primary outcome was cirrhosis, defined based on ICD-9 and –10 codes for cirrhosis or portal hypertensive complications which are highly specific for diagnosis of cirrhosis (Supp. Table 1)^{29–31}. Diagnosis codes are available through linkage to the electronic medical record in MGI and to National Health Service data in UKBB. For patients with multiple diagnosis codes for cirrhosis, the earliest was used. Both inpatient and outpatient ICD codes are available in MGI, but only inpatient codes are available in UKBB. We excluded patients with prevalent cirrhosis defined as first diagnosis of cirrhosis 1 year after the NAFLD index date or earlier. Patients were followed until onset of cirrhosis, death, or last follow-up date, whichever was earliest. For UKBB, last follow-up date was May 31, 2017, and for MGI, December 31, 2021.

As a sensitivity analysis, we also used as the outcome liver-related events (LREs), defined as hepatic decompensation or hepatocellular carcinoma (Supp. Table 1); specifically, compensated cirrhosis would not count as an LRE in this analysis.

Statistical methods

Other predictors—We generated a “clinical risk score” based on presence of diabetes, obesity, and ALT ≥ 2 x upper limit of normal (ULN), which were the clinical predictors most strongly associated with incident cirrhosis. Each patient received 2 points for presence of diabetes, and 1 each for presence of obesity or ALT ≥ 2 x ULN, for a range of 0–4 points. These values were assigned approximately based on the beta values in multivariable analysis in MGI (Table 1; $\beta = 0.41$ for obesity, $\beta = 0.40$ for ALT ≥ 2 x ULN, and $\beta = 0.70$ for diabetes), except that to simplify the score, obesity and ALT ≥ 2 x ULN were each assigned 1 point and diabetes was assigned 2 points. The same score was used in both the MGI and UKBB cohorts. We then divided patients into low, intermediate, and high risk (0–1, 2–3, and 4 points, respectively) groups; these cutoffs were chosen because cumulative incidence of cirrhosis was similar in patients with 0 vs. 1, or 2 vs. 3 points (Supp. Figure 1).

We conducted subgroup analyses in which effects of genetic predictors significantly associated with incident cirrhosis were stratified by presence of environmental risk factors for incident cirrhosis. Finally, we conducted sensitivity analyses to stratify patients based on presence or absence of advanced fibrosis/cirrhosis based on FIB4 score cutoffs <1.3 , 1.3 – 2.67 , or >2.67 ^{32, 33}.

Comparing diagnostic accuracy of genetic predictors—We compared the diagnostic accuracy of *PNPLA3*-rs738409-GG genotype with a cirrhosis PRS¹⁶ using a time-dependent receiver operating characteristic (ROC) framework³⁴. Here, cases were defined as those diagnosed with cirrhosis within 8 years after the index date, and controls were defined as those with at least 8 years of follow-up who did not develop cirrhosis. We computed area under the curve (AUC) for *PNPLA3*-rs738409-GG genotype or for the cirrhosis polygenic risk score, plus age, sex, and genetic principal components 1–10. In UKBB, principal components were calculated using 407,219 unrelated, high-quality samples and 147,604 high-quality genetic variants chosen to minimize linkage disequilibrium³⁵. In MGI, principal components were derived from a Human Genome Diversity Panel reference panel and approximately 650,000 genetic variants³⁶. We also calculated sensitivity, specificity, and positive/negative likelihood ratios³⁷. AUC values were compared and corresponding confidence intervals generated using the DeLong method³⁸.

Survival models—In time to event analyses, the primary outcome was incident cirrhosis, modeled as a Fine-Gray analysis with competing risk of death without cirrhosis³⁹. Follow-up time was defined as time from first ALT elevation (in MGI) or ALT elevation (in UKBB) to first diagnosis of cirrhosis or loss to follow-up, whichever came first. Patients lost to follow-up were censored (removed from further analysis) at last follow-up date. All models were adjusted for age, sex, and genetic principal components 1–10 to account for ancestry (principal components are variables that summarize overall genetic variation and, therefore, population stratification by ancestry). In addition, we conducted multivariable models which included age, sex, and principal components, as well as all predictors associated with incident cirrhosis at $P < .1$ in univariable analysis. We used competing risk methods to calculate incidence rate and cumulative incidence of cirrhosis, which in this case is defined as the product of the probability of having cirrhosis and the probability of having neither cirrhosis nor death without cirrhosis, over the follow-up period^{40, 41}. In these models, we reported hazard ratios (HR) for predictors³⁹.

In addition, we reported incidence of cirrhosis both as incidence rate (and incidence rate ratios [IRRs]) and 10-year cumulative incidence (10YCI). Incidence rates were defined as number of events (i.e. incident cirrhosis cases) divided by person-years (PY) of follow-up. Incidence rates were compared using a Poisson test with number of events and PY of follow-up as the input parameters⁴². IRRs were computed by dividing incidence rates, with 95% confidence intervals defined as $e^{\log(\text{IRR}) \pm 1.96 * \left(\frac{1}{\text{events}_1} + \frac{1}{\text{events}_2}\right)}$, where events_1 and events_2 are the number of incident events in the two groups whose incidence rates are being compared⁴³. 10YCI was calculated as proportion of patients with an event at 10 years using competing risk methods, with confidence intervals (CI) computed as $10\text{YCI}^{e^{\pm 1.96 * \frac{\sqrt{\text{variance}}}{10\text{YCI} * \log(10\text{YCI})}}}$ as previously reported⁴⁴. Note that incidence rate and IRRs do not take into account competing risks, while 10YCI does. Annotated code is shown in Supp. Data 1.

