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Combined effect of ADH1B rs1229984, rs2066702 and ADH1C rs1693482/ rs698 alleles on alcoholism and chronic liver diseases

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Abstract. The aim of this study was to analyze the combined effect of the most frequent alcohol dehydrogenase polymorphisms (Arg48His and Arg370Cys in ADH1B, Arg272Gln and Ile350Val in ADH1C) on the alcohol use habits, alcohol dependence and chronic liver diseases in Hungary.
The study included men, aged 45–64 years. Altogether, 241 cases with chronic liver disease (CLD) and 666 randomly selected controls without CLD were analysed for all four polymorphisms. Associations between the polymorphisms, individually, and in combination, and excessive and problem drinking and CLD, were assessed using logistic regression.
In this study we have identified a novel mutation, called ADH1B Arg370His. The ADH1C Arg272Gln and Ile350Val showed almost complete linkage. The 272Gln/350Val allele increased the risk of excessive and problem drinking in homozygous form (OR = 1.582, $p = 0.035$, CI = 1.034–2.421, OR = 1.780, $p = 0.016$, CI = 1.113–2.848, respectively). The joint analysis showed that when combined with the wild type ADH1C Arg272/Ile350 allele, the ADH1B 48His is protective against CLD (OR = 0.368, $p = 0.019$, CI = 0.159–0.851).
The results obtained in the study help not only to clarify the effects of different ADH SNPs but to better understand how these polymorphisms modify each other's effects in the development of alcoholism and related diseases.

Keywords: Genetic epidemiology, genetic, case-control study, alcohol, chronic liver disease

1. Introduction

The burden of alcohol-related disease is especially high in the countries of Central and Eastern Europe [1]. In Hungary, the mortality from cirrhosis is more than 3 times higher than in Western European countries [2]. The risk of alcohol dependency (AD) and chronic liver diseases (CLDs), at an individual level, is affected by genetic factors [3].

Ingested alcohol is mainly metabolized in the liver, where the first step of oxidative degradation to acetaldehyde, is catalysed by the alcohol dehydrogenases (ADHs) mainly those in Class I [4]. Since the aldehyde dehydrogenase, catalysing the oxidation of acetaldehyde to acetate, has a low K_m, the acetaldehyde produced is eliminated shortly after being formed [5]. Class I ADHs consist of α, β and γ subunits, encoded by the genes ADH1A, ADH1B, and ADH1C. Single nucleotide polymorphisms (SNPs) have been described

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in both ADH1B and ADH1C that modify the enzymatic properties.

The ADH1B Arg48His allele (rs1229984) gives rise to the β_2 subunit with a 40-fold higher V_{max} in homozygous form than the β_1 subunit that is encoded by the wild allele. The ADH1B Arg370Cys (rs2066702) encodes the β_3 subunit. In its homodimer form, it has 30-fold higher V_{max} than β_1 [4,6]. The wild type ADH1C (γ_1 subunit) contains arginine at 272nd and isoleucine at 350th position, while the ADH1C rs1693482/ rs698 variant, which encodes the γ_2 subunit, contains glutamine (rs1693482) and valine (rs698), respectively. The V_{max} of γ_1 is 2–2.5 times higher than that of γ_2 [4]. These two SNPs are in high linkage disequilibrium [7]. Variants with higher activity (γ_1, β_2 and β_3) are considered to give rise to acetaldehyde accumulation via faster ethanol degeneration, leading to adverse effects such as facial flushing, nausea and tachycardia [4,7]. These effects may deter further drinking, but if individuals persist with alcohol consumption, hepatotoxicity and other tissue damage may occur [3,4,7].

Meta-analyses [8,9] have concluded that ADH1B Arg48 allele is associated with a significantly increased risk of alcoholism but is protective against liver disease only in Asian populations. ADH1C 272Gln/350Val allele seems to have little or no effect on alcoholism or liver disease in European populations. The difference between Asian and Caucasian populations may arise from the presence of different haplotypes [10].

