Biomarker and Genomic Risk Factors for Liver Function Test Abnormality in Hazardous Drinkers

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Background: Alcohol dependence and long-term excessive alcohol use may cause liver damage, but only some patients develop cirrhosis. Similarly, high alcohol intake without evident liver disease often but not always produces abnormal enzymatic liver function tests (LFTs), particularly gamma-glutamyl transferase (GGT). We postulate that the factors predisposing to cirrhosis in alcoholics and to liver enzyme abnormality in drinkers are similar, and that biochemical LFTs could therefore be useful as markers of risk of alcoholic liver disease in excessive drinkers.

Methods: Data from participants in twin and twin-family studies on alcohol use and dependence were used to identify 1,003 people who had reported excessive alcohol intake (28 drinks or more per week). A total of 962 of these provided blood for biochemical tests at the same time. Body mass index (BMI) and biomarkers of metabolic syndrome, inflammation, and iron stores were used in logistic regression with abnormality in serum GGT, alanine aminotransferase (ALT), or aspartate aminotransferase (AST) as outcomes. We conducted genome-wide association analyses for GGT, ALT, and AST separately in the group reporting excessive alcohol intake ($N = 951$) and a low-intake group reporting 14 drinks or fewer per week ($N = 8,716$), and compared results.

Results: Abnormal GGT and ALT among excessive drinkers were associated with higher BMI, triglycerides, insulin, uric acid, C-reactive protein, ferritin, and transferrin saturation; and with lower high-density-lipoprotein cholesterol. Abnormal AST was associated with triglycerides, ferritin, and transferrin saturation. ALT was significantly associated with variants at reported genetic loci for alcoholic liver disease (*PNPLA3*, rs738409, $p = 0.0076$; *TM6SF2*, rs10401969, $p = 0.0076$; *HSD17B13*, rs10433879, $p = 0.0024$).

Conclusions: Known risk factors for alcoholic cirrhosis including obesity and markers of metabolic syndrome, iron overload and inflammation are associated with liver enzyme abnormality in excessive drinkers.

Key Words: Alcohol, Alanine Aminotransferase, Aspartate Aminotransferase, Cirrhosis, Gamma-Glutamyl Transferase.

EXCESSIVE ALCOHOL USE is associated with risks including intoxication, development of addiction, organ damage, and increased mortality. The liver is particularly susceptible to alcohol-related damage, possibly because it is the main site of alcohol metabolism.

Elevation of the serum enzymes used as liver function tests (LFTs) is a common consequence of high alcohol intake. This is most evident for gamma-glutamyl transferase (GGT) but also occurs with alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Agarwal et al., 2016;

Conigrave et al., 2003; McDonald et al., 2013; Niemela et al., 2017; Rosalki et al., 1970; Whitfield et al., 1978). The dose–response relationships in the general population show increases in mean values and in the prevalence of abnormal values with increasing alcohol intake (Alatalo et al., 2009; Liangpunsakul et al., 2010; Sillanaukee et al., 2000; Steffensen et al., 1997; Whitehead et al., 1996; Whitfield et al., 2013). Mean differences between drinker and abstainer groups become apparent when alcohol intake is above 20 to 40 grams per day or 14 to 28 drinks per week (Whitfield et al., 2013).

There are similar dose-related effects of drinking on risk of alcoholic liver disease (Becker et al., 1996; Pequignot et al., 1978; Tuyns and Pequignot, 1984). Meta-analysis of data from 9 studies on cirrhosis (Corrao et al., 2004) showed that people consuming more than 25 grams of alcohol per day are at significantly increased risk compared to nondrinkers. However, not all alcoholics, or drinkers exceeding the thresholds for hazardous intake, show clinical or laboratory evidence of liver damage (Grant et al., 1988; Lelbach, 1976; Mann et al., 2003). Despite the statistical association between intake and cirrhosis, alcoholics with cirrhosis may have consumed similar amounts of alcohol to those who

have only steatosis (Grove et al., 1998) or no detectable liver disease (Whitfield et al., 2015). It has been estimated that over half of excessive drinkers develop fatty liver but less than 20% develop cirrhosis (Gramenzi et al., 2006). About half show abnormality of LFTs, particularly GGT (Conigrave et al., 2003). This suggests that there is variation in susceptibility to liver damage from alcohol, both in the occurrence of clinical problems and in the occurrence of test abnormality, and it would be useful to know whether abnormality of GGT, AST, or ALT in at-risk drinkers is a precursor and potential predictor of alcoholic liver disease.

Known risk factors for alcoholic liver disease include obesity and metabolic syndrome (Iturriaga et al., 1988; Naveau et al., 1997) and iron overload (Ganne-Carrie et al., 2000). A variant in the *PNPLA3* gene, known to be associated with fatty liver and with serum ALT activity, is associated with risk of alcoholic liver disease and cirrhosis (Tian et al., 2010). This was confirmed and additional loci near *TM6SF2* and *MBOAT7* were identified in a genome-wide study (Buch et al., 2015). A subsequent genome-wide study investigating loci which affect serum AST or ALT (Abul-Husn et al., 2018) found that a locus on chromosome 4 near the *HSD17B13* gene contains a protective variant affecting risk of both alcoholic and nonalcoholic liver diseases, including cirrhosis.