For descriptive statistics, quantitative variables were reported as mean (standard error) and categorical variables as percentages (%). Continuous variables were compared with the

Student t test if normally-distributed and the Mann-Whitney test if not normally-distributed. Categorical variables were compared with a chi-squared statistic.

In the primary analysis in the discovery cohort, we used Bonferroni-adjusted P values for genetic predictors for 9 comparisons. Otherwise, a two-sided P value < .05 was used to determine statistical significance. R version 4.0.2 (Vienna, Austria) was used for all analyses, with the *cmprsk* package used for time to event analyses. For each analysis, patients with missing datapoints were excluded.

RESULTS

Cohorts

The derivation cohort was MGI. Baseline characteristics are shown in Supp. Table 2. The MGI cohort included 7,893 participants with NAFLD, median age was 52 years, and 57% were female. There was a high prevalence of diabetes (36%) and obesity (58%). Incident cirrhosis developed in 194 (2.5%) MGI participants during a median follow-up of 71.6 (45.3–100.9 months), yielding an incidence rate of 4.0 per 1,000 PY overall. Of the remaining patients, 600 died during follow-up, 4,514 were lost to follow-up during the study period, and the remainder reached the end of the study period. Number of distinct outpatient ALT measurements in the MGI cohort is shown in Supp. Figure 2; the median number of measurements was 9.

The validation cohort was UKBB. Unlike MGI, UKBB is a community-based cohort and as expected had a lower prevalence of comorbidities such as diabetes and obesity, and lower FIB4 scores (Supp. Table 2). The UKBB cohort included 46,880 patients. During median follow-up of 106.3 (IQR 97.3–114.3 months), 248 (0.53%) developed incident cirrhosis, yielding an incident rate of 0.60 per 1000 PY overall. Of the remaining patients, 1,601 died during follow-up, 141 were lost to follow-up during the study period, and the remainder reached the end of the study period.

Validation of NAFLD and cirrhosis phenotypes

The NAFLD phenotype was validated in MGI as detailed in Methods. We also evaluated in a Michigan Medicine cohort sensitivity for ICD-10 codes for cirrhosis by evaluating patients with NAFLD (defined by ALT as above) who had an ultrasound, computed tomography, or magnetic resonance imaging study that reported a cirrhotic-appearing liver. We found that 973/1251 (77.8%) of these patients had an ICD code for cirrhosis within 12 months of the date of the imaging study showing cirrhosis, suggesting that ICD codes have acceptable sensitivity for cirrhosis. In UKBB, we validated the ALT definition of NAFLD among the subset of participants who underwent liver magnetic resonance imaging with proton density fat fraction measurement and found that specificity of ALT elevations was 93.0% (3,272/3,515) for liver fat fraction >5.5%. As additional validation that NAFLD defined by ALT level is clinically relevant, we found that UKBB participants with elevated ALT as defined above had markedly increased risk of incident cirrhosis than those without elevated ALT (Supp. Figure 3) with HR 7.38 (95% CI 6.21–8.78, P<.0001). We were unable to

validate sensitivity of ICD codes for cirrhosis in UK Biobank as we do not have access to a “gold standard” metric of cirrhosis.

Predictors of incident cirrhosis in Michigan Genomics Initiative

In models adjusted only for age, sex, and genetic principal components 1–10, FIB4 score was a strong predictor of incident cirrhosis (HR 4.34 [95% CI 2.82–6.70, $P < .0001$] for >2.67 vs. ≤ 2.67 (Supp. Table 3, Supp. Figure 4). Other risk factors included diabetes (HR 2.14 [95% CI 1.60–2.85, $P < .0001$]), higher body mass index (HR 1.83 [95% CI 1.26–2.64, $P = .0014$] for obese vs. lean/overweight), and elevated ALT (HR 2.00 [95% CI 1.48–2.69], $P < .0001$ for vs. $<2 \times$ ULN) (Table 1, Figure 1).

We systematically evaluated genetic variants previously associated with NAFLD (Methods). In MGI, only two of these individual variants were associated with increased rate of progression to cirrhosis: *PNPLA3*-rs738409-GG (vs. CC) with HR 3.48 (95% CI 2.32–5.22, $P_{\text{adjusted}} < .0001$) and *TRIB1*-rs28601761-CC (vs. GG) with HR 2.15 (95% CI 1.30–3.53, $P_{\text{adjusted}} = .023$) (Supp. Table 3, Figure 1). A previously reported PRS for cirrhosis was associated with incident cirrhosis, but we observed an effect only at the highest risk quartile: HR 2.30 (95% CI 1.53–3.46, $P_{\text{adjusted}} = .00058$) vs. lowest quartile (Supp. Table 3, Supp. Figure 4). Variants in *TM6SF2*, *HSD17B13*, and other previously reported risk loci were not significantly associated with incident cirrhosis (Supp. Table 3, Supp. Figure 4). We then generated a multivariable model for incident cirrhosis that additionally included the most consistent predictors of incident cirrhosis in MGI, namely *PNPLA3*-rs738409-G, *TRIB1*-rs28601761-C, diabetes, obesity (categorized as obesity vs. lean/overweight), and ALT level (categorized as vs. $<2 \times$ ULN) and all predictors remained significantly associated with incident cirrhosis with similar HRs compared to the univariable analysis (Table 1).