The frequency of the polymorphic alleles varies among geographical regions. The ADH1B 48His allele is relatively rare in Caucasians with a frequency of 0–10% [6,11–15] except in Russia where one study found it in 41% [16]. This allele is more frequent among Asian populations, where its frequency varies from 50 to 90 percent, depending on ethnicity [15,17]. The ADH1B 370Cys allele is mainly found among African Americans, with a prevalence of 10–35% but it is rare or absent in Caucasian and Asian populations [6,10].

ADH1C 272Gln/350Val allele frequencies range from about 30 to 50% in Caucasians and strongly varies in Asian populations [6,11–14,17].

Although there has been considerable research among Caucasians on the effects of these SNPs in the recent years, only one study [18] analysed the combined effects of these polymorphisms, using only one outcome in each analysis, alcohol use or dependence. Moreover, almost all studies only investigated patients with alcoholic liver disease and alcoholic controls. In this study we describe not only the combined effect of the most frequent ADH polymorphisms both on CLDs, AD and alcohol use but with including non-alcoholic cases and controls, we can also assess the possible effect of these polymorphisms on CLDs in moderate or rare drinkers. The results help not only to an improved understanding of the high prevalence of chronic liver disease in Hungary but it may contribute to better understanding of how these polymorphisms modify each other's effects in relation to alcohol consumption and liver disease.

2. Materials and methods

2.1. Sample recruitment

Subjects were recruited from 57 general practices in four Hungarian counties (Györ-Moson-Sopron, Zala, Hajdú-Bihar and Szabolcs-Szatmár-Bereg) participating in the General Practitioners' Morbidity Sentinel Stations Program [19]. The present study was restricted to men, aged 45-64 years. Cases were all subject identified as having chronic liver diseases while controls were selected at random from those with no diagnosed CLDs. The case definition, described previously [20], is the presence of at least two of the following pre-specified criteria based on physical examination: spider naevi; ascites; palmar and plantar erythema; jaundice; enlarged, firm liver with rounded or nodular edge; and at least one of the following laboratory findings: increased level of serum bilirubin, elevated aspartate transferase activity, elevated alanine transferase activity, elevated gamma-glutamyl-transpeptidase activity, elevated alkaline-phosphatase activity, or decreased serum albumin. These physical and laboratory examinations were conducted both on case and control patients. Where the findings of the tests were contradictory with the previous diagnosis, patient was transferred to the proper group according to the test results. Among cases, more than 65% were previously diagnosed by hepatologists, where the image examinations (ultrasound, CT, MRI) and liver biopsy were used to confirm the diagnosis. In case of the newly identified cases, the diagnostic tests were conducted also by specialists. In our study only screening, not diagnostic examinations were carried out just to define the presence or absence of liver diseases. Patients with hepatitis B or hepatitis C infection were excluded from the analysis. The patients were not screened for Cytomegalovirus or Epstein-Barr virus infections in the framework of the study; their serological status was classified later, during the detailed diagnostic examinations. The final

study population contained 666 controls and 241 cases. The age distribution of controls was representative for the overall population ($p = 0.424$) and cases were significantly older than controls (55.17 years vs. 53.89 years, respectively, $p = 0.0023$).

EDTA anti-coagulated blood samples were taken by general practitioners for routine biochemical tests and genotyping. Written informed consent was obtained from each patient. The study was approved by the Regional and Institutional Ethics Committee, Medical and Health Science Center, University of Debrecen. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

2.2. Questionnaire-based data collection

Detailed information on alcohol consumption was gathered using a self-completed questionnaire, yielding the following variables.

Frequency of drinking, with seven possible outcomes: (i) has never drunk alcohol, (ii) didn't drink in the last twelve months, (iii) drinks less than once a month, (iv) drinks 1 to 3 times in a month, (v) 1 to 2 times in a week, (vi) 3 to 4 times in a week and (vii) at least 5 times in a week.

Summary drinking data, in 4 categories: (i) non-drinker; (ii) infrequent drinker, consuming alcohol less than 3 times per month, independently from the quantity of the consumed alcohol; (iii) moderate drinker, if consumption is at least weekly, the weekly total is 14 units or less, and the daily amount is never more than 5 units; (iv) heavy drinker, if more than 14 units are consumed weekly or if the amount more than 5 units is consumed on at least one day of a week (1 unit = 15 g pure ethanol).

