Research Article
NAFLD and Alcohol-Related Liver Diseases

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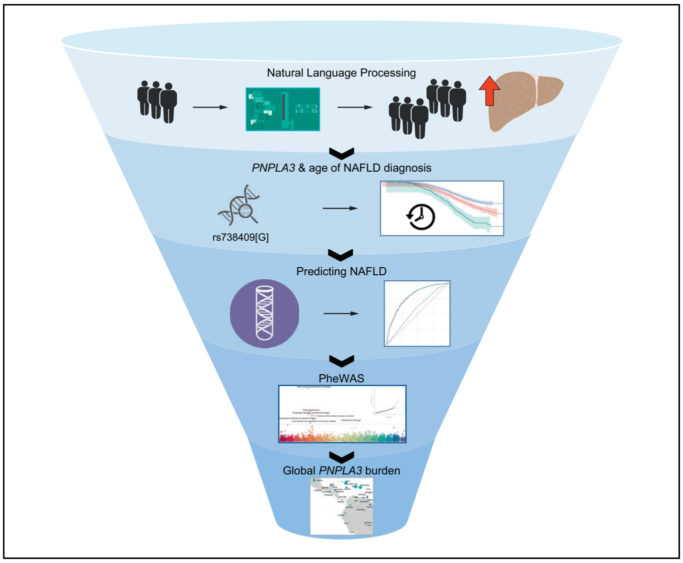
A common variant in *PNPLA3* is associated with age at diagnosis of NAFLD in patients from a multi-ethnic biobank

Graphical abstract


Natural Language Processing
PNPLA3 & age of NAFLD diagnosis
rs738409[G]
Predicting NAFLD
PheWAS
Global *PNPLA3* burden

Highlights

- Natural language processing increased NAFLD detection over ICD codes by 2.5 times.
- rs738409 “GG” carriers were diagnosed with NAFLD 3 years earlier than non-carriers.
- PNPLA3 rs738409 effects were most pronounced in Hispanics.
- PheWAS showed rs738409 was associated with other NAFLD-related liver diseases.
- rs738409 was associated with earlier diagnosis for PheWAS-identified diseases.

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Lay summary

Despite clear associations between the *PNPLA3* rs738409 variant and elevated risk of progression from non-alcoholic fatty liver disease (NAFLD) to more severe forms of liver disease, it remains unknown if *PNPLA3* rs738409 plays a role in the age of NAFLD onset. Herein, we found that this risk variant is associated with an earlier age of NAFLD and other liver disease diagnoses; an observation most pronounced in Hispanic Americans. We conclude that *PNPLA3* rs738409 could be used to better understand liver disease risk within vulnerable populations and identify patients that may benefit from early prevention strategies.

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A common variant in *PNPLA3* is associated with age at diagnosis of NAFLD in patients from a multi-ethnic biobank

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Background & Aims: The Ile138Met variant (rs738409) in the *PNPLA3* gene has the largest effect on non-alcoholic fatty liver disease (NAFLD), increasing the risk of progression to severe forms of liver disease. It remains unknown if the variant plays a role in age of NAFLD onset. We aimed to determine if rs738409 impacts on the age of NAFLD diagnosis.

Methods: We applied a novel natural language processing (NLP) algorithm to a longitudinal electronic health records (EHR) dataset of >27,000 individuals with genetic data from a multi-ethnic biobank, defining NAFLD cases (n = 1,703) and confirming controls (n = 8,119). We conducted i) a survival analysis to determine if age at diagnosis differed by rs738409 genotype, ii) a receiver operating characteristics analysis to assess the utility of the rs738409 genotype in discriminating NAFLD cases from controls, and iii) a phenome-wide association study (PheWAS) between rs738409 and 10,095 EHR-derived disease diagnoses.

Results: The *PNPLA3* G risk allele was associated with: i) earlier age of NAFLD diagnosis, with the strongest effect in Hispanics (hazard ratio 1.33; 95% CI 1.15–1.53; p <0.0001) among whom a NAFLD diagnosis was 15% more likely in risk allele carriers *vs.* non-carriers; ii) increased NAFLD risk (odds ratio 1.61; 95% CI 1.349–1.73; p <0.0001), with the strongest effect among Hispanics (odds ratio 1.43; 95% CI 1.28–1.59; p <0.0001); iii) additional liver diseases in a PheWAS (p <4.95 × 10^{-6}) where the risk variant also associated with earlier age of diagnosis.

Conclusion: Given the role of the rs738409 in NAFLD diagnosis age, our results suggest that stratifying risk within populations known to have an enhanced risk of liver disease, such as Hispanic carriers of the rs738409 variant, would be effective in earlier identification of those who would benefit most from early NAFLD prevention and treatment strategies.

Lay summary: Despite clear associations between the *PNPLA3* rs738409 variant and elevated risk of progression from non-alcoholic fatty liver disease (NAFLD) to more severe forms of liver disease, it remains unknown if *PNPLA3* rs738409 plays a role in the age of NAFLD onset. Herein, we found that this risk variant is associated with an earlier age of NAFLD and other liver disease diagnoses; an observation most pronounced in Hispanic Americans. We conclude that *PNPLA3* rs738409 could be used to better understand liver disease risk within vulnerable populations and identify patients that may benefit from early prevention strategies.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is estimated to impact 24% of the global population and is a leading impetus for liver transplantation, representing over 75% of all chronic liver disease.[1,2] In the United States, the prevalence of NAFLD has steadily increased over the last 3 decades, and to date, population estimates range from 30-45%.[3,4] NAFLD is characterized by a continuum of severity, ranging from simple steatosis, a relatively benign condition, to fibrosis and hepatocyte death. Up to 30% of histologically defined cases of NAFLD include inflammation and can lead to higher rates of liver-related death.[5] NAFLD may progress to steatohepatitis, cirrhosis and hepatocellular carcinoma, but the etiologies of these progressions are complex and poorly understood.

To date, through genome-wide association study (GWAS) discoveries, more than 10 variants have been significantly associated with NAFLD. Of these, a coding variant in *PNPLA3* (patatin-like phospholipase domain-containing protein 3) *p*.Ile138Met (rs738409 C>G) is the strongest genetic risk factor for NAFLD and its severity, with odds ratios (ORs) ranging from 2.08 to 18.23 (combined OR 3.41; 95% CI 2.57–4.52; p <0.00001)[6–9] across

Keywords: NAFLD; Non-alcoholic fatty liver disease; *PNPLA3*; Natural language processing; Electronic health record; Phenome-wide association study; PheWAS; Genetic; Survival; Biobank; Hispanic.

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different populations. *PNPLA3* encodes a protein belonging to the patatin-like phospholipase family with putative triglyceride and phospholipid remodeling functions within hepatic lipid deposits.[10] The rs738409 polymorphism has been repeatedly associated with NAFLD risk and elevated liver fat content,[7–9] presumably through the variant's putative functional role in reducing hepatic triglyceride hydrolytic activity by nearly 80%.[11,12]

The *PNPLA3* "G" risk allele has a frequency of 23.1% in the general population (NHLBI TopMed; n = 125,568). However, the risk allele is more common in Hispanic/Latino (HA) populations (47.16%; 95% CI 47.1–47.2)[7,13,14] than in African American (AA 13.7%) and European American (EA 22.8%) populations,[15] and has a larger impact on NAFLD risk in HA populations.[7,9] This may contribute, in part, to a higher estimated prevalence of NAFLD in HA (45–58%), compared to AA (24–35%) and EA (33–44%),[3,4] and supports previous findings that HA are more likely to progress to more severe forms of liver disease than other ancestries.[16] Despite clear associations of *PNPLA3* rs738409 with elevated risk of progression from NAFLD to more severe forms of liver disease,[17,18] particularly among HA, it remains unknown if *PNPLA3* rs738409 plays a role in age of NAFLD onset. This is likely due to the asymptomatic nature of NAFLD and a general lack of longitudinal population cohorts for which genetic information is available. To prevent the progression of NAFLD to non-alcoholic steatohepatitis (NASH) and other more severe pathologies, it is critical to better classify the onset of NAFLD. Early screening for NAFLD within populations with high genetic (or other) risk could lead to more focused prevention, interventions and/or treatments.

