THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The effect of alcohol dehydrogenase gene polymorphisms on alcohol consumption and chronic liver diseases in Hungary

By Reka Toth

Supervisor: Roza Adany, MD, PhD, DSc

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF HEALTH SCIENCES

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Table of contents

List of abbreviations

AD: alcohol dependence

ADH: alcohol dehydrogenase

ALD: alcoholic liver disease

CAGE: **C**ut, **A**nnoyed, **G**uilty, **E**ye-opener

CEE: Central-Eastern European

CI: confidence interval

CLD: chronic liver disease

CYP450: cytochrome P450

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

EDTA: ethylenediaminetetraacetic acid

GP: general practitioner

GPMSSP: General Practitioners' Morbidity Sentinel Station Program

HNE: 4-hydroxy-2-nonenal

ICD: International Classification of Diseases

LC: LightCycler

LD: linkage disequilibrium

MAA: malondialdehyde-acetaldehyde adducts

MDA: malondialdehyde

MEOS: microsomal ethanol oxidizing system

NAD: Nicotinamide adenine dinucleotide

OR: odds ratio

PCR: polymerase chain reaction

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

SD: standard deviation

SDR: standardized death rates

SNP: single nucleotide polymorphism

TNFα: tumor necrosis factor-alpha

RFLP: restriction fragment length polymorphism

Magyar nyelvű összefoglaló

Bár az utóbbi években a krónikus májbetegség és cirrózis standardizált halálozási aránya csökkenést mutatott, a betegség által okozott korai halálozás Magyarországon még mindig jelentősen magasabb, mint Nyugat-Európában. Bár a krónikus májbetegségeknek az alkoholfogyasztás a legfőbb meghatározója, a kockázat kialakításában egyértelműen részt vesz a genetikai meghatározottság is. Célunk volt, hogy felmérjük a leggyakrabban előforduló alkohol dehidrogenáz (ADH) polimorfizmusok (Arg48His és Arg370Cys az ADH1B génben, Arg272Gln és Ile350Val az ADH1C génben) kombinált hatását az alkoholfogyasztási szokásokra, alkoholfüggőségre és krónikus májbetegségekre Magyarországon. A vizsgálat során 241 májbeteg és 666 véletlenszerűen kiválasztott, 45-64 éves férfi bevonásával történt. A genotipizálás után a polimorfizmusok egyedi és kombinált hatását a mértéktelen és problémás alkoholfogyasztásra és a májbetegségre logisztikus regresszióval vizsgáltuk.

A vizsgálat során azonosítottunk egy új mutációt, az ADH1B Arg370His-t. Az ADH1B*2 allél jelenléte szignifikánsan csökkentette az esélyhányadost (EH) az alkoholfogyasztást leíró változók esetében (alkoholfogyasztás gyakorisága, alkoholizmus a CAGE kérdőív szerint). Szignifikánsan csökkentette az allél a májbetegségek kialakulásának az esélyét is (EH=0.47; p=0.003), de ez az összefüggés eltűnt az alkoholfogyasztási szokásokra történő korrigálás után. A nagyivók között ez az allél nem növelte a májbetegségek kialakulásának esélyét. Az ADH1C Arg272Gln és Ile350Val majdnem teljes kapcsoltságot mutatott. A 272Gln/350Val változat homozigóta formában növelte a gyakori ivás és az alkoholizmus esélyét (EH=1.51, p=0.028, EH=1.78, p=0.016, rendre). A kombinált analízis azt mutatta, hogy az ADH1B 48Arg allél csökkenti a májbetegség kialakulásának az esélyét, de csak a vad típusú ADH1C Arg272/Ile350 alléllal kombinálva (EH=0.37, p=0.019).

Ezek az eredmények nem csak a legfontosabb ADH polimorfizmusok egyéni hatását segítenek megérteni, hanem azt is, hogyan módosítják egymás hatását a krónikus májbetegség és az alkoholizmus kialakulására a magyar lakosság körében. Ez segítheti a helyi viszonyoknak megfelelő népegészségügyi programok kialakítását, ezáltal a krónikus májbetegség magas halálozásának csökkentését.

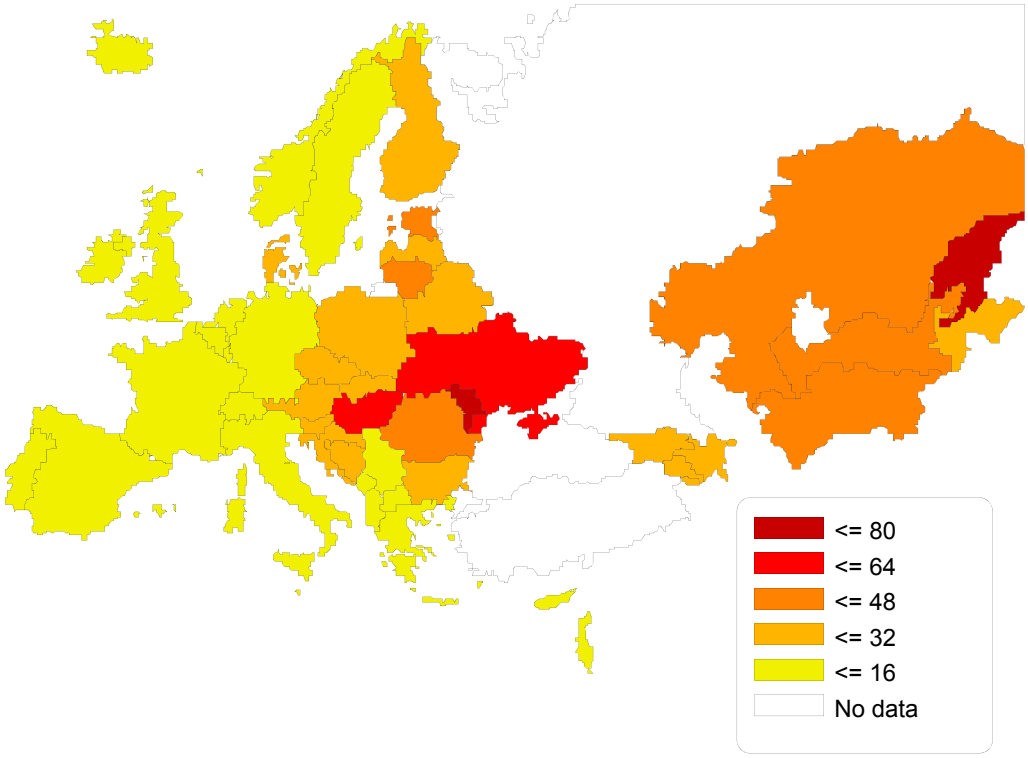
Introduction

Epidemiology of liver diseases

Although standardized death rates have recently declined, premature mortality from chronic liver disease and cirrhosis (Figure 1.) remains markedly higher in the countries of Central and Eastern Europe – especially in Hungary – than in their Western European neighbors.

Figure 1.

Standardized death rates (SDR) chronic liver disease and cirrhosis, 0-64 years, per 100000, male, last available

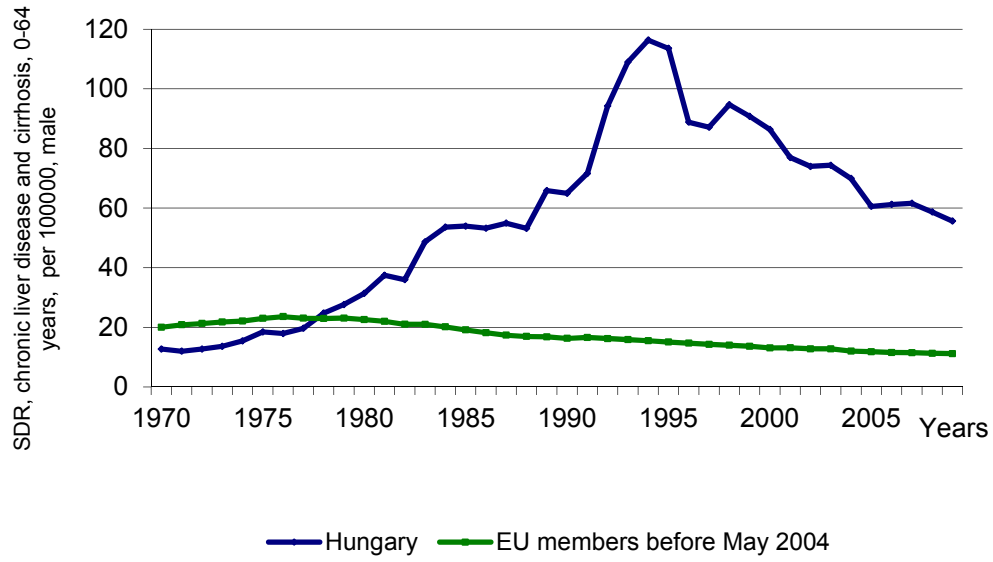


Source: WHO, European health for all database (HFA-DB), updated in 2011 January [1]

The mortality from chronic liver diseases (CLDs) and liver cirrhosis is particularly high among Hungarian males; at its peak in 1994, it was over five times higher than the rate seen in Western European countries at any time in the previous decades (Figure 2).

Figure 2.