Secondary analyses in Michigan Genomics Initiative

In models including gene-environment interaction terms (e.g. *PNPLA3*-rs738409-G dosage * diabetes status), the interaction terms were not significant for either *PNPLA3* or *TRIB1* genotype and any of the environmental predictors ($P > .05$ for all). Consistent with this, HRs of *PNPLA3* and *TRIB1* genotype across different clinical risk categories (e.g. people with vs. without diabetes) were not significantly from one another ($P_{\text{heterogeneity}} > .05$ for all) (Supp. Figure 5).

We conducted a sensitivity analysis where LREs rather than cirrhosis were the primary outcome and found that the main clinical and genetic predictors identified in the primary analysis were associated with LREs, other than *TRIB1* genotype (Supp. Table 4). Another sensitivity analysis stratifying by patients without baseline advanced fibrosis (FIB4 < 1.3) or with indeterminate FIB4 (1.3–2.67) showed concordant directions of effects of *PNPLA3* genotype, *TRIB1* genotype, diabetes, obesity, and ALT elevation, though statistical significance was weaker than in the overall cohort (Supp. Table 5).

Combined impact of *PNPLA3* or *TRIB1* genotype and clinical risk factors on incident cirrhosis

Next, we sought to integrate genetic and clinical risk factors for risk stratification in patients with low (<1.3) or indeterminate FIB4 values (1.3–2.67) who are not considered to be at high risk for incident cirrhosis by current guidelines³³. *PNPLA3*-rs738409-GG genotype was associated with higher incidence rate of cirrhosis compared to CC/CG genotypes, among both patients with low FIB4 (incidence rate 6.7 [95% CI 3.2–12.3] vs. 2.4 [95% CI 1.8–3.1] events per 1,000 PY; incidence rate ratio 2.8 [95% CI 2.2–3.6]; P=.0057) and with indeterminate FIB4 (incidence rate 11.4 [95% CI 6.4–18.8] vs. 4.3 [95% CI 3.3–5.5] cases per 1,000 PY; incidence rate ratio 2.7 [95% CI 2.3–3.1]; P=.0017) (Table 2, Supp. Table 6). The association between *PNPLA3* genotype and cirrhosis risk was also significant when stratified by diabetes status, obesity status, and ALT level. Notably, in patients with indeterminate FIB4 and diabetes, *PNPLA3*-rs738409-GG had incidence rate ratio of 4.4 (95% CI 3.4–5.8) vs. CC/CG genotype (28.8 [95% CI 13.8–52.9] vs. 6.5 [95% CI 4.2–9.5]/1000 PY) (Table 2). In patients with indeterminate FIB4, *PNPLA3*-rs738409-GG had incidence rate ratio 3.8 (95% CI 2.4–5.8) in those with intermediate clinical risk scores (17.6 [95% CI 6.5–38.4] vs. 4.7 [95% CI 2.7–7.5]/1000 PY; P=.011) and 3.3 (95% CI 1.2–8.7) in those with clinical high risk scores (30.5 [95% CI 6.3–89.2] vs. 9.3 [95% CI 3.4–20.3]/1000 PY; P=.11) (Table 2), compared to CC/CG genotype. *TRIB1*-rs28601761-CC genotype was associated with higher incidence rate of cirrhosis in patients with low FIB4 but not in those with indeterminate FIB4 scores (Supp. Table 7).

Validation in UK Biobank

On univariable analysis, diabetes, obesity, elevated ALT, *PNPLA3*-rs738409-GG genotype were associated with incident cirrhosis, as was the case in MGI (Supp. Table 8). The association between the *TRIB1*-rs28601761-C allele and cirrhosis was not statistically significant. In UKBB unlike in MGI, TM6SF2-rs58542926-T associated with incident cirrhosis while the cirrhosis PRS did not (Supp. Table 8). Other genetic predictors were not significantly associated with incident cirrhosis (data not shown; P>.05 for all). On multivariable analysis, the association between obesity and incident cirrhosis was no longer statistically significant (P=.09) but there were otherwise no meaningful changes in the results with diabetes, ALT level, and *PNPLA3*-rs738409 genotype continuing to have significant associations and *TRIB1*-rs28601761-C not (Table 1).

We conducted sensitivity analyses in UKBB similar to those in MGI. After stratification by FIB4 score, associations between diabetes, obesity, ALT, and *PNPLA3* genotypes and incident cirrhosis within each FIB4 category were similar to those in the overall cohort though P values were less significant due to a smaller number of events (Supp. Table 9). The factors associated with incident LREs in the overall UKBB cohort were the same as those associated with incident cirrhosis (Supp. Table 10). Unlike in MGI, HRs for effects of *PNPLA3* genotype on incident cirrhosis were larger in higher-risk subgroups such as diabetes, obesity, and ALT >2x ULN (Supp. Figure 6).

Next, we validated in UKBB the combined effects on cirrhosis incidence of *PNPLA3*-rs738409 genotype and the non-genetic factors of diabetes, obesity, ALT elevations, and the

clinical risk score, in patients with low (<1.3) or indeterminate (1.3–2.67) FIB4 scores. The associations between *PNPLA3* genotype and metabolic risk factors, and incident cirrhosis were similar to the findings in MGI (Table 2, Supp. Table 11). Among patients with FIB4 1.3–2.67, *PNPLA3*-rs738409-GG genotype was associated with incidence rate ratio for cirrhosis of 2–3 among patients with diabetes, obesity, or intermediate or high clinical risk scores (Table 2).