CAGE score, from 0 to 4, according to the number of positive answers on the CAGE questionnaire, a widely used tool to detect problem drinking [21].

CAGE status is negative if the number of positive answers on the CAGE questionnaire is 0 or 1, and positive if it is 2 or more.

Patterns of alcohol use and demographic, financial and educational characteristics in this sample have been published previously [22]. In the present study we analysed data only obtained on samples genotyped for all the 4 polymorphisms.

Education is categorised from 0 to 3, where 0 signifies an individual with 8 years of education or less, 1 signifies having attended secondary school without doing the school leaving exam, 2 and 3 signify completed secondary and higher education, respectively.

Financial status (self-assessed) has 3 possible values: 0 (bad/ very bad), 1 (adequate) and 2 (good/ very good). This has been found to have greater validity than questions on income or wealth in studies in this region [23].

Demographic, financial and educational characteristics and alcohol use habits of the study population are shown in Table 1.

2.3. Genotyping

DNA was isolated from leukocytes with the use of MagNA Pure LC DNA Isolation Kit – Large Volume (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Genotyping was performed on LightCycler real time PCR System (Roche Diagnostics GmbH, Mannheim, Germany) by melting curve analysis.

The genotyping for ADH1B Arg48His (rs1229984) was performed as described previously [22]. ADH1C Arg272Gln (rs1693482) and Ile350Val (rs698) polymorphisms were screened together in a duplex reaction, while ADH1B Arg370Cys (rs2066702) was analysed separately. Sequencing, where needed, was conducted by Biomi Ltd., Hungary, with one of the adequate PCR primers. The LightCycler reactions were performed with LightCycler DNA Master HybProbe (Roche Diagnostics, GmbH, Mannheim, Germany, Cat. No. 12 015 102 001), according the manufacturers' instructions. The probes applied in the melting curve analysis were used in 0.2 μmol/l concentrations, while the primers were in 0.5 μmol/l concentrations. The sequence of the primers, probes and the reaction conditions are shown in the Table 2.

2.4. Statistical analyses on samples used for:

2.4.1. Single SNP analysis

Logistic regression calculating odds ratios (ORs) was applied to test the association between each genotype and the outcome variables, presence of chronic liver disease, frequency of drinking, summary drinking data, number of positive CAGE answers given and CAGE status. Results were adjusted for age, financial and educational status. Where variables were non-binary, ordered logistic regression was used which is appropriate to estimate the relationship between an ordinal dependent variable and a set of independent variables.

With the ADH1B Arg48His and the newly identified ADH1B Arg370His, the dominant model was used by virtue of the low number of homozygous mutant samples while for ADH1C Arg272Gln and Ile350Val the additive model proved the best fit.

Table 1
Demographic, educational, financial characteristics and alcohol use in the study population

		Controls	Cases
Age	Mean ± SD	53.89 ± 5.59	55.17 ± 5.49
Education			
Data availability (n)		659	239
		%	%
	8 years of education	21.85	28.87
	Secondary school without exam	42.64	49.37
	Secondary school	21.70	17.15
	Higher education	13.81	4.60
Financial status			
Data availability (n)		662	241
		%	%
	Bad/very bad	16.92	22.82
	Adequate	68.13	67.22
	Good/very good	14.95	9.96
Frequency of drinking			
Data availability (n)		644	236
		%	%
	Never	13.66	7.20
	Did not drink in the last 12 month	2.80	3.39
	Less than 1 time in a month	7.14	2.54
	1–3 times in a month	13.04	3.39
	1–2 times in a week	22.20	6.78
	3–4 times in a week	15.84	17.80
	At least 5 times a week	25.31	58.90
Summarised drinking data			
Data availability (n)		625	212
		%	%
	Non-drinker	20.16	13.68
	Infrequent drinker	11.20	4.72
	Moderate drinker	21.44	13.68
	Heavy drinker	47.20	67.92
Number of positive CAGE answers given			
Data availability (n)		538	201
		%	%
	0	55.20	15.42
	1	18.03	18.41
	2	10.59	19.40
	3	9.67	27.36
	4	6.51	19.40
CAGE status			
Data availability (n)		538	201
		%	%
	Negative	73.23	33.83
	Positive	26.77	66.17

2.4.2. Linkage analysis

To map the linkage between the SNPs, LD, D' and r^2 values were calculated using Haploview Software [24].