SDR for chronic liver disease and cirrhosis, 0-64 years, per 100000, male

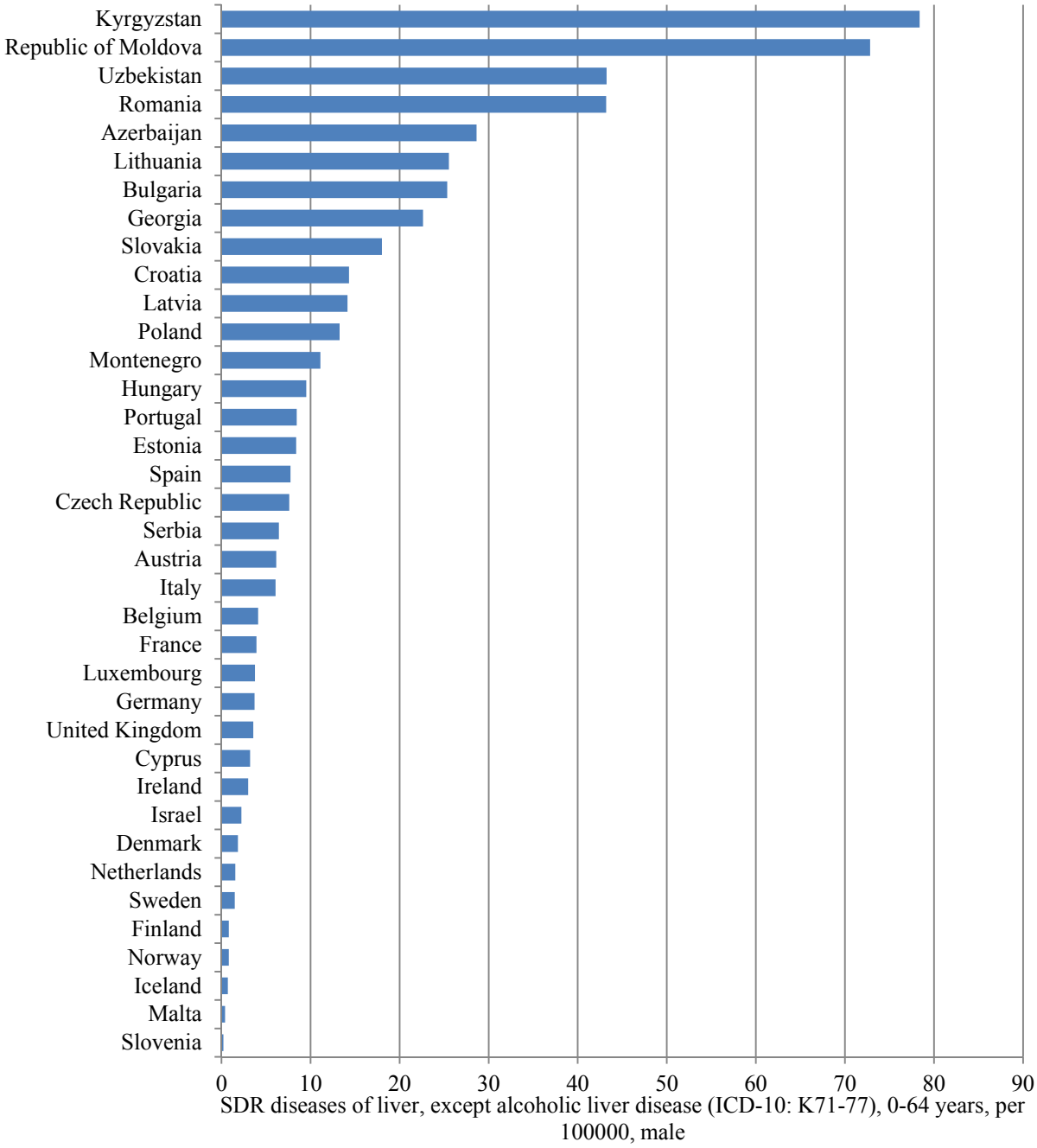


Source: WHO, European health for all database (HFA-DB), updated in 2011 January [1]

Alcohol is the major cause of liver cirrhosis in the Western world [2]. According to the European Detailed Mortality Database, among Hungarian males, the alcoholic liver disease (ALD) was responsible for more than 80% of the deaths from liver diseases (ICD-10 code: K70-K77) in 2009 [3]. As it is indicated on Figures 3 and 4, the alcoholic liver disease causes the high mortality from chronic liver diseases. These figures show that while Hungary is in the middle range among the European countries in terms of non-alcoholic liver diseases the country leads the mortality list in case of alcoholic liver diseases.

Figure 3.

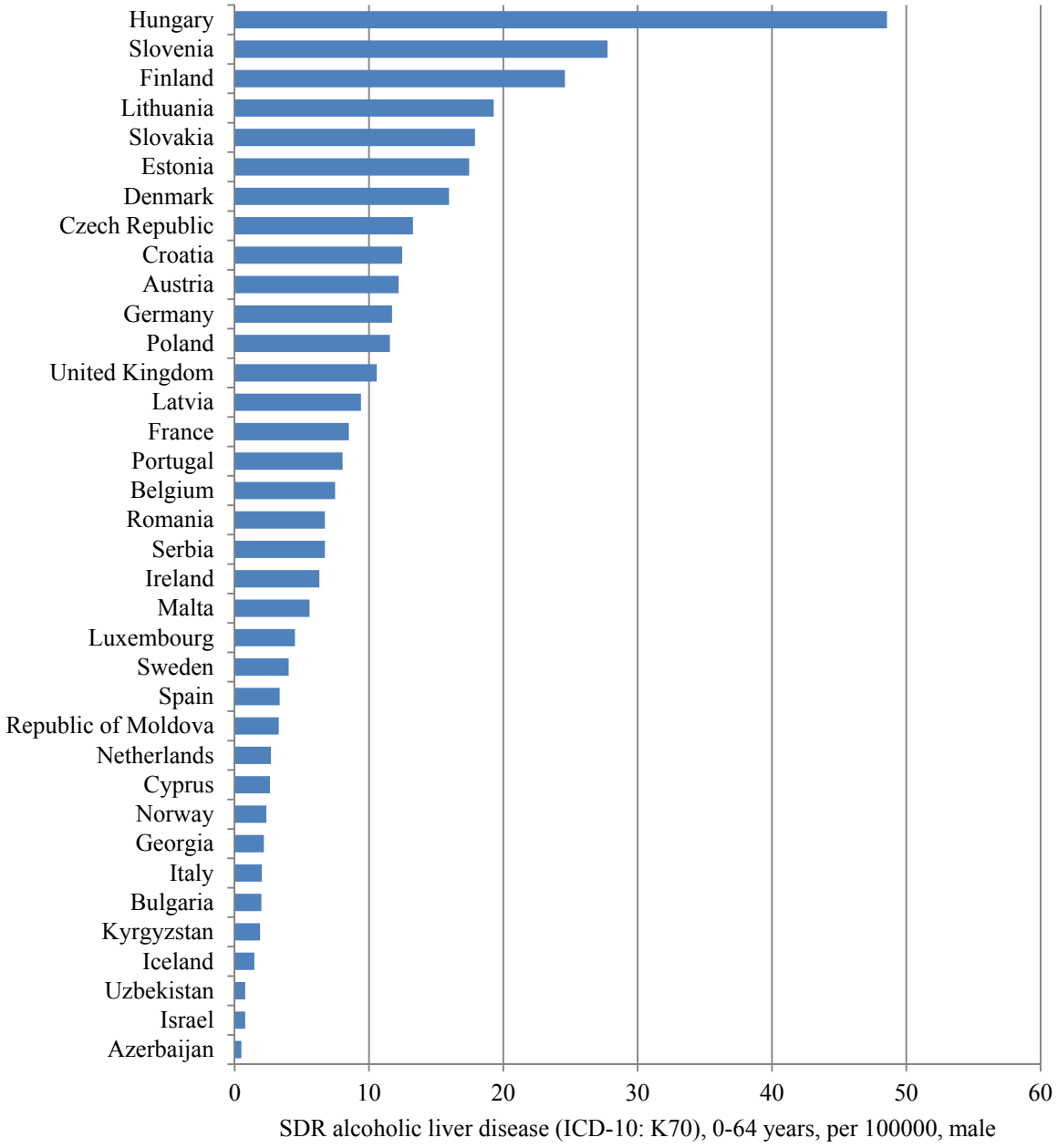
SDR for diseases of liver except alcoholic liver diseases (ICD-10: K71-77), 0-64 years, per 100000, male



Source: WHO, European detailed mortality database, updated in 2011 June [3]

Figure 4.