To address this knowledge gap and clinical need, we leveraged large-scale, longitudinal electronic health record (EHR) information, sequencing data and novel natural language processing (NLP) techniques to determine the effect of *PNPLA3* rs738409 on age at disease diagnosis in a diverse ancestry population of over 27,000 individuals. To gain insight into potentially novel variant-disease associations, we performed a phenome-wide association study (PheWAS) to unselectively examine the relationship of the *PNPLA3* rs738409 variant with a broad spectrum of EHR-derived health outcomes. Our findings contribute to the understanding of the impact of *PNPLA3* rs738409 on liver disease progression, and highlight a pronounced disease risk in HA populations.

Patients and methods

Participants

Mount Sinai's Bio*Me* is an EHR-linked clinical care biobank and comprises over 50,000 multi-ethnic participants (as of June, 2019), characterized by a broad spectrum of longitudinal biomedical traits. Enrolled participants consent to be followed throughout their clinical care (past, present, and future) in real-time, allowing integration of their genomic information with their de-identified EHR data for discovery research and clinical care implementation. Bio*Me* is an unselected population-based biobank, and participants are predominantly recruited from outpatient primary care practice. EHR adoption began in 2006, and all outpatient clinics are linked to EHRs. The present study population is comprised of 27,744 AA, HA and EA participants from Bio*Me* for whom EHR clinical measures and genetic data were available (Fig. S1). The Bio*Me* Biobank is IRB approved and fully complies with Health Information Privacy and Portability Act (HIPAA) regulations.

Genetic data

The Bio*Me* biobank is a longitudinal cohort allowing for analysis of time course data from the EHR that is linked to genetic data. Genetic data for 31,676 Bio*Me* biobank participants, was obtained through a collaboration with the Regeneron Genetics Center. All samples were tested for sufficient DNA concentrations by picogreen-based quantification, and processed with Kapa library prep reagents, the IDT xGen capture platform, and genotyped on the Illumina Global Screening Array. Samples were blacklisted for gender discordance (genotype sex-check results with homozygosity rates more than 0.2 but less than 0.8 were excluded), low sequencing coverage, heterozygosity rates (samples falling outside of 3 standard deviations from the mean were excluded), contamination, low call rate and the discovery of duplicates. Variants were removed for excessive missingness (>3%). Finally, pairwise identity by descent (IBD) levels were checked and individuals from the pairs with IBD greater than 0.185 were removed. The *p*.Ile138Met variant (rs738409 C>G) on chromosome 22 in *PNPLA3* was extracted from all participants passing QC filters (n = 31,676).

In the total population, the frequency of the (G) risk allele was 25.5% with frequencies in self-identified HA, AA and EA of 35%, 14.4% and 23.1%, respectively, consistent with prior reports for global,[15] EA and AA populations.[7] HA participants in Bio*Me* in New York City are predominantly of Caribbean origin, which likely explains why the G allele frequency is lower compared to frequencies reported in other HA populations of predominantly Mexican descent (~50%).[7,13,19,20] The rs738409 variant was tested for Hardy Weinberg equilibrium (HWE) using a χ^2 test prior to analysis. Within AA and EA genotype distributions were in HWE (AA χ^2 p = 0.31, EA χ^2 p = 0.61), while the HA genotype distribution violated HWE (χ^2 p = 1.6×10^{-5}). Observations were similar among the control (non-NAFLD) population (AA G = 14.1%, χ^2 p = 0.36; EA G = 22.7%, χ^2 p = 0.4; HA G = 34.1%, χ^2 p = 0.0002). Given the high accuracy of the genotyping, it is unlikely that violation of HWE is due to technical error. HA controls had similar genotype frequencies, indicating that the HWE violation is unlikely to be the primary cause of any association with NAFLD, and we proceeded with the analysis of the variant.[21] Our final data set was restricted to AA, HA and EA participants (n = 27,744).

Phenotypic data from electronic health records

For this study, age was defined as age at enrollment in the biobank and was restricted to >18 years. Anthropometry measures and serum lab values for alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, platelets, alkaline phosphatase and total bilirubin (markers commonly used in the diagnosis of liver diseases) were available in a subset of participants (Table S1). For all continuous measures other than age (*i.e.* body mass index (BMI), lab values), we generated a median value of all outpatient measures on record for a given participant, excluding emergency and inpatient measures.

We used NLP methods to query EHR medical charts and imaging reports for terminology indicating a diagnosis of NAFLD, an approach that has been shown to improve sensitivity and overall accuracy of case definition. Details of the NLP approach including its validation are published elsewhere.[22] Briefly, we used CLiX, a general-purpose stochastic parser (Clinithink, Atlanta, USA), which maps patient facts described in clinical narrative to post-coordinated SNOMED expressions. SNOMED expressions are based on SNOMED CT, a hierarchical, general-purpose clinical terminology (SNOWMED International, London, UK). We used the NLP engine to map SNOMED concepts for NAFLD (including concepts such as steatosis, NAFLD *etc.*) for individual patients. The algorithm was iterated and improved using manual validation as the gold standard. Two clinicians, without knowing case/control status, independently reviewed all records on 200 patients; 100 case patients identified as having NAFLD and 100 randomly selected patients identified as controls. Patients were classified as cases or controls based on clinical criteria. A clinical diagnosis of NAFLD required i) the presence of fatty infiltration of the liver on imaging or physician documentation in the notes that such imaging was performed/diagnosis was confirmed, ii) exclusion of hepatitis C infection, iii) absence of documented alcohol abuse. Inter-rater agreement was high (kappa = 0.95) and disagreements were reviewed by consensus in consultation with a third clinician. Further details are provided elsewhere.[22] This approach significantly improved sensitivity (93% *vs.* 32%) compared to ICD codes. We identified 2,228 cases (compared to 893 with ICD). Similarly, we used NLP to classify controls from EHR who had radiology imaging of the liver, but with confirmed absence of excess liver fat or steatosis (n = 9,886). We then used ICD codes to remove participants with a diagnosis of hepatitis B or C from cases and controls, resulting in 1,703 NAFLD cases and 8,119 screened controls available for analyses (Fig. S1). Age at diagnosis, represented in years from birth, was determined by subtracting participant date of birth from the first disease diagnosis date on record.

Statistical analyses

Population characteristics

Unadjusted means for age, sex, BMI and lab values are reported in Table S1. Linear regression of *PNPLA3* rs738409 on traits adjusted for age, sex and 6 ancestry principal components was performed and *p* values represent the effect of each G risk allele. Unadjusted means for age, sex, BMI and lab values were stratified by NAFLD status and a 2-sided Cochran independent *t* test was used to test for differences (adjusted for age, sex and 6 principal components) between cases and controls in all participants and individual ancestry groups. *p* values for age are from chi-square test (Table S2). To examine the role of *PNPLA3* rs738409 on NAFLD diagnosis, logistic regression was used to estimate ORs with Wald CIs and *p* values for the G risk allele in adjusted (age, sex, BMI and 6 principal components) and unadjusted models.

Survival analyses

We leveraged the availability of longitudinal EHR data to further characterize the clinical impact of the *PNPLA3 p*.Ile138Met genotype on NAFLD diagnosis. We conducted a survival analysis to determine if age at diagnosis differed by *PNPLA3* genotype using computed product-limit estimates of the survivor function and Kaplan-Meier survival curves to visualize results. A Cox proportional hazards model was used to assess the additive effect of the *PNPLA3* risk allele on the hazard age of diagnosis for NAFLD. NLP-defined NAFLD cases (n = 1,703) were analyzed with screened controls (n = 8,119). The first record indicating a diagnosis was considered the "event", while undiagnosed controls were censored upon reaching current age. In survival analyses, the Log-rank test (χ^2, *p* value) was used to test for homogeneity in age at diagnosis survival functions for NAFLD. The Sidák test for multiple pairwise comparisons was used for comparison of age at diagnosis across genotype strata, with Hall-Wellner confidence intervals. Cox proportional hazards models (adjusted for age, sex and 6 principal components) generated hazard ratios with confidence limits. Following the PheWAS analysis (see below), we conducted similar survival analyses to determine if age at diagnosis for ICD9-derived phenome wide significant diseases differed by *PNPLA3* genotype. In these analyses, all individuals without ICD9 codes for a given disease were considered controls.