2.4.3. Multivariate SNP analysis

To detect the combined effect of the three main polymorphisms (48His, 272Gln, 350Val) and the possible epistasis between them, they were entered into a multivariate logistic regression model. Altogether, five groups were formed according to the genotype status of the polymorphisms investigated: the samples in the first group (named wild/wild) were homozygous wild for all three mutations and served as reference group; in the second (wild/heterozygous) they were wild for Arg48His and heterozygous for Arg272Gln and Ile350Val; the members of the third group (wild/mutant) were also wild for Arg48His, but homozygous mutant for Arg272Gln/ Ile350Val; the fourth group (heterozygous/wild) contained carriers for 48His and wild samples for Arg272Gln/ Ile350Val, while the fifth group (heterozygous/heterozygous) was formed from samples carrying the 48His allele and were heterozygous for Arg272Gln/ Ile350Val (Table 3).

Table 2

Primers, probes and conditions of the PCR reactions

	ADH1B Arg370Cys/His		ADH1C Arg272Gln		ADH1C Ile350Val	
Forward primer	CAACAAGCATGTGGGTTGTCTAA		CCCTCAAGACTACAAGAAACCCATT		CAGTCTGGAATGCAGCACT	
Reverse primer	CACTTGAATTTTAAATTTTCCTGAA		CAAGCCAGGTAACAAAAAGATGAC		TAGAATACAAAGCAAAACAAAAAAAC	
Sensor probe	AAAACGTCAGGACGGTACAGATAC		TGTCAAGCCGACCGATGACT		TAAAACATTTGTTATTAATGCATCCAGTGA	
Anchor probe	GCAATAGGAAAGAAGAGACATTGTGTTAACA		CAAACGAAAAATCCACACCTCCATCAGTC		CTTCTTAGCCATAAAGTCAGCCACAAGTTTG	
PCR Reaction	temperature (°C)	Time (sec.)	temperature	time	temperature	time
Initial Denaturation	95	60	95	60	95	60
Dentauration	95	0	95	0	95	0
10 x Annealing	58	15	58	15	58	15
Amplification	72	20	72	20	72	20
Dentauration	95	0	95	0	95	0
10 x Annealing	56	15	56	15	56	15
Amplification	72	20	72	20	72	20
Dentauration	95	0	95	0	95	0
20 x Annealing	54	15	54	15	54	15
Amplification	72	20	72	20	72	20

The sensor probes are labelled with fluorescein on their 3' ends. The anchor probes are labelled with LC640® for ADH1C Arg272Gln and LC705® for ADH1B Arg370Cys/His and ADH1C Ile350Val on their 5' ends.

Table 3
Genotypes of polymorphisms in groups used for multivariate SNP analysis

	ADH1B Arg48His	ADH1C Arg272Gln	ADH1C Ile350Val
First group (wild/wild)*	homozygous wild	homozygous wild	homozygous wild
Second group (wild/heterozygous)	homozygous wild	heterozygous	heterozygous
Third group (wild/mutant)	homozygous wild	homozygous mutant	homozygous mutant
Fourth group (heterozygous/wild)	heterozygous	homozygous wild	homozygous wild
Fifth group (heterozygous/heterozygous)	heterozygous	heterozygous	heterozygous

*served as reference type.

Any other combinations of these mutations and the other polymorphisms involved in this study were present in such a low number as to generate potentially ambiguous results, therefore they were excluded from the analyses. Logistic regression was used to measure the association between these genotype combinations and CLDs or alcohol consumption habits. Results are expressed with both 95% confidence intervals and p values at the 5% significance level. Data were analysed using STATA 10.0 statistical software (StataCorp LP, Texas, USA).

3. Results

Data on alcohol use and the demographic, financial and educational characteristics of the study population have been shown previously in Table 1.

