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RESEARCH ARTICLE

Nesic et al: PNPLA3 and TM6SF2 gene variants in ALC

Genetic risk of alcohol-related liver cirrhosis: Associations of *PNPLA3*, *TM6SF2*, and a two-variant polygenic risk score

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ABSTRACT

A minority of individuals who consume excessive alcohol develop cirrhosis. Variants in the patatin-like phospholipase domain-containing protein 3 gene (*PNPLA3*) and the transmembrane 6 superfamily member 2 gene (*TM6SF2*) have been previously identified as associated with alcohol-related cirrhosis (ALC). This study aimed to examine the variants of *PNPLA3* and *TM6SF2* and to develop and assess a polygenic risk score (PRS) for ALC. We enrolled 118 patients diagnosed with ALC and 131 control subjects, who were either abstainers or low-level alcohol consumers without evidence of liver disease. Genotyping of risk variants was performed using PCR-RFLP methodology. PRS, based on independent allelic effect size estimates from genotyped genetic loci, were computed and compared across groups. The development of ALC was significantly associated with CG and GG genotypes of *PNPLA3* (CG: OR: 1.82; 95% CI: 1.05-3.17; p=0.033; GG: OR: 7.64; 95% CI: 3.06-19.07; p<0.001) and the CT genotype of *TM6SF2* (OR: 2.43; 95% CI: 1.27-4.63; p=0.007), controlling for age and sex. Patients with cirrhosis exhibited a significantly higher mean PRS compared to controls (0.32 vs. 0.167, p = 1.8e-07). The odds ratios (ORs) and 95% confidence intervals for the group with the highest PRS score compared to the reference group were 6.707; 95% CI: 3.313-13.581, p<0.001. In our ALC patient cohort, the *PNPLA3* rs738409 and *TM6SF2* rs58542926 variants were associated with an increased risk of ALC development. Moreover, the PRS derived from these two variants effectively identified the genetic components linked to cirrhosis within the study population.

Keywords: Alcohol-related liver cirrhosis, ALC, genetic variants, PNPLA3, TM6SF2, polygenic risk score, PRS.

INTRODUCTION

Excessive alcohol consumption is one of the major causes of prolonged hepatic inflammation, which can gradually lead to the disease progression from alcohol-related fatty liver to alcoholic steatohepatitis and in 10-15% of long-term excessive drinkers, to alcohol-related cirrhosis (ALC) [1]. Cirrhosis is an irreversible, progressive disease associated with high mortality rates due to liver failure and a high rate of hepatic malignancies [2]. It is a multifactorial disease impacted by genetic and environmental risk factors and their interplay. In Europe, almost 60% of cirrhosis cases are alcohol related [3].

The amount of alcohol intake, the pattern of alcohol drinking and the unequal harm attributable to alcohol between males and females [4-6] are risk factors that contribute to onset of the disease and its progression. In addition, there is a significant interindividual variability at risk attributed to genetic components in disease susceptibility [7, 8].

Variations in several genes, whose products play a role in alcohol metabolism, ethanol-induced oxidative stress, inflammation and lipid metabolism were investigated as candidate genetic markers for possible association with ALC [9, 10]. Several studies reported that non-synonymous variants in the *Patatin-like phospholipase domain-containing protein 3* gene (*PNPLA3;* rs738409, I148M) that encodes PNPLA3 protein and *transmembrane 6 superfamily member 2* gene (*TM6SF2*; rs58542926, E167K), that encodes TM6SF2 protein, seem to play an important role in the pathogenesis and progression of the spectrum of liver diseases, regardless of etiology [11-14]. These two proteins are implicated in lipid metabolism and given that fatty liver is an initial manifestation of alcohol-related liver disease (ALD), it is hypothesized that they contribute to the pathogenesis of cirrhosis [10].

The PNPLA3 protein is an enzyme that exhibits lipase activity towards triglycerides and retinyl esters and acyltransferase activity on phospholipids. In humans, *PNPLA3* expression is the highest in the liver, predominantly in hepatocytes [15]. The I148M (rs738409) variant of the *PNPLA3* is the most studied variant associated with the increased risk for liver disease [16], as it is linked to the accumulation of triglycerides in liver cells. This is a loss-of-function single-nucleotide change of a cytosine to guanine that leads to an isoleucine to methionine substitution at position 148 (I148M)

of the protein [17]. Carriers of this variant are at a higher risk of developing steatosis (fatty liver) and progressing to the more severe liver damage, including cirrhosis [18-20]. The TM6SF2 protein is involved in the export of lipids from liver cells and lower levels of this protein are associated with the accumulation of hepatic triglycerides and reduced secretion of very low-density lipoprotein triglycerides (VLDL TG) [21]. Variant *rs58542926* (E167K) in the *TM6SF2* gene, an adenine to guanine substitution in coding nucleotide 499, which replaces glutamate at position 167 with lysine [14], has been associated with altered lipid metabolism, the accumulation of triglycerides, elevated serum aminotransferases, and lower serum lipoproteins, leading to the fatty liver and potentially the progression to cirrhosis [22, 23].

Due to the global increase in alcohol consumption, the expansion of ALD is expected in the near future [24]. Preventing ALD involves challenging tasks such as reducing alcohol consumption worldwide. However, clinical experience has proven that when the patients are informed of their high risk for the disease, they are more likely to reduce or cease their alcohol consumption [25]. Therefore, recognition of excessive alcohol users at the most significant risk of developing cirrhosis is crucial for reducing the incidence of ALC development.

Aim of the study

We genotyped the risk variants in *PNPLA3* and *TM6SF2* genes in ALC patients and evaluated their impact on ALC development by analyzing associated clinical and biochemical parameters, including standard liver function tests. We aimed to investigate the relationship between *PNPLA3* and *TM6SF2* genetic variants, as well as a two-variant polygenic risk score (PRS), and the presence of ALC within our case–control cohort.

