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Brief Report: Genetics of Alcoholic Cirrhosis—GenomALC Multinational Study

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Background: The risk of alcohol-related liver cirrhosis increases with increasing alcohol consumption, but many people with very high intake escape from liver disease. We postulate that susceptibility to alcoholic cirrhosis has a complex genetic component and propose that this can be dissected through a large and sufficiently powered genomewide association study (GWAS).

Methods: The GenomALC Consortium comprises researchers from Australia, France, Germany, Switzerland, United Kingdom, and United States, with a joint aim of exploring the genetic and genomic basis of alcoholic cirrhosis. For this National Institutes of Health/National Institute on Alcohol Abuse and Alcoholism funded study, we are recruiting high-risk drinkers who are either cases (with alcoholic cirrhosis) or controls (drinking comparable amounts over similar time, but free of significant liver disease). Extensive phenotypic data are obtained using semistructured interviews and patient records, and blood samples are collected.

Results: We have successfully recruited 859 participants including 538 matched case-control samples as of September 2014, using study-specific inclusion-exclusion criteria and data collection protocols. Of these, 580 are cases (442 men and 138 women) and 279 are controls (205 men and 74 women). Duration of excessive drinking was slightly greater in cases than controls and was significantly less in women than men. Cases had significantly lower lifetime alcohol intake than controls. Both cases and controls had a high prevalence of reported parental alcohol problems, but cases were significantly more likely to report that a father with alcohol problems had died from liver disease (odds ratio 2.53, 95% confidence interval 1.31 to 4.87, $p = 0.0055$).

Conclusions: Recruitment of participants for a GWAS of alcoholic cirrhosis has proved feasible across countries with multiple sites. Affected patients often consume less alcohol than unaffected ones, emphasizing the existence of individual vulnerability factors. Cases are more likely to report liver disease in a father with alcohol problems than controls, consistent with a potential genetic component to the risk of alcoholic cirrhosis.

Key Words: Alcoholic Liver Disease, Genomewide Association, Cirrhosis, Genetic Risk Factors, High-Risk Drinkers.

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BACKGROUND

THE OVERALL RELATIONSHIP between excessive alcohol use and risk of liver damage is well established. Hepatic steatosis can be found on biopsy in most high-risk drinkers, but only a minority of these drinkers progress to alcoholic hepatitis, cirrhosis, or hepatoma. The reasons for this variation in susceptibility to liver damage, apart from quantity of alcohol and sex differences (Becker et al., 1996; Bellentani et al., 1997; Pequignot et al., 1978; Tuyns and Pequignot, 1984) and perhaps obesity (Iturriaga et al., 1988; Liu et al., 2010; Naveau et al., 1997), are unknown.

Some evidence for a genetic basis for alcoholic liver cirrhosis (ALC) comes from early twin studies (Hrubec and Omenn, 1981) and from the wide interethnic variation in mortality rates due to ALC (Caetano and Clark, 1998; Stinson et al., 2001). Many nonalcoholic liver diseases (non-ALDs) have been shown to have a genetic component, and loci affecting risk of hepatitis B or C infection, primary biliary cirrhosis, drug-associated hepatotoxicity, or nonalcoholic fatty liver disease (see <http://www.genome.gov/gwastudies/#searchForm>. Accessed May 27, 2014) have been identified by genomewide association studies (GWAS). One locus (*PNPLA3*, rs738409) discovered through GWAS for nonalcoholic fatty liver disease was also shown in independent studies to be associated with ALD severity and was an independent risk factor for ALC (Nischalke et al., 2011; Seth et al., 2010; Stickel et al., 2011; Tian et al., 2010; Trepo et al., 2011, 2012). However, several other candidate gene approaches to identify risk factors for ALC/ALD remain inconclusive (Stickel and Hampe, 2012). Moreover, no genomewide association approaches have yet been pursued to identify loci contributing to risk for alcoholic cirrhosis.

We have initiated a multicenter international program to collect DNA samples from thousands of high-risk drinkers, half of whom will have alcoholic cirrhosis (cases) while the other half will have no clinical evidence of significant liver disease (controls). We will conduct a GWAS by genotyping and comparing allele frequencies for single-nucleotide polymorphisms (SNPs) between the 2 groups to identify genetic factors that predispose drinkers to, or protect them against, alcoholic cirrhosis. We postulate that identification of genetic risk factors that predispose some drinkers to develop ALC may lead to an improved understanding of how alcohol damages the liver, strategies to prevent liver disease, and new treatment modalities.

