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# PNPLA3 Association with Alcoholic Liver Disease in a Cohort of Heavy Drinkers

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## Abstract

**Aims:** Prior studies have established variation at the *PNPLA3* gene to be associated with a risk of developing alcoholic liver disease (ALD). We attempt to replicate this finding and other potential genetic variations previously associated with ALD utilizing a case-control design in a cohort of subjects with alcohol use disorders.

**Short summary:** This case-control study performed in a US clinical sample of heavy drinkers, replicates the previously reported association between ALD and rs738409 polymorphism in the *PNPLA3* gene in heavy drinkers. This association persisted after accounting for the subject's diabetes status.

**Methods:** Patients of European ancestry with a history of ALD were identified ($n = 169$). Controls consisted of patients without ALD who were from the same cohorts and were $\geq 30$ years of age, had lifetime total years drinking $\geq 20$ and lifetime maximum drinks per day $\geq 12$ ($n = 259$). Patients were genotyped for 40 candidate single nucleotide polymorphisms (SNPs) selected for the purpose of testing their association with ALD. The association of each SNP with ALD was tested using a logistic regression model, assuming log-additive allele effects. Bonferroni correction was applied and multivariable logistic regression models were used to account for relevant covariates.

**Results:** Age, sex, and body mass index (BMI) distributions were similar between cases and controls. Diabetes was more prevalent in the ALD cases. Three SNPs were associated with ALD at the nominal significance level (rs738409 in PNPLA3, $P = 0.00029$; rs3741559 in AQP2, $P = 0.0185$; rs4290029 in NVL, $P = 0.0192$); only *PNPLA3* rs738409 SNP was significant at the Bonferroni-corrected *P*-value threshold of 0.00125. Association results remained significant after adjustment for diabetes status.

**Conclusion:** Our case-control study confirmed that *PNPLA3* rs738409 SNP is associated with ALD. This is an important replication in a US clinical sample with control subjects who had long histories of alcohol consumption.

## INTRODUCTION

Cirrhosis of the liver is associated with progressive fibrosis and distortion of the hepatic architecture. Liver cirrhosis is irreversible under most circumstances and is a leading indication for liver transplantation (Bonis *et al.*, 2001). Alcoholic liver disease (ALD) is the second most common etiological factor responsible for liver cirrhosis among patients awaiting a liver transplant and is associated with significant morbidity, mortality and health care expenditure (Yoon *et al.*, 2014; Wong *et al.*, 2015).

While consumption of large quantities of alcohol over prolonged periods of time is associated with ALD, only ~10–15% of patients with heavy alcohol consumption go on to develop these complications (Mann *et al.*, 2003). This suggests that other factors play a role in mediating this risk. There is a growing base of evidence linking a variant in the *PNPLA3* gene (rs738409: C > G, NP_079,501.2:p.I148M) with ALD (Salameh *et al.*, 2015). A small number of other single nucleotide polymorphisms (SNPs) have also been shown to be associated with an increased risk of ALD (Anstee *et al.*, 2016).

Thus far, at least six studies have examined the association between the alcoholic cirrhosis and the variation in the *PNPLA3* gene. While most of these studies have examined European subjects, none of them were performed in a clinical sample in the USA. Also, studies that included controls with heavy drinking, utilized a cut off of at least 10 years of alcohol consumption. This might lead to some bias as prior studies have shown that patients with alcohol-related liver disease usually have around a 20-year history of drinking (Naveau *et al.*, 1997).

In this case-control study utilizing a clinical sample of patients with European ancestry from the USA, we attempt to replicate these findings by testing the association between ALD and a set of candidate SNPs selected based on prior findings. We also aimed to utilize controls with a longer history of alcohol consumption.

## METHODS

### Population and setting

Clinical data for the cases and controls selected for this study were retrieved from a database including a total of nearly 900 alcohol dependent subjects, who were recruited for three studies examining genetic influences on ALD and alcohol use disorders, and had undergone genotyping as detailed below. In addition, chart review was conducted to retrieve additional information. Patients who either self-reported a diagnosis, or alternately, received a clinical diagnosis of type II diabetes following their evaluation were considered to have diabetes. Subjects included patients with alcohol use disorders participating in a DNA Repository for Genomic Studies of Addiction ($n = 166$), those recruited for a study of genetic predictors of severe alcohol withdrawal ($n = 141$) and those listed for liver transplantation who were recruited to a study examining risk factors for potential relapse ($n = 121$).