MATERIALS AND METHODS

Subjects

We recruited 118 patients with ALC diagnosed between 2015 and 2018 at the Clinic of Gastroenterology and Hepatology, University Hospital Medical Center "Zvezdara", Belgrade, Serbia.

The presence of cirrhosis was determined by combined clinical criteria involving laboratory parameters and clinical examination. Aspartate aminotransferase (AST),

alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), serum albumin, bilirubin concentrations and coagulation tests (international normalized ratio-INR) were determined in blood test (liver function tests, LFT) and their abnormal results were indicators of liver failure. Cirrhosis was radiologically confirmed using ultrasonography and, when required, computed tomography to identify nodular liver morphology, splenomegaly, collateral vessels, and ascites. Esophagogastroduodenoscopy was used to assess esophageal varices, while neuropsychological testing evaluated hepatic encephalopathy symptoms, including confusion, asterixis, and fetor hepaticus. Moreover, we calculated each patient's Child-Pugh score (CP) based on the LFT results and results of the clinical examination to assess the severity of the cirrhosis. We classified patients into A, B and C classes based on the CP scores. Patients from class C had the advanced hepatic dysfunction and the worst prognosis.

Other causes of liver disease (e.g., hepatitis C or hepatitis B infection, autoimmune liver disease, metabolic liver diseases) were excluded from the study by standard serological and biochemical tests. Viral hepatitis was ruled out by screening for hepatitis B surface antigen (HBsAg), total anti-HBc, and anti-HCV antibodies (Abs). Autoimmune liver disorders were excluded based on negative findings for antinuclear (ANA), anti–smooth muscle (ASMA), and anti-mitochondrial (AMA) antibodies, as well as normal serum immunoglobulin levels. Metabolic causes were excluded by measuring serum iron, ferritin, total iron-binding capacity, and transferrin saturation (for hereditary hemochromatosis); serum ceruloplasmin and 24-hour urinary copper excretion (for Wilson's disease); and serum α1-antitrypsin levels (for α1-antitrypsin deficiency). Patients with suspected drug-induced, cholestatic, or cryptogenic liver disease were excluded based on their clinical history and biochemical findings.

Detailed information about age at onset of at-risk alcohol consumption, duration of at-risk alcohol consumption, daily alcohol consumption, the type of beverage and drinking patterns were collected during the interview at the first clinical examination in the hospital. Duration of at-risk alcohol consumption was calculated from self-reported age at the start of regular drinking to the age at the cirrhosis diagnosis. An average daily alcohol consumption of >2 standard drinks for women and >3 standard drinks for men was defined as at-risk alcohol consumption. One

standard drink is a glass of beer, a glass of wine, or a shot of spirits, and amounts to 10g of ethanol [26]. Average daily alcohol consumption in grams was calculated by multiplying the volume of drinks by strength of drinks and the conversion factor (0.789).

We selected 131 control subjects, age and sex matched with cases, recruited from voluntary blood donors and individuals undergoing routine health examinations, who self-reported as either abstainers or individuals with a daily alcohol consumption of less than one standard drink. In particular, the control subjects exhibited no signs indicative of chronic liver disease and had no documented history of chronic hepatic illnesses or other primary pathological conditions. Laboratory assessments of standard liver function tests, including ALT, AST, ALP, and bilirubin, were within normal ranges, and no abdominal ultrasound or other imaging modalities were conducted.

Genotyping

Whole blood from study participants were collected in K2EDTA-coated vacutainers, and genomic DNA was extracted with a DNA isolation method based on silica-membrane spin columns, according to the manufacturer's protocol (Gene JET Whole Blood Genomic DNA Purification Mini Kit, Thermo Scientific, Massachusetts, USA). Isolated DNA was stored at -20°C until further analysis. For both investigated variants genotyping was performed using polymerase chain reaction followed by the restriction fragment length polymorphism (PCR-RFLP) analysis. The PCR reactions were performed for each variant separately as previously described [27, 28]. PCR mixture (total volume 50 µl for each reaction) contained 2xMultiplex Master Mix (Qiagen, Germany), 0.5µM of each primer (Metabion, Germany), and 0.2 µg of genomic DNA. The PCR reactions were performed in thermal cycler Tgradient (Biometra GmbH, Göttingen, Germany) The presence of PCR product were verified on 2% agarose gel, stained with SYBR Safe DNA gel stain (Invitrogen, California, USA) and visualized by UV light. Restriction endonucleases, BseGI (BtsCI) for *PNPLA3* (rs738409 C>G) and Hpy188I for *TM6SF2* (rs58542926 C>T) (New England BioLabs, Massachusetts, USA) were used to digest PCR products, according to the manufacturer's instructions and digested fragments were electrophoresed on 3% agarose gel to obtain genotypes according to fragments length. (Supplementary Table 1).

All subjects were successfully genotyped for both tested variants. For procedure quality validation, 10% of samples were randomly selected and re-genotyped using the same PCR-RFLP methods. Samples were processed in several laboratory batches with a balanced distribution of cases and controls across batches. Throughout all phases of DNA processing, (PCR, digestion, and gel scoring) laboratory personnel were blinded to the case–control status. Sex mismatches and potential sample swaps were excluded by two independent investigators (one clinician and one scientist) through verification of the consistency between the participant records and laboratory documentation.

Ethical statement

All patients and control subjects were of self-reported Serbian ancestry. All procedures were conducted in accordance with the Helsinki Declaration of 1975, and the study protocol was approved by the Ethics Committee of the University Hospital Medical Center „Zvezdara“ (Approval No. 8-6-2018, dated June 1, 2018) Informed consent was obtained from all study participants.