A combination of biomarker and genomic data from our twin/family studies on alcohol use in the general population allows us to address the causes of the observed variation in GGT, AST, and ALT in excessive drinkers. We focus on these because the dose–response relationships in our data between reported alcohol intake and other LFTs are weaker or biphasic (for bilirubin, alkaline phosphatase) or, for albumin, in the opposite direction to that expected (Whitfield et al., 2013). Our primary aim was to investigate whether liver enzyme abnormalities in at-risk drinkers who are not known to have significant liver disease at the time of blood collection are associated with risk of developing alcoholic cirrhosis later (assuming they continue drinking). We approach this by assessing whether and how far (i) the existence of liver enzyme abnormalities in an at-risk drinker and (ii) alcoholic cirrhosis share the same predisposing factors. The rationale is that if they do, then the group who show liver enzyme abnormality will overlap with those who develop cirrhosis, and the liver enzyme abnormalities may be useful as predictors, or as surrogates for clinical outcomes in studies of differences in vulnerability. To address this question, we first focus on obesity or metabolic syndrome and on iron overload, and then test whether genetic loci associated with alcoholic cirrhosis are important in determining the LFT response to alcohol in hazardous drinkers. Second, we aim to identify any novel risk factors or loci whose effects only become apparent under conditions of high alcohol exposure. We approach this by testing whether the biomarker associations and genetic causes of GGT, AST, and ALT variation in excessive drinkers are similar to those in low-risk drinkers.

MATERIALS AND METHODS

Participants and data were from our studies on the genetics of alcohol and nicotine dependence and on the biological consequences of excessive alcohol use. Initial contact was through a voluntary twin registry (the Australian Twin Registry), with invitations to participate later extended to twins' relatives and to their spouses and spouses' families. Patterns of alcohol use, psychiatric morbidity, and parental education (before selection on criteria outlined below) were consistent with those expected for the Australian population. All individuals gave informed consent, and the studies were approved by the appropriate ethics review committees.

Nicotine-Alcohol Study

The largest and most comprehensive source of data and samples was a study on the genetics of alcohol and nicotine use and dependence, which recruited twins who had participated in our previous studies on alcohol consumption and their spouses, parents, siblings, and adult children (Heath et al., 2011; Whitfield et al., 2013). This group of studies, each having the same questionnaire and sample handling protocols but differing in targeting of potential participants, took place between 2002 and 2005. The substudies focused on families of probands known (from previous information) to be (i) heavy smokers, or (ii) alcohol-dependent or scoring highly for a heaviness-of-drinking factor score, or (iii) from large sibships (with 4 to 14 full siblings). This group will be referred to as the Nicotine-Alcohol Study.

Information was collected in a telephone interview which included questions on lifetime and past-year alcohol or nicotine dependence, and on the usual quantity and frequency of alcohol use over the previous 12 months. Blood was collected between 2002 and 2005 from 9,031 people (3,998 men and 5,033 women) aged 18 to 92 (mean 51.6, SD 13.8) years. At the time of blood collection, participants reported on their alcohol intake and smoking over the past 7 days, their height and weight, and the time since their last meal. Alcohol and smoking data were compiled from the self-report diaries by summing the number of alcoholic drinks or the number of times tobacco products were used over the 7-day period. Anyone who used any tobacco products, including snuff and chewing tobacco, was characterized as a "smoker" but practically all tobacco use (98%) was as cigarettes. Body mass index (BMI) was calculated as kg/m^2 from self-reports of weight and height.

The participants lived throughout Australia, and blood samples were sent for processing to the Queensland Institute of Medical Research in Brisbane via courier for next-day delivery. After centrifugation, serum was obtained from tubes without anticoagulant and plasma from fluoride–oxalate tubes (for glucose measurement). Aliquots were stored at $-70°C$ until analysis. Serum ALT, AST, cholesterol, C-reactive protein (CRP), ferritin, high-density-lipoprotein cholesterol (HDL-C), GGT, glucose, iron, transferrin, triglycerides, and urate were measured using Roche reagents on a Roche 917 or Modular P analyzer (Roche Diagnostics, Risch-Rotkreuz, Switzerland). Serum insulin, hepatitis B surface antigen, and hepatitis C antibodies were measured using Abbott reagents on an Abbott AxSym analyzer (Abbott Diagnostics, Santa Clara, CA). We calculated low-density-lipoprotein cholesterol (LDL-C) using the Friedewald equation, and transferrin saturation from iron and transferrin.

Additional Studies

Additional data and samples, including information on alcohol use, biochemical test results, and DNA, were available from 2 earlier studies. The range of tests performed and the information on smoking status were less complete than for the Nicotine-Alcohol

Study described above. Data from these 2 additional studies were mainly used to maximize the number of people available for genome-wide association analysis in subgroups defined by alcohol intake (as described below). They also allowed us to assess repeatability across occasions for GGT, AST, and ALT results in excessive drinkers.

In the first of these additional studies, data on alcohol use and potentially comorbid conditions were collected using the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) interview (Bucholz et al., 1994). In the SSAGA Blood Study (Whitfield et al., 2002), which took place between 1993 and 1996, blood was collected from a subset of the interview participants comprising 1,048 male and 2,012 female twins between 28 and 89 years old (mean 44.4, SD 11.7). At the time of blood collection, participants reported on their alcohol intake over the past 7 days, their height and weight, and the time since their last meal. BMI was calculated from weight and height. Alcohol data were compiled from the self-report diaries by summing the number of alcoholic drinks over the 7-day period. Blood was processed and stored as described above, except that approximately a third of the samples were processed within 2 hours of collection rather than being sent to a central laboratory. Serum ALT, AST, and GGT were measured using Boehringer reagents on a Hitachi 747 analyzer (Hitachi Ltd, Tokyo, Japan).