This report summarizes our approach to establish the first international database of clinical and genetic data and biological samples from heavy drinkers with and without liver cirrhosis and provides descriptive data for the first 859 study participants from our data collection (recruited up to the end of September 2014).

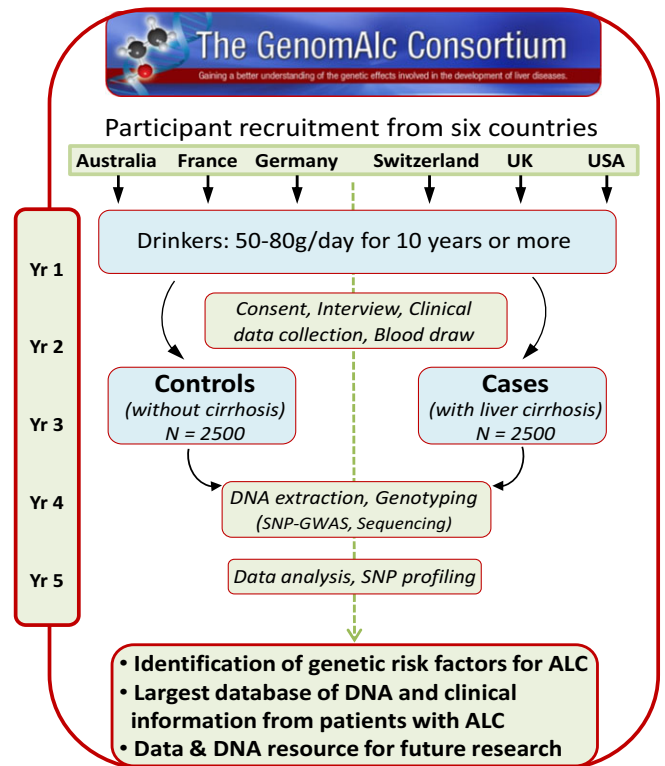


Fig. 1. GenomALC Consortium workflow to conduct genomewide association study (GWAS) for alcoholic cirrhosis. ALC, alcoholic liver cirrhosis; SNP, single-nucleotide polymorphism.

MATERIALS AND METHODS

Study Design

This case-control study is conducted by the international GenomALC Consortium comprising researchers from Australia, France, Germany, Switzerland, the United Kingdom, and the United States (Fig. 1). This multicenter study aims to recruit equal numbers of cases and controls, with approval from respective sites' institutional review boards, informed consent of the participants, and following National Institutes of Health (NIH) guidelines. All international sites began active recruitment of participants in September 2012 with an aim to collect a total of 5,000 participants, recruited both prospectively and from existing repositories of participating centers.

An important task in such association studies is a clear definition of the cases and controls. For ethical reasons, liver biopsy in alcoholics without clinical or biochemical evidence of liver disease is not justifiable. Consequently, we have adopted the approach used by prior genetic studies in ALD, namely to select control patients who have a history of heavy drinking but with normal liver tests and no current or prior evidence of cirrhosis nor, if liver biopsy has been performed, of any liver fibrosis. A control group of high-risk drinkers, rather than a control group from the general population, is necessary to clearly distinguish the genetics of alcoholic cirrhosis from the genetics of alcohol dependence.

Recruitment and Inclusion/Exclusion Criteria

Patients presenting to liver or alcohol treatment clinics who fulfill the recruitment criteria are invited to enroll in the study.