Accuracy of *PNPLA3* genotype vs. a cirrhosis PRS in predicting incident cirrhosis

We compared the accuracy of *PNPLA3* genotype vs. the cirrhosis PRS¹⁶ in predicting incident cirrhosis using a time-dependent ROC model, in patients with diabetes and FIB4 1.3–2.67 (Methods)³⁴. In MGI, AUC of *PNPLA3*-rs738409-GG genotype and the cirrhosis PRS were 0.78 (95% CI 0.71–0.85) and 0.73 (95% CI 0.65–0.81), respectively (P=.057), and in UKBB, 0.67 (95% CI 0.59–0.75) and 0.65 (95% CI 0.58–0.73), respectively (P=.33). All models included age, sex, and principal components 1–10. Sensitivity, specificity, positive likelihood ratio, and negative likelihood ratio of *PNPLA3*-rs738409-GG genotype for predicting incident cirrhosis were 0.30, 0.93, 4.40, 0.75, respectively, in MGI, and 0.21, 0.91, 2.33, and 0.87 in UKBB.

FIB4, *PNPLA3* genotype, and clinical risk factors for risk stratification

Finally, we evaluated whether *PNPLA3* genotyping could identify patients with low or indeterminate baseline FIB4 (<1.3 or 1.3–2.67) who are at high risk of disease progression, i.e. risk similar to that of those with FIB4 >2.67. We focused on patients with lower FIB4 scores but diabetes, based on our findings that diabetes has the most consistent effect on cirrhosis risk of the clinical risk factors (Table 2).

Among patients with indeterminate baseline FIB4 scores (1.3–2.67) and diabetes, *PNPLA3* genotype identified a high-risk subpopulation. In MGI, *PNPLA3*-rs738409-GG carriers had cumulative incidence of cirrhosis similar to that of patients with high baseline FIB4 (HR = 1.96 [95% CI 0.95–4.17], p = .068), and nearly five-fold higher risk than those with rs738409-CC or CG genotypes (HR 4.76 [95% CI 2.22–10.00], P<.0001) (Figure 2). Similarly, in UKBB, persons with indeterminate baseline FIB4 score and diabetes who carried the *PNPLA3*-rs738409-GG genotype had incidence of cirrhosis similar to that of the patients with baseline FIB4 >2.67 (HR = 0.67 [95% CI 0.33–1.37], P=.28) and nearly three-fold greater than those with indeterminate FIB4, diabetes, but *PNPLA3*-rs738409-CC or CG genotypes (HR = 2.85 [95% CI 1.33–6.25], P=.0076) (Figure 2).

In contrast, in patients with FIB4 <1.3, *PNPLA3* genotype and diabetes status did not identify a high-risk group. FIB4 >2.67 was associated with a markedly increased risk of incident cirrhosis compared to low FIB4 (<1.3), diabetes, and low-risk *PNPLA3* genotype, with HR 3.9 (95% CI 2.2–6.9) and 40.4 (9.9–164.0) in MGI and UKBB, respectively (p<.0001 for both; Supp. Figure 7). Further, among patients with low FIB4 and diabetes, *PNPLA3* genotype was not significantly associated with increased cumulative incidence of cirrhosis in either MGI or UKBB (p>.05 for both).

DISCUSSION

FIB4 is becoming increasingly used for risk stratification and incorporated into clinical pathways for management of NAFLD³³. However, in the primary care setting, the vast majority of patients do not have advanced fibrosis or elevated FIB4, and further methods for risk stratification are necessary. We found that *PNPLA3* genotyping in conjunction with clinical risk factors may improve risk stratification in patients with NAFLD. Patients with indeterminate FIB4 (1.3–2.67), elevated genetic risk (*PNPLA3*-rs738409-GG), and diabetes have an overall incidence rate of cirrhosis comparable to that of patients with high FIB4 (>2.67) for whom closer monitoring and hepatology evaluation is recommended³³. In contrast, patients with low baseline FIB4 (<1.3) were at much lower risk even if diabetes was present and *PNPLA3* genotype did not meaningfully affect this risk. Thus, routine *PNPLA3* genotyping identifies a subgroup of patients with NAFLD and indeterminate FIB4 who are not otherwise considered to have high risk for advanced liver disease, but who may nonetheless warrant more regular follow-up and more aggressive treatment (Figure 3).

Contrary to other studies, we found that heterozygotes for the *PNPLA3*-rs738409-G allele had no difference in cirrhosis incidence than those who did not carry the risk allele. One of the earliest studies evaluating impact of *PNPLA3* genotype on cirrhosis in NAFLD found a dose-dependent effect of the rs738409-G allele on fibrosis stage⁴⁵. Other studies have found a dose-dependent effect of the *PNPLA3* risk allele on mortality, liver-related events, and liver-related death^{46,47}. These differences may be in part due to choice of statistical modeling (time-to-event in our study vs. case-control in other studies) and how baseline fibrosis stage was accounted for (biopsy in other studies vs. FIB4 in our study). We found that a previously-reported cirrhosis PRS had similar AUC for incident cirrhosis compared to *PNPLA3* genotype alone¹⁶. PRSs have clear advantages compared to individual variants in that they better capture the polygenic nature of genetic risk of common diseases and may have greater statistical power, but may not be generalizable across ancestry and may obscure biological mechanisms behind gene-trait associations^{48,49}. While it may be possible to develop more complex PRSs for cirrhosis, these findings suggest that genotyping of *PNPLA3* alone, which is less expensive than genome-wide genotyping and easier to understand, may have similar clinical applicability for NAFLD.