Statistical analysis

Data analysis was performed using Statistical Package for the Social Sciences (SPSS version 20.0, SPSS Inc., Chicago, USA). In all performed analyses, a P-value <0.05 was considered significant. Multiplicity was addressed by applying of Benjamini-Holchberg FDR method, with statistical significance defined at FDR $\leq$ 0.05 and secondary significance considered at FDR $\leq$ 0.1 Allele and genotype frequencies were determined by direct counting. Hardy-Weinberg equilibrium (HWE) was tested for ALC patients and the control group. Continuous variables are presented as means with standard deviations or medians with 25 and 75 percentiles, depending on their distribution normality; the distribution was tested using mathematical and graphical methods. Group differences for normally distributed continuous variables were analyzed using the independent t-test and one-way ANOVA as appropriate. The Kruskal-Wallis Test was used for non-normally distributed variables. Categorical variables are presented as the number and the percentage of cases. The Chi-square or Fisher's exact test, as appropriate, was used to assess differences between groups for categorical variables. Effect size and 95% confidence intervals were generated using nonparametric bootstrap procedure with 2000 resamples, performed in Python (SciPy,

pandas). . Correlations between variables were tested using Pearson's and point-biserial correlations, as applicable. Polygenic risk score (PRS) based on two selected variants was computed in software R v.4.3.0 following the function:

$$f_\beta(x) = \left(\Sigma_{i=1}^{n} \beta_i \cdot g(x_i) \right) / \left(\Sigma_{i=1}^{n} \beta_i \right)$$

For each of the two genes, the $g(x_i)$ represented points 0, 0.5 and 1, given to the participants based on their genotype. The individuals with two reference alleles were assigned 0 points, the heterozygous carriers of the risk allele associated with ALC 0.5 points, and homozygous carriers of the risk allele were assigned 1 point. Maximum $g(x_i)$ value would be 2, in case that an individual was homozygote for both assessed variants. Furthermore, β coefficients representing allelic effect weights were extracted from the PGS Catalog searched for the term alcoholic liver cirrhosis (PRS ID: PGS000704; https://www.pgscatalog.org/score/PGS000704; accessed on 8th April 2025) [29]. Effect weights for tested variants are shown in the Table 1. Differences in PRS distribution between controls and ALC patients were assessed using a non-parametric Wilcoxon rank sum test for continuous data. The association between genotypes/PRS and ALC was tested using binary logistic regression. Results are expressed as odds ratios (OR) with 95% confidence intervals (CI). Because diabetes and alcohol use were exclusion criteria for control subjects, and BMI is influenced by cirrhosis these variables were not included as confounding factors to avoid introducing bias. Association between *TM6SF2* genotypes and ALC was evaluated using Firth regression due to sparse genotype category (few *TM6SF2* TT genotypes). To comprehensively assess potential inheritance patterns, each variant was analyzed using the additive, dominant, and recessive models. To evaluate the association of PRS with ALC, participants were stratified into three balanced PRS-based groups (tertiles) according to empirical distribution of PRS values. The reference group (low risk group) represents patients with PRS=0, which includes subjects with wild-type (wt) genotypes for both tested variants. We used ROC curve analysis to evaluate the PRS model's performance. Internal validation was performed using 1000 bootstrap resamples, and the optimism-corrected AUC and 95%CI were obtained. Model calibration was examined by the calibration slope and Hosmer-Lemeshow test. The same β-weighted and normalized PRS was used consistently for all analyses, including the Wilcoxon comparisons, Figure 1, tertile classification, and ROC

analyses. For bootstrap validation and calibration slope estimation, we used Python 3.13 with pandas, NumPY and scikit-learn libraries. All analyses were restricted to complete cases.

RESULTS

Our study included 249 subjects, out of which 118 were patients diagnosed with ALC and 131 were control subjects. The gender representation was similar in both groups (P=0.540); the ALC group comprised 104 (88.1%) men and 14 (11.9%) women, and the control group comprised 112 (85.5%) men and 19 (14.5%) women. ALC patients had a mean age of 58.6±9.6 years, ranging from 32 to 80 years, while the mean age in the control group was 58.4±10.8 (ranging from 30 to 87), and this difference was not statistically significant (P=0.846). The median age at the start of at-risk alcohol consumption in ALC patients was 23 years, with a median daily alcohol consumption of 72g, and the average duration of alcohol consumption before cirrhosis diagnosis was 34.6±10.98 years. Other characteristics of the patients with ALC are presented in Table 2.

Pearson's correlation coefficient was calculated to evaluate the association between variables related to alcohol consumption. Patients who began at-risk alcohol consumption at an older age exhibited a more rapid progression to cirrhosis (r (118) = –.513; P<0.001). However, no significant correlation was observed between the amount of daily alcohol consumption (median, 72 g/day) and either the duration of alcohol consumption prior to the diagnosis of ALC, nor the age at which the at-risk alcohol consumption commenced. A point-biserial correlation analysis was performed to investigate the association between the average daily alcohol consumption and the type of beverage consumed. It was found that patients who consumed spirits, either alone or in combination with other beverages, had a higher daily alcohol intake (rpb = 0.262, n = 116, P = 0.004).