3.1. Single SNP analysis

3.1.1. Identification of a new SNP variant

During the analysis of the ADH1B Arg370Cys (rs2066702) mutation an unexpected polymorphism was detected, causing approximately 2°C lowering in the melting temperature compared to the wild type (the probe was specific for the mutant type). Sequencing revealed this to be a recently identified gene variant in which the same amino acid is affected but with substitution of histidine instead of cysteine. This variant is Arg370His (rs75967634), caused by a G to A substitution in the 1193rd mRNA position. Figure 1 shows the results of the sequencing. Although this mutation has been submitted recently to PubMed's SNP database, no data are yet available on its frequency or its effect, so it was included in the further analysis. It was present in 1.98% of the study population.

3.1.2. Analysing the included SNPs

The allele frequencies and genotype frequencies of ADH polymorphic regions studied are shown in Table 4. All mutations examined were in Hardy-Weinberg equilibrium in both cases and controls.

Odds ratios were calculated by using data obtained on samples analysed for all 4 polymorphisms (Table 5).

Carriage of the ADH1B 48His allele was associated with significantly lower odds ratio for drinking frequency, the number of positive answers on CAGE assessment, and positive CAGE status. There was a significant association between ADH1B 48His and CLDs, but it disappeared after adjusting for CAGE status and scores (OR $= 0.61$, $p = 0.101$; OR $= 0.63$, $p = 0.123$, respectively).

The ADH1B 370His allele was not associated with any of the examined outcomes.

The ADH1B Arg370Cys was excluded from further analysis as a consequence of its low frequency ($< 1\%$).

The frequency of ADH1C 272Gln was almost the same as ADH1C 350Val and there were only two samples with different genotypes for these two mutations (Table 4) therefore they were excluded from further analysis and the effect of these polymorphisms were calculated together in the single SNP analysis. These two mutations showed no association with CLDs, but they were associated with increased odds ratio for frequency of drinking, summary drinking data and CAGE status. However, these associations were only significant when the mutations were homozygous.

The results of the single SNP analyses can be seen in Table 5.

3.2. Linkage analysis

The results of the linkage analysis and the frequency of the main haplotypes are shown in Fig. 2. As expected, there is a very high linkage between the two

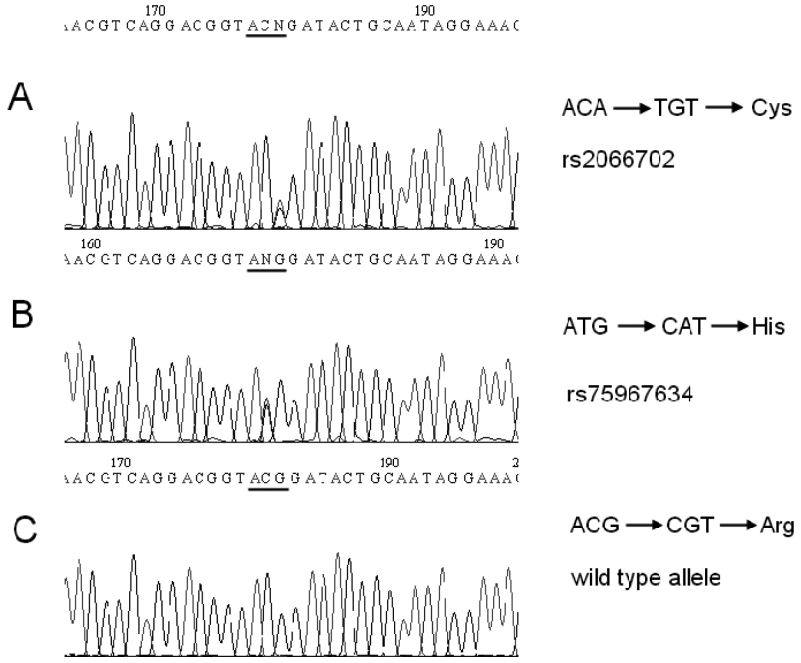


Fig. 1. The results of the sequencing of the ADH1B Arg370Cys/Arg370His polymorphism. Part A shows the coding sequence of a sample heterozygous for 370Cys, while on part B, the sequence coding a heterozygous 370His variant can be seen. Part C represents the wild type (homozygous for Arg370).