A strength of our study is that the central finding that *PNPLA3* status and diabetes together allowed for risk stratification of cirrhosis among patients with NAFLD was validated in both a tertiary care cohort with high prevalence of comorbidities (MGI) and a community-based cohort that is overall healthier than the general population (UKBB)⁵⁰. Cirrhosis was also defined differently between cohorts in that UKBB included only inpatient diagnosis codes (which could lower sensitivity, especially for detecting compensated cirrhosis) while MGI included inpatient or outpatient codes, thus better capturing patients with compensated cirrhosis. These differences manifested themselves clearly in a higher cumulative incidence of cirrhosis in MGI, though the cumulative incidence of cirrhosis was comparable in patients with high FIB4 across cohorts implying that this difference in cirrhosis definition is less important in higher risk patients. Importantly, our key finding was validated despite differences in cohort characteristics and definitions of NAFLD and cirrhosis. In addition, we evaluated the impact of *PNPLA3*-rs738409 genotype in conjunction with a clinical

risk score and found it had prognostic significance independent of metabolic risk factors. Notably, *PNPLA3*-rs738409-GG carriers with diabetes had a risk similar to those with baseline high FIB4 scores, indicating that it identifies a group that would not be prioritized with current guidelines. Finally, this is one of the first studies evaluating the impact of genetics on the rate of disease progression in patients with NAFLD. While understanding the lifetime risk of cirrhosis as it relates to genetics is important, rate of progression is arguably more important in NAFLD because of the high prevalence of non-hepatic comorbidities among patients with NAFLD that may limit life expectancy⁵¹.

One limitation of this study is that both the UKBB and MGI cohorts are predominantly Caucasian. We note that the *PNPLA3*-rs738409-G allele frequency varies across ancestry, and approximately 25% of patients of Asian or Hispanic ancestry have the highest risk -GG genotype (vs. 7% in the UKBB and MGI cohorts). These differences in allele frequency imply that our findings may be even more important in some non-Caucasian populations, but this remains to be determined and validation in ethnically diverse cohorts will be necessary. In addition, ascertainment of cirrhosis relied on ICD codes rather than “objective” evidence of cirrhosis. We and others have shown that ICD codes for cirrhosis have high specificity and positive predictive value^{29, 30}. In this study we report that in a Michigan Medicine cohort the sensitivity is >75% among those with imaging evidence of cirrhosis though we acknowledge that patients with abdominal imaging may represent a biased cohort and sensitivity may be lower among the general population with NAFLD. Unfortunately, we were unable to evaluate sensitivity of ICD codes for cirrhosis in UKBB. Patients in MGI may have been lost to follow-up at Michigan Medicine but subsequently diagnosed with cirrhosis at another medical center, which may result in decreased sensitivity for detecting incident cases of cirrhosis. We used a surrogate definition of NAFLD based on ALT elevations, though we and others have shown high specificity of this definition^{26, 28, 52}. Duration of NAFLD, which presumably is linked to risk of cirrhosis, was not known in our study⁵³. Finally, we did not have access to histology or elastography data to determine baseline fibrosis stage. We accounted for baseline fibrosis severity using FIB4 score which is well-validated for ruling out advanced fibrosis and is part of major society clinical care pathways³³, and to our knowledge there are no available cohorts of patients with transient elastography or histology of comparable size to our cohorts.

In summary, risk of cirrhosis among patients with NAFLD without baseline advanced fibrosis can be stratified using three readily available clinical datapoints: BMI, diabetes status, and ALT level. *PNPLA3* genotype can further refine risk stratification. We suggest that patients with indeterminate FIB4 and metabolic risk factors (especially diabetes) routinely undergo *PNPLA3* genotyping, followed by referral to hepatology if the rs738409-GG allele is present (Figure 3).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

The authors acknowledge the Michigan Genomics Initiative participants, Precision Health at the University of Michigan, the University of Michigan Medical School Central Biorepository, the University of Michigan Medical School Data Office for Clinical and Translational Research, and the University of Michigan Advanced Genomics Core for providing data and specimen storage, management, processing, and distribution services, and the Center for Statistical Genetics in the Department of Biostatistics at the School of Public Health for genotype data curation, imputation, and management in support of the research reported in this manuscript.

Grant support:

VLC was supported in part by a Clinical, Translational and Outcomes Research Award from the American Association for the Study of Liver Diseases and K08 DK132312. EKS, YC, XD, and AO are supported by NIH grants R01 DK106621, R01 DK107904 (to EKS) and The University of Michigan Department of Internal Medicine.

Abbreviations:

ALT	alanine aminotransferase
CI	confidence interval
FIB4	Fibrosis-4
HR	hazard ratio
MGI	Michigan Genomics Initiative
NAFLD	non-alcoholic fatty liver disease
PRS	polygenic risk score
PY	person-year
UKBB	UK Biobank
ULN	upper limit of normal

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Author names in bold designate shared co-first authorship.