Association of variant genotypes *PNPLA3* rs738409 and *TM6SF2* rs58542926 with the development of the ALC

The frequency of the G allele of the *PNPLA3 rs*738409variant was 0.45 and 0.24 in the ALC and the control group, respectively, and the prevalence of the T allele of the *TM6SF2 rs58542926* variant was significantly higher in the patient group (0.177) compared to the control subjects (0.084) (P=0.002). The genotype GG (*PNPLA3*) was

more frequent in patients with ALC than in the control group (P<0.001). Regarding the *TM6SF2* variant, the CC genotype was more prevalent in the control group than in the ALC group (84.7% vs. 68.7%, p = 0.003). The CT genotype was more frequent in the ALC group than in the control group (27.1% vs. 13.8%, p = 0.009). The genotype distributions for each of the studied variants were in Hardy-Weinberg equilibrium (Supp.Table 2). The G allele in the *PNPLA3* and the T allele in the *TM6SF2* gene were strongly associated with the disease (P<0.001 and P=0.002, respectively, Table 3.). We examined the association between genotypes of the studied genes and ALC. To investigate the independent effect of each genotype, we included sex and age as covariates in the multiple logistic regression models. Subjects with CG and GG *PNPLA3* genotypes, compared to the CC genotype, had an almost two and almost eight times higher risk of developing cirrhosis, respectively (CG: OR=: 1.82; 95% CI= 1.05-3.17; P=0.033; GG: OR=: 7.64; 95% CI= 3.06-19.07 ; P<0.001). As shown in Table 3, the *PNPLA3* variant was significantly associated with the ALC under both dominant and recessive models (P < 0.001 for both). Considering that the TT genotype of the *TM6SF2* gene was present in only five patients and two individuals in the control group (P = 0.261), we employed Firth logistic regression. Subjects with CT genotype of *TM6SF2* gene had 2.43 times higher chances for the disease (OR= 2.43; 95% CI= 1.27-4.63; P=0.007). The dominant genetic model for this variant showed that the possibility of developing cirrhosis was 2.5 times higher in the carriers of the CT or TT genotype of the *TM6SF2* gene compared to CC (OR=: 2.52; 95%CI= 1.36-4.66; P=0.003). The results are presented in Table 3. After controlling for multiple testing with the Benjamini-Hochberg method at FDR=0.05 the BH thresholds were P ≤ 0.0375 for the χ^2 tests, and P value ≤ 0.0444 for the regression models.

Estimation of ALC development risk using a polygenic risk score (PRS)

We generated variant-based PRS for the two tested variants, and the risk of developing ALC was assessed by comparison of the scores between the studied groups. The distribution of PRSs among patients with ALC and the control subjects showed significance with P =1.8e-07. The mean PRS for the ALC and the control group were 0.32 and 0.167, respectively (Figure 1). For logistic regression analysis, patients were divided into three risk groups (low, moderate, high) according to the PRS values. The reference group (low risk group) represents patients with PRS=0,

which includes subjects with wild-type (wt) genotypes for both tested variants. The high-risk group (the highest PRS group) had seven times higher risk for ALC development compared to the reference (low risk) group after adjusting for age and sex (P<0.001) (Table 4). The model demonstrated moderate discriminatory power, with an AUC of 0.684 (95% CI 0.617-0.750). Internal validation using 1000 bootstrap resamples yielded an optimism-corrected AUC of 0.684 (95%CI: 0.616-0.745), indicating minimal overfitting and high stability of model performance. Calibration slope of 1.360 showed mild underfitting and Hosmer-Lemeshow goodness-of-fit test indicated excellent agreement between observed and predicted risk (χ^2=3.441; P=0.904).

Clinical characteristics of the ALC group according to *PNPLA3* and *TM6SF2* genotypes

Comparing drinking profiles, laboratory parameters and clinical characteristics of ALC patients across *PNPLA3* rs738409 and *TM6SF2* rs58542926 genotypes, the average daily dose of consumed alcohol differed significantly across the *PNPLA3* genotypes, where carriers of the CC genotype consumed the highest amounts compared to the carriers of CG and GG genotypes (P=0.002). A significant trend toward higher levels of ALT was detected as the number of *G* alleles of the *PNPLA3* gene increased (Kruskal–Wallis H(2) = 8.10, p = 0.017), The effect size was η^2 = 0.052, with a bootstrap confidence interval raging from -0.005 to 0.182, indicating a small to moderate effect. However, this association did not remain statistically significant after applying the Benjamini-Hochberg procedure, at either FDR = 0.05 or FDR = 0.1. Other laboratory parameters did not vary between subgroups (P>0.05 for all parameters, Table 5). Characteristics across the *TM6SF2* genotypes did not differ between the subgroups (P>0.05 for all parameters, Table 5).

DISCUSSION

Alcohol-related liver cirrhosis is a multifactorial disease, with high variability in progression and outcomes due to environmental and genetic factors and their mutual interactions. The amount of alcohol intake [30, 31] as well as the pattern of drinking [4, 5] has been shown to be directly proportional to the risk of liver disease. Compared to men, women exhibit similar effects at lower levels of alcohol

consumption [6, 32]. Additionally, genetic risk factors, along with other chronic and acute conditions, may affect the progression to different stages of ALD [2, 33].

The *PNPLA3* rs738409 variant was the first to be consistently and strongly associated with various liver pathological conditions such as steatosis, fibrosis, cirrhosis and hepatocellular carcinoma (HCC) in liver diseases regardless of the etiologies [12, 16, 17, 20, 34-37]. It significantly enhances lipid accumulation in the liver by promoting the conversion of lysophosphatidic acid into phosphatidic acid and leads to increased lipotoxicity [38]. An association between this variant and hepatic fat concentration was first identified in the study concerning metabolic dysfunction–associated steatotic liver disease (MASLD) [17]. In later studies, a strong association between this variant and ALD [20], ALC [12], and HCC [34] was documented. Our results showed that the carriers of both the CG and GG genotypes of the *PNPLA3* rs738409 variant had a higher risk of developing cirrhosis when compared to carriers of the CC genotype, and indicate that this variant is strongly associated with ALC, which is in line with other studies [18, 20, 39-42]. The *TM6SF2* gene has emerged as a candidate gene for various liver diseases [12]. Protein TM6SF2 is involved in VLDL secretion. The rs58542926 variant was associated with increased intracellular lipid accumulation leading to fatty liver and with ALC [12], HCC [13], and MASLD [43] development. Our findings showed that the probability of developing cirrhosis was more than 2 times higher in carriers of the CT or TT genotype (compared to CC) of the *TM6SF2* variant rs58542926. We detected only seven individuals with the TT genotype (5 ALC patients and 2 control subjects). Our results are in line with the previously reported study [13]. The frequencies of the G allele of the *PNPLA3* rs738409 variant and the T allele of the *TM6SF2* variant rs58542926 were higher in patients with cirrhosis, confirming that the carriers of these alleles were at higher risk of the disease. The frequencies of minor alleles of the *PNPLA3* and *TM6SF2* genes in our control group align with frequencies reported for the European population in the Genome Aggregation Database (gnomAD) [44, 45]. However, in our cohort, the interpretation of *TM6SF2* effects is limited by the very small number of TT homozygotes, which resulted in wide CIs and unstable estimates for TT in the analysis. Therefore, these findings should be considered exploratory. Validation of the TT-associated risk will require larger or pooled cohorts for confirmation.