ADH1C mutations. The high D' combined with the low r^2 values in case of ADH1C 272Gln and 350Val versus ADH1B 48His indicate that the combination of wild-wild-mutant and mutant-mutant-wild alleles is more frequent in the population than could be expected from the allele frequencies themselves.

3.3. Multivariate SNP analysis

The results of the multivariate analysis are shown in Table 6.

For drinking habits, only the frequency of drinking was significantly associated with the heterozygous/wild group, with an odds ratio of 0.550 ($p = 0.011$, CI = 0.346–0.874).

There was no significant difference for either the summary drinking data or the number of positive CAGE answers.

Both wild/heterozygous and wild/mutant groups resulted in higher odds ratios for positive CAGE status (OR = 1.540, $p = 0.024$, CI = 1.058–2.241; OR = 1.859, $p = 0.014$, CI = 1.136–3.042). Interestingly, neither the heterozygous/wild, nor the heterozygous/heterozygous showed difference for CAGE status.

Significant association with CLDs was found only in case of groups containing the mutant allele for Arg48His. Both appeared to be protective, with odds ratios of 0.398 (heterozygous/wild, $p = 0.010$, CI = 0.197–0.806) and 0.461 (heterozygous/heterozygous, $p = 0.043$, CI = 0.218–0.975).

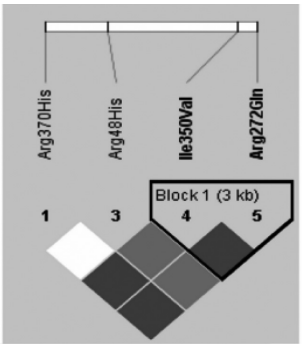
To clarify whether these combinations act directly on the risk of chronic liver disease or via drinking habits (i.e. CAGE status), these outcome variables were entered together into a logistic regression model to assess their effect when controlling for the other outcome variable. When CAGE status was used as outcome and the presence of chronic liver disease was adjusted for, the association with wild/heterozygous and wild/mutant groups remained significant (OR = 1.756, $p = 0.006$, CI = 1.171–2.633; OR = 2.204, $p = 0.004$, CI = 1.287–3.774). When CLD was used as a dependent variable and results were adjusted for CAGE status, only the effect of the heterozygous/wild group on CLD remained significant (OR = 0.368, $p = 0.019$, CI = 0.159–0.851).

To assess the possible effects of these combinations on CLDs among non-alcoholics, we entered them into a logistic regression model restricted only to CAGE negative cases and controls. Only the heterozygous/wild group showed significant association with CLDs (OR = 0.116, $p = 0.039$, CI = 0.015–0.900).

Table 4
Genotype and allele frequencies of the polymorphisms studied

	Allele frequency				Genotype frequency					
	wt		mt		wt/wt		wt/mt		mt/mt	
	Cases	Cont.	Cases	Cont.	Cases	Cont.	Cases	Cont.	Cases	Cont.
ADH1B Arg48His (rs1229984)	0.956	0.914	0.044	0.086	0.913	0.832	0.087	0.165	0.000	0.003
ADH1B Arg370His (rs75967634)	0.990	0.990	0.010	0.010	0.979	0.981	0.021	0.020	0.000	0.000
ADH1B Arg370Cys (rs2066702)	1.000	0.999	0.000	0.001	1.000	0.997	0.000	0.003	0.000	0.000
ADH1C Arg272Gln (rs1693482) / Ile350Val (rs698)	0.613	0.613	0.387	0.387	0.375	0.367	0.475	0.492	0.150	0.141

wt = wild type, mt = mutant type, cont. = controls.



Marker 1	Marker 2	D'	LOD	r^2
Arg370His	Arg48His	0.057	0.1	0
Arg370His	Ile350Val	1	3.85	0.016
Arg370His	Arg272Gln	1	3.87	0.016
Arg48His	Ile350Val	0.859	8.2	0.038
Arg48His	Arg272Gln	0.858	8.11	0.037
Ile350Val	Arg272Gln	1	387.06	0.995

Fig. 2. The results of the linkage analysis. The upper part shows the LD plot, where the intensity of the colour indicates the D'/LOD values. The lower part contains the D', LOD and r^2 values between the SNPs.