Prediction analyses

We used receiver operating characteristics (ROC) analyses to assess the utility of common clinical measures (age, sex, BMI and "lab values": ALT, AST, albumin, platelets, alkaline phosphatase and total bilirubin) and *PNPLA3* rs738409 genotype as predictors to discriminate between NAFLD cases and controls.[23] Lab values were not normally distributed and were $\log_{10}$ transformed. Importantly, all data used for NAFLD cases in ROC models were obtained prior to the first diagnosis. These analyses were carried out using cases (n = 1,095) for whom BMI and laboratory values were available prior to the first diagnosis date. For controls, we used data values from the time of enrollment in the Bio*Me* biobank. Area under the curve (AUC) values for ROC are presented with 95% Wald confidence limits and ROC contrast estimation tests were used to assess improvements in disease discrimination between i) *PNPLA3* genotype, ii) Age + sex + BMI + 6 Principal components + "lab values", iii) Age + sex + BMI + 6 Principal components + *PNPLA3* genotype and iv) a full model containing all predictors; Age + sex + BMI + 6 Principal components + "lab values" + *PNPLA3* genotype.

In addition, we used net reclassification improvement (NRI) to assess changes in predicted risks between models among NAFLD cases and controls.[24] The NRI analysis evaluates the net number (%) of individuals correctly reclassified as "events/cases" or "nonevents/controls" when using the disease predictor groups described above. Integrated discrimination improvement (IDI) analysis was used to compare prediction models and to assess improvement in average sensitivity.[24] Absolute IDI is dependent on the overall "event" rate. Since the NAFLD event-rates in our cohort are relatively small, the absolute IDI value will be small. Therefore, we report relative IDI values, which represent a percent relative improvement between models. Survival, ROC, NRI and IDI analyses were conducted in SAS 9.4/SAS Studio 3.8.

Phenome-wide association study

We used a linear mixed model[25] to perform PheWAS association between *PNPLA3* rs738409 and 10,095 EHR-derived ICD-9 codes. Cases for a given disease were defined as having one or more relevant ICD-9 codes on record. PheWAS analyses were conducted using the GCTA software via the "*–mlma*" flag, adjusting for sex encoded as a discrete covariate, as well as age, BMI, and the first 6 eigenvectors of principal components encoded as continuous covariates. A linear mixed model in GCTA was chosen for the PheWAS analysis as this method has been shown to best account for population structure and distant relatedness as well as case:control imbalances typical to diverse biobank study populations.[26] To control for relatedness, a genetic relationship matrix (GRM) was calculated across all 31,676 participants from

281,666 common (minor allele frequency [MAF] >1%), non-palindromic single-nucleotide polymorphisms. Both the GRM and principal components were calculated using the GCTA software using the "*–make-grm.gz*" and "*–pca*", flags respectively. For visualization purposes, ICD9 coding structure was used to group individual ICD9 codes into 17 broad clinical disease categories, derived from the ICD-9 codes. Of the 10,095 EHR-derived ICD-9 codes, 7,846 intersected with available genetic information, thus the Bonferroni corrected significance threshold was $p < 6.37 \times 10^{-6}$. Naïve ORs, for the impact of *PNPLA3* rs738409 "G", are based on unadjusted case:control counts. Cramer's V statistic was used to test for correlation between the most significant ICD9 codes among risk allele carriers and a matrix of the top ICD9 phenotype hits across all individuals was generated to visualize disease co-occurrence.

Using the PheWAS methods described above, we also tested for associations in 2 additional sets of EHR-derived procedure codes. Current Procedural Terminology (CPT) codes are a proprietary coding system developed by the American Medical Association to code services provided by health care professionals.[27] Similarly, ICD9 Procedure codes represent medically billable procedures, a separate classification system from diagnosis codes.[28] We conducted procedure-wide association studies on 12,302 CPT codes and 5,721 ICD9 procedure codes within the participants. Of the 12,302 CPT codes and 5,721 ICD9 procedure codes, 4,391 and 2,227 intersected with available genetic information, thus the Bonferroni corrected significance thresholds were $p < 1.13 \times 10^{-5}$ and $p < 2.25 \times 10^{-5}$, respectively.

Global PNPLA3 *rs738409 frequencies*

We examined risk allele frequency of rs738409 within the multi-ethnic PAGE Study, which includes 45,255 unrelated participants from across the Americas, Africa, East and South Asia, and Oceania.[29] As part of the PAGE Study, 99 distinct populations were assembled, based on self-identified race or ethnicity or country of origin (Sorokin, Belbin, Wojcik *et al.*, 2019) within PAGE (n = 38 labels), the Human Genome Diversity Panel populations (n = 53 labels), and additional global reference panels from within the PAGE Study (n = 8 labels). We also assembled a super-population label for self-identified Hispanics. Allele frequencies for the rs738409 G allele were calculated using PLINK 1.90.[30] Frequencies were visualized in R using the maps (https://cran.r-project.org/web/packages/maps/maps.pdf) and plotrix (https://cran.r-project.org/web/packages/plotrix/plotrix.pdf) packages.

Results

PNPLA3 rs738409 genotype and NAFLD diagnosis

To determine whether *PNPLA3* rs738409 influenced the age at which NAFLD was diagnosed, we conducted survival analyses on our longitudinal EHR data. Notably, our novel NLP methods improved true NAFLD case identification by 150%, increasing the number of cases from 893 (with ICD alone) to 2,228 and accurately defined 9,986 NAFLD-free controls. Among these individuals, the *PNPLA3* G risk allele was associated with earlier age of NAFLD diagnosis in all participants (hazard ratio [HR] 1.49; 95% CI 1.35–1.64; p <0.0001) (Fig. 1A) and the mean age of diagnosis in carriers of both risk alleles was 3.1 years earlier than in non-carriers (58.4 years *vs.* 55.3 years). This corresponds to a 5% greater probability of being diagnosed with NAFLD at age 60 among carriers of both risk alleles compared to non-carriers (18% *vs.* 13%, respectively). In individual ancestry groups the overall effect of the *PNPLA3* G risk allele on NAFLD diagnosis age was similar (HA: HR 1.33, 95% CI 1.15–1.53, p <0.0001; AA: HR 1.51, 95% CI 1.23–1.85, p <0.0001; EA: HR 1.34, 95% CI 1.08–1.65, p = 0.005). However, the probability of being diagnosed with NAFLD by age 60 was 15% higher in HA carriers of both risk alleles compared to non-carriers (31% *vs.* 16%, respectively). Results were similar for AA (28% *vs.* 10% non-carriers) and EA (23% *vs.* 12% non-carriers) (Fig. 1B,C). Consistent with what others have shown, *PNPLA3* G risk allele carriers had an increased risk of NAFLD compared to non-risk carriers (OR 1.61; 95% CI 1.349–1.73; p <0.0001), which was somewhat attenuated after adjusting for age, sex, BMI and 6 principal components (OR 1.48; 95% CI 1.35–1.60; p <0.0001). The effect of *PNPLA3* was more pronounced among HA (OR 1.43; 95% CI 1.28–1.59; p <0.0001) and EA (OR 1.51; 95% CI 1.27–1.80; p <0.0001) than among AA (OR 1.43; 95% CI 1.16–1.77; p = 0.0008).

Prediction of NAFLD

To assess whether *PNPLA3* genotype can be used to predict risk of developing NAFLD, we compared its predictive ability to that of clinical measures (BMI, age, sex, principal components and measurable components of blood such as ALT and AST) for which we had data before the NAFLD diagnosis. *PNPLA3* had a limited ability as a sole predictor of NAFLD (AUROC = 0.574) (Table S3). The use of clinical measures alone yielded a higher NAFLD discriminatory ability (AUROC = 0.785) than *PNPLA3* alone, and improved sensitivity by 34%. When *PNPLA3* was added to clinical measures, NAFLD discrimination improved marginally (AUROC = 0.788, p <0.0001) (Fig. 2), and resulted in 19% of controls being correctly reclassified (p <0.0001), but only 1% of cases correctly reclassified (Table S4). When stratified by ancestry, *PNPLA3* had the strongest individual AUC in HA (AUROC = 0.566) and improved NAFLD prediction when added to clinical measures in HA, however the effect was modest (AUC 0.777 *vs.* 0.782, p = 0.03) (Fig. 2 and Fig. S3). Similarly, the addition of *PNPLA3* to clinical measures in HA correctly reclassified 13% of cases (p = 0.001) and accounted for a 2.2% increase in sensitivity over clinical measures alone (Tables S4 and S5). *PNPLA3* did not significantly improve prediction of NAFLD over clinical measures in AA or EA, nor did it improve the percent of cases correctly classified or improve sensitivity. The full model containing clinical measures and *PNPLA3* performed best in EA compared to HA and AA (Table S3).