SDR for alcoholic liver diseases (ICD-10: K70), 0-64 years, per 100000, male



Source: WHO, European detailed mortality database, updated in 2011 June [3]

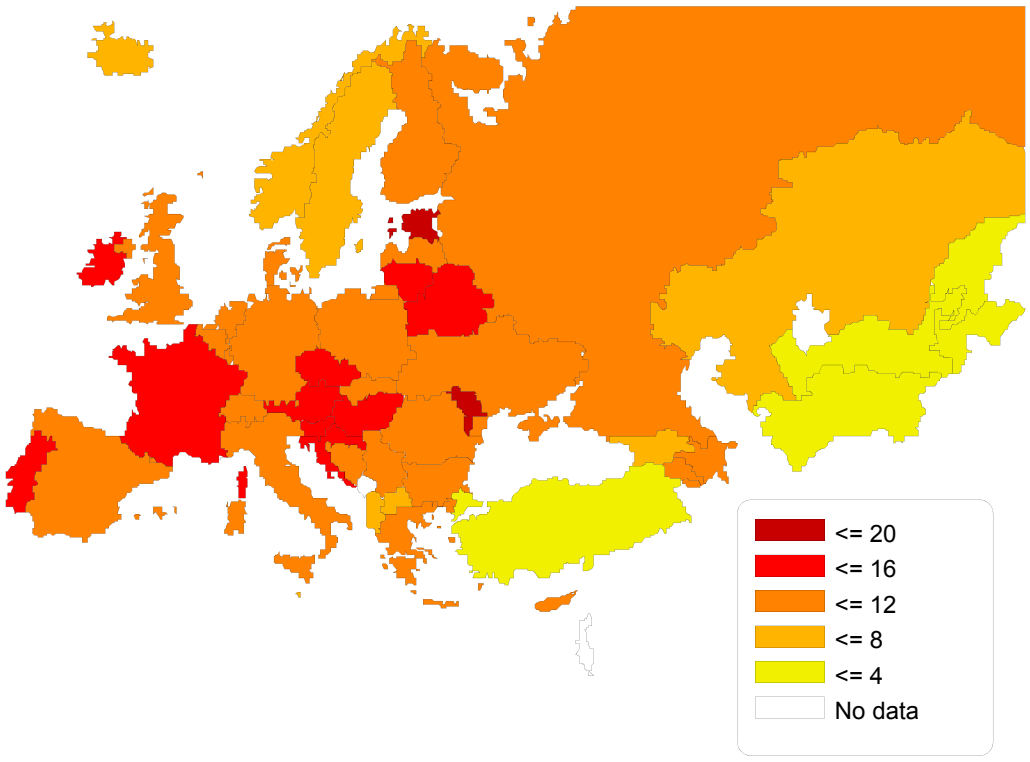
The suggested safe limits for alcohol intake is about 21 units per week for men and 14 units per week for women [2]. However, a previous prospective study on Danish subjects has found significant increase in the relative risk of developing liver disease if

the alcohol consumption was higher than 7 to 13 beverages (84-156 g alcohol) per week for women and 14 to 27 beverages (168-324 g alcohol) per week for men [4]. At least 80% of heavy drinkers develop fatty liver, 10-35% develop alcoholic hepatitis and only 10% will develop cirrhosis [5].

Despite that 87.2% of males consuming alcohol and more than 20% of drinkers have heavy drinking episodes [6], the high death rates for CLDs observed in Hungary cannot simply be explained by the elevated alcohol consumption rates because among males, the per capita consumption is not remarkably higher than in the Western European countries (Figure 5).

Figure 5.

Total adult (15+) per capita consumption of pure alcohol (drinkers only) in liters, males, last available

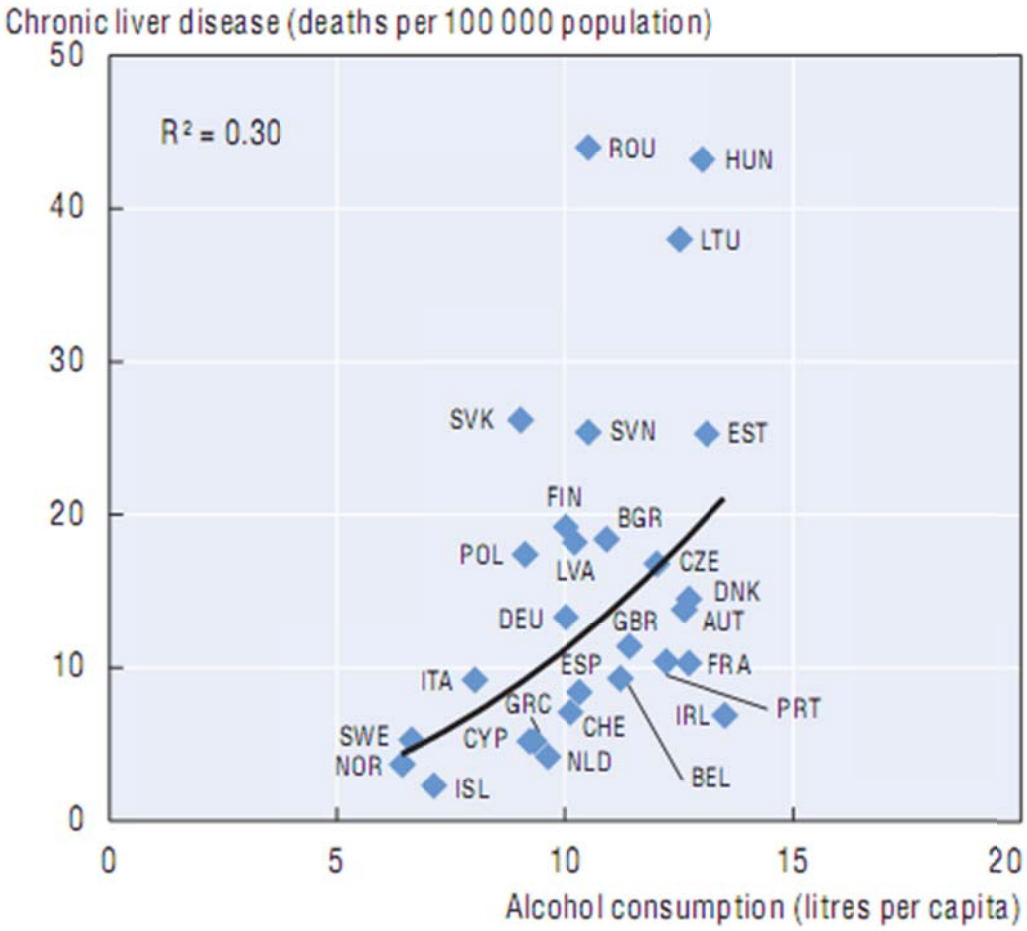


Source: WHO, European health for all database (HFA-DB), updated in 2011 January [1]

This alteration from the trend can also be observed on Figure 6, where both alcohol consumption data and death rates from chronic liver diseases are represented.

Figure 6.

Alcohol consumption and chronic liver disease (last available) in the 27 EU member states, Iceland, Norway, Switzerland and Turkey



R^2: determination coefficient, gives the proportion of the variance of one variable that is predictable from the other variable.

Source: OECD, Health at a Glance: Europe 2010 [7]

According to these data, it has long been suspected that, aside from the alcohol, other factors may also contribute to the high alcoholic liver disease mortality in Hungary and other Central-Eastern European (CEE) countries.

Having excluded causes of liver disease, possible explanations have focused on two potentially complementary factors, the nature of the alcohol consumed and the genetic composition of the population. Regarding the quality of alcohol beverages, Szucs et al. showed that the concentrations of methanol, isobutanol, 1-propanol, 2-butanol and isoamyl alcohol are significantly higher in homemade spirits, which suggest that the consumption of these spirits may have contributed to high level of liver cirrhosis mortality in Central and Eastern Europe, as an additional risk factor for alcoholic liver diseases [8]. There has, however, been no research so far on the contribution of genetic factors in Hungary.

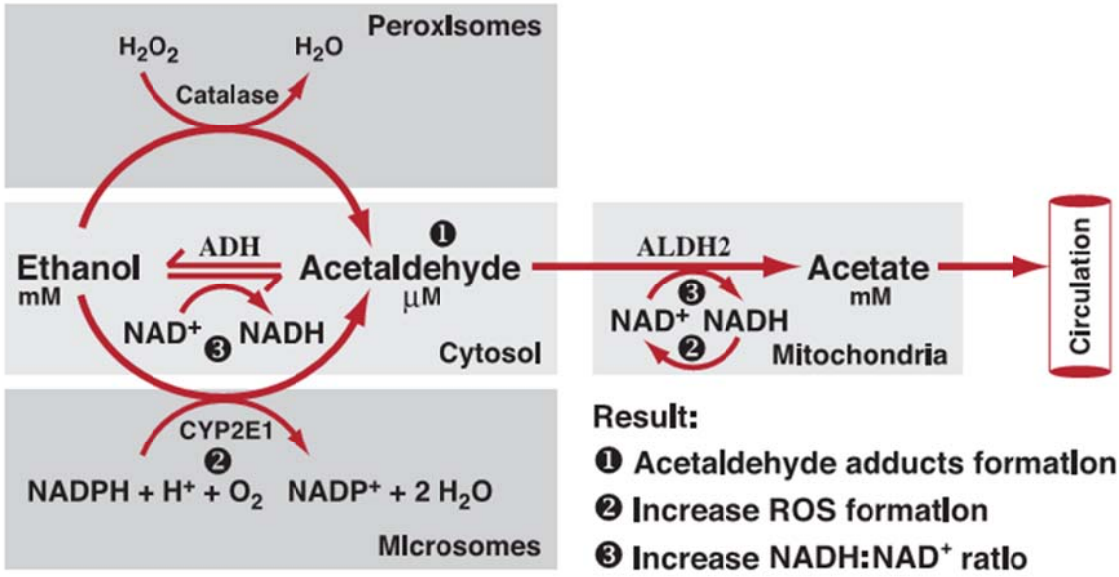
Effects of alcohol consumption on ALD

Chronic alcohol abuse can cause liver disease which progresses from simple steatosis through steatohepatitis, fibrosis and cirrhosis to liver disease. To understand the molecular mechanisms underlying the pathogenesis, the pathway of ethanol degradation have to be reviewed.