4. Discussion

Although there have been recent declines, death rates from chronic liver diseases and cirrhosis remain much higher in the majority of the Central-Eastern European countries – among them Hungary – than in the countries of western Europe. In previous years, the potential role of the quality of alcohol consumed in this region has been examined [25,26] but potential genetic factors have received less attention. Although research has been conducted in other European countries [12–14,16, 18,27–32], the combined effects of the main polymorphisms remain to be clarified. In a previous study [22], we have taken a first step by mapping the prevalence of ADH1B Arg48His in Hungary. In this study, our goal was not only to wider this investigation to other, also important SNPs, but to examine their joint impacts on chronic liver diseases, alcohol dependence and alcohol drinking habits not only among alcoholic cases and controls but among moderate and rare drinkers as well. The results of this complex analysis begin to offer insights into the genetic background of alcohol consumption and alcohol-related diseases not only among Hungarians but among other Caucasian populations.

An interesting, unexpected finding of our study was the identification of a novel mutation, the ADH1B Arg370His (rs75967634), and assessed its frequency. This polymorphic sequence has already been submitted into the PubMed's SNP database, but without any information on the population studied, its frequency, or possible effects. Because of its close proximity to ADH1B Arg370Cys, it cannot be detected with either the original hybridization probe assay [33] or with newer methods such as Illumina GoldenGate or Sequenom MassArray techniques and RFLP. A highly specific melting point analysis as used in this study is required to detect

Table 5

The results of single SNP analysis

		Chronic liver diseases		Alcohol drinking frequency		Summarised drinking data		Number of positive CAGE answers given		CAGE status	
		OR (CI)	p	OR (CI)	p	OR (CI)	p	OR (CI)	p	OR (CI)	p
ADH1B rs1229984	c	**0.474 (0.287–0.781)**	**0.003**	**0.625 (0.453–0.864)**	**0.004**	0.850 (0.591–1.222)	0.380	**0.522 (0.346–0.788)**	**0.002**	**0.531 (0.330–0.853)**	**0.009**
ADH1B rs75967634	c	1.046 (0.361–3.033)	0.934	2.179 (0.887–5.351)	0.089	2.710 (0.880–8.346)	0.082	0.776 (0.317–1.901)	0.579	0.652 (0.224–1.897)	0.432
ADH1C rs1693482/ rs698	ht	0.906 (0.650–1.261)	0.557	1.118 (0.862–1.449)	0.401	1.021 (0.771–1.352)	0.885	1.282 (0.953–1.724)	0.101	1.350 (0.960–1.897)	0.084
	mt	0.956 (0.599–1.525)	0.851	**1.513 (1.045–2.192)**	**0.028**	**1.582 (1.034–2.421)**	**0.035**	1.412 (0.942–2.116)	0.094	**1.780 (1.113–2.848)**	**0.016**

c = carriers, ht = heterozygous, mt = homozygous mutant.
In case of genotypes, homozygous wild types were regarded as reference types, while for drinking habits, they were the following: never, non-drinker, 0 and negative, respectively.
Significant results are marked with bold type.

Table 6

The results of the multivariate SNP analysis

	Chronic liver diseases		Alcohol drinking frequency		Summarised drinking data		Number of positive CAGE answers given		CAGE status	
	OR (CI)	p	OR (CI)	p	OR (CI)	p	OR (CI)	p	OR (CI)	p
wild/ heterozygous	0.813 (0.568–1.162)	0.256	1.017 (0.761–1.361)	0.907	0.978 (0.716–1.336)	0.890	1.338 (0.918–1.694)	0.078	**1.539 (1.058–2.241)**	**0.024**
wild/mutant	0.823 (0.508–1.334)	0.430	1.281 (0.869–1.888)	0.211	1.518 (0.974–2.367)	0.065	1.353 (0.888–2.063)	0.160	**1.859 (1.136–3.042)**	**0.014**
heterozygous/ wild	**0.398 (0.197–0.806)**	**0.010**	**0.550 (0.346–0.874)**	**0.011**	0.823 (0.492–1.378)	0.459	0.703 (0.389–1.271)	0.244	1.014 (0.494–1.664)	0.967
heterozygous/ heterozygous	**0.461 (0.218–0.975)**	**0.043**	0.760 (0.466–1.239)	0.270	0.983 (0.563–1.717)	0.951	0.570 (0.306–1.061)	0.076	0.490 (0.221–1.090)	0.080