The Anxiety Study took place between 1993 and 1996 and included 943 male and 1,533 female subjects, twins and their siblings, between 24 and 96 years old (mean 40.8, SD 11.2) at the time of blood collection. Serum ALT, AST, and GGT were measured using Roche reagents on a Roche 917 analyzer. Information on alcohol intake and smoking was taken from earlier questionnaire-based studies.

Genotyping

Genotype data were derived from several genotyping projects with Illumina 317K, 370K, 610K, Omni Express, or Infinium CoreExome chips (Illumina, Inc., San Diego, CA). After quality control of sample and single nucleotide polymorphism (SNP) data, imputation of SNP genotypes was performed using SNPs common to these platforms and haplotype data on ~65,000 chromosomes from the Haplotype Reference Consortium (http://www.haplotype-reference-consortium.org/, accessed 2018-02-13). Imputed SNPs with a minor allele frequency below 1%, or with the imputation quality measure R^2 <0.3, were excluded, leaving approximately 7.8 million autosomal and X-chromosome markers.

Data Analysis

Frequency distributions of the variables were checked and where necessary log-transformation was carried out (for ALT, AST, CRP, ferritin, GGT, insulin, triglycerides). Reported alcohol intake was transformed as $\log(N$ of drinks $+ 1)$ to allow inclusion of people who reported no drinks. Glucose and insulin results were adjusted for time between the last meal and blood collection, by creating intervals of fasting time and normalizing the results for each person by subtracting the mean and dividing by the standard deviation for that time period. Because of the possible effect of past or current hepatitis on LFTs, data from 13 people in the excessive drinking group with positive results for either hepatitis B or C tests were excluded.

Data analysis initially used IBM SPSS, release 22 (IBM Corp., Armonk, NY) for data management and estimation of means and correlations. However, because our studies emphasized twins and their families, there is a genetic overlap between many of the subjects. This means that, to the extent that family members are similar to each other for genetic reasons (which will vary according to the heritability of the phenotypes), the number of independent observations is less than the number of participants and the standard errors for calculated statistics will be underestimated. However, the estimates themselves, including means, correlation coefficients, and hazard ratios, are not changed; only the standard errors and therefore the confidence intervals and p-values are affected. To overcome this problem, we repeated the logistic regression analysis using Stata (StataCorp LLC, College Station, TX) with the "clustered robust standard error" option, grouping subjects by family to generate robust standard errors for the regression coefficients and for estimation of confidence intervals for hazard ratios.

Allelic association analysis for log-transformed GGT, AST, and ALT was done using an additive model in GEMMA (Genome-wide Efficient Mixed Model Association algorithm; Zhou and Stephens, 2012) with sex, age, BMI, number of drinks in previous week, smoking status, and 10 ancestry-informative principal components as covariates.

RESULTS

There were 11,985 people with data on alcohol intake and biochemical results from 1 or more of the 3 studies, of whom 1,003 reported alcohol intake ≥28 drinks/wk in any study. Of the 1,003, 962 had GGT, ALT, and AST results, and 951 had information on alcohol consumption, liver enzyme results from the time of reporting high alcohol intake, and genotyping. Because the Nicotine-Alcohol Study had the most comprehensive set of test results and self-report data, our main focus is on this.

Table 1 shows the characteristics of 8,382 participants from the Nicotine-Alcohol Study who had results for GGT, AST, and ALT, and who had provided data for the 7-day alcohol recall, and also for subgroups of 860 excessive drinkers who reported 28 drinks or more in the 7 days before blood collection and 4,908 low-risk drinkers who reported 14 drinks or fewer. Similar information is given for 3,060 participants in the SSAGA study and 2,351 participants in the Anxiety Study in Table S1; because some people participated in more than 1 study, the total number of results from Table 1 and Table S1 can be greater than the total number of participants.

Alcohol intake was assessed by the 7-day recall method, which has good repeatability across occasions, correlates with quantity–frequency measures, and is associated with all-cause mortality (Whitfield et al., 2004, 2018). The choice of the cutoff values of ≥28 for "excessive" drinking and ≤14 drinks per week for "low-risk" drinking for both men and women was based on the dose–response curves presented below.

Effect of Reported Alcohol Intake on Liver Enzymes

Participants in the Nicotine-Alcohol Study were divided into 6 alcohol intake groups based on the reported number of alcoholic drinks taken in the 7 days before blood collection (none, 1 to 7, 8 to 14, 15 to 28, 29 to 56, and over 56). The means and 95% confidence intervals for log-transformed GGT, ALT, and AST were calculated for each