We further validated the combined impact of examined variants through the PRS calculation to identify individuals at risk of developing ALC. In our study, the mean PRS was two times higher in the group of patients with ALC than in the group of control subjects. Further stratification of subjects in three risk groups according to their PRS values enabled more accurate identification of individuals at the highest risk of developing cirrhosis, where those in the group with the highest scores had seven times higher risk of ALC compared to the reference. Although the AUC value of 0.684 indicates moderate predictive accuracy, this level of discrimination is consistent with other studies [46]. The PRS model demonstrated stable performance and good calibration in the internal dataset. A broader panel of variants for PRS calculation, including comorbidities associated with ALC, such as diabetes mellitus, has been reported [46–48]. Whitfield et al. demonstrated that a three-variant risk score, when combined with diabetes, enhanced the discrimination of ALC among heavy alcohol consumers [46]. Furthermore, a 20-SNP PRS further improved prediction when integrated with the clinically known predictors of ALC risk in the drinking population [48]. In contrast to these approaches, our study utilized only two nucleotide variants, excluded diabetes and BMI, and employed non-drinking controls, thereby estimating genetic susceptibility without the confounding effects of alcohol exposure or metabolic comorbidities. This model identifies genetic association and simple risk stratification within our case-control study. While it may suggest potential for earlier risk identification in broader populations, any use of these genetic variants and our simple PRS for early stratification among excessive drinkers or prediction before heavy drinking begins remains hypothetical and requires further studies. Although simpler and potentially less discriminative in high-risk alcohol drinkers, the 2-SNP model provides a practical and cost-effective approach that can be integrated into broader multivariable risk-prediction tools.

We analyzed the relationship between the studied genotypes and the characteristics of ALC patients. Carriers of the CC genotype of the *PNPLA3* gene variant rs738409 had a lower risk for ALC despite significantly higher daily alcohol consumption, compared to carriers of other genotypes. Therefore, carriers of either one or two G alleles were more susceptible to cirrhosis even with lower alcohol consumption, which agrees with the findings that individuals with this variant are at a higher risk of developing fatty liver and even cirrhosis [18-20].

As the number of G alleles of the *PNPLA3* rs738409 increased, there was a significant trend toward higher serum levels of ALT enzyme. However, this association did not remain statistically significant after Benjamini-Hochberg correction for multiple comparisons. The effect size indicated a small-to-moderate effect, so this result should be interpreted with caution. The higher levels of ALT were previously linked with the *PNPLA3* genotypes [49, 50]. ALT is a highly sensitive and specific marker of liver function, localized in the hepatocytes, and the exact mechanism through which *PNPLA3* influences ALT elevation is still not fully understood [51]. It is possible that an altered protein causes fat accumulation in the liver, leading to elevated liver enzyme levels or increased ALT levels by inducing hepatocyte necrosis and impairing liver function.

No significant differences were observed for any other biochemical parameter across the genotypes, suggesting that the examined biochemical markers were not associated with the explored genetic variants in our study.

It is important to note that this study is a single-center study, and future research should aim to include a larger sample size to improve the generalizability of these findings. Our patients were not diagnosed with liver biopsy. Although the liver biopsy is the golden standard for cirrhosis diagnosis, it is possible to accurately diagnose liver cirrhosis by non-invasive methods in the majority of patients with chronic liver disease [52]. In our study, the diagnosis was established based on the clinical symptoms and laboratory test results, alongside a history of long-term excessive alcohol consumption, while excluding other potential causes of cirrhosis.

The control group was limited to individuals without diabetes or significant alcohol consumption to ensure a homogeneous comparison. Since alcohol intake and diabetes were exclusion criteria for one group, and BMI was influenced by cirrhosis, incorporating these variables into regression models would have introduced bias. Therefore, we used only sex and age adjustments in the regression models. A limitation of the study design is the inability to exclude gene-environment interactions. The estimated ORs for variants and PRS should be interpreted as associations within this study design, rather than fully independent genetic effects, and no further sensitivity analysis including BMI, diabetes or alcohol consumption habits were feasible without introducing bias. Recruiting individuals with significant alcohol

consumption as controls would allow demonstrating an independent association of variants with cirrhosis, adjusted for alcohol consumption. This approach would require liver biopsy for control participants to prevent misclassification, which was not feasible. While these design choices limit generalizability, they were necessary to ensure clear assessment of genotype associations within a defined population. Another concern regarding the study participants is the potential inaccuracy of self-reported alcohol consumption data, which may be affected by recall bias or intentional misreporting. The amounts of alcohol are usually under-reported because of consumer awareness of the harmful effects of alcohol and the social desirability [53]. It is reasonable to believe that patients with cirrhosis reporting ongoing drinking are providing accurate information, while the group reporting abstinence or low levels of alcohol consumption may include some individuals who are drinking excessive amounts of alcohol. _It is important to consider that our PRS represents an initial, proof-of-concept model that should be subject to future optimization. For the clinical use of the risk score to identify at-risk patients, the risk score should include additional genetic variants alongside other known risk factors.