Consenting participants are given a unique de-identified study-specific number as the only identifier used for tracking samples. De-identified study-specific numbers are centrally generated as barcode labels and human readable codes and distributed to participating sites. The study inclusion–exclusion criteria require that participants must have had alcohol consumption averaging at least 80 g per day (for men) or 50 g per day (for women), for at least 10 years. This criterion is based on epidemiological evidence of the alcohol–cirrhosis relationship. The cutoff was set at a relatively high level so as to minimize the chance that cirrhosis was caused by factors other than alcohol. However, it is not essential for either cases or controls to meet the Diagnostic and Statistical Manual of Mental Disorders (DSM) criteria for alcohol dependence (American Psychiatric Association, 1994). In practice, most will do so, but the key criterion is prolonged high-risk alcohol intake rather than dependence per se. Cases have evidence of cirrhosis as per clinical signs and/or noninvasive transient elastography (Fibroscan®; Echosens, Paris) and/or histopathology by biopsy, with exclusion of hepatitis B or C, autoimmune liver disease, hemochromatosis, Wilson’s disease, and liver transplantation for liver disease other than ALC. Unequivocal evidence of cirrhosis is defined as imaging results (sonography, computed tomography, magnetic resonance imaging) compatible with cirrhosis together with detectable ascites by imaging or paracentesis and/or grade 2 or higher spontaneous hepatic encephalopathy and/or moderate or large esophageal varices on upper gastrointestinal endoscopy. Histological cirrhosis on biopsy is defined as Metavir fibrosis stage F4 or Ishak fibrosis stage 5 or 6. Controls have normal results for aspartate aminotransferase, alanine aminotransferase, total bilirubin, albumin, platelet count, and international normalized ratio while actively drinking or within 7 days of stopping, the most recent episode of heavy alcohol use, and no evidence of liver disease (clinical and/or Fibroscan® and/or histopathology showing F1, F2, F3, or F4 fibrosis, pericellular/intrasinusoidal fibrosis, perivenular fibrosis, or alcoholic hepatitis). HIV infection is an exclusion criterion for both cases and controls.

Instruments for Conducting the Study

We have developed standard operating procedures to maintain uniformity across sites for the operational and administrative steps to be followed during the study. These are distributed through the Consortium’s website www.genomalc.org. The website provides an overview of the study, which is open to all, and will provide a means for public dissemination of research outcomes as these emerge. The protocols and resources are restricted to researchers involved in the study. A data entry spreadsheet designed for the study records data collected through the questionnaires. The data entry design has built in formulae and conditional formatting that take into account the units and normal ranges of biochemical data that vary across the 6 countries and converts these into standard units. It also has built in warnings if data are out of range, incorrect, or misclassified. This design also assists in extracting data in a format that can be used for matching of cases and controls for age, gender, and ethnicity, and for extraction for data analysis (e.g., SPSS). To ensure that case and control groups overall are comparable for age and gender, we have adopted a stringent strategy to match cases to controls at the time of collection. For this, a semi-automated Excel-based algorithm is designed to match each case with a control for age, gender, and ethnicity. The system can match hundreds of controls to cases simultaneously, both site by site and globally. Participants with mismatched ethnicity are excluded from matching. Controls are matched for age within -3 to $+5$ years of cases. We have chosen to match the age at diagnosis for the cases to the age at study participation of controls.

Data and Sample Collection

Data collection uses a combination of face-to-face interview and medical-record sources. Data are collected regarding demographics, ancestry, alcohol and tobacco history, education, clinical symptoms, blood biochemistry, severity and date of diagnosis of cirrhosis, medication, drug use, anthropometrics, and other potential covariates that might affect development of cirrhosis (Table S1). We have set up a central database at the QIMR Berghofer Medical Research Institute (QIMR) in Brisbane, Australia, using existing facilities for managing epidemiological data with appropriate data security (backup, encryption, and access control). Data from the collection centers are uploaded after local entry and stored at QIMR. Each data file is checked by the site PI followed by the study coordinator to control quality of data received. Tools to review enrollment and matching of enrolled participants (case to control, male and female, age) are used to monitor recruitment monthly.

Blood is drawn and stored appropriately in specific tubes for DNA (essential), RNA, and serum/plasma (optional) extraction. All prospectively collected blood samples for DNA are shipped frozen to our laboratory in Indiana, USA, for central processing. DNA isolation is centralized to maintain the quality standard. Nanodrop measurements for 260/280 optical density ratio and concentration are used to determine the quality of DNA. If quality is not up to specification, DNA isolation is repeated from the remaining blood sample.

Sample Numbers, Power, and Effect Size

To determine the optimum case to control proportions for this study, we used Genetic Power Calculator (<http://pngu.mgh.harvard.edu/~purcell/gpc/>). The best power for any genotypic relative risk was obtained when the number of cases and controls were equal, reaching 86% (at a per-allele relative risk of 1.3) with 2,500 cases and 2,500 controls (Table S2, Model 1). Assuming a loss of up to 15% of the samples due to a variety of reasons (insufficient DNA quality, unavailability of phenotypic data, inappropriate matching with controls, etc.), Model 2 (Table S2) shows that $\sim 72\%$ power is retained to identify SNPs associated with a per-allele relative risk of 1.3. Our aim of obtaining a total of 5,000 participants, more than the minimum requirement of $N = 4,937$ for 80% power (Table S2), provides sufficient power to opt for genomewide SNP genotyping. We will prospectively recruit from the participating multinational sites 1,250 cases and 1,250 control samples during the first 4 years. We also have access to $>2,500$ retrospective samples from high-risk drinkers with or without liver disease from previous studies which we plan to include in the genotyping.