Clinical characterization of *PNPLA3* risk allele status using electronic health records

In order to explore the broader clinical impact and comorbidities of the *PNPLA3* risk allele, we performed a PheWAS to correlate ICD9 codes with *PNPLA3* risk allele status in the Bio*Me* biobank participants. As anticipated, the *PNPLA3* risk allele was most significantly associated with "Other chronic non-alcoholic liver disease" [ICD9: 571.8] ($\beta = 0.01$/per allele [±0.002]; $p = 1.91 \times 10^{-17}$), a sub-classification of the broader category "diseases of the digestive system" (Fig. 3). It is used to describe fatty liver without mention of alcohol (NAFLD), but can also serve as a diagnosis code for NASH.[31] Additional liver or liver-related diseases also reached significance, including "portal hypertension" [ICD9: 572.3] ($\beta = 0.008$ [±0.001]; $p = 7.43 \times 10^{-10}$), "non-alcoholic cirrhosis" [ICD9: 571.5] ($\beta = 0.01$ [±0.002]; $p = 1.1 \times 10^{-8}$) "esophageal varices" [ICD9: 456.21] ($\beta = 0.006$ [±0.001]; $p = 2.34 \times 10^{-7}$), "alcoholic cirrhosis" [ICD9: 571.2] ($\beta = 0.005$ [±0.001]; $p = 3.1 \times 10^{-7}$) and

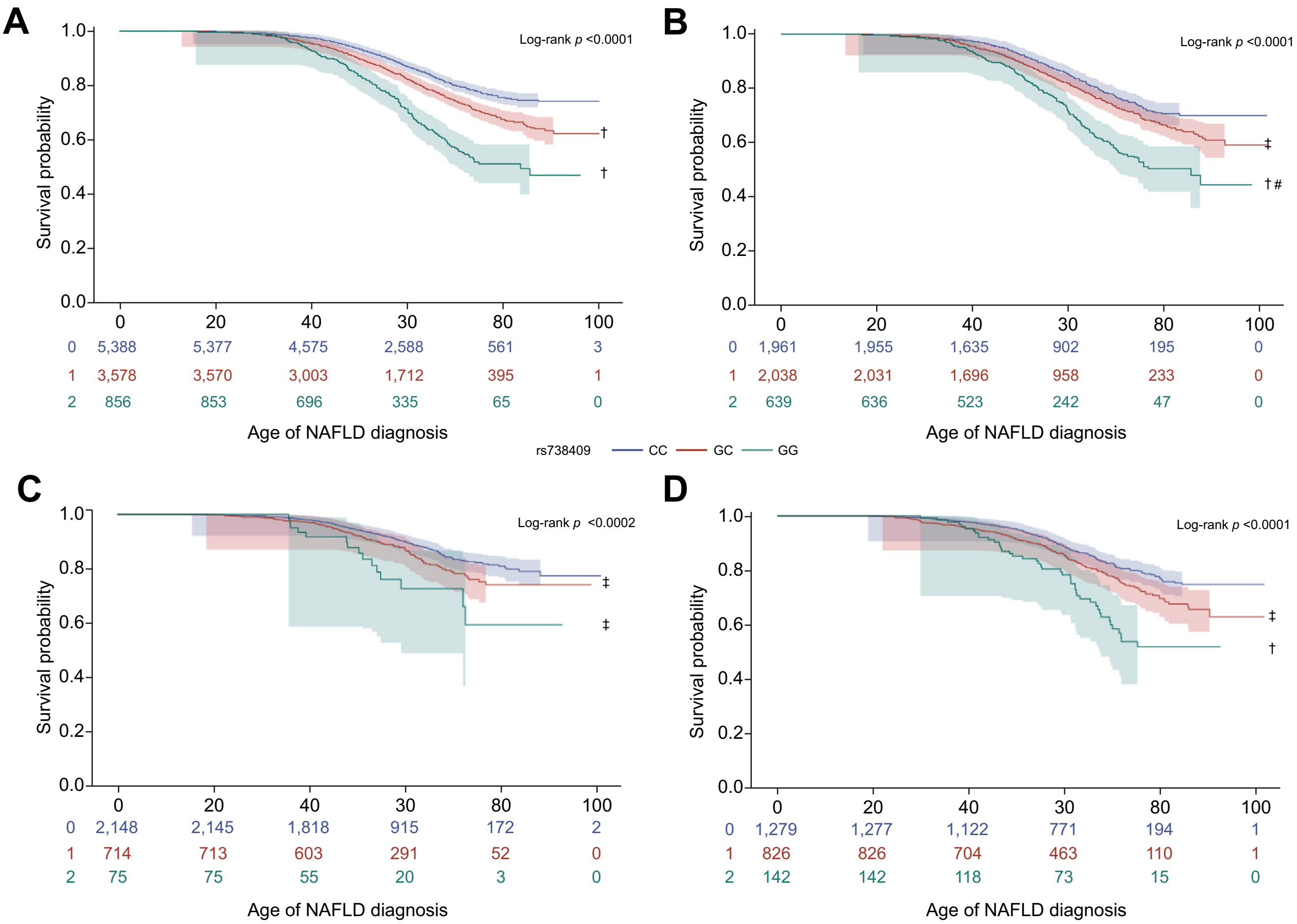

Fig. 1. Kaplan-Meier survival curves comparing age at diagnosis for NAFLD by *PNPLA3* rs738409 genotype. (A) All, (B) HA, (C) AA and (D) EA. X-axis displays the number of years to NAFLD diagnosis (age of diagnosis). Y-axis displays the Kaplan-Meier survival probability by genotype. G is the effect allele; CC (blue curve); GC (red curve); and GG (green curve). The log-rank *p* value denotes the strength of significant differences among survival curves. Symbols denote *p*-values from Sidak-adjusted log-rank multiple comparisons: †significantly different from CC (p <0.0001), ‡significantly different from CC (p <0.01), #significantly different from GC (p = 0.001). Shaded areas represent confidence intervals. Censoring marks have been removed for visualization. NAFLD, non-alcoholic fatty liver disease. (This figure appears in color on the web.)

“other chronic liver disease“ [ICD9: 572.8] (β = 0.0048 [±0.001]; $p = 2.56 \times 10^{-6}$) (Table 1). PheWAS analyses adjusted for BMI did not notably change association results for NAFLD or the other top phenotypes (Fig. S3, Table S6). NLP-defined NAFLD had a low correlation and co-occurrence with other ICD9-defined liver diseases (Figs. S4 and S5). To assess whether our PheWAS findings were influenced by the fact that NAFLD cases may more often be affected by related diseases, we performed sensitivity analyses in which we removed all NAFLD cases. This attenuated disease associations (Table S6), but liver phenotype hierarchy was maintained. We observed a significant association of one ICD9 procedure code (42.33: Endoscopic excision) with *PNPLA3* (Tables S7 and S8).