The liver is the main organ responsible for ethanol metabolism. The major route of alcohol degradation is the oxidation of ethanol to acetaldehyde mediated by three enzyme systems. The dominant enzymes involved in the oxidation of ethanol to acetaldehyde are alcohol dehydrogenases (ADH) which have high affinity to alcohol and are present in the cytoplasm. Catalyzing the same step, the cytochrome P450 system – also called the microsomal ethanol oxidizing system or MEOS – only induced by chronic alcohol consumption in the peroxisomes, while the catalases in the microsomes oxidizing only a small part of the consumed ethanol. The second step of alcohol degradation is the oxidation of acetaldehyde to acetate catalyzed by aldehyde dehydrogenase (ALDH) enzyme system located in the mitochondria. The produced acetate spontaneously breaks down to water and CO_2. Both ADH and ALDH transfer electrons to NAD^+, while the MEOS system's electron acceptor is the molecular oxygen (O_2). Catalases reduce hydrogen peroxide (H_2O_2) to water [9-11]. The step of the ethanol oxidation and their possible harmful results on liver can be seen on Figure 7.

Figure 7.

Oxidative pathways of alcohol metabolism



ROS: reactive oxygen species, CYP2E1: cytochrome p450 2E1

Source: [9]

The ethanol can take its effect on the development and progression of liver diseases on at least three ways: (i) via the acetaldehyde and other toxic byproducts of the ethanol degradation pathway, (ii) via the caused biochemical changes and (iii) via oxidative stress.

Toxic byproducts of the ethanol pathway

The oxidation of acetaldehyde to acetate is relatively slow and therefore allows the accumulation of acetaldehyde in the body. Due to the toxicity of acetaldehyde several systematic effects of ethanol abuse are mediated, at least in part, by the direct or indirect effects of the elevated acetaldehyde levels [12]. Because acetaldehyde is chemically reactive, it can interact with proteins, lipids and DNA [13, 14].

The alcohol degradation by CYP450 2E1 generates reactive oxygen species (ROS) which promotes the formation of lipid peroxidation products, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE). Similar to acetaldehyde these compounds also

form adducts with various other molecules, moreover, MDA and acetaldehyde can react with each other forming malondialdehyde-acetaldehyde adducts (MAA) [9, 14].

The adduct of these molecules with DNA – N^2-ethyl-2′-deoxyguanosine and 1,N^2-propano-2′-deoxyguanosine – can result in replication errors, therefore, in cell death or carcinogenesis [13].

These compounds also form adducts with proteins, primarily through their lysine amino acids. The main proteins appear to be preferentially modified by aldehydes are hemoglobin, albumin, tubulin, lipoproteins, collagen, CYP450 2E1 and ketosteroid reductase [15]. Although the formation of aldehyde adducts during alcohol consumption has been well established, the effects of these adducts and their role in the pathogenesis of liver diseases still needs to be clarified. The calmodulin and tubulin adducts can result in impaired microtubular function and subsequently a disorganization in the hepatocytes that is characterized by structural changes in the liver. The presence of the aldehydes increases the collagen production of the liver cells, possibly via an adduct-stimulated way. These disturbances in the extracellular matrix production may lead to formation of scar tissue in the liver (i.e. hepatic fibrosis). These adducts could also elicit an immune response, in which they may trigger harmful immune processes or inflammation that could lead to liver damage [14, 16].

Biochemical changes

Steatosis, one of the earliest hepatic changes, could play a crucial role both in the initiation and the progression of ALD. As it was mentioned earlier, the oxidation of ethanol to acetaldehyde and subsequently acetate utilizes NAD^+ as an electron acceptor, causing a shift in the NADH:NAD^+ ratio. This reduced state can be involved in the accumulation of lipids during alcohol ingestion with increasing the rate of fatty acid synthesis and esterification and decreasing the ß oxidation of free fatty acids [11, 17].

Oxidative stress

A lot of different factors and processes can cause alcohol induced oxidative stress. Some examples are [18]:

- the shift in the NADH:NAD^+ ratio
- the effects of the produced acetaldehyde

- alcohol induced hypoxia
- alcohol's effect on the immune system (e.g. the cytokine production)
- alcohol induced increase in the activity of CYP450 2E1

Reactive oxygen and nitrogen species (ROS and RNS, respectively) are produced in the normal cellular metabolism as well. However, normally they are balanced with antioxidants. The above mentioned processes (among others) can cause the tipping of this balance and, as a consequence, oxidative stress.

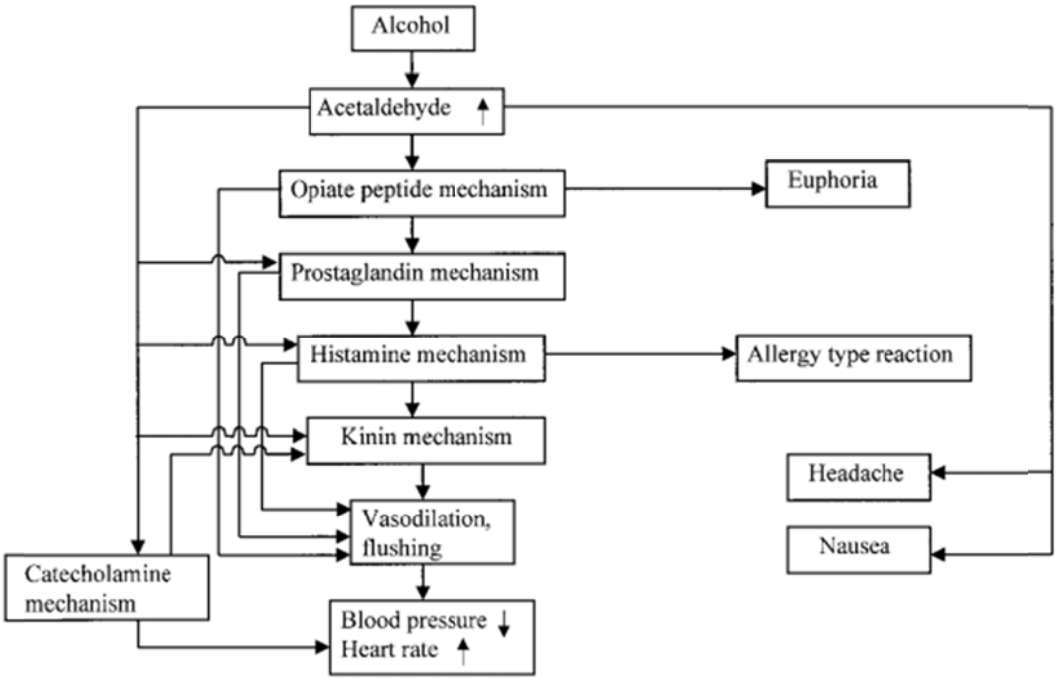
The reduction of superoxide ($O_2^{\bullet -}$) leads to formation of hydroxyl radicals ($OH^{\bullet}$) while its reaction with $NO^{\bullet}$ results peroxynitrite ($ONOO^-$). These are the most important ROS and RNS molecules in alcohol induced oxidative stress. They can alter and/or interfere with processes within the cell, causing cellular injury. They can also modify the signaling cascades within the cell. The major oxidant sensitive cascades include small molecules (e.g. Ca^{2++}), stress activated protein kinases and transcription factors. Oxidative stress has also a very important role in the activation of inflammation and the increase in apoptotic hepatocyte death primarily through the enhanced production of TNFα [11, 19].

Pharmacological and behavioral effects of acetaldehyde

Acetaldehyde accumulation in the periphery produce the symptoms of the so-called "alcohol sensitivity". These symptoms are often observed in people with deficient ALDH enzyme. These include the vasodilatation, increased skin temperature, facial flushing, tachycardia, lowered blood pressure, dry mouth or throat associated with bronchoconstriction, nausea, headache and euphoria. These adverse effects of alcohol consumption can deter from further drinking therefore reduce the susceptibility of developing alcohol dependence (AD). This effect is the basis of the therapeutic use of disulfiram (Antabuse), which is a synthetic inhibitor of ALDH [20]. These peripheral effects are caused by the number of pathways modulated by acetaldehyde (Figure 8).

Figure 8.

Pathways for acetaldehyde effects



Source: [20]

Although the adverse effects of acetaldehyde are well known, its role in brain is still matter of debates. As a highly reactive compound, it can react not only with proteins, but can form complexes with biogenic amines which include, among others, neurotransmitters such as serotonin and dopamine [21]. Salsolinol, the complex formed with dopamine, exhibits reinforcing properties in animals, emerging the possible role of acetaldehyde in the development of alcoholism. However, some researchers suggest that the level of the acetaldehyde remains low in the brain, mainly its limited diffusion through the blood-brain barrier. By contrary, others claim that in case of high acetaldehyde concentration, it can cross the barrier and takes part in the reinforcing mechanism of alcohol consumption [22].