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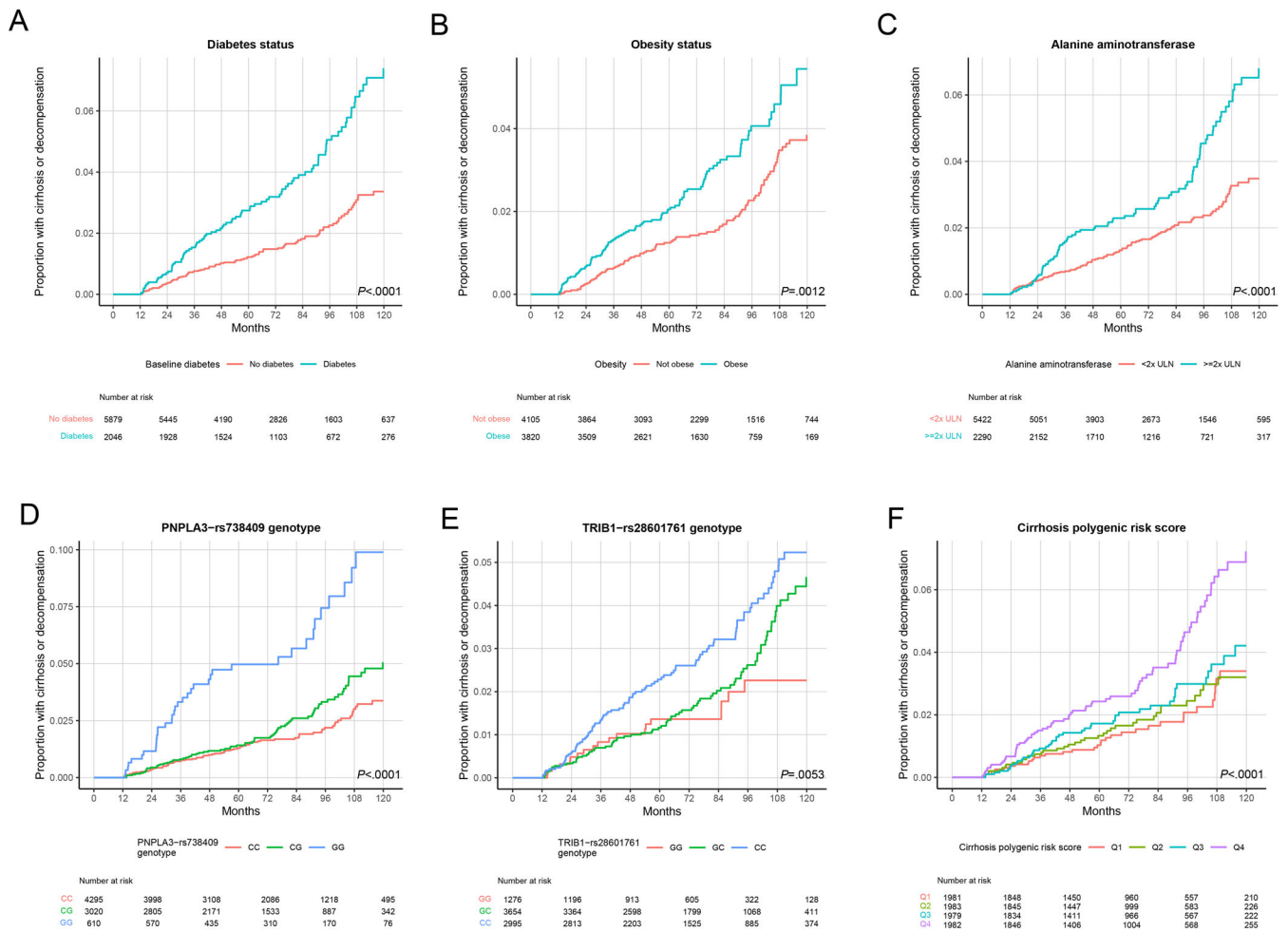
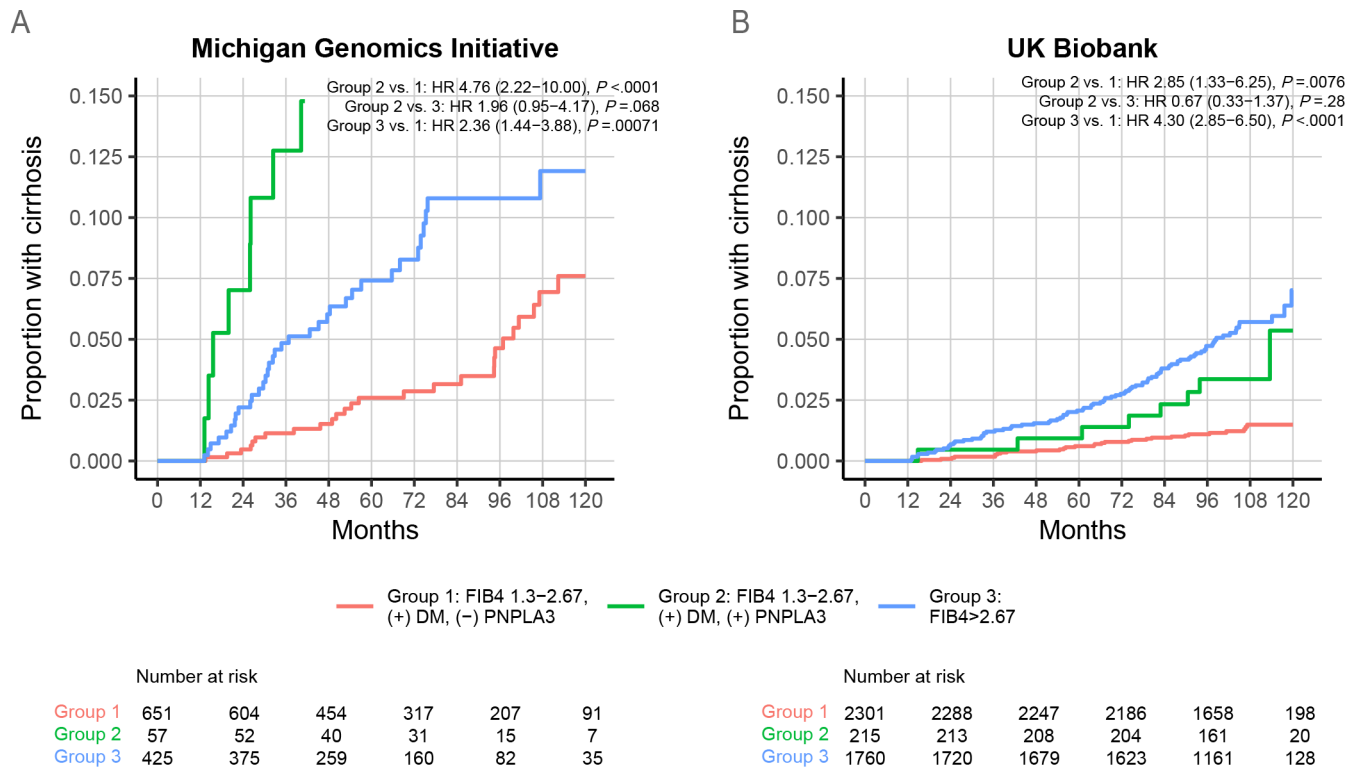