CONCLUSION

Understanding the genetic underpinnings of chronic liver diseases and identifying an individual's genetic susceptibility is crucial for the development of management strategies, including lifestyle modifications and monitoring for disease progression. In our cohort, *PNPLA3* rs738409 and *TM6SF2* rs58542926 variants were associated with an elevated risk of alcohol-related cirrhosis, and a polygenic risk score based on these loci effectively identified individuals with increased genetic susceptibility to the disease. Given the case-control design, these findings represent preliminary cohort-specific risk associations. Future research with heavy-drinking controls without ALC will be needed to assess disease severity and the potential clinical significance of this two-variant PRS.

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TABLES AND FIGURES WITH LEGENDS

Table 1. Parameters utilized in the polygenic risk score analysis

Genetic variant (risk allele)	*PNPLA3* rs738409 (G)	*TM6SF2* rs58542926 (T)
β (logOR)	0.19895	0.186567

A risk allele refers to the genetic variant associated with alcohol-related liver cirrhosis. In this study, homozygous carriers of alleles linked to alcohol-related liver cirrhosis received 1 point for each gene analyzed, while heterozygous carriers were assigned 0.5 points, and non-carriers received 0 points. The β values utilized in this research were obtained from the PGS Catalog (PRS ID: PGS000704, accessed on April 8, 2025). Abbreviations: PNPLA3: Patatin-like phospholipase domain-containing protein 3; TM6SF2: Transmembrane 6 superfamily member 2; β: Regression coefficient; OR: Odds ratio; logOR: Natural logarithm of the odds ratio; PRS: Polygenic risk score; PGS: Polygenic score.

Table 2. Characteristics of ALC patients

Drinking profile			Clinical characteristics		Laboratory parameters	
Age at onset of at-risk alcohol consumption, years		23 (19-30)	Diabetes, N (%)	32 (27.1)	AST, IU/L	68.5 (43.5-113)
Duration of at-risk alcohol consumption, years		34.54 (10.98)	Child-Pugh class	A, N(%) 18 (15.3)	ALT, IU/L	36 (22-54.25)
Daily alcohol consumption, g		72 (56-90)		B, N(%) 47 (39.8)	ALP, IU/L	117 (79.75-156.5)
Type of beverage	Beer, N (%)	82 (69.5)		C, N(%) 53 (44.9)	GGT, IU/L	128 (70-261.25)
	Wine, N (%)	25 (21.2)	Ascites, N(%)	84 (71.2)	Albumin, g/L	28 (25-32.25)
	Spirits, N (%)	87 (73.7)	Encephalopathy, N(%)	63 (53.4)	Bilirubin, μmol/L	43.7 (21.35-90.8)
			Esophageal varices, N(%)	71 (60.2)	INR	1.5 (1.30-1.79)

The data are presented as means ± standard deviations or medians (25th - 75th percentiles), unless otherwise specified. It is important to note that beverage categories are non-mutually exclusive, resulting in percentages that may exceed 100%. Abbreviations: N: Number of subjects; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transpeptidase; INR: International normalized ratio.

Table 3. Association of analyzed alleles/genotypes in variants *PNPLA3* rs738409 and *TM6SF2* rs58542926 with the development of alcohol-related liver cirrhosis

	Odds Ratio [95% CI]	***p* value**
PNPLA3		
G vs. C	2.55 [1.74-3.74]	<0.001
CG vs.CC	1.82 [1.05-3.17]	0.033
GG vs. CC	7.64 [3.06-19.07]	<0.001
Dominant model CG/GG vs. CC	2.54 [1.51-4.26]	<0.001
Recessive model GG vs. CC/CG	5.73 [2.4.-13.7]	<0.001
TM6SF2		
T vs. C	2.34 [1.35-4.06]	0.002
CT vs. CC	2.43 [1.27-4.63]	0.007*
TT vs.CC	3.33 [0.63-17.68]	0.158*
Dominant model CT/TT vs. CC	2.52 [1.36-4.66]	0.003
Recessive model TT vs. CC/CT	2.76 [0.52-14.58]	0.232*

The odds ratio is adjusted for sex and age. The 95% CI and *p* value are reported. Although only raw *p* values are presented, multiple testing was controlled using the Benjamini-Hochberg procedure, with a FDR set at 0.05. The significance threshold for the Benjamini-Hochberg procedure was $p \leq 0.0444$. * *p* values are derived from Firth logistic regression. Abbreviations: PNPLA3: Patatin-like phospholipase domain-containing protein 3; TM6SF2: Transmembrane 6 superfamily member 2; CI: Confidence interval; FDR: False discovery rate.

Table 4. Adjusted ORs for the two-SNP polygenic risk score (*PNPLA3* + *TM6SF2*)

Risk group	Score	Odds Ratio*	Lower 95% CI	Upper 95% CI	*p* value
Low, N=94	0	Reference			
Moderate, N=87	>0 ≤ 0.26	1.731	0.937	3.199	0.080
High, N=68	> 0.26	6.707	3.313	13.581	<0.001

*The odds ratio is adjusted for sex and age. A threshold of 0.26 represents the first tertile cut-off from the empirical PRS distribution. The low-risk group comprises subjects with a PRS of 0 or carriers of wild-type (wt) genotypes for both tested variants. The moderate-risk group includes subjects with a PRS greater than 0 but less than 0.26. The high-risk group consists of subjects with a PRS greater than 0.26. Abbreviations: OR: Odds ratio; SNP: Single-nucleotide polymorphism; PNPLA3: Patatin-like phospholipase domain-containing protein 3; TM6SF2: Transmembrane 6 superfamily member 2; N: Number of subjects; CI: Confidence interval; PRS: Polygenic risk score.