Table 1. Characteristics of the Nicotine-Alcohol Study participants

| | All participants | | | | | | Excessive drinkers only | | | | | | Low-risk drinkers only | | | | | |
| | Male | | | Female | | | Male | | | Female | | | Male | | | Female | | |
	N	Median	90% range	N	Median	90% range	N	Median	90% range	N	Median	90% range	N	Median	90% range	N	Median	90% range
Age (years)	3,741	49	34 to 76	4,641	48	33 to 76	729	47	33 to 71	131	43	33 to 69	1,583	51	34 to 78	3,325	49	34 to 77
Total drinks (past week)	3,741	10	0 to 53	4,641	3	0 to 22	729	41	28 to 93	131	35	28 to 73	1,583	1	0 to 7	3,325	1	0 to 7
Current smokers (%)	3,741	20%		4,641	18%		729	37%		131	54%		1,583	15%		3,325	15%	
BMI (kg/m²)	3,741	26.6	21.4 to 34.6	4,641	25.4	19.8 to 37.1	729	26.6	21.2 to 34.1	131	25.8	19.7 to 36.1	1,583	26.6	21.2 to 35.2	3,325	25.7	19.8 to 37.8
GGT (units/l)	3,741	27	13 to 100	4,639	17	9 to 60	729	39	16 to 174	131	29	12 to 132	1,583	24	12 to 81	3,325	17	8 to 58
ALT (units/l)	3,741	26	13 to 61	4,641	18	10 to 40	729	29	15 to 75	131	20	11 to 72	1,583	25	13 to 59	3,325	18	10 to 40
AST (units/l)	3,741	25	17 to 43	4,641	21	14 to 34	729	27	18 to 58	131	24	16 to 55	1,583	25	16 to 40	3,325	21	14 to 34
Triglyceride (mmol/l)	3,740	1.89	0.86 to 4.85	4,640	1.44	0.69 to 3.46	729	1.92	0.84 to 5.77	131	1.43	0.76 to 5.31	1,583	1.86	0.88 to 4.61	3,325	1.5	0.69 to 3.56
HDL-C (mmol/l)	3,741	1.31	0.85 to 2.03	4,640	1.63	1.04 to 2.44	729	1.53	0.98 to 2.32	131	1.88	1.10 to 2.83	1,583	1.2	0.8 to 1.85	3,325	1.57	1.01 to 2.28
LDL-C (mmol/l)	3,535	3.33	1.94 to 4.90	4,565	3.15	1.88 to 4.83	671	3.44	2.05 to 4.92	123	2.98	1.57 to 4.76	1,508	3.26	1.86 to 4.86	3,270	3.19	1.92 to 4.89
Glucose (mmol/l)	3,034	4.9	3.7 to 8.6	3,765	4.6	3.6 to 7.0	535	5.0	4.0 to 8.9	93	4.8	3.9 to 6.2	1,286	4.91	3.64 to 9.30	2,668	4.62	3.50 to 7.22
Insulin (μmol/l)	1,046	60	14 to 371	1,099	57	18 to 265	129	52	13 to 324	129	50	16 to 250	158	112	26 to 495	354	70	23 to 297
Urate (μmol/l)	3,741	0.340	0.230 to 0.480	4,639	0.250	0.160 to 0.390	729	0.360	0.230 to 0.500	131	0.270	0.176 to 0.400	1,583	0.33	0.22 to 0.47	3,325	0.25	0.16 to 0.39
C-reactive protein (mg/l)	3,731	1.8	0.3 to 10.4	4,631	2.2	0.3 to 13.7	728	1.9	0.3 to 10.6	131	2.6	0.4 to 15.1	1,578	1.80	0.3 to 12.6	3,320	2.3	0.3 to 14.7
Ferritin (mg/l)	3,740	218	44 to 634	4,640	79	15 to 320	729	268	83 to 752	131	121	23 to 574	1,582	200	36 to 617	3,325	77	14 to 320
Transferrin saturation (%)	3,738	31	18 to 51	4,637	27	12 to 45	728	33	20 to 56	131	30	14 to 51	1,582	30.2	16.9 to 49.2	3,323	25.8	11.5 to 43.9

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma-glutamyl transferase. Excessive drinkers are defined as those who reported 28 drinks or more in the previous week at the time of blood collection, and low-risk drinkers as those who reported 14 drinks or fewer. The 90% ranges are based on the 5th and 95th percentiles for each variable.

group and then back-transformed to the original units. The dose–response relationships for men and women (Fig. 1) were essentially parallel, and there was no significant sex × alcohol group interaction for GGT, ALT, or AST. Based on data shown in Fig. 1, we defined excessive drinkers (for the purpose of identifying those at risk of abnormal liver enzymes) as those reporting ≥28 drinks in the previous week (40 grams alcohol per day or more), for both men and women.

Reference Ranges

A reference group was defined, using conservative criteria with respect to both alcohol and obesity, as people with low or no alcohol intake (reporting ≤7 drinks/wk) and also with normal BMI (≤25.0 kg/m²). The upper reference intervals (95th percentile) were estimated for each enzyme and separately for men and women. For GGT, ALT, and AST, these were 61, 44, and 36 units/l in men and 43, 32, and 32 units/l in women, respectively. These limits were used in the definition of abnormal GGT, ALT, and AST for logistic regression analysis.

Phenotypic Predictors of Abnormal Enzyme Results in Hazardous Drinkers

Using logistic regression to define the variables associated with abnormal enzyme results in the excessive drinkers (Table 2), we found that a cluster of variables related to obesity and metabolic syndrome, the 2 measures of iron status, and the inflammation marker CRP, each had significant effects. This pattern was consistent across the 3 enzymes, although the odds ratio estimates and p-values suggested stronger associations for GGT and ALT than for AST. The significant predictors at $p < 0.01$ were (in increasing order of p-values, i.e., starting with the most robust association) triglycerides, CRP, ferritin, BMI, insulin, transferrin saturation, urate, and glucose for GGT; triglycerides, ferritin, insulin, BMI, CRP, transferrin saturation, HDL-C, and LDL-C for ALT; and transferrin saturation, ferritin, triglycerides, and urate for AST. When analyzed separately, results were similar in men and women but the preponderance of men among the excessive drinkers led to less statistically significant associations in the women. The reported number of drinks, even in this group selected for high alcohol intake, showed significant associations with GGT and AST but smoking status (smoker or nonsmoker at the time of blood collection) did not. Multivariate analysis, in which all the postulated predictors were entered in order to identify independent effects, gave results shown in Table S2.