From this study, subjects with a clinical documented history of alcoholic liver disease, defined as having either a diagnosis of alcoholic cirrhosis or requiring liver transplantation due to alcohol-related liver injury, were designated as cases. All included controls were over 30 years of age and reported a history of heavy drinking, defined as ≥20 years of lifetime drinking and a lifetime maximum drinks per drinking day ≥12. Twenty cases and 20 controls were randomly identified and their electronic medical records were thoroughly reviewed to ensure accurate designation. This review revealed that all 40 cases and controls were correctly classified.

### Gene and SNP selection

A total of 44 ALD candidate SNPs were selected for this study. These included five SNPs previously reported to be associated with cirrhosis in patients with chronic hepatitis C by Huang *et al.* (2015), Eleven tag SNPs covering the *TLR4* gene, which was one of the genes implicated in ALD in the study by Huang *et al.*, (2015) as well as 10 SNPs in *AQP1*, 7 SNPs in *AQP2*, and one SNP in *PNPLA3* (rs738409) were selected based on the findings of Stickel, *et al.* (2011). In addition, 10 SNPs in the *MTHFR* gene encoding methylenetetrahydrofolate reductase were also genotyped. Thus in total, 44 SNPs were genotyped for the purpose of testing association with ALD. Additional information including the genotype counts are provided in the online supplement (See Supplementary Table S1).

### Genotyping and quality control

Genotyping was conducted as part of prior studies of genetic predictors of alcohol dependence, and included the candidate SNPs described above as well as candidate SNPs for alcohol dependence and 55 ancestry informative markers (Biernacka *et al.*, 2013; Karpyak *et al.*, 2013, 2014). One SNP failed (rs2290351 in *AP3S2*) and three *AQP1* SNPs had very low minor allele frequencies (MAF ≤ 0.005) and were excluded from analysis.

Genotyping was performed at the Mayo Clinic on the Illumina BeadXpress™ platform using a VeraCode™ SNP panel following the manufacturer's protocol. For quality control, a CEPH family trio (Coriell Institute) was genotyped six times, and DNA from four cases was included in duplicate. Concordance between replicates was 100% and there were no Mendelian inheritance errors. One of the candidate SNPs failed genotyping, while all remaining markers had call rates >97%. Analysis was restricted to subjects that self-reported their race as Caucasian, and structure (Pritchard *et al.*, 2000) analysis of the ancestry informative markers was used to verify self-reported race.

### Analyses

Analyses were performed using data from subjects with European ancestry who were successfully genotyped. The association of each SNP with ALD was tested using a logistic regression model, assuming log-additive allele effects (i.e. with SNPs coded in terms of the number of copies of the minor allele). Multivariable logistic regression models were used to test for genetic association while accounting for covariates including diabetes status.

## RESULTS

The cases and controls were similar in terms of their sex, age and BMI distribution and obesity rates. The cases had a higher prevalence of diagnosis of diabetes mellitus as compared to the controls

**Table 1.** Demographic characteristics

| | Alcoholic controls $N = 259$ (%) | ALD cases $N = 169$ (%) | *P*-value |
|---|---|---|---|
| Age | 51.4 ± 9.5 | 52.8 ± 8.8 | 0.0550 |
| Gender (M) | 195 (75.3) | 134 (79.3) | 0.3374 |
| BMI | 27.4 ± 6.1 | 27.2 ± 5.5 | 0.8225 |
| Obesity | 71 (28.3) | 44 (28.0) | 0.9545 |
| Diabetes | 43 (16.7) | 37 (27.8) | **0.0058** |

**Table 2.** SNPs associated with ALD at the nominal 0.05 significance level

| SNP | Gene | OR | *P*-value | $OR_{adj}$ | $P\text{-value}_{adj}$ |
|---|---|---|---|---|---|
| rs738409 | PNPLA3 | 1.75 | 0.00029 | 1.76 | 0.0003 |
| rs3741559 | AQP2 | 0.64 | 0.019 | 0.62 | 0.016 |
| rs4290029 | NVL | 1.63 | 0.019 | 1.69 | 0.014 |

Without adjustment for diabetes (OR, *P*-value) and after adjustment for diabetes ($OR_{adj}$, $P\text{-value}_{adj}$).