Table 5. Comparison of characteristics of ALC patients across *PNPLA3* and *TM6SF2* genotypes

	PNPLA3			***p* value**	***TM6SF2***			***p* value**
	CC (N=40)	CG (N=49)	GG (N=29)		CC (N=81)	CT (N=32)	TT (N=5)	
Gender, *n* (%)				0.067				0.315
Female	7 (17.5)	7 (14.3)	0 (0)		12 (14.8)	2 (6.3)	0 (0)	
Male	33 (82.5)	42 (85.7)	29 (100)		69 (85.2)	30 (93.8)	5 (100)	
Age	60.25 (8.96)	58.0 (9.3)	57.5 (10.8)	0.424	58.5 (9.6)	58.1 (9.9)	63.3 (6.6)	0.522
Drinking profile								
Age at onset of at-risk alcohol consumption, years	23.5(19.2-30)	23(19-28)	21(18-25)	0.446	22(19-27.5)	24 (18.2-30)	25 (19-30.5)	0.824
Duration of at-risk alcohol consumption,	35.1 (11.2)	33.7 (11)	35.0 (11)	0.774	34.6 (10.6)	33.8 (12.4)	38.5 (8.9)	0.674

years									
Daily alcohol consumption, g		80(60-100)	60(37-75)	74(48-100)	**0.002**	72(48-90)	75 (58.7-100)	60 (45-80)	0.611
Type of beverage	Beer, n (%)	29 (72.5)	33 (67.3)	20 (69)	0.766	57 (70.4)	21 (65.6)	4 (80)	0.745
	Wine, n (%)	9 (22.5)	10 (20.4)	6 (20.7)	0.969	21 (25.9)	4 (12.5)	0 (0)	0.144
	Spirits,n (%)	32 (80)	34 (69.4)	21 (72.4)	0.518	61 (75.3)	22 (68.8)	4 (80)	0.735
Laboratory parameters									
AST, IU/L		63(36.8-119)	59(41-100)	84(51.5-127)	0.277	66(42-111.5)	68.5 (44.2-112.5)	72 (39-377)	0.876
ALT, IU/L		30.5(21.3-41.5)	35(22.5-57.5)	47(32-72)	**0.017**	36(22-50)	36 (22.2-57.2)	30 (19.5-421)	0.863
ALP, IU/L		120.5(78.3-157)	110(79.5-154.5)	115(76-171.5)	0.958	122(81.5-159.5)	106 (74-	104 (79.5-	0.657

							154.7)	269)	
GGT, IU/L		141(71-226)	120(54.5-301.5)	131(84-392)	0.555	124(70.5-249)	136.5 (71-274.7)	131 (38-400)	0.912
Albumin, g/L		29(26.0-32.8)	27(25-32.5)	27(24-32)	0.465	28(25-32)	27 (24-34.7)	28 (26.5-32.5)	0.951
Bilirubin, μmol/L		42.65(22.4-88.4)	43.8(20.2-111.4)	40.8(21.3-90.5)	0.907	40.8(22.2-90)	50.2 (19.2-91.5)	58.5 (23.3-196)	0.835
INR		1.48(1.3-1.8)	1.52(1.3-1.7)	1.42(1.3-1.8)	0.897	1.44(1.3-1.7)	1.51 (1.2-1.8)	1.7 (1.3-2.3)	0.636
Clinical characteristics									
Diabetes N (%)		9 (22.5)	12 (24.5)	11 (37.9)	0.314	21 (25.9)	10 (31.3)	1 (20)	0.793
Child-Pugh class	A, N(%)	6 (15)	7 (14.3)	5 (17.2)	0.939	11 (13.6)	6 (18.8)	1 (20)	0.754
	B,	15 (37.5)	20 (40.8)	12 (41.4)	0.933	37 (45.7)	9 (28.1)	1 (20)	0.149

N(%)								
C, N(%)	19 (47.5)	22 (44.9)	12 (41.4)	0.88	33 (40.7)	17 (53.1)	3 (60)	0.386
Ascites, N(%)	27 (67.5)	37 (75.5)	20 (69)	0.677	61 (75.3)	19 (59.4)	4 (80)	0.219
Encephalopathy, N(%)	26 (65)	23 (46.9)	14 (48.3)	0.193	41 (50.6)	18 (56.3)	4 (80)	0.411
Esophageal varices, N(%)	22 (55)	35 (71.4)	14 (48.3)	0.093	49 (60.5)	19 (59.4)	3 (60)	0.994

The data are presented as means ± standard deviations or medians (25th - 75th percentile), unless otherwise specified. Beverage categories are non-mutually exclusive; therefore, percentages may exceed 100%. Normally distributed continuous variables were evaluated using the independent t-test and one-way ANOVA, as appropriate. For non-normally distributed variables, the Kruskal-Wallis Test was employed. The Chi-square test or Fisher's exact test, as appropriate, was utilized to assess differences between groups for categorical variables. Multiple testing was controlled using the Benjamini–Hochberg procedure. None of the *p* values met the significance criteria at FDR of 0.05 or 0.1. Abbreviations: PNPLA3: Patatin-like phospholipase domain-containing protein 3; TM6SF2: Transmembrane 6 superfamily member 2; P: Probability; N: Number of subjects; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transpeptidase; INR: International normalized ratio; FDR: False discovery rates.