Impact of *PNPLA3* Ile138Met genotype on age of diagnosis in PheWAS identified liver diseases

To gain insight into a potentially novel role for *PNPLA3* in the diagnosis of additional liver diseases, we performed survival analyses for 4 conditions that reached significance in our PheWAS. The *PNPLA3* risk allele was significantly associated with earlier age of diagnosis in all participants for “non-alcoholic cirrhosis” [ICD9: 571.5] (HR 1.35; 95% CI 1.22–1.49; p <0.0001) and “portal hypertension” [ICD9: 572.3] (HR 1.52; 95% CI 1.12–2.08; p = 0.007) (Fig. 4A,B), with carriers of both risk alleles being diagnosed 1 year earlier than non-carriers. The diagnostic age of “esophageal varices” [ICD9: 456.21], although significantly associated with PNPLA3 in PheWAS, was not impacted by the *PNPLA3* risk allele (HR = 1.69; 95% CI 0.98–2.89; p = 0.055) which may be due to a small number of cases for this disease. Interestingly, age of diagnosis for “ascites” [ICD9: 789.59], which was sub-phenome wide significant, was earlier in carriers of the G allele for *PNPLA3* (HR 1.29; 95% CI 1.04–1.61; p = 0.01) (Fig. 4C,D). Consistent with our observations in NAFLD, stratification of non-alcoholic cirrhosis cases by ancestry showed *PNPLA3* genotype to be associated with earlier age of diagnosis in HA (HR 1.42; 1.26–1.62; p <0.0001) and AA (HR 1.42; 1.1–1.82; p = 0.005), but not in EA. Analyses for other diseases were not stratified by ancestry due to sample size.

Frequency of *PNPLA3* risk variant worldwide

Understanding that the *PNPLA3* risk variant plays a role in susceptibility to and progression of NAFLD, we sought to investigate which global populations may be at a heightened genetic risk. We examined patterns of segregation of the rs738409[G] allele

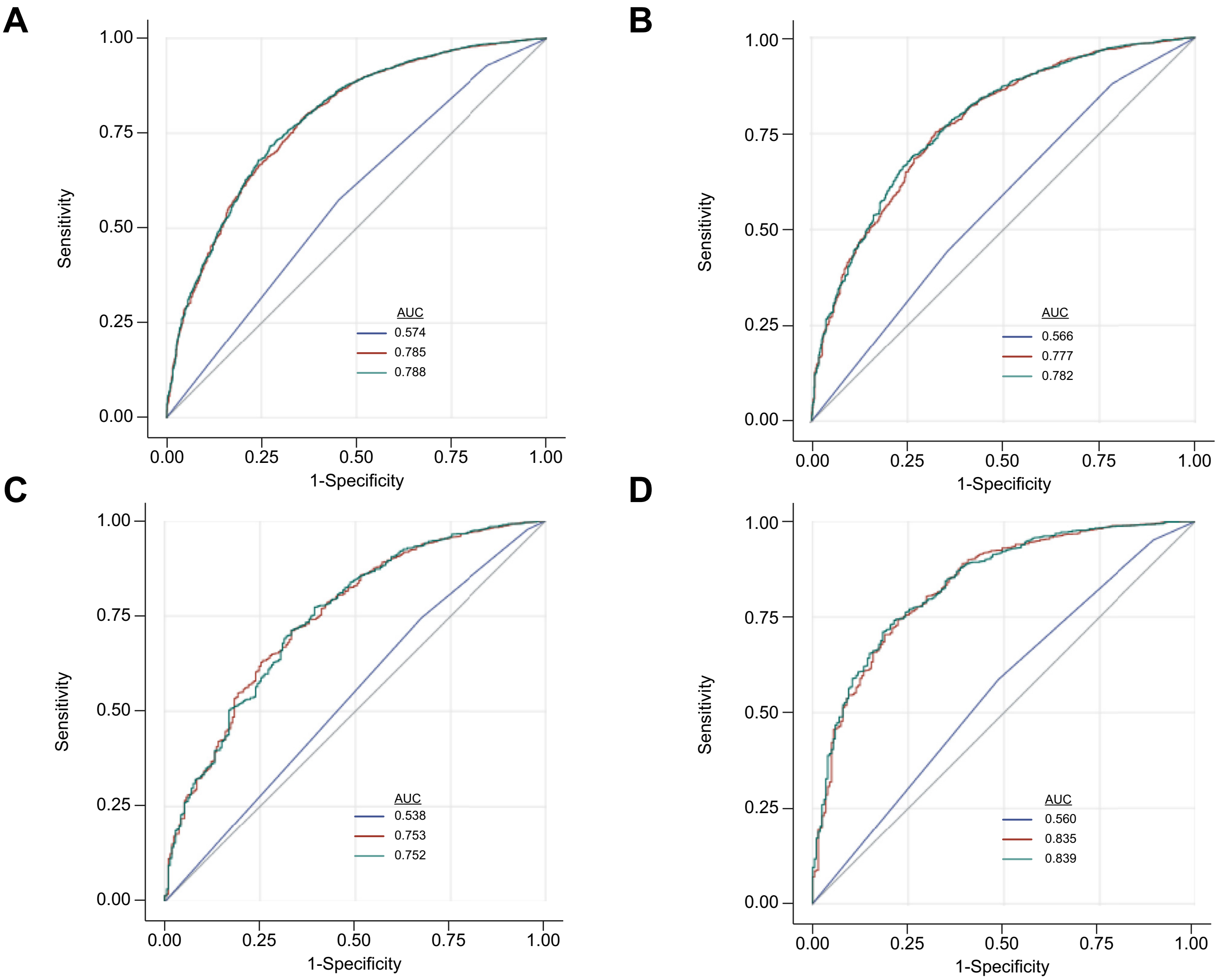

Fig. 2. Receiver operating characteristic curves for disease discrimination. AUROC for 3 models on NAFLD by ancestry: Blue line = *PNPLA3* genotype; Red line = age, sex, 6 principal components, BMI and "lab values"; Green line = age, sex, 6 principal components, BMI, "lab values" and PNPLA3 genotype. (A) all participants (956 NAFLD, 7,248 control), (B) African American (214 NAFLD, 2,392 control), (C) Hispanic (540 NAFLD, 3,279 control), (D) European American (210 NAFLD, 1,577 control). 95% Wald confidence limits and ROC contrast estimation test *p* values for NAFLD are presented in Fig. S2. BMI, body mass index; NAFLD, non-alcoholic fatty liver disease. (This figure appears in color on the web.)

within the PAGE Study, which represents global diversity across the Americas, Africa, East Asia, Oceania, and South Asia.[29] The G allele segregated commonly (MAF >0.05), within all populations of the PAGE Study with exception of 2 HGDP populations, Mbuti Pygmy and Melanesians, where the variant was monomorphic (MAF 0%; 95% CI 0–16.0%, in both populations) (Fig. 5 and Table S9). The G allele segregated at highest frequencies within PAGE Global reference populations from Central and South America, notably the Warao (MAF 85.0%; 95% CI 64.0–94.8%), Puno (MAF 85.4%; 95% CI 81.7–88.5%), Zapotec (MAF 85.7%; 95% CI 68.5–94.3%), and Bari, where the variant was fixed (MAF 100%; 95% CI 89.8–100%). The G variant was the major allele in PAGE populations from Central and South America, including Peru (MAF 66.7%; 95% CI 58.8–73.7%), El Salvador (MAF 60.8%; 95% CI 49.4–71.1%), Guatemala (MAF 58.3%; 95% CI 46.8–69.0%), Ecuador (MAF 54.1%; 95% CI 49.7–58.6%) and Mexico (MAF 49.9%; 95% CI 48.9–50.8%). In a super-population of self-identified Hispanics from the PAGE Study, the variant also segregated commonly (MAF 39.6%; 95% CI 39.2–40.1%). Elsewhere in PAGE, the variant segregated commonly in populations of East Asian ancestry, notably Japan (MAF 45.7%; 95% CI 44.4–46.9%), Korea (MAF 45.1%; 95% CI 36.5–53.9%), and China (MAF 35.5%; 95% CI 30.5–40.8%). Geographical regions within Central and South America, as well as South Eastern Asia show enrichment for the *PNPLA3* G allele, which is consistent with population-based epidemiological studies demonstrating a heightened prevalence of NAFLD in individuals with these genetic backgrounds.[2,6,19] This data illustrates how populations currently under represented in *PNPLA3* research may also be impacted by the risk variant.