In excessive drinkers who participated in one of the other studies (Anxiety or SSAGA Blood) as well as in the Nicotine-Alcohol Study, enzyme levels showed significant repeatability across occasions (Fig. S1); results on one occasion predicted results on another.

Comparison of Predictors of Abnormal Enzyme Results in Excessive and Low-Risk Drinkers

Information on factors associated with abnormal results in low-risk drinkers (those reporting 14 drinks or fewer in the preceding 7 days) is shown in Table S3. Comparing these results with those for the excessive drinkers, we find that once again the obesity, and metabolic and lipid results are associated with GGT, AST, and ALT. Higher CRP, and ferritin but not transferrin saturation, is also associated with increased probability of abnormal enzyme results.

These results based on logistic regression, with the emphasis on classification of enzyme results as normal or abnormal, were supplemented by analyses based on correlation analyses (Table S4).

Candidate Genes and Genome-Wide Association Study

We assessed the effects of SNPs in *PNPLA3*, *TM6SF2*, *MBOAT7*, and *HSD17B13*, which have been shown to affect risk for alcoholic cirrhosis, on GGT, ALT, and AST results in excessive drinkers. These results are summarized in Table 3, and results for the same SNPs in low-risk drinkers are shown for comparison. In the excessive drinker group, variation at rs738409 at *PLPNA3* showed at least nominally significant ($p < 0.05$) association with ALT ($p = 0.0076$) and AST ($p = 0.018$); in each case, the less frequent G allele was associated with higher enzyme results. SNPs at *TM6SF2* and *HSD17B13* were associated with ALT ($p = 0.0076$ and 0.024, respectively) but not

AST; for rs10401969 at *TM6SF2* the less frequent C allele was associated with higher ALT but for rs10433879 at *HSD17B13* the less frequent C allele was associated with lower ALT results. None of these alcoholic cirrhosis loci significantly affected GGT in the excessive drinkers. Conversely, we used publicly available data to assess whether SNPs shown to affect ALT or GGT at $p < 5 \times 10^{-8}$ in the general population (Chambers et al., 2011) showed associations in the summary statistics of a genome-wide association study (GWAS) of alcoholic cirrhosis (Buch et al., 2015). No data were available for AST. Results are shown in Table S5. The only overlap after allowing for multiple testing was for the *PNPLA3* SNP rs738409 which affected ALT at $p = 1.17 \times 10^{-28}$ and alcoholic cirrhosis at $p = 1.54 \times 10^{-48}$; none of the significant GGT SNPs showed even nominal association with alcoholic cirrhosis.

After performing genome-wide association analyses for GGT, AST, and ALT separately in the excessive and low-risk drinking groups, no SNPs reached genome-wide significance in the excessive-drinkers group. Four loci for GGT and 1 for ALT were significant in the low-risk group. The 4 GGT loci were in or near the genes *HNF1A* (rs7979478, chr. 12, $p = 3.02 \times 10^{-15}$), *EXOC3L4* (previously *C14orf73*) (rs7151779, chr. 14, $p = 2.06 \times 10^{-12}$), *RORA* (rs340005, chr. 15, $p = 3.66 \times 10^{-9}$), and *GGT1* (rs28509371, chr. 22, $p = 1.84 \times 10^{-28}$), which had been recognized in previous studies. The significant locus for ALT was again at *PNPLA3* ($p = 2.68 \times 10^{-9}$ for rs3747207, which has $r^2 = 0.983$ with rs738409), and this SNP was also marginally significant ($p = 5.71 \times 10^{-8}$) for AST.
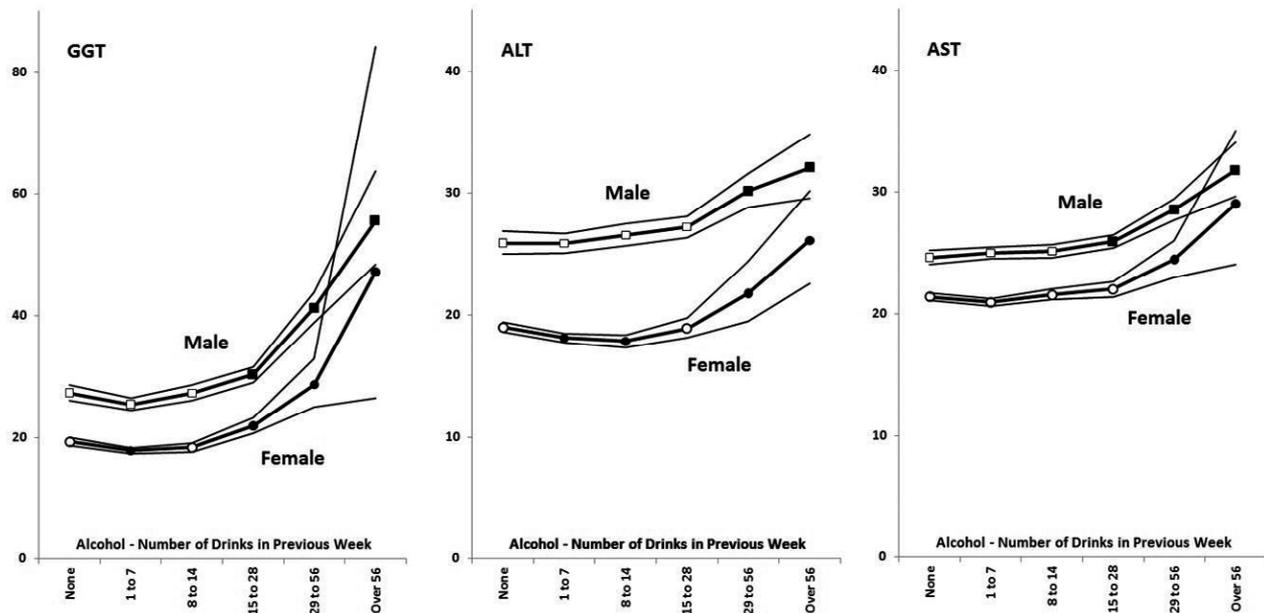