Once all samples have been collected, which is expected to be in 2016, we will utilize the most appropriate genomewide chip available to genotype the study population. We propose to undertake this in partnership with the Centre for Inherited Disease Research, a NIH-funded central facility that provides genotyping for investigators seeking to identify genes that contribute to human disease.

Clinical Data Analysis

The preliminary analysis presented on the first 859 subjects is to test the feasibility of our study design for recruitment across multiple centers internationally and to determine whether our recruitment strategy and criteria for defining cases and controls is valid. Statistical tests were performed using SPSS, version 22 (IBM Corporation, Armonk, NY). Because our main aim at this stage is to demonstrate equivalence between cases and controls, reported p -values are not adjusted for multiple comparisons.

RESULTS

Recruitment, Demographics, Alcohol Data, and DNA Quality

Initial screening identified 943 potential subjects (cases or controls) of which 16 did not meet the alcohol intake criteria, and another 25 have incomplete data. These are excluded from current analysis. The frequency distribution on the cohort profile is on the remaining 902 including $n = 43$ with non-Caucasian ancestry (Fig. S1A). The clinical analysis is performed only on those eligible for matching, $n = 859$ ($902 - 43$). So far, we have recruited more cases ($N = 580$) than controls ($N = 279$) and more males ($N = 647$) than females ($N = 212$). However, we have been successful in obtaining a similar ratio of males to females in the 2 groups (73% men among controls, 76% men among the cases). Clinical evidence of cirrhosis in cases was verified by the presence of ascites and/or grade 2 or higher hepatic encephalopathy and/or moderate to large esophageal varices. Our data show that 76% of the cases had ascites, 37% grade 2 or higher hepatic encephalopathy, and 57% esophageal varices. Eleven percent of cases had been diagnosed with hepatoma (in addition to cirrhosis), and 95% had at least 1 of these complications. This cohort is thus largely comprised of advanced liver disease with complications. The cohort predominantly consists of European Caucasian (>95%) participants, and in this cohort, approximately 63% (538/859) could be matched for age, gender, and ethnicity, identifying 269 case-control pairs (Fig. S1A).

DNA extracted from ~53% of the samples ($n = 455$) has resulted in good quality as shown by OD260/280 ratio and concentration (Fig. S1B). Approximately 1% of samples (5 of 455 extractions) that needed repeat processing also resulted in desired quality and concentration appropriate for genotyping for all samples tested so far. The distribution of DNA concentration across samples is shown in Fig. S1C.

Lifetime Alcohol Intake in Cases and Controls

Quantitative data on duration and amount of excessive drinking, allowing estimation of lifetime alcohol intake, were available for 895 of the participants. Cases and controls reported very similar duration of excessive drinking. For men, the mean duration was 25.4 years in the cases and 23.7 years in the controls and for women 19.4 and 18.7 years, respectively. The controls reported more alcohol intake per day than cases; the means for men were 213 g per day for cases and 256 g per day for controls and for women 158 g per day for cases and 218 g per day for controls. Male cases had consumed a mean lifetime amount of 1,965 kg of ethanol (EtOH, 1,073 kg in women), and controls had consumed significantly more (mean lifetime amount of 2,212 kg of EtOH for men and 1,495 kg in women). Details of mean and median amounts are shown in Table 1, and the cumulative frequency distributions for male and female cases and controls are shown for duration (Fig. S2A), estimated alcohol intake per day (Fig. S2B), and estimated lifetime alcohol