Samples were homozygous wild for both ADH1B Arg48His, and ADH1C Arg272Gln and Ile350Val (wild/wild) were regarded as reference types.
The first part of the groups' name indicates the sample's genotype for ADH1B Arg48His, while the second means the genotype for both ADH1C polymorphisms.
Significant results are marked with bold type.

it. Although we did not find any significant association with alcohol drinking habits and liver diseases, there may be possible associations that could not be identified in the present study because of its low prevalence. Consequently, the effect of this SNP needs further investigation, including studies in other populations.

The results for ADH1B Arg48His in the samples used in this study show concordance with the findings of our previous study [22], as was expected.

The two main polymorphisms of ADH1C, the Arg272Gln and Ile350Val have an almost complete linkage between each other, as it was expected from previous studies [34]. However, the results regarding the effect of these mutations are not so obvious among Caucasians. Although previous meta-analyses and other studies [8,9,13,14] did not find an association between these alleles and alcoholism or liver disease, some researchers have described associations with alcohol drinking habits and/or alcoholism among Europeans [18,30,32]. However, while our results confirm the lack of association with CLDs, we did find a significantly increased OR for problem drinking (i.e. positive CAGE status) and heavy drinking in ADH1C 272Gln/350Val homozygous patients. This ambiguity may be due to the differences in categorizing drinking habits in different studies and the relatively minor effect of this genotype. Our results of the linkage disequilibrium completely concur with previous studies [11,14, 18], which showed that ADH1B 48His allele is associated with the Arg272/Ile350 ADH1C allele.

To clarify the independent effect of the main polymorphisms of ADH1B and ADH1C, a multivariate analysis was conducted. When CLD and alcohol use habits were used as an outcome variable without controlling for each other, the results were what were expected on the basis of the single SNP analysis, except that being heterozygous for the ADH1B 48His allele did not reduce the odds of problem drinking. This can be due that this allele has an adverse effect on CAGE status in cases and controls, e.g. in cases it did not proved to be protective against CAGE positivism ($p = 0.371$). Combined with the relatively low number of subjects in these categories it can result in the lack of significant association.

Interestingly, when CAGE status was used as an outcome variable and adjustment was made for the presence of CLD, only the association with homozygous ADH1B Arg48 status remained significant. When CLD was the outcome variable and CAGE status was controlled for, the association with carriage of ADH1B 48His and homozygous for ADH1C Arg272/Ile350 remained significant. These results suggest that it is reasonable to suppose that the polymorphisms of ADH1C have a direct impact only on drinking habits. The outcome of the single SNP analyses also supports this hypothesis. On the contrary, while the ADH1B 48His allele seemed to impact only on drinking habits on the basis of previous individual analyses [22], these results suggest that the absence of the controversial ADH1C 272Gln/350Val alleles are significantly protective against chronic liver disease. This is also supported by the significantly protective effect against CLDs of the group that was heterozygous for ADH1B 48His allele and homozygous for ADH1C Arg272/Ile350 alleles in non-alcoholics (i.e. negative CAGE status). However, this hypothesis needs to be confirmed by enzymatic tests to assess enzyme activity and the consequent alcohol elimination rate in subjects with these allelic combinations or by other case-control studies involving non-alcoholic cases and controls as well.

It is important to recognize that our study may underestimate the real effect of the genotypes studied as we have no data about individuals who have stopped drinking after being diagnosed with liver disease. Nevertheless, this work represents an important step in determining the genetic background of alcoholism and chronic liver diseases in Caucasians as it is one of the most complex studies conducted in Europe, involving a remarkably high number of cases and controls and yielding novel and interesting findings, including new interactions between ADH SNPs, alcohol dependence and CLDs.

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