Fig. 1. Dose–response relationships for serum gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) activity by alcohol intake in men and women. Points and lines show geometric means and 95% CIs for those means, estimated from the means and standard errors for log-transformed enzyme activity and converted back to enzyme units for clarity. Note the different scale on the *y*-axis for GGT. Open squares (for men) and circles (for women) show groups where means do not differ significantly ($p > 0.01$) from the group reporting no drinks, and filled squares and circles show groups whose means do differ (at $p < 0.01$) from the no-drinks means.

Table 2. Results of Logistic Regression with Abnormality in GGT, ALT, or AST as the Outcome and Predictor Variables as Listed

Univariate	N	GGT OR	95% CI	p	AST OR	95% CI	p	ALT OR	95% CI	p
Age (years)	860	1.019	1.009 to 1.030	4.16×10^{-4}	0.996	0.983 to 1.010	0.617	0.989	0.975 to 1.003	0.114
Total drinks (log)	860	27.45	10.91 to 69.04	1.92×10^{-12}	8.043	2.937 to 22.02	4.91×10^{-5}	2.452	0.864 to 6.959	0.091
Nonsmoker/ smoker	860	1.314	0.977 to 1.768	0.070	0.925	0.664 to 1.290	0.646	1.000	0.715 to 1.399	1.000
BMI	860	1.107	1.062 to 1.154	1.67×10^{-6}	1.054	1.009 to 1.100	0.018	1.128	1.079 to 1.180	9.82×10^{-8}
Triglyceride (log)	860	16.61	8.856 to 31.16	$<1.0 \times 10^{-15}$	3.279	1.649 to 6.521	6.99×10^{-4}	11.18	5.222 to 23.93	4.97×10^{-10}
HDL-C	860	0.676	0.482 to 0.947	0.023	0.988	0.655 to 1.489	0.952	0.483	0.290 to 0.804	0.0051
LDL-C	794	1.145	0.957 to 1.371	0.139	1.167	0.932 to 1.462	0.180	1.374	1.103 to 1.712	0.0047
Glucose	628	1.256	1.075 to 1.467	0.0041	1.126	0.964 to 1.315	0.136	1.217	1.026 to 1.443	0.024
Insulin (log)	851	1.445	1.238 to 1.686	3.01×10^{-6}	0.972	0.804 to 1.176	0.772	1.604	1.368 to 1.881	6.25×10^{-9}
Urate (mmol/l)	860	44.84	6.144 to 327.3	1.77×10^{-4}	24.64	2.677 to 226.8	0.0047	38.26	4.193 to 349.1	0.0012
C-reactive protein (log)	857	2.974	2.158 to 4.098	2.74×10^{-11}	1.501	1.043 to 2.161	0.029	2.138	1.513 to 3.021	1.63×10^{-5}
Ferritin (log)	860	5.545	3.322 to 9.258	5.75×10^{-11}	7.156	3.492 to 14.67	7.45×10^{-8}	9.263	4.643 to 18.48	2.62×10^{-10}
Transferrin saturation (%)	859	1.026	1.013 to 1.040	1.23×10^{-4}	1.043	1.029 to 1.058	2.68×10^{-9}	1.026	1.013 to 1.040	1.04×10^{-4}

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase.
Excessive drinkers (28 or more drinks per week) only. Glucose and insulin are adjusted for time between blood collection and the last meal (fasting time).

To contrast the allelic association results in the 2 groups, we selected "suggestive" SNPs (with $p < 5 \times 10^{-6}$) for each enzyme either in excessive drinkers or in low-risk drinkers (listed in Table S6), and compared their effect sizes between the 2 groups. Results of these comparisons are shown in Figs S2–S4, restricted to SNPs with $p < 5 \times 10^{-7}$ for clarity. There are loci that appear to show differences in allelic effects between excessive and low-risk drinkers but none reach genome-wide statistical significance ($p < 5 \times 10^{-8}$) for heterogeneity, and the apparent differences in allelic associations between the 2 drinking groups may be due to the large number of variants tested.

DISCUSSION

Relationships Between Alcohol Intake and GGT, ALT, AST

All 3 enzymes showed increasing mean values as self-reported alcohol intake increased above 14 drinks per week or an average of 2 drinks per day. As we reported previously (Whitfield et al., 2013) and as expected from previous studies, the alcohol-induced change was greater for GGT than for ALT or AST. Our selection of 28 drinks per week (4 drinks or 40 grams per day) as a definition of excessive drinkers was based on several considerations. This was the lowest level of alcohol intake giving a significant difference in mean results from the no-alcohol group for all 3 enzymes and in both sexes. The epidemiological data on alcoholic liver disease risk (Becker et al., 1996; Corrao et al., 2004; Pequignot et al., 1978), and most previous data on liver enzymes in drinkers, are consistent with our alcohol/enzyme dose–response curves (Fig. 1). We chose to combine data from men and women into 1 analysis for each enzyme with separate cutoff values defining abnormality, rather than sex-specific analyses. The parallel curves for men and women and the absence of significant sex × alcohol group interaction support this approach for the enzyme results, even though sex differences in susceptibility are expected for alcoholic liver disease.