Table 1. Comparison of Alcohol Use History Between Cases and Controls

	Men						Women						Case vs. control: sex- and country-adjusted p -value								
	Case			Control			Case			Control											
	Mean	SD	N	SEM	Median	Mean	SD	N	SEM	Median	Mean	SD		N	SEM	Median					
Age started excessive drinking	26.2	9.3	442	0.4	24	24.3	9.1	205	0.6	22	29.8	10.6	138	0.90	30	27.6	9.9	74	1.1	26	0.0019
Years of excessive drinking	25.4	11.0	442	0.5	25	23.7	9.2	205	0.6	23	19.4	9.3	138	0.79	17	18.7	7.3	74	0.8	16	0.013
Total alcohol, g/d ^a	213	146	442	6.9	168	256	137	205	9.6	225	158	110	138	9.4	139	218	109	74	12.7	200	6.31×10^{-12}
Total lifetime alcohol, kg ^a	1,965	1,658	442	79	1,503	2,211	1,573	205	110	1,848	1,073	837	138	71	899	1,495	1,023	74	119	1,258	1.70×10^{-5}
Number of drinks in past 12 months	1,154	1,312	431	63	126	2,381	1,040	203	73	3,000	1,204	1,290	133	112	875	2,280	961	73	112	3,000	1.08×10^{-46}
AUDIT score	12	11	441	0.5	9	26	11	204	0.7	29	14	13	137	1.1	14	27	10	73	1.2	30	5.22×10^{-64}

AUDIT, the Alcohol Use Disorders Identification Test (AUDIT).

^a p -Values for total ethanol (EtOH) per day, total lifetime EtOH, and past-year number of drinks are based on log-transformed data. Data for total daily alcohol intake are grams per day for periods of excessive drinking.

Table 2. Family History of Alcohol Problems and Death from Liver Disease

(A) Prevalence of reported parental alcohol problems by case-control status				
	Yes	No	Percent yes	<i>p</i> -Value Odds ratio 95% CI
Did father have alcohol problems?				
Cases	239	312	43.4	<i>p</i> = 0.061 Odds ratio 1.33 95% CI 0.99 to 1.78
Controls	134	132	50.4	
Did mother have alcohol problems?				
Cases	88	469	15.8	<i>p</i> = 0.0078 Odds ratio 1.63 95% CI 1.14 to 2.34
Controls	64	209	23.4	
(B) Prevalence of reported parental death from liver disease, in parents with alcohol problems, by case-control status				
	Yes	No	Percent yes	<i>p</i> -Value Odds ratio 95% CI
Did father die of liver disease?				
Cases	52	160	24.5	<i>p</i> = 0.0055 Odds ratio 2.53 95% CI 1.31 to 4.87
Controls	13	101	11.4	
Did mother die of liver disease?				
Cases	14	62	18.4	<i>p</i> = 0.653 Odds ratio 1.25 95% CI 0.50 to 3.14
Controls	9	50	15.3	
(C) Logistic regression: case-control status by whether father or mother died of alcoholic liver disease (in subjects reporting that their father or mother, respectively, had alcohol problems); adjusted for age, sex, and log-transformed lifetime alcohol intake				
	<i>B</i>	SE (<i>B</i>)	<i>p</i>	Exp (<i>B</i>)
Father died of liver disease	0.983	0.343	0.0042	2.67
Mother died of liver disease	-0.231	0.474	0.626	0.79

intake (Fig. S2C). Past-year alcohol intake, estimated from the frequency and quantity questions in the Alcohol Use Disorders Identification Test (AUDIT), and the overall AUDIT score were both significantly lower in the cases than controls (Table 1). Case-control comparison by country, for grams of EtOH per day and lifetime kg of EtOH, confirms that amount of alcohol consumed per day and over lifetime was consistently higher for controls than cases at all recruitment countries (Fig. S3).

History of Alcohol Problems and Liver Disease in Parents

We asked participants about alcohol problems in their father or mother and whether their father or mother died from liver disease if they did have alcohol problems. The prevalence of alcohol problems was higher for the fathers than mothers; about half the participants reported problems in fathers and about a fifth reported problems in mothers. The prevalence of both paternal- and maternal-reported alcohol problems was slightly greater in the controls (Table 2A).

Taking only subjects who reported alcohol problems in a parent (because only those parents were at risk of alcoholic cirrhosis), the risk of cirrhosis is significantly ($p = 0.0055$) increased if a subject's father died of liver disease, but not

significantly if their mothers died from liver disease (perhaps due to lower numbers; Table 2B). However, the trends were in the same direction, with higher rates of reported liver deaths in both the fathers and the mothers of cases. Logistic regression, adjusted for sex, age at diagnosis, and lifetime alcohol intake, gave similar results ($p = 0.0042$ for father having died of liver disease if he had an alcohol problem, $p = 0.626$ for mother having died of liver disease if she had an alcohol problem; Table 2C).