The alcohol dehydrogenase gene family

There are seven ADH genes, located at the chromosome region 4q22 in humans. They are dimeric, cytosolic zinc-metalloenzymes and their subunits' molecular weight is about 40 kDa. These enzymes are classified in 5 classes based on their enzymatic properties and sequence similarities (Table 1). Note that in some previous articles, the former nomenclature was used. In this work we use the gene names as accepted by the HUGO Gene Nomenclature Committee. Only enzymes belong to the same class can heterodimerize [23]. Alcohol degradation in the liver primarily involves Class I enzymes [24]. Class I ADHs consist of α, β and γ subunits, encoded by the genes ADH1A, ADH1B and ADH1C. Since these enzymes have a low K_m, the produced acetaldehyde is normally eliminated shortly after being formed [25].

Table 1.

Properties of alcohol dehydrogenases (ADH) in human

Gene locus	Subunit	Class	K_m (ethanol) (mM)	% liver contribution at 22 mM ethanol	Tissue distribution
ADH1A	α	I	4	8.1	liver
ADH1B*1	β	I	0.05	21.8	liver. lung
ADH1C*1	γ	I	1	41.5	liver, stomach
ADH4	π	II	34	28.6	liver, cornea
ADH5	χ	III	1000	<1	most tissues
ADH6	μ, σ	IV	20	<1	stomach, oesophagus, mucosas
ADH7		V	30	<1	liver, stomach

Source: [24, 26]

Allelic variants occur mainly in ADH1B and ADH1C genes, encoding the β and γ subunits.

Genetic polymorphisms of ADH1B and ADH1C

Although the level of the alcohol consumption is the main determinant of alcohol dependency and chronic liver diseases, their risk, at an individual level, is also affected by genetic factors [27]. Results from twin studies and experimental work on a range of receptors have produced an estimate of 40 to 60% heritability for alcohol dependence [28-30], while 49% of the variability of alcohol elimination rate is genetically determined [23]. Coding variations in the corresponding genes influence the kinetic properties of the enzymes and may result in acetaldehyde accumulation. Single nucleotide polymorphisms (SNPs) causing amino acid changes in the NAD^+ coenzyme-binding domain have been described in both ADH1B and ADH1C that modify the enzymatic properties.

The ADH1B*2 allele (rs1229984) results in an arginine to histidine change in the 48th amino acid residue that 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 type ADH1B*1 allele. The ADH1B*3 (rs2066702) encodes the β_3 subunit, containing an arginine to cysteine change at the 370th amino acid residue. In its homodimer form, it has 30-fold higher V_{max} than β_1 [24, 31]. The wild type ADH1C allele, the ADH1C*1 (γ_1 subunit) contains arginine at 272nd and isoleucine at 350th position, while the ADH1C*2 variant, which encodes the γ_2 subunit, contains glutamine (rs1693482) and valine (rs698), respectively. The V_{max} of γ_1 is 2.0-2.5 times higher than that of γ_2 [24]. The two SNPs forming the ADHC*2 allele are in high linkage disequilibrium [32]. Taking into account these polymorphisms, hepatic class I alcohol dehydrogenase in humans can exist as 21 different possible forms (homo- and heterodimers). The number of possible forms in a given person is depending on his or her genotype [33]. The enzymatic properties of the above mentioned polymorphisms compared with the wild type allele can be seen in Table 2.

Table 2.

Properties of polymorphic forms of human alcohol dehydrogenase

Gene locus	Subunit type	K_m (ethanol) (mM)	V_{max} (turnover number/min)
ADH1B*1	β1	0.05	9
ADH1B*2	β2	0.9	400
ADH1B*3	β3	34	300
ADH1C*1	γ1	1	87
ADH1C*2	γ2	0.63	35

Source: [24]

These values means that in case of being homozygous for ADH1B*1 and ADH1C*2 the total ethanol degradation capacity is about 80% of the homozygous wild type alleles. For a man homozygous for ADH1B*2 and ADH1C*1 alleles, the oxidizing capacity is almost eight times higher than with the reference alleles. Carrying the ADH1B*3 allele in homozygous form results a more than two times higher oxidizing capacity. Interestingly, the activity is the same in men with ADH1B*2/*2-ADH1C*1/*1 and in men with ADH1B*2/*2-ADH1C*2/*2 genotype, however, the ADH1C*2/*2 genotype, in itself, lowers the activity. The exact values of hepatic ethanol oxidizing values of the izozymes, calculated by Lee et al. [34], are shown in Table 3.

Table 3.

Ethanol metabolism in humans with different genotypes

Genotype combination	Ethanol oxidizing capacity at various ethanol concentrations (mmol/min)	
	10 mM	20 mM
ADH1B*1/*1-ADH1C*1/*1	2.1	2.3
ADH1B*1/*1-ADH1C*2/*2	1.7	1.9
ADH1B*2/*2-ADH1C*1/*1	17	18
ADH1B*2/*2-ADH1C*2/*2	17	18
ADH1B*3/*3-ADH1C*1/*1	3.4	5.1
ADH1B*3/*3-ADH1C*2/*2	3.0	4.6

Activities are calculated for 70 kg men.

Variants with higher activity (γ_1, β_2 and β_3) are considered to give rise to acetaldehyde accumulation via faster ethanol degeneration, because the ALDH with unchanged activity is not able to cope with the increased speed of ethanol degradation. The accumulation of acetaldehyde may lead to the previously mentioned adverse effects of this toxic byproduct [24, 32]. These effects may deter from further drinking but, as it is hypothesized, if individuals persist with alcohol consumption, hepatotoxicity and other tissue damage may occur due to the effects of acetaldehyde [24, 27, 32]. However, some researchers have not reported significantly elevated acetaldehyde levels in Asian populations in those with the ADH1B*2 polymorphism [35, 36].

The most important meta-analysis on the effect of alcohol dehydrogenase gene polymorphisms was conducted in 2006 by Zintzaras et al. [37]. This study, combined with previous meta-analyses by Whitfield et al. [38, 39], have concluded that ADH1B*1 allele is associated with significantly increased risk of alcoholism (it also means that ADH1B*2 allele is associated with lower risk of alcoholism) both in Caucasians and East Asians but the association is stronger in Asian populations, perhaps because of the different haplotypes [40]. For liver disease, only Whitfield et al. found significant association with ADH1B*2 allele, but this work included only 3 studies, all of them on

Asian populations. The meta-analysis conducted by Zintzaras et al., did not shown significant association either in Asian or in Caucasian populations. It is important to recognize that since these analyses were conducted using patients with alcoholic liver disease and alcoholic controls, the ADH1B*1 allele was hypothesized to be protective against liver diseases. This also means that the ADH1B*2 allele is thought to be susceptible for liver diseases among alcoholics.

The effect of the ADH1C*2 allele was concluded in the same meta-analyses [37-39]. The ADH1C*2 allele showed significant association with alcohol dependence only among Asians. The most relevant odds ratios are shown in Table 4.

Table 4.

The effect of ADH1B and ADH1C polymorphisms on alcoholism and liver diseases

	Whitfield et al. [38, 39]		Zintzaras et al. [37]	
	ADH1B*1 OR (CI)	ADH1C*2 OR (CI)	ADH1B*1 OR (CI)	ADH1C*2 OR (CI)
Alcoholic liver disease				
Caucasian	n.a.	n.a	0.74 (0.45-1.19)	1.02 (0.83-1.26)
Asian	0.57 (0.39-0.83)	1.01 (0.46-2.31)*	0.68 (0.37-1.25)	1.13 (0.57-3.00)
Alcohol dependence				
Caucasian	2.1 (1.32-3.44)	n.a.	1.62 (1.22-1.89)	1.03 (0.88-1.20)
Asian	5.19 (3.74-7.26)**	0.38 (0.29-0.51)	2.23 (1.81-2.74)	1.91(1.45-2.53)