Figure 1: Genetic and environmental factors associated with progression to cirrhosis in Michigan Genomics Initiative. Models were run as Fine-Gray competing risk analyses. (A) Diabetes status. (B) Obesity status. (C) Alanine aminotransferase (ALT), with upper limit of normal (ULN) defined as 19 U/L in women and 30 U/L in men. (D) PNPLA3-rs738409 genotype. (E) TRIB1-rs28601761 genotype. (F) Cirrhosis polygenic risk score, divided into quartiles (Q), with Q1 indicating the lowest quartile.

**Figure 2:**

PNPLA3 genotype identifies a subgroup of patients with indeterminate FIB4 with cirrhosis incidence comparable to that of patients with high FIB4. (A-B) Patients were divided into three groups: high FIB4 (>2.67), indeterminate FIB4 (1.3–2.67) with diabetes [(+) DM] and PNPLA3-rs738409-GG genotype [(+) PNPLA3], and indeterminate FIB4 with diabetes and PNPLA3-rs738409-CC or -CG genotype [(-) PNPLA3]. (A) Michigan Genomics Initiative. (B) UK Biobank.

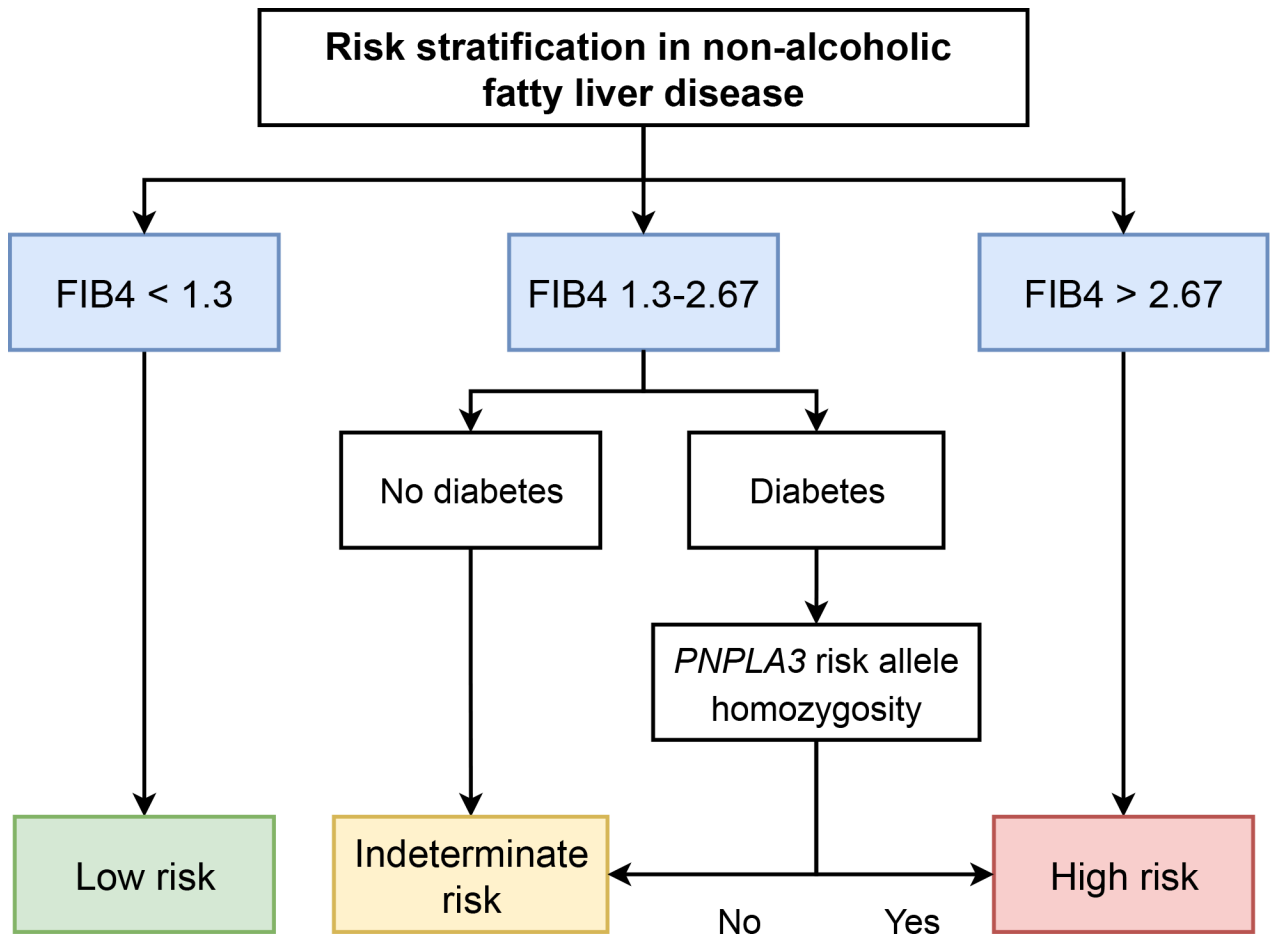


Figure 3: Schematic for incorporating *PNPLA3* genotype into risk stratification in non-alcoholic fatty liver disease. "*PNPLA3* risk allele homozygosity" refers to *PNPLA3*-rs738409-GG genotype. FIB4, fibrosis-4 score.