(27.8% vs. 16.7%; $P = 0.0058$). The demographic characteristics of the cases and controls are detailed in Table 1.

Of the total 40 SNPs that were tested for association with ALD, three SNPs showed association at the nominal significance level of $P < 0.05$ (Table 2). The association with SNP rs738409 in PNPLA3 was significant after a Bonferroni correction for multiple testing ($P_{corrected} = 0.012$). Further, after adjusting for diabetes status using a multivariable logistic regression model, *PNPLA3* SNP rs738409 remained significantly associated with ALD.

## DISCUSSION

Our case-control study replicates the previously reported association between ALD and rs738409 polymorphism in the *PNPLA3* gene in heavy drinkers. This association persisted after accounting for the subject's diabetes status. Out of the remaining candidate SNPs only two (*AQP2* rs3741559 and *NVL* rs4290029) showed a nominally significant association with ALD, which did not survive Bonferroni correction. Thus this study performed in a US clinical sample of heavy drinkers, provides an important validation of the previous candidate gene and genome wide association findings associating PNPLA3 variation with alcohol-related liver injury, specifically alcoholic cirrhosis.

The I148M variation (rs738409: C > G) in the *PNPLA3* gene represents a cytosine to guanine substitution, this in turn results in an isoleucine to methionine switch at codon 148. This gene encodes a 481-amino acid protein that is part of the patatin-like phospholipase domain-containing family. While the true physiologic and biologic function of the protein encoded by this gene is unclear, it has been shown that this substitution results in increased triglyceride accumulation in the liver in murine models (He *et al.* 2010). It has been speculated that *PNPLA3* protein normally hydrolyzes triglycerides. When there is the I148M substitution, the protein loses its normal enzymatic activity and leads to accumulation of triglycerides in the liver, resulting in the downstream effects of alcoholic liver disease, alcoholic cirrhosis and hepatocellular carcinoma (He *et al.*, 2010).

Previous research has shown that *PNPLA3* gene polymorphisms are associated with predisposition to alcoholic liver disease in heavy drinkers. In addition, these gene polymorphisms have been shown to influence the severity of alcohol-related liver damage (Hassan *et al.*, 2013; Burza *et al.*, 2014; Salameh *et al.*, 2015). Our effect size is in line with previous reports and suggests that this finding is consistent across populations and clinical and non-clinical samples and could potentially be part of risk stratification in heavy drinkers to determine risk of developing alcohol-related liver injury.

Type 2 diabetes mellitus (T2DM) can accelerate the progression of fibrosis in the liver (Adams *et al.*, 2005). In addition, T2DM has been shown to increase the risk of development of cirrhosis of the liver (Adams *et al.*, 2010). Also, being overweight/obese and an elevated BMI can increase the risk of liver disease and its progression to end-stage liver disease in subjects who are heavy drinkers (Loomba *et al.*, 2009). In our sample, as expected, cases with ALD were significantly more likely to have diabetes as compared to the controls. We accounted for diabetes status in our cases and controls, and *PNPLA3* SNP rs738409 remained associated with ALD after accounting for T2DM status. Both BMI and obesity rates were similar between cases and controls.

Our study has to be viewed in light of some limitations. Firstly, this was a candidate gene analysis and not a genome wide association study. Thus, we had to make an *a priori* assumption of which SNPs to include in the study and were therefore unable to identify any new potential genetic associations with ALD. Nonetheless, this study provides another important replication of the association between PNPLA3 polymorphism and ALD in a clinical cohort of heavy drinkers. Second, our sample consisted only of subjects with white European ancestry, thus limiting the generalizability of the findings. Other studies have examined this association in subjects with different ancestry and found similar results (Tian *et al.*, 2010). Finally, our dataset was limited in terms of other alcohol consumption related data precluding the investigation of other potential investigations.

## CONCLUSION

In our case-control study of heavy drinkers with European ancestry, the *PNPLA3* rs738409 SNP was significantly associated with alcoholic liver disease. This study provides a replication of the previously identified association in a clinical sample of heavy drinkers from the USA, and it further adds to the growing literature implicating the association of polymorphisms in the *PNPLA3* gene contributing to a risk of ALD.

## SUPPLEMENTARY MATERIAL

Supplementary data are available at *Alcohol And Alcoholism* online.

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## CONFLICT OF INTEREST STATEMENT

None declared.

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