Discussion

Using longitudinal HER data, collected for more than 10 years, and NLP approaches, we retrospectively defined NAFLD cases and controls in the multi-ethnic Bio*Me* Biobank in New York City. We found evidence to show that individuals who carried the *PNPLA3* *p*.Ile138Met (rs738409) G variant, a known NAFLD-associated disease allele, had a significantly earlier age of

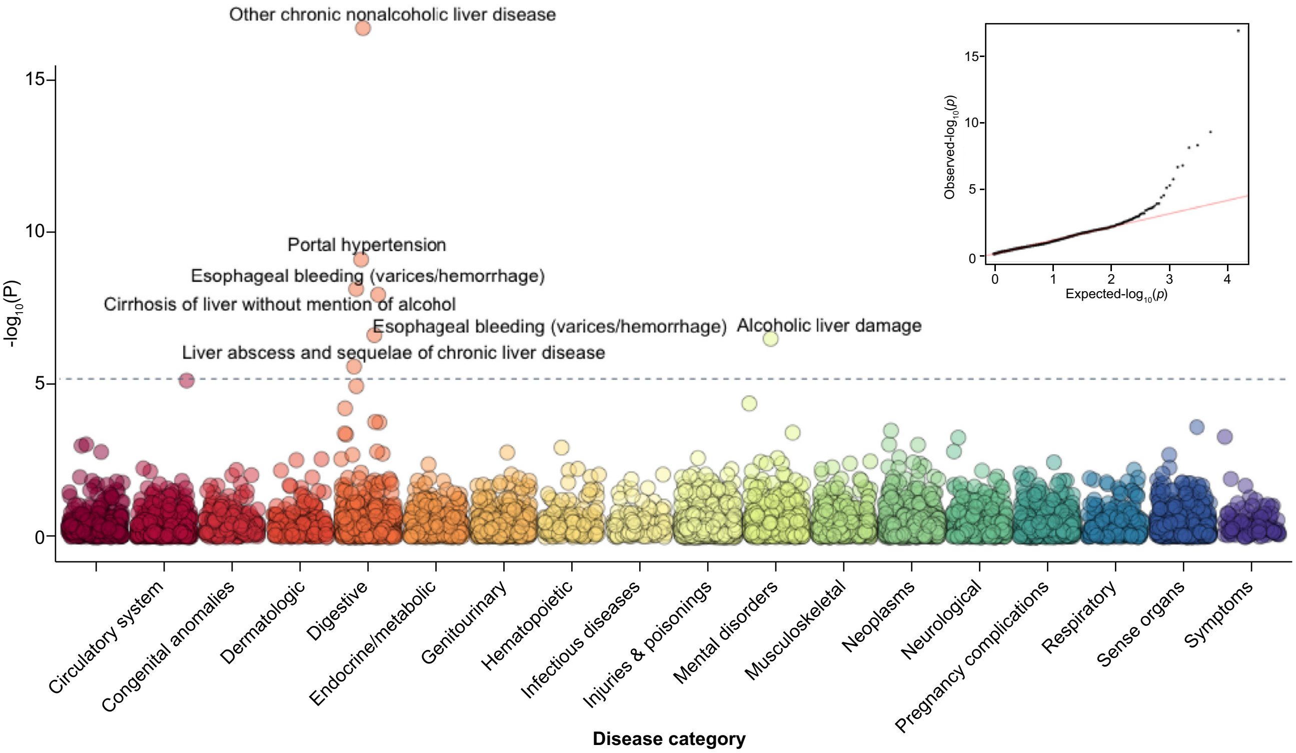

Fig. 3. PheWAS Manhattan plot of PNPLA3 Ile138Met *vs.* ICD9 diagnosis codes. The x-axis represents categories of disease as defined by ICD9 coding hierarchy. The y-axis is the -log10 *p* value for the association. Adjustment for age, sex, PCs 1-6. Bonferroni significance for multiple testing was $p < 6.37 \times 10^{-6}$. PheWAS, phenome-wide association study. (This figure appears in color on the web.)

Table 1. Top 15 PheWAS ICD9 phenotypes for *PNPLA3* rs738409 (adjusted for age, sex and principle components).

ICD-9	Beta	Standard error	Odds ratio	*p* value	Description	Organ system
571.8	0.0160	0.0019	1.66	1.91E-17	Other chronic non-alcoholic liver disease	Digestive
572.3	0.0084	0.0014	1.65	7.43E-10	Portal hypertension	Digestive
456.1	0.0072	0.0012	1.69	7.18E-09	Esophageal varices without mention of bleeding	Digestive
571.5	0.0125	0.0022	1.34	1.10E-08	Cirrhosis of liver without mention of alcohol	Digestive
456.21	0.0063	0.0012	1.61	2.34E-07	Esophageal varices, without bleeding, elsewhere	Digestive
571.2	0.0050	0.0010	1.91	3.10E-07	Alcoholic cirrhosis of liver	Mental disorders
572.8	0.0048	0.0010	1.58	2.52E-06	Other sequelae of chronic liver disease	Digestive
456.8	0.0033	0.0007	1.90	7.26E-06	Varices of other sites	Circulatory system
789.59	0.0061	0.0014	1.37	1.11E-05	Other ascites	Digestive
303.9	0.0056	0.0014	1.30	4.12E-05	Alcohol dependence, unspecified drinking behavior	Mental disorders
573.8	0.0048	0.0012	1.30	5.91E-05	Other specified disorders of liver	Digestive
456.2	0.0026	0.0007	1.88	0.00017	Esophageal, with bleeding	Digestive
537.89	0.0068	0.0018	1.29	0.00017	Other specified disorders of stomach and duodenum	Digestive
369.69	0.0007	0.0002	3.15	0.00024	One eye: profound vision impairment; other eye: normal vision	Sense organs
141.90	0.0012	0.0003	2.73	0.00031	Malignant neoplasm of tongue, unspecified	Neoplasms
571.1	0.0019	0.0005	1.89	0.00037	Acute alcoholic hepatitis	Mental disorders
794.80	0.0050	0.0014	1.32	0.00039	Non-specific abnormal results of function study of liver	Digestive
572.4	0.0020	0.0006	1.74	0.00043	Hepatorenal syndrome	Digestive
790.6	0.0097	0.0028	1.13	0.00050	Other abnormal blood chemistry	Symptoms

Shaded cells represent phenome-wide significant hits ($p < 6.37 \times 10^{-6}$). Naïve odds ratios, for the impact of *PNPLA3* rs738409 "G", are based on case:control counts. PheWAS, phenome-wide association study.

NAFLD diagnosis. The effect of rs738409 on the age of diagnosis of NAFLD was most pronounced in the HA population, among whom the prevalence of NAFLD is also highest.[3,20] However, our results show AA carriers of the *PNPLA3* risk allele, a population thought to have a lower prevalence of chronic liver disease (3.9 *vs.* 6.7% in AA and HA, respectively), also incur risk for NAFLD and other liver diseases.[32] This observation is consistent with a prior report in which the rs738409 polymorphism conferred high risk for NAFLD and NASH development across ethnicities.[9] Consequently, our findings provide the first evidence for a role of *PNPLA3 p.*Ile138Met in the timing of progression to NAFLD.

NAFLD is the most common cause (52%) of chronic liver disease across ancestries[32] and populations at heightened risk, such as HA and AA,[33] encounter disparities in the prevalence and