Quantitative Predictors of Liver Enzyme Abnormality Among Excessive Drinkers

Three groups of quantitative variables are postulated to affect risk of abnormal enzyme results: alcohol intake, variables associated with obesity and metabolic syndrome, and markers of iron status (ferritin, transferrin saturation). Higher reported amount of alcohol (even when above the 28 drinks limit) increased risk of abnormal results for all 3 enzymes. Obesity- or lipid-related phenotypes affected the risk of abnormal results; triglyceride for all 3 enzymes and HDL-C and BMI for 1 or more. Iron status (ferritin, transferrin saturation) affected risk of abnormality for all 3. In addition, CRP, a marker of inflammation, was strongly associated with abnormal results for GGT. Two of these 3 domains affecting enzyme abnormality, obesity, and its associated metabolic changes and iron status were predicted from published work on risk factors for liver disease in drinkers (Ganne-Carrie et al., 2000; Iturriaga et al., 1988; Majumdar et al., 1991; Naveau et al., 1997). Inflammation is also recognized as part of the pathological process leading to alcoholic liver disease (Gao and Tsukamoto, 2016; Wang et al., 2012), and both high CRP and positive correlations between CRP and GGT, AST, and ALT in excessive drinkers have been reported previously (Qu et al., 2016). From this pattern of associations, we conclude that the quantitative risk factors for alcoholic liver disease and for enzyme abnormality in excessive drinkers are similar.

Table 3. Allelic Associations for Serum GGT, AST, and ALT in Excessive Drinkers and in Low-Risk Drinkers, at Loci Which Have Been Shown to Affect Risk of Alcoholic Cirrhosis

Locus	SNP	Drinking	GGT $\beta \pm$ SE	AST $\beta \pm$ SE	ALT $\beta \pm$ SE
PNPLA3	rs738409	Excessive	0.02139 ± 0.01688, $p = 0.205$	0.02099 ± 0.00887, $p = 0.018$	0.03256 ± 0.01216, $p = 0.0076$
		Low-risk	0.00033 ± 0.00502, $p = 0.947$	0.01403 ± 0.00262, $p = 9.11 \times 10^{-8}$	0.02335 ± 0.00399, $p = 4.85 \times 10^{-9}$
TM6SF2	rs10401969	Excessive	0.04399 ± 0.02755, $p = 0.111$	0.02130 ± 0.01460, $p = 0.145$	0.05341 ± 0.01996, $p = 0.0076$
		Low-risk	-0.00655 ± 0.00797, $p = 0.412$	0.00358 ± 0.00417, $p = 0.391$	0.00336 ± 0.00634, $p = 0.596$
MBOAT7	rs626283	Excessive	0.00309 ± 0.01411, $p = 0.827$	-0.00445 ± 0.00744, $p = 0.549$	-0.00445 ± 0.01020, $p = 0.663$
		Low-risk	0.00171 ± 0.00413, $p = 0.679$	-0.00087 ± 0.00217, $p = 0.689$	-0.00072 ± 0.00329, $p = 0.827$
HSD17B13	rs10433879	Excessive	-0.02701 ± 0.01572, $p = 0.086$	-0.00921 ± 0.00830, $p = 0.268$	-0.03446 ± 0.01134, $p = 0.0024$
		Low-risk	-0.00814 ± 0.00468, $p = 0.082$	-0.00144 ± 0.00245, $p = 0.557$	-0.01155 ± 0.00372, $p = 0.0019$

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase.
Covariates were sex, age, number of drinks in previous week, body mass index, and smoking status (current vs. never or ex-smoker).

We also tested whether the known risk factors for alcoholic liver disease (obesity- and iron-related measures) affected the probability of abnormal liver enzymes in a group of participants who reported low-risk alcohol intake (defined for our purposes as people who reported 14 or fewer drinks in the week preceding blood collection). Similar to results in the excessive drinkers, risk of abnormal GGT, AST, and ALT results in the low-risk drinkers was associated with BMI, triglycerides, glucose, insulin, urate, CRP, and ferritin (Table S3).

Genetic Variation in Relation to Liver Enzyme Abnormality Among Excessive Drinkers

We next tested whether the small number of genetic variants previously reported to affect risk of cirrhosis in alcoholics had significant effects on GGT, AST, or ALT results in the excessive drinkers. Compared with a genome-wide approach, this diminishes the multiple-testing problem and allows a less stringent *p*-value. The only notable association was for SNPs in *PNPLA3* (Table 3), which are already known to affect ALT (Chambers et al., 2011) and also to affect risk for both alcoholic cirrhosis (Buch et al., 2015) and nonalcoholic fatty liver disease (Xu et al., 2015). Our results confirm that this locus affects ALT and AST in both excessive and low-risk drinkers.