Table 1:

Multivariable predictors of incident cirrhosis

Predictor	Michigan Genomics Initiative (n=6,482)		UK Biobank (n=49,751)	
	Hazard ratio (95% confidence interval)	P value	Hazard ratio (95% confidence interval)	P value
Diabetes	2.01 (1.43–2.83)	<.0001	3.05 (2.28–4.08)	<.0001
Body mass index				
Lean/overweight	(Referent)		(Referent)	
Obese	1.50 (1.04–2.18)	0.031	1.25 (0.96–1.62)	0.094
Alanine aminotransferase				
<38/60 U/L in women/men	(Referent)		(Referent)	
≥ 38/60 U/L in women/men	1.49 (1.06–2.10)	0.024	3.06 (2.32–4.02)	<.0001
PNPLA3-rs738409 genotype				
CC	(Referent)		(Referent)	
CG	1.43 (1.00–2.06)	0.052	1.05 (0.80–1.38)	.73
GG	3.24 (2.01–5.23)	<.0001	1.99 (1.38–2.88)	.00024
TRIB1-rs28601761 genotype				
GG	(Referent)		(Referent)	
GC	1.20 (0.69–2.11)	0.52	1.24 (0.83–1.86)	.29
CC	1.91 (1.10–3.32)	0.022	1.33 (0.88–2.00)	.18

Models were run as Fine-Gray competing risk analyses. Results are shown as hazard ratio (95% confidence interval). In univariable models, effect of each specific predictor is shown after adjustment for age, sex, and genetic principal components 1–10 to account for ethnic variation. Multivariable results indicate hazard ratios for each predictor additionally adjusted for all of the other predictors shown in this table.

Table 2:

Incidence rate of cirrhosis stratified by PNPLA3 genotype, in patients in indeterminate Fibrosis-4 score, in the Michigan Genomics Initiative and UK Biobank

Cohort	Incidence rate based on PNPLA3-rs738409 genotype		Incidence rate ratio	P value
	CC (lowest risk) or CG	GG (highest risk)		
Michigan Genomics Initiative				
All (n=2,601)	4.30 (3.29–5.52)	11.38 (6.37–18.76)	2.65 (2.25–3.11)	.0017
Diabetes (n=712)	6.50 (4.24–9.52)	28.76 (13.79–52.90)	4.43 (3.37–5.81)	.00039
Obesity (n=1,169)	4.22 (2.31–7.08)	16.83 (7.70–31.95)	3.46 (2.60–4.60)	.0031
ALT \geq 2x ULN (n=797)	5.14 (3.29–7.65)	14.73 (6.36–29.02)	2.87 (2.07–3.97)	.015
Clinical risk score				
Low (n=1,394)	3.40 (2.15–5.09)	4.88 (1.01–14.27)	1.44 (0.69–3.01)	.47
Intermediate (n=710)	4.71 (2.74–7.54)	17.64 (6.48–38.40)	3.75 (2.41–5.83)	.0011
High (n=130)	9.33 (3.42–20.31)	30.53 (6.30–89.23)	3.27 (1.23–8.72)	.011
UK Biobank				
All (n=23,114)	0.61 (0.50–0.74)	0.91 (0.52–1.48)	1.49 (1.30–1.71)	.16
Diabetes (n=2,538)	1.53 (1.03–2.19)	4.39 (1.89–8.65)	2.87 (2.10–3.91)	.013
Obesity (n=10,375)	0.68 (0.51–0.88)	1.85 (1.04–3.05)	2.73 (2.31–3.23)	.0013
ALT \geq 2x ULN (n=9,693)	0.84 (0.65–1.08)	1.13 (0.54–2.08)	1.34 (1.07–1.68)	.34
Clinical risk score				
Low (n=16,619)	0.47 (0.36–0.60)	0.17 (0.02–0.61)	0.36 (0.13–0.99)	.17
Intermediate (n=5,499)	0.83 (0.58–1.15)	2.06 (0.99–3.80)	2.49 (1.94–3.21)	.022
High (n=752)	2.28 (1.22–3.91)	5.77 (1.57–14.76)	2.52 (1.33–4.79)	.010

Incidence rate is shown as events per 1,000 person-years (95% confidence interval), in the overall cohort and key subgroups. Clinical risk score: low risk includes patients with no diabetes and no more than one of ALT \geq 2x ULN or obesity; high risk includes those with diabetes, obesity, and ALT \geq 2x ULN; and intermediate risk indicates all other patients. Incidence rate ratios are shown as ratio for rs738409-GG vs. CC/CG genotype (95% confidence interval). Incidence rate ratio is for the incidence rate of PNPLA3-rs738409-GG genotype relative to CC/CF genotype. P value is for the association between *PNPLA3* genotype (defined as rs738409-CC or -CG vs. -GG) and incidence rate of cirrhosis within each subgroup. Incidence rate confidence intervals and p values were computed using a Poisson statistic with number of events and follow-up duration as the parameters (see Methods for details). ULN, upper limit of normal, defined as 19 U/L for women or 30 U/L for men. Indeterminate Fibrosis-4 score was defined as 1.3–2.67.