* *Based on only one study,* ** *Han Chinese population, n.a.: not available*

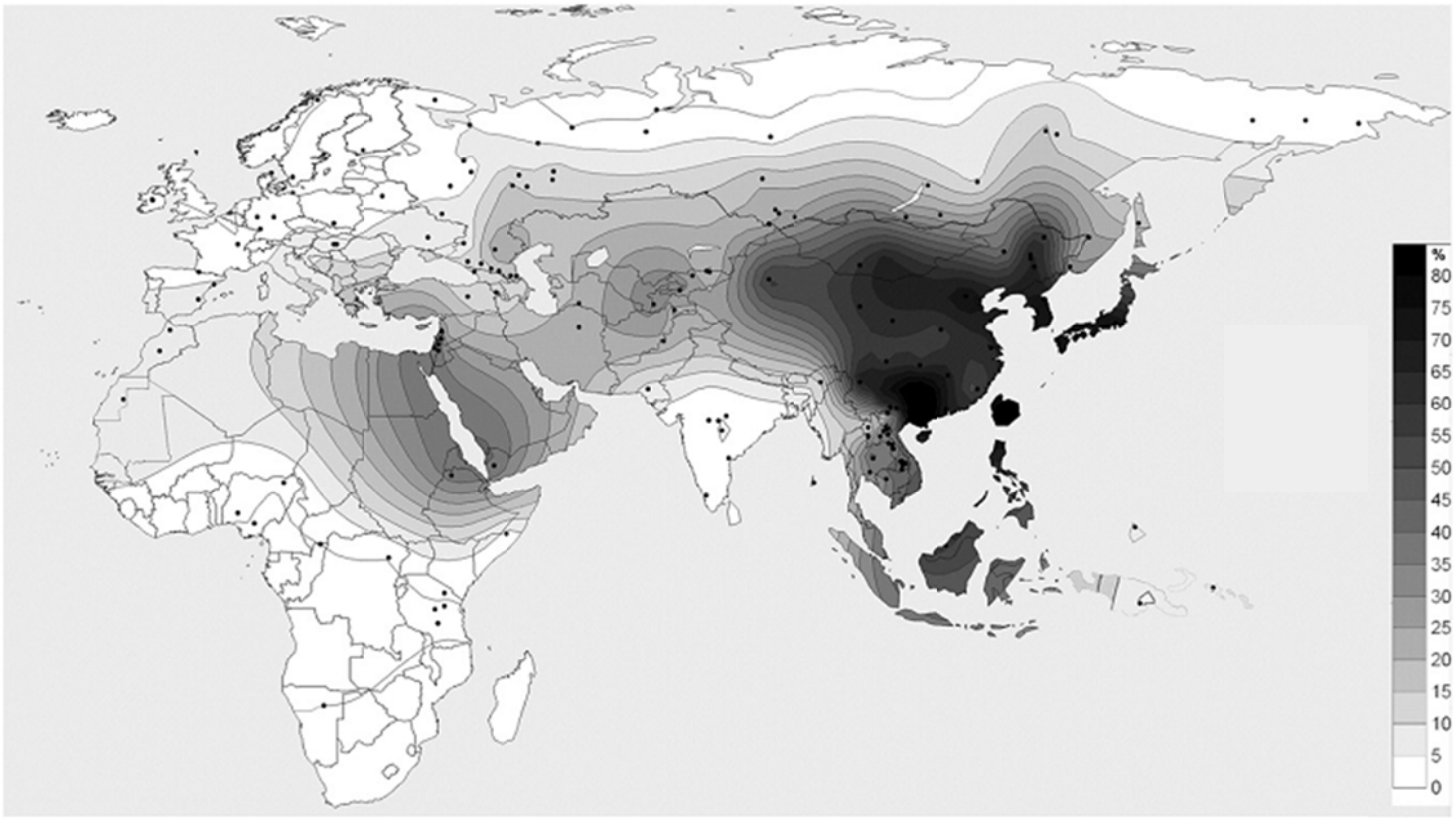
Rather less data is available on ADH1B*3 allele. No significant association of ADH1B*3 with alcohol dependence has been found in African Americans, although this allele was associated with a negative family history of alcoholism [41]. However, this allele proved to be protective against alcohol dependence in Mission Indians in California [42] and in African-American families [31].

The frequency of the polymorphic alleles varies among geographical regions, exhibiting an East-West gradient. The ADH1B*2 allele is relatively rare in Afro-Americans and Caucasians with a frequency of 0-10% [31, 43-47]. This allele is more frequent among Asian populations, where its frequency varies from 50 to 90 percent,

depending on ethnicity [47, 48]. Borinskaya et al. [49] modeled the geographic distribution of the ADH1B*2 with refining the map of Li et al. [50] and making additional genotyping. This distribution map can be seen on Figure 9.

Figure 9.

*The geographic distribution of the ADH1B*2 allele*



The dots represent the populations with known frequency data. Source: [49]

This specific pattern of the distribution might be due to local selective factors on this allele or it simply represents the migration processes in Asia [49, 50].

The ADH1B*3 is mainly found among African Americans, with a prevalence of 10-35% but it is rare or absent in Caucasian and Asian populations [31, 40]. It is also present in Native Mission Indians with a low frequency [42].

ADH1C*2 allele frequencies range from about 30 to 50% in Caucasian populations. It is strongly varies in Asian populations, with a higher frequency among

East-Asia and lower in Southwest-Asia. In Africa, the frequency of the allele is higher than among Caucasians, but lower than East-Asians [31, 40, 43-46, 48]. This interesting pattern of allele distribution probably evolved due to the linkage disequilibrium between the different ADH genes and the possible selection mechanisms on ADH1C*2 or other alleles, such as ADH1B*2 [40].

Aldehyde dehydrogenase gene family

There are three classes of ALDHs. The most important enzymes for acetaldehyde oxidation are the cytosolic ALDH1 and the mitochondrial ALDH2. Both enzymes are tetrameric and have low K_m for acetaldehyde. The ALDH2 gene is polymorphic [24].

The Glu504Lys polymorphism of ALDH2

This polymorphism, encoded by a G to A substitution, results in replacement of glutamate with lysine at position 504 (the official name of the polymorphism has recently changed from Glu487Lys). The wild allele named ALDH2*1, the mutant allele is the ALDH2*2. Homozygotes for ALDH2*2 have essentially no ALDH2 activity, while heterozygotes have markedly reduced activity, hence this is a dominant trait [51]. The presence of an ALDH2*2 allele is strongly protective against alcohol dependence due to the severe adverse effects of the accumulated acetaldehyde [26, 52]. But, as in case of ADH1B*2 allele, if heterozygotes tolerate heavy drinking may suffer from the harmful effects of the elevated level of acetaldehyde [24]. The inactive allele is relatively common in people of Asian descent, but it is rare or absent in people with Caucasian and African descent [26]. Interestingly, Higuchi et al. [53] showed that the protective effect of this allele is decreasing while the number of alcoholic carriers is increasing. So the ALDH2 deficiency may become an important risk factor.

In our study we have screened our study population for ALDH2 Glu504Lys, although it is very rare in European populations; the mortality of chronic liver diseases is so high in Hungary that we cannot dismiss any of the possible explanations.

Aims

The aim of our study was to determine if the genetic background plays significant role in the high prevalence of chronic liver disease in Hungary. To assess the frequency and the effect of the most frequent ADH polymorphisms, a case-control study was undertaken. By mapping the alcohol consumption patterns of the involved population, the aim of this study was to measure whether these mutations have any effect of drinking habits. Apart from describing the single effects of these polymorphisms, our goal was to analyse their combined effect both on CLDs, AD and alcohol use. The results may help not only to an improved understanding of the high prevalence of chronic liver diseases 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.

With better understanding of the genetic polymorphisms' role in determining the individual drinking habits and in the development of liver diseases, more personal therapies can be applied. This is very important, since in Hungary, alcoholism is also a very serious issue. On the level of the population, mapping the frequency of allelic variants can help in evolving public health programs more suitable to local specialties to lower the high level of cirrhosis and liver disease.

Materials and methods

Sample collection

In 1998 the school of Public Health in the University of Debrecen and the National Public Health and Medical Officer Service set up a surveillance system in Hungary's four counties (Szabolcs-Szatmár-Bereg, Hajdú-Bihar, Zala and Győr-Moson-Sopron). The General Practitioners' Morbidity Sentinel Station Program (GPMSSP) was established to monitor the morbidity of non-communicable diseases with major public health importance, such as hypertension, diabetes, stroke and chronic liver diseases. These four counties also represent the well-known differences in socio-economic and health status between the eastern and western part of Hungary. During data collection, the involved general practitioners provided data about not only the previously diagnosed prevalent, but during the monitoring the incident cases as well. The population registered to these practices was representative in terms of age and sex of both the participating counties and the overall Hungarian population [54]. This program also provides a framework for epidemiological researches.

The source population of our study was based on the GPMSSP, but restricted to men aged 45-64 years at the time of data collection. Controls were selected with random systematical sampling. Potential cases were patients with previously diagnosed chronic liver disease. Both cases and controls underwent physical and laboratory tests to verify the previous diagnosis or the absence of the disease. The diagnostic criteria of chronic liver disease were the following: having at least two of the following pre-specified criteria: 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, decreased serum albumin [55]. A laboratory finding was regarded as elevated if it exceeded the upper reference limit (URL) even with one unit. In case of contradiction between the previous diagnosis and the laboratory results, the affected patients were categorized according the latter.

Blood samples were taken from the involved persons for laboratory and genetic tests and they filled in a questionnaire which contained questions about their financial, marital and educational status, health behaviors and alcohol consumption habits. Patients with hepatitis B or hepatitis C infection were excluded from the analysis.

Altogether, 666 controls and 241 cases were involved in our study, the participation rate was 60%. As for age distribution, controls were representative for the overall Hungarian population (p=0.424), while cases were significantly older (55.17 years vs. 53.89 years, respectively, p=0.0023). Written informed consent was obtained from each patient. The study was approved by the Regional and Institutional Ethics Committee, Medical and Health Science Centre, University of Debrecen.

Questionnaire-based data collection

Detailed information on education and financial status was gathered with a self-completed questionnaire, yielding the following variables [55].

Education is categorized 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 signifies completed secondary education and 3 signifies higher education.

Financial status has 3 possible values, from 0 (bad/ very bad) and 1 (adequate) to 2 (good/ very good) based on self-administration.

The educational and financial status of the study population can be seen on Table 5.

Table 5.
Demographical, educational and financial status of the study group

		Controls	Cases
Age (years)	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

Detailed information on alcohol consumption was gathered with a self-completed questionnaire, yielding the following variables [55].

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 a week and (vii) at least 5 times a week.

Summary drinking data contains 4 categories: (i) non-drinker; (ii) infrequent drinker, consuming alcohol less than 3 times per month; (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).

The number of problem drinkers was measured with the CAGE questionnaire [56]. This questionnaire contains four indirect questions to detect drinking problems. The word CAGE is an acronym according the names of the questions (**C**ut, **A**nnoyed, **G**uilty, **E**ye-opener). The questions are the following.