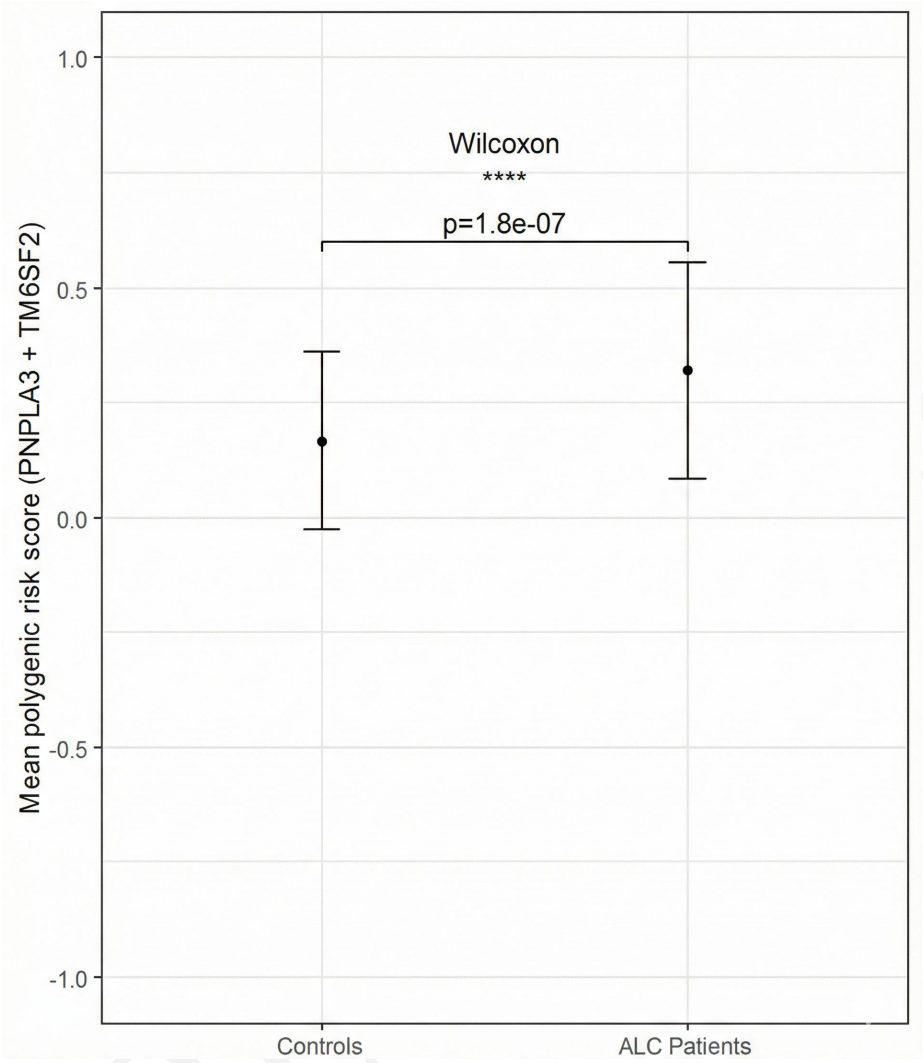

Figure 1. Distribution of PRS in ALC patients and control subjects. The means for the control and ALC groups are denoted by dots (0.17 for the control group, 0.32 for the ALC group). Error bars indicate the standard deviation (0.19 for the control group, 0.23 for the ALC group). Given that the standard deviation exceeded the mean in the control group, suggesting a non-normal distribution, differences were analyzed using the non-parametric Wilcoxon rank sum test for continuous data. Abbreviations: PRS: Polygenic risk score; ALC: Alcohol-related liver cirrhosis.

SUPPLEMENTAL DATA

Supplementary table 1. PCR-RFLP conditions and restriction fragment patterns for analyzed *PNPLA3* and *TM6SF2* variants.

Gene (variant)	Forward primer (5'→3') Reverse primer (5'→3')	PCR conditions (35 cycles)	PCR product size (bp)	Restriction enzyme	Fragment pattern (bp)
PNPLA3 (rs738409)	TGGGCCTGAAGTCCGAGGGT CCGACACCAGTGCCCTGCAG	ID:94°C/2min; D:94°C/30s; A:66°C/30s; E:72°C/30s; FE:72°C/5min	333	BseGI (BtsCI)	C allele: 200+133 G allele: 333
TM6SF2 (rs58542926)	ACGGGGAAAGTTCAGGCACATTG CCTGGGCAGCATGGTGAAACC	ID:94°C/2min; D:94°C/30s; A:62°C/30s; E:72°C/30s; FE:72°C/5min	429	Hpy188I	C allele: 178+33+85 T allele: 251+178

Abbreviations: ID: Initial denaturation; D: Denaturation; A: Annealing; E: Elongation; FE: Final elongation; PCR-RFLP: Polymerase chain reaction–restriction fragment length polymorphism; bp: Base pairs; PNPLA3: Patatin-like phospholipase domain-containing protein 3; TM6SF2: Transmembrane 6 superfamily member 2.

Supplementary table 2. Genotype distribution and allele frequencies of variants *PNPLA3* rs738409 and *TM6SF2* rs58542926 in the ALC and control group

Variant	Allele/genotype	ALC group N=118		Control group N=131		*p*
PNPLA3	C, N(%)	129	(54.7)	198	(75.6)	**<0.001**
	G, N(%)	107	(45.3)	64	(24.4)	
	CC, N(%)	40	(33.9)	74	(56.5)	**<0.001**
	CG, N(%)	49	(41.5)	50	(38.2)	0.589
	GG, N(%)	29	(24.6)	7	(5.3)	**<0.001**
HWE^p		0.078		0.699		
TM6SF2	C, N(%)	194	(82.2)	240	(91.6)	**0.002**
	T, N(%)	42	(17.8)	22	(8.4)	
	CC, N(%)	81	(68.7)	111	(84.7)	**0.003**
	CT, N(%)	32	(27.1)	18	(13.8)	**0.009**
	TT, N(%)	5	(4.2)	2	(1.5)	0.261
HWE^p		0.427		0.221		

Differences between allele and genotype frequencies were assessed using the Chi-square test or Fisher's exact test in cases of sparse cell counts. Raw *p* values are presented. Multiple testing corrections were applied using the Benjamini-Hochberg procedure (FDR = 0.05), with results deemed significant at *p* values ≤ 0.0375.
Abbreviations: ALC: Alcohol-related liver cirrhosis; N: Number of subjects; HWE^p: Hardy–Weinberg equilibrium *p* value; FDR: False discovery rates.