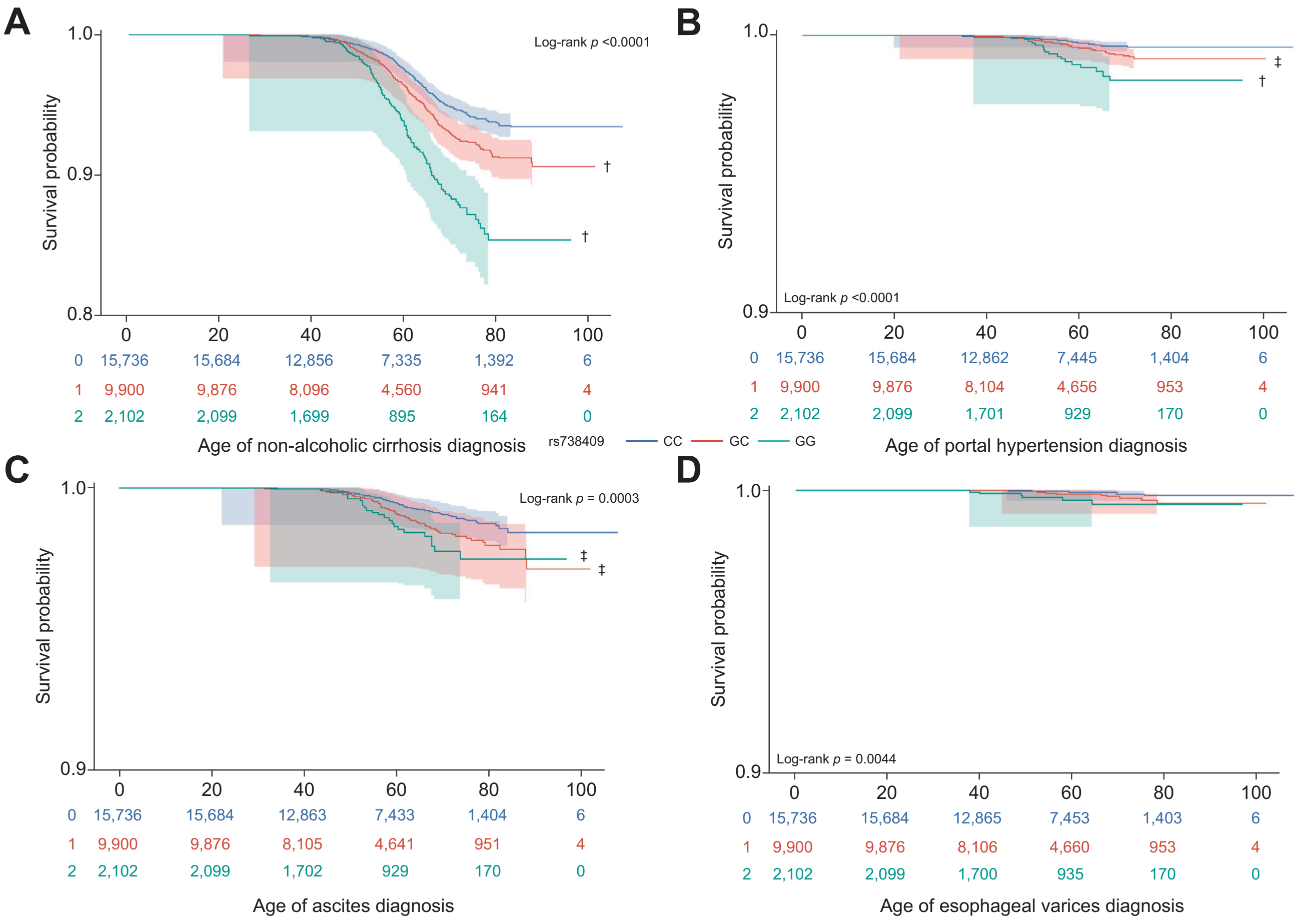

Fig. 4. Kaplan-Meier survival curves comparing time to diagnosis for ICD9-defined liver diseases. (A) non-alcoholic cirrhosis (571.5, n = 941), (B) portal hypertension (572.3, n = 92), (C) ascites (789.59, n = 201) and (D) esophageal varices (456.21, n = 31). *X*-axis displays the number of years to NAFLD diagnosis (age of diagnosis). *Y*-axis displays the Kaplan-Meier survival probability by genotype and has been adjusted to visualize results due to high percentage of censoring. G is the effect allele; CC (blue curve); GC (red curve); and GG (green curve). The log-rank *p* value denotes the strength of significant differences among survival curves. Symbols denote *p* values from Sidak-adjusted log-rank multiple comparisons: †significantly different from CC (p <0.0001); ‡significantly different from CC (p <0.01). Shaded areas represent confidence intervals. Censoring marks have been removed for visualization. NAFLD, non-alcoholic fatty liver disease. (This figure appears in color on the web.)

treatment of liver disease[34] which could have clinically meaningful consequences on liver-related morbidity and mortality in these populations.[34] NAFLD is often asymptomatic and is in many cases not diagnosed until ~40 years, yet the disease is becoming increasingly prevalent in HA children and adolescents, with NAFLD diagnosis occurring as early as 8 years.[2] NAFLD commonly progresses to other, more severe liver pathologies[5] and an early NAFLD diagnosis identifies individuals at risk of more severe liver disease in adulthood. Early phase animal studies have provided intriguing evidence for a causal role of *PNPLA3* in the progression of NAFLD to NASH,[35,36] suggesting translational therapies could be developed to mitigate progression to NASH in at-risk populations. Therefore, identifying NAFLD earlier in at-risk populations is an important first step. This need highlights the importance of enhanced understanding of the role of *PNPLA3* rs738409 in the timing of NAFLD development.

Although PNPLA3 had strong associations with age of NAFLD diagnosis in our study, and is a well-established risk variant for NAFLD, its ability to discriminate NAFLD cases from controls alone was limited compared to clinical measures. Prediction of NAFLD marginally improved when *PNPLA3* genotype data was added to clinical measures, but this modest improvement was only observed in HA participants at a magnitude similar to prior work in an HA population for which the rs738409 risk allele also had a modest impact in discriminating NAFLD.[37] Currently, genetic testing is not clinically recommended in the diagnosis of NAFLD.[38] Yet, there are clear associations of *PNPLA3* with disease severity and, as we now show, with age of diagnosis. Our global analysis of *PNPLA3* rs738409 risk allele frequency revealed enrichment of the variant in several ancestries localizing to Central/South America and South/East Asia. This suggests that individuals with ancestral ties to these locations are at an elevated risk for NAFLD, a concept consistent with GWASs findings showing strong association between rs738409 and NAFLD risk in Hispanic, Japanese and Korean individuals.[6,7,39,40] Our data suggests that *PNPLA3* rs738409 genotype could be combined with commonly measured clinical data present in the EHR (BMI, liver enzymes) to target individuals at increased risk of liver disease and more accurately stratify disease risk in a clinical setting. This approach could aid in earlier clinical prevention strategies, namely before the onset of liver disease, within existing health care populations and improve identification of individuals at risk of developing NAFLD and/or other chronic liver diseases.