1. Have you ever felt that you should cut down on your drinking?

2. Have people annoyed you by criticizing your drinking?
3. Have you ever felt guilty or bad about drinking?
4. Have you ever had a drink first thing in the morning to steady your nerves or to get rid of a hangover (eye opener)?

According the number of the positive answers we formed two variables.

CAGE score, from 0 to 4, according to the number of positive answers on the CAGE questionnaire.

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

The alcohol use habits in the control and case group are shown in Table 6.

Table 6.
Alcohol use habits in the study population

		Controls	Cases
Frequency of drinking			
Data availability		746	274
		%	%
	Never	13,14	6,93
	Did not drink in the last 12 month	2,82	3,65
	Less than 1 time in a month	7,10	2,55
	1-3 times in a month	12,47	4,01
	1-2 times in a week	23,06	8,39
	3-4 times in a week	16,49	17,52
	At least 5 times a week	24,93	56,93
Summary drinking data			
Data availability		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
CAGE score			
Data availability		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		538	201
		%	%
	Negative	73.23	33.83
	Positive	26.77	66.17

DNA preparation

DNA isolations were performed from the leukocytes of blood with EDTA anticoagulation taken by the GPs. We used MagNA Pure LC DNA Isolation Kit –Large

Volume (Roche Diagnostics, GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Genotyping

Genotyping was performed on LightCycler 1.5 System (Roche Diagnostics, GmbH, Mannheim, Germany) by real-time polymerase chain reaction (PCR) followed by a melting curve analysis.

For every polymorphisms, LightCycler DNA Master HybProbe (Roche Diagnostics, Mannheim, Germany, Cat. No. 12 015 102 001) was used in 1x concentration, according to the manufacturer's instructions. Five percent dimethyl sulfoxide (DMSO) was used, while $MgCl_2$ was present in 3 mM concentration. The primers were used in 0,5 μM, while the hybridization probes were used in 0,2 μM concentration. The 3' ends of the "anchor" probes were labeled with fluorescein, while the 5' ends were labeled with LightCycler Red 640 or LightCycler Red 705 fluorescent dyes. The 3' ends of the "sensor" probes were closed with a phosphate group. The primers and probes were produced by TIB MolBiol (Berlin, Germany). The primers used for the PCR are seen on Table 7.

Table 7.

Primers used for PCR

Polymorphism	Forward primer	Reverse primer
ALDH Glu504Lys	ACCCTTTGGTGGCTACAA	CCAACAGACCCCAATCC
ADH1B Arg48His	CAAAACCCTCAAATACATTTTAGAAA	GGCCTAAAATCACAGGAAGG
ADH1B Arg370Cys	CAACAAGCATGTGGGTTGTCTAA	CACTTGAATTTTAAATTTTCCTGAA
ADH1C Arg272Gln	CCCTCAAGACTACAAGAAACCCATT	CAAGCCAGGTAACAAAAAGATGAC
ADH1C Ile350Val	CAGTCTGGAATGCAGCACT	TAGAATACAAAGCAAAACAAAAAAAC

All primers are indicated in 5'-3' direction

The sequences of the hybridization probes used to detect the presence of the polymorphisms are seen in Table 8.

Table 8.

Hybridization probes used for genotyping

Polimorphism	Sensor probe	Anchor probe
ALDH Glu504Lys	CAGTTTCACTTCAGTGTSTGCC-FL	LC705-GCAGCCCGTACTCGCCCAA-PH
ADH1B Arg48His	TTCTCTTTATTCTGTAGATGGTGGC TGTAGG-FL	LC640-ATCTGTCACACAGATGACCACG-PH
ADH1B Arg370Cys	AAAACGTCAGGACGGTACAGATA C-FL	LC705- GCAATAGGAAAGAAGAGACATTGTGTTAACA-PH
ADH1C Arg272Gln	TGTCAAGCCGACCGATGACT-FL	LC640- CAAACGAAAAATCCACACCTCCATCAGTC-PH
ADH1C Ile350Val	TAAAACATTTGTTATTAATGCATC CAGTGA-FL	LC705- CTTCTTAGCCATAAAGTCAGCCACAAGTTTG-PH

LC640 - LightCycler Red 640, LC705 - LightCycler Red 705, PH - phosphate group

ADH1C Arg272Gln (rs1693482) and Ile350Val (rs698) polymorphisms were screened together in a duplex reaction, while ADH1B Arg370Cys (rs2066702) and ADH1B Arg48His (rs1229984) were analyzed in a simplex reaction. The reaction conditions are indicated in Table 9. and 10.

Table 9.

PCR conditions for genotyping ADH1B Arg370Cys/His, ADH1C Arg272Gln and Ile350Val

		ADH1B Arg370Cys/His		**ADH1C Arg272Gln and Ile350Val**	
PCR Reaction		temperature (°C)	Time (sec.)	temperature	time
Initial	Denaturation	95	60	95	60
	Dentauration	95	0	95	0
10 x	Annealing	58	15	58	15
	Amplification	72	20	72	20
	Dentauration	95	0	95	0
10 x	Annealing	56	15	56	15
	Amplification	72	20	72	20
	Dentauration	95	0	95	0
20 x	Annealing	54	15	54	15
	Amplification	72	20	72	20

Table 10.

PCR conditions for genotyping ADH1B Arg48His

		ADH1B Arg48His	
PCR Reaction		temperature (°C)	Time (sec.)
Initial Denaturation		95	60
	Dentauration	95	0
35 x	Annealing	52	10
	Amplification	72	20

Melting curve analysis was performed under the following conditions: PCR products were denaturated for 30 sec at 95 °C, then cooled down to 40 °C for 2 min, and warmed up to 72 °C ramping 0.2 °C/s with continuous fluorescence detection, followed by a final cooling step (40 °C for 30 sec);

The sequencing, where needed, was conducted by Biomi Ltd., Hungary, with one of the adequate PCR primers.

Statistical analysis

Single SNP analysis

Logistic regression 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. 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.

The logistic regression was either bifactorial (containing only one of the above mentioned explanatory variables) or multifactorial (as well as the genotype, taking into account one of the variables describing alcohol consumption habits as independent risk factors for developing chronic liver disease), including interactions where applicable.

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.

Linkage analysis

To map the linkage between the SNPs, Logarithm of Odds (LOD), D' and r² values were calculated using Haploview Software [57]. Under the assumption of random-mating, maximum likelihood estimates of gametic frequencies can be obtained with the use of EM (expectation-maximization) algorithm. The LOD is the log of the likelihood odds ratio, a measure of confidence in the value of D'. The usual threshold of LOD is 3. Above this, we can say that two loci are linked. D' is defined as, according to Lewontin [58]:

$$D' = \begin{cases} \dfrac{\pi_{11}\pi_{22} - \pi_{12}\pi_{21}}{\min(\pi_{1+}\pi_{+2}, \pi_{+1}\pi_{2+})} D > 0 \\ \dfrac{\pi_{11}\pi_{22} - \pi_{12}\pi_{21}}{\min(\pi_{1+}\pi_{+1}, \pi_{+2}\pi_{2+})} D > 0 \end{cases}$$

Where $D = \pi_{11}\pi_{22} - \pi_{12}\pi_{21}$, and π_{11} is the frequency of the genotype carrying the first allele for both polymorphism and likewise of the other alleles in the population, while π_{1+} is the frequency of the genotypes with the first allele of the first polymorphism and likewise of the other alleles. The correlation coefficient (r^2) between the two loci, calculated as follows [59]:

$$r^2 = \frac{D^2}{\pi_{1+}\pi_{2+}\pi_{+1}\pi_{+2}}$$

Multivariate SNP analysis

To detect the combined effect of the three main polymorphisms (ADH1B*2 and the two polymorphism of ADH1C*2) 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 homozygous for ADH1B*1 and heterozygous for ADH1C*2; the members of the third group (wild/mutant) were also homozygous for ADH1B*1, but they were also homozygous for ADH1C*2; the fourth group (heterozygous/wild) contained carriers for ADH1B*2 and homozygous samples for ADH1C*1, while the fifth group (heterozygous/heterozygous) was formed from samples carrying the ADH1B*2 allele and were heterozygous for ADH1C*2 (Table 11).

Table 11.
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 so they were excluded from the analyses. Logistic regression was used to measure the association between these genotype combinations and CLDs or alcohol consumption habits.

Odds ratios (ORs) were calculated to estimate the association between risk factors and outcome variables. Odds ratios are indicated in adjusted form, where adjustment included age, education and financial status. Results are expressed with both 95% confidence intervals and p values.

Data were analyzed using STATA 9.0 statistical software (StataCorp LP, Texas, USA).

Results

Effect of alcohol consumption on chronic liver diseases

The relationship between alcohol consumption and chronic liver disease was also examined. As expected, there was a clear dose response effect, with a greater probability of disease in those drinking alcohol 3-4 times in a week (OR=2.25; p=0.013; CI 1.186 – 4.280) compared to non-drinkers, increasing further in those drinking at least 5 times a week (OR=4.18; p<0.001; CI 2.350 – 7.444). This was also seen with CAGE, which captured alcohol dependence. The presence of even one positive answer increased the probability of liver disease (OR=3.49; p<0.001; CI 2.018 – 6.027), with additional positive answers increasing it further (Table 12).