DISCUSSION

While genomewide screening has been applied for numerous diseases including chronic liver diseases, such approach has been lacking for ALD until now. ALC has an enormous impact on the burden of liver disease; therefore, knowing its genetic background would be extremely valuable for both practical screening considerations and research purposes. As ALC is a disease linked to xenobiotic exposure, it is possible that relatively strong single polymorphism effects will be found, as in smaller studies where large and highly significant effects have been detected for single polymorphisms (Daly et al., 2009; Newton-Cheh et al., 2009). There are also examples where suggestive GWAS has resulted in a list of possibilities for further studies (Purcell et al., 2009). Our

GenomALC study design and approach have strong potential to generate new information on the genetic architecture and the heritability of ALC. Hence, conducting a GWAS in ALC seems timely and necessary.

To address this task, the GenomALC Consortium was established whose aims, design, and progress are described, and information provided on alcohol use in the first 859 case and control subjects enrolled to date. Several points emerge from this preliminary analysis. The cases were diagnosed with cirrhosis at a mean age of 52.6 for men and 49.7 for women, after a mean of 25.4 and 19.4 years of excessive drinking. However, the ranges were wide: 26 to 73 years for age and 10 to 57 years for excessive drinking. Moreover, there was a wide range of lifetime alcohol intake among the cases, suggesting variation in vulnerability. Admittedly, these estimates of lifetime intake are retrospective and may have substantial error, but the differences are striking, ranging 7-fold from 511 kg (10th centile) to 3,500 kg (90th centile) of EtOH intake to achieve cirrhosis. The data on lifetime alcohol intake in cases and controls across all recruitment sites confirm that it is perfectly possible to progress to cirrhosis after a lifetime alcohol intake, which is lower than that reported by control subjects without cirrhosis.

The cases also had lower past-year alcohol intake and AUDIT scores compared to the control group, which may be due to their recognition of the implications of continuing drinking for their liver disease. It could also be due to the inclusion of currently abstinent drinkers recruited under transplant settings in the case, but not in the control group. However, this does not affect our conclusion about lifetime alcohol consumption being no greater in the cases than in matched controls. Overall, our results strengthen the case for the existence of individual susceptibility factors in ALD.

It is possible that our case-control definition and recruitment instruments, in the absence of liver biopsy, may result in inclusion of some patients with fibrosis as controls; however, controls are highly unlikely to have cirrhosis. As cases are matched for age at diagnosis with controls, it is unlikely that factors (such as gene polymorphisms) that contribute significantly to the development of cirrhosis will be obscured by inclusion of some patients with fibrosis in the control group. We also acknowledge that it is possible, but uncommon, to develop ALC with alcohol intakes <80 g per day (men) and <50 g per day (women). Such patients would be at the extreme of individual risk, but do not meet the recruitment criteria for this study. We set these entry criteria to be confident that cases included in the study did indeed have alcohol-induced cirrhosis. The attribution of alcohol as the etiology for cirrhosis is less clear for individual cases at lower levels of consumption.

The association between case-control status and family history of an alcoholic parent dying from liver disease is a novel and potentially important finding. Although the most likely interpretation is that this is due to genetic transmission of risk, effects of shared environment or recall bias cannot be

excluded. At this stage, the effect is not statistically significant for maternal history. This result adds to the justification for performing a GWAS, assuming it is confirmed with the increased numbers as recruitment proceeds. We also found that slightly more parental alcohol problems were reported by controls than cases. For both groups, the rate was above that expected for the general population (Grant, 1997; Heath et al., 1997), consistent with previous studies showing familial aggregation and significant heritability of alcohol problems.

In conclusion, we report that through the establishment of our international GenomALC Consortium, it has proven feasible to recruit and collect data and samples from chronic high-risk drinkers for our overall aim to identify genetic loci for risk of ALC. In future, identification of relevant loci is expected to give insight into the mechanisms of ALD and may (depending on the size of allelic effects) allow identification of at least some high-risk people. In the meantime, our recruitment of a substantial number of people allows us to assess disease associations and potential risk factors using self-report, clinical, and laboratory data. Analysis of these aspects on the entire collection including this cohort is anticipated once recruitment is complete. A significant benefit of this study is the establishment of a central database of clinical characteristics and a biorepository of DNA from a large cohort of heavy drinkers, with consequent opportunities for future collaborations and research.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Study cohort and DNA profiles of GenomALC participants.

Fig. S2. Comparison of alcohol consumption in cases and controls.

Fig. S3. Case–control comparisons by country: (A) for grams of alcohol per day; (B) for lifetime kg of alcohol consumption.

Table S1. Data collection

Table S2. Power calculation and sample size at various relative risks