A recent report (Abul-Husn et al., 2018) implicated rs72613567, an insertion–deletion variant in *HSD17B13*, in liver disease (including alcoholic liver disease and alcoholic cirrhosis) and also showed it is associated with variation in AST and ALT (but not GGT) levels. This variant was not imputed in our data but a close proxy (rs10433879, $r^2 = 0.958$) was available and it was nominally associated (at $p < 0.05$) with ALT in both excessive ($p = 0.0025$) and low-risk ($p = 0.0019$) drinkers but not with AST or GGT.

Turning to genome-wide association results, no SNPs reached the accepted significance level of $p < 5 \times 10^{-8}$ among the excessive drinking participants. However, the number of people in this group with genotype and enzyme data was 951 and although this is a substantial cohort given the precondition of excessive drinking, the power to detect effects at genome-wide significance is limited. Only loci accounting for more than about 4% of trait variance would be detectable with 80% power. The larger low-risk drinking group showed significant associations for GGT at 4 loci which are already known from population-based data without differentiation by alcohol intake (Chambers et al., 2011), and for ALT and probably AST at *PNPLA3*. Based on the number of subjects available, we did not find evidence for significantly different genetic effects on enzyme results in excessive and low-risk drinkers.

Implications

It would be useful to be able to conclude that factors affecting the liver enzymes also affect the development of

liver disease in people drinking too much, because the enzyme results might then serve as markers of future risk. Several older papers compared patients' GGT with their liver histology (Banciu et al., 1983; Frezza et al., 1989; Wu et al., 1976), finding a consistent association between higher GGT and structural abnormality. Despite a large literature on GGT and mortality, information related to liver disease mortality is sparse and even papers which report positive associations between mortality from liver disease and GGT (Breitling et al., 2011; Ruhl and Everhart, 2009) do not distinguish between alcoholic and other causes.

We found that phenotypic risk factors for alcoholic liver disease and for liver enzyme abnormality are similar. We also found that both the phenotypic and genetic influences on the enzyme results are similar between excessive (28 or more drinks per week) and low-risk (14 or fewer drinks per week) drinking groups. However, our results (and a literature-based comparison of GWAS findings for enzymes and for alcoholic cirrhosis) provide only limited support for shared genetic risk factors for abnormal liver enzyme results in excessive drinkers and for alcoholic cirrhosis, and mainly for ALT. Ideally, a prospective study which enrolled excessive drinkers without evidence of liver disease at baseline and used clinical liver disease as an end point is needed to assess the predictive value of GGT, AST, or ALT in relation to the development of alcoholic liver disease.

In relation to the use of enzyme results, particularly GGT, as markers of alcohol intake or of abstinence in alcoholics, the quantitative risk factors related to obesity and iron status can indicate which patients are more likely, or less likely, to have abnormal results while drinking. The fact that many of the influences on GGT are common to the excessive and low-risk drinking groups suggests that those who have low results while drinking will also have low results while abstinent, and reinforces the clinical value of within-person comparisons across time rather than a universal reference range.

This study is subject to a number of limitations. The number of people reporting excessive drinking is limited, which means that only substantial effects can be detected at the stringent p-values required for genome-wide genetic studies. The study is not prospective, so although we can say that certain characteristics are associated with abnormal enzyme results, we cannot claim that these characteristics are predictive or causative. We do not have data on the enzyme results in the same subjects during both low-risk and excessive drinking, for within-person comparisons. The results refer to liver enzyme abnormality and not directly to effects on alcoholic liver disease. Abnormal results may be signaling the presence of fatty liver, an early phenomenon reversible with abstinence, rather than irreversible changes, although at least for GGT there is previous evidence of association with fibrosis and cirrhosis in alcoholics. Some of these limitations, particularly for power, will be overcome in the foreseeable future: Information on the genetics of these liver enzymes (in the general population and in the subset of excessive drinkers) can be expected from UK Biobank data (http://biobank.ctsu.ox.ac.uk/˜bbdatan/biomarkers.pdf, accessed 2018-08-22). Prospective or nested case–control data on predictive performance will become more accessible with the spread of electronic medical records, and linkage of laboratory results to outcomes.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. GGT, ALT, and AST repeatability across occasions in excessive drinkers.

Fig. S2. Comparison of allelic effects on GGT in low-risk and excessive drinking groups, for SNPs which show $p < 5 \times 10^{-7}$ in either group.

Fig. S3. Comparison of allelic effects on AST in low-risk and excessive drinking groups, for SNPs which show $p < 5 \times 10^{-7}$ in either group.

Fig. S4. Comparison of allelic effects on ALT in low-risk and excessive drinking groups, for SNPs which show $p < 5 \times 10^{-7}$ in either group.

Table S1. Characteristics of the Nicotine-Alcohol Study participants.

Table S2. Multivariate logistic regression for abnormality in GGT, AST, and ALT results in excessive drinkers (28 drinks per week or more).

Table S3. Logistic regression results for 'low-risk' drinkers, up to 14 drinks per week.

Table S4. Comparison of correlations of selected variables with GGT, ALT, AST in excessive (XS) and low-risk (LR) drinkers.

Table S5. Loci, and lead SNPs, identified by Chambers et al. (Nature Genetics 2011;43:1131–1138) as significantly affecting plasma ALT or GGT; with allelic effects for these SNPs on risk of alcoholic cirrhosis as estimated by Buch et al. (Nature Genetics 2015;47:1443–1448).

Table S6. List of significant ($p < 5 \times 10^{-8}$) or suggestive ($p < 5 \times 10^{-6}$) SNPs for GGT, ALT, and AST, in either excessive or in low-risk drinkers.