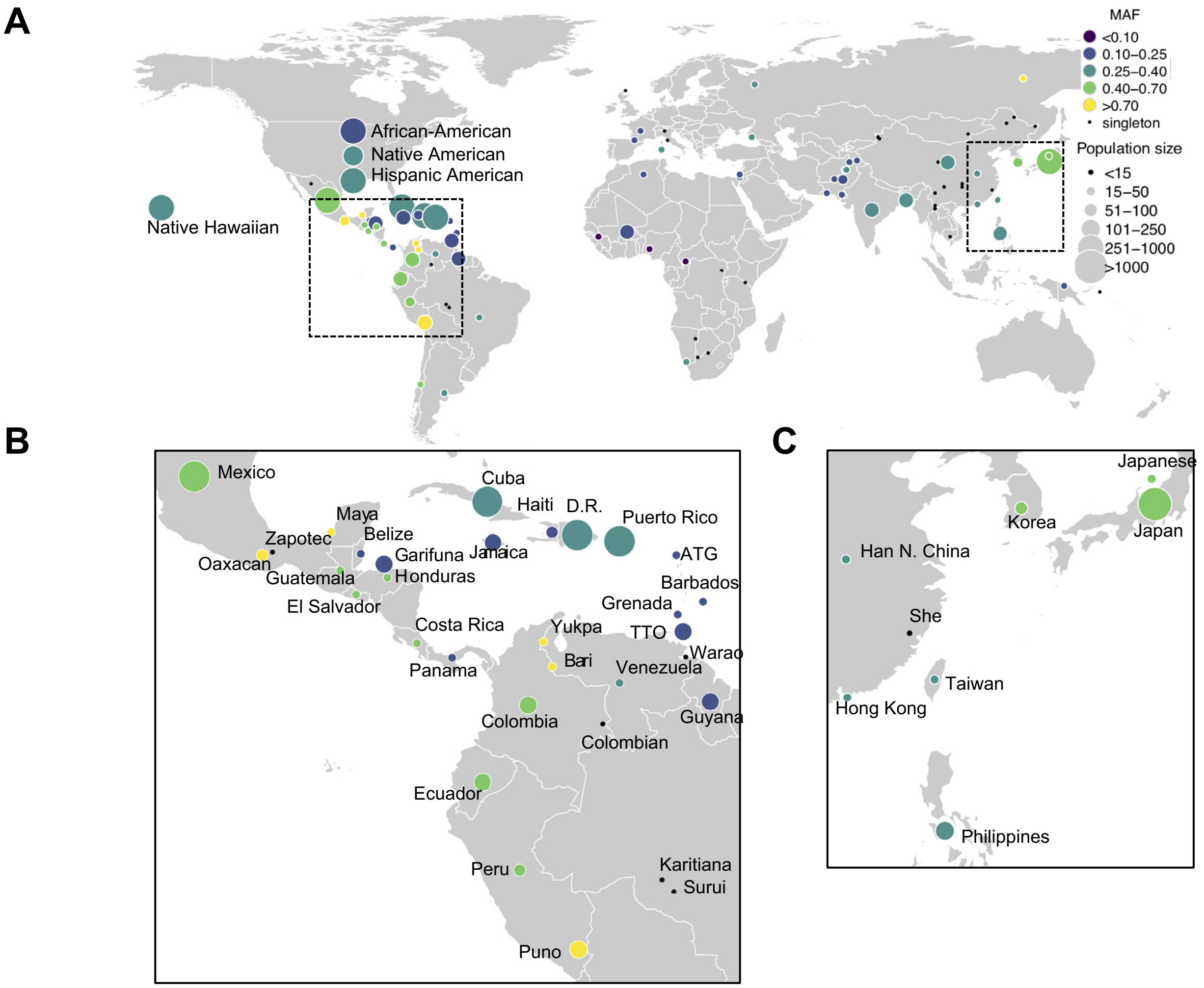

Fig. 5. Global allele frequency of *PNPLA3* rs738409 in the populations of the PAGE study. (A) Global allele frequencies for the minor and risk allele (G allele) are shown. (B) Enlarged image of Central and South America from panel A. (C) Enlarged image of East Asia from panel A. Color denotes allele frequency and size denotes the number of individuals in each of 99 populations from PAGE. (This figure appears in color on the web.)

We showed that *PNPLA3* was most significantly associated with NAFLD in a PheWAS of our cohort, consistent with prior GWASs in NAFLD showing strong associations with rs738409.[6–8] Additionally, we identified phenome-wide significant associations with portal hypertension, esophageal varices, non-alcoholic cirrhosis, and alcoholic cirrhosis; all known sequelae of chronic liver disease. NAFLD is thought to represent the proximal end of a spectrum of liver disease and severity, and it is common for patients with more severe forms of liver disease such as NASH and cirrhosis to first present with NAFLD.[3] The *PNPLA3* rs738409 G allele has previously been associated with increased risk of cirrhosis (with ORs ranging from 2.08 to 3.41),[17,41] portal hypertension and esophageal varices (in the context of NAFLD or cirrhosis severity, respectively).[42,43] *PNPLA3* rs738409 may be directly impacting on the risk of other chronic liver diseases, however the associations may also be explained by the role of *PNPLA3* rs738409 on enhancing the risk for NAFLD, which greatly increases the probability of progressing to more severe disease.

Additionally, we show evidence of *PNPLA3* rs738409 associating with earlier ages of liver disease diagnosis identified through PheWAS, suggesting potential independent effects of the variant and justifying a closer examination of the biological role of the variant in more severe aspects of liver disease. In addition to being associated with liver diseases, *PNPLA3* rs738409 has been reported to play a role in lipoprotein metabolism, liver enzyme levels and dietary energy balance, suggesting a potential pleiotropic role in liver disease.[7,44,45] It is premature to assume that *PNPLA3* rs738409 directly contributes to other liver diseases independently of NAFLD. But, the application of further PheWAS to other variants identified in NAFLD GWASs (*i.e. NCAN, PPP1R3B, GCKR* and *LYPLAL1*)[8,37] could potentially further inform biological connections between NAFLD and other liver diseases and contribute to our understanding of how NAFLD progresses to more severe pathologies.

The overall prevalence of NAFLD in our cohort was below general population level estimates, despite NLP methods utilizing radiological imaging. Had all individuals had radiology data, NAFLD prevalence would likely be higher. The low observed prevalence suggests that [a] NAFLD is under diagnosed and/or [b] ICD9 codes used for the determination of a disease phenotype can be incomplete and cases in our cohort were "missed"[46,47] which may, in part, explain why there is a generally low co-occurrence of NAFLD with other liver diseases. Recently, a large meta analysis reported that the global prevalence of NAFLD (55.5%) in older patients with type 2 diabetes (T2D) is 2-fold higher than in the general population and T2D appears to accelerate the progression to NASH.[48] Given the overall prevalence of T2D (28%) and obesity (35%) in our study population, a higher prevalence of NAFLD was expected. This further illustrates the critical need to assess NAFLD risk factors, such as *PNPLA3* genotype and T2D status in younger populations as an approach to slow or prevent progression to NASH. Despite a lower than expected NAFLD prevalence, we were able to show a clear pattern of genetic effects on age of diagnosis for a variety of liver diseases. Similarly, several novel interesting sub-phenome wide significant threshold diseases, whose non-significance may simply be limited by power, were identified. Our prediction analyses were tempered by the availability of pre-diagnosis predictor variable data and reduced case sizes, yet we still show some predictive utility of *PNPLA3* and other clinical measures. Replication of our findings in younger populations would further clarify the role of *PNPLA3* rs738409 in age of liver disease diagnoses.

By leveraging longitudinal EHR data, we showed that *PNPLA3* rs738409 contributes to an earlier diagnosis of NAFLD and several other liver diseases. Our findings suggest that *PNPLA3* rs738409 could be used to stratify risk within populations known to have an enhanced risk for liver disease, an approach that may help identify those who would benefit most from early prevention and/or treatment strategies. A PheWAS approach to our data contributed to enhanced understanding of the relationship between clinical phenotypes and *PNPLA3* rs738409 and these new associations may help improve our understanding of the underlying *PNPLA3* rs738409 biology. Similar disparities in disease risk have been observed in AA populations, in which risk variants for *APOL1* are both more frequent and confer increased risk of kidney disease, cardiovascular disease and early-onset hypertension.[49,50] Ancestry-specific disparities such as these highlight the need for a greater understanding of how *PNPLA3 p.*Ile138Met impacts the timing of NAFLD development and, more broadly, the strong need for diversity in translational genomic research focusing on clinically relevant variants. Overall, approaches like ours can be used to identify at-risk populations within a health care network. Leveraging genetic variants to predict disease is an area of intense study in personalized medicine,[51] yet other critical components such as ethnicity, clinical measures, environmental factors and family history must be considered in risk-stratification approaches. Disease prediction begins to have meaningful clinical utility only when treatment(s) exists.[52] NAFLD currently has no standard clinical treatment recommendations beyond general diet counseling and weight loss, which have had some success,[53] however the pre-identification of individuals most at risk of developing NAFLD and related liver disease would allow clinicians to target those poised to benefit the most from these approaches. Due to the pervasiveness of pediatric obesity, most overweight and obese children should be screened for NAFLD. Hispanic populations exhibit an elevated frequency of the *PNPLA3* rs738409 variant, which also seems to have a stronger impact in this population, coupled with earlier ages of diagnosis. Although the variant showed an effect in other ethnic groups, specific attention must be paid to Hispanic children and adolescents. Our findings suggest that attempts to stratify NAFLD risk in younger age groups, and intervene accordingly, may contribute to mitigating negative medical outcomes in adulthood.

Abbreviations

AA, African American; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; BMI, body mass index; CPT, Current Procedural Terminology; EA, European American; EHR, electronic health record; GRM, genetic relationship matrix; GWAS, genome-wide association study; HA, Hispanic/Latino; HR, hazar ratio; HWE, Hardy Weinberg equilibrium; IBD, identity by descent; IDI, integrated discrimination improvement; NAFLD, non-alcoholic fatty liver disease; NLP, natural language processing; NRI, net reclassification improvement; OR, odds ratios; PheWAS, phenome-wide association study; ROC, receiver operating characteristic.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

RWW concept and design, analyses, interpretation of data, writing of article; GMB analyses, interpretation of data, writing of article; EPS analyses, writing of article; TVV analyses, writing of article; GLW analyses; AM analyses, writing of article; CRG analyses, writing of article; JC interpretation of data, writing of article; NSA interpretation of data, writing of article; GN interpretation of data, analyses, writing of article; RFJL concept and design, interpretation of data, writing of article; EEK concept and design, interpretation of data, writing of article.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2020.01.029.

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