Table 12.

Effect of alcohol consumption on liver diseases

	OR	p	CI 95%
Frequency of drinking			
Never	1		
Did not drink in the last 12 month	1.994	0.196	0.701 - 5.673
Less than 1 time in a month	0.774	0.620	0.282 – 2.127
1-3 times in a month	0.495	0.128	0.200 - 1.224
1-2 times in a week	0.605	0.185	0.288 - 1.272
3-4 times in a week	**2.253**	**0.013**	**1.186 - 4.280**
At least 5 times a week	**4.183**	**<0.001**	**2.350 - 7.444**
Summarised drinking data			
Non-drinker	1		
Infrequent drinker	0.617	0.247	0.273 - 1.396
Moderate drinker	0.910	0.752	0.508 - 1.632
Heavy drinker	**2.131**	**0.001**	**1.343 - 3.383**
Number of positive CAGE answers given			
0	1		
1	**3.488**	**<0.001**	**2.018 - 6.027**
2	**7.058**	**<0.001**	**3.987 - 12.496**
3	**10.729**	**<0.001**	**6.169 - 18.660**
4	**11.855**	**<0.001**	**6.327 - 22.214**
CAGE status			
Negative	1		
Positive	**5.782**	**<0.001**	**3.990 - 8.378**

Single SNP analysis

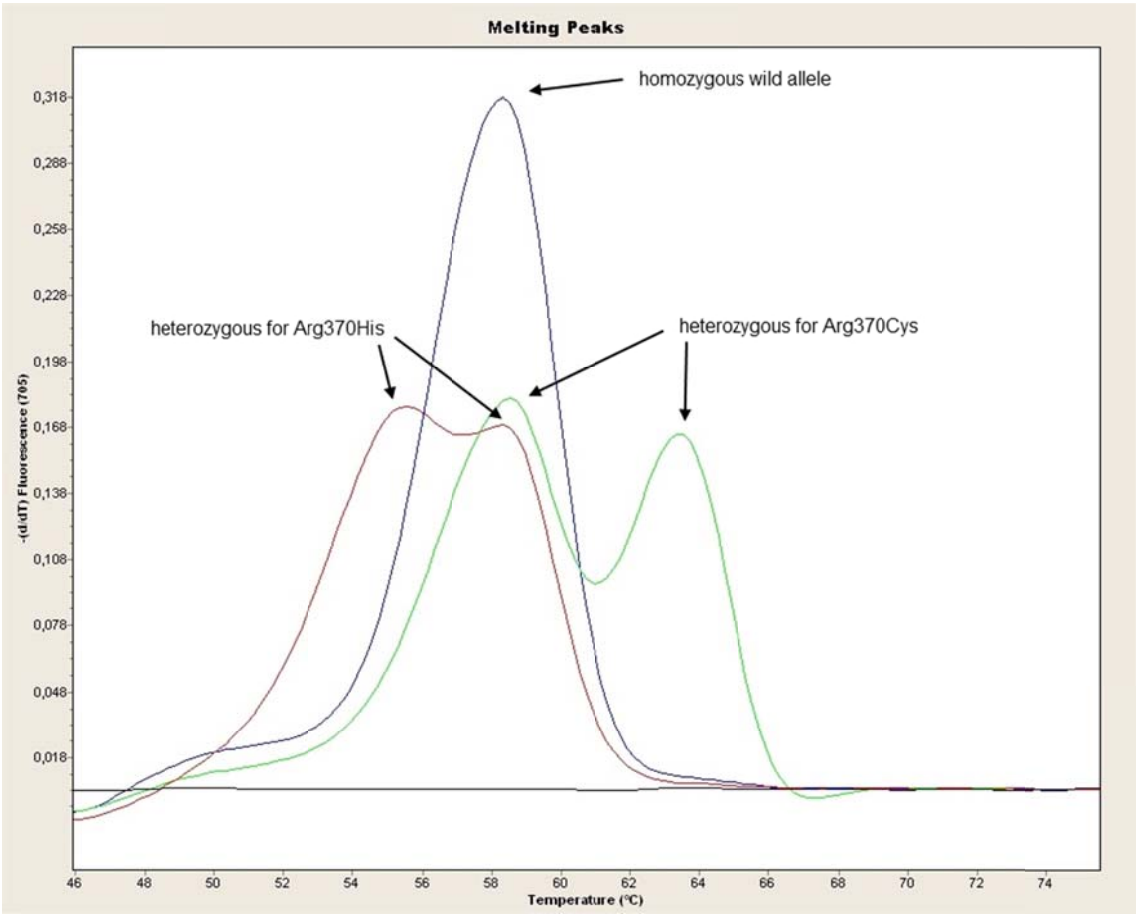
A SNP in the ALDH gene, the ALDH2*2 (Glu504Lys) was also investigated in our study population. This virtually inactive allele has a similar, but stronger effect than the ADH1B Arg48His and it is frequent among Asian populations [24]. In the Hungarian population, no carrier was found, so this allele was excluded from the further analysis.

Identification of a new SNP variant

During the analysis of the ADH1B Arg370Cys (rs2066702) mutation an unexpected polymorphism was detected, causing an approximately 2 °C lowering in the melting temperature compared to the wild type (the probe was specific for the mutant type). Sequencing revealed that this allele is 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 10 shows the shift in the melting point, as it was seen in the LightCycler analysis, while Figure 11 shows the result of the sequencing.

Figure 10.

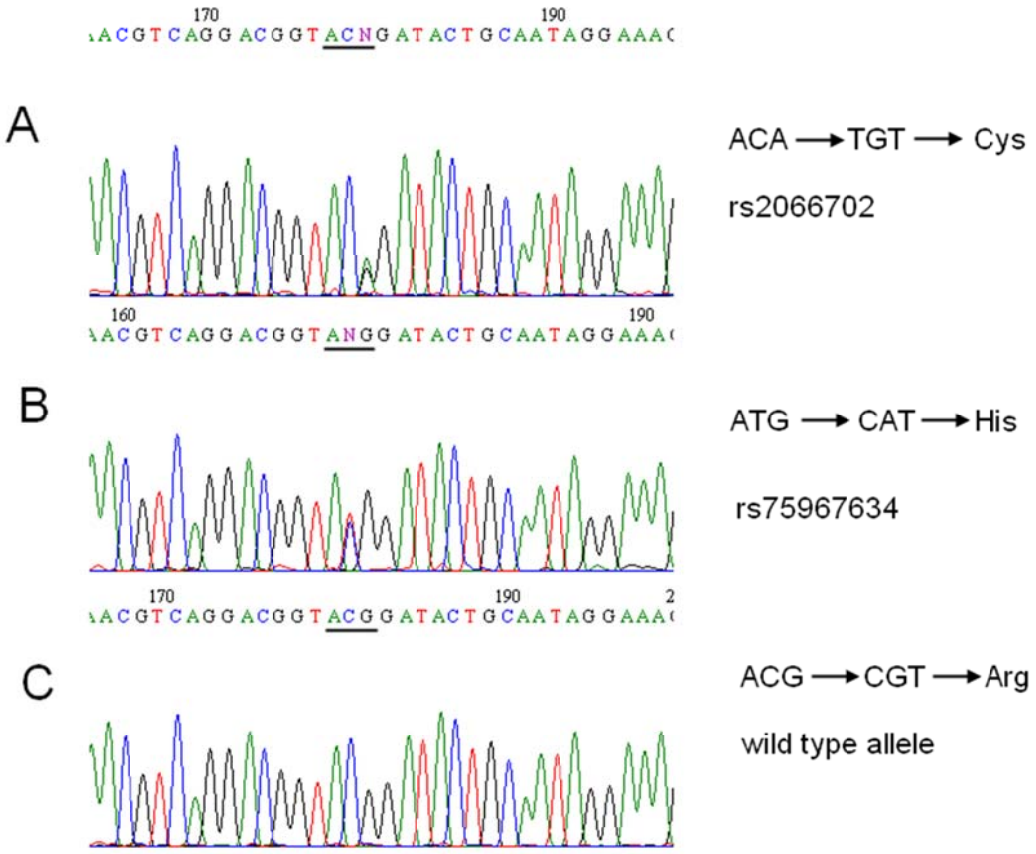
The result of the melting point analysis



Homozygous sample for 370His or 370Cys was not found in our study population.

Figure 11.

The result of the sequencing



Note that the result of the sequencing is in reverse orientation.

Although this mutation has been submitted recently to PubMed's SNP database, no data are available on its frequency or its effect, so it was included in the further analysis. It was present in 1 % both in cases and controls.

Analysing the included SNPs

The allele frequencies and genotype frequencies of ADH polymorphic regions studied are presented in Table 13. Since the ADH1C Arg272Gln and Ile350Val showed the

same genotype in all but two samples, they were analyzed together, excluded the samples with different genotype, except in the linkage analysis.

Table 13.

Allele and genotype frequencies in the study population

	Allele frequency				**Genotype frequency**					
	wt		mt		wt/wt		wt/mt		mt/mt	
	Cases	Cont.	Cases	Cont.	Cases	Cont.	Cases	Cont.	Cases	Cont.
ADH1B Arg48His	0.956	0.914	0.044	0.086	0.913	0.832	0.087	0.165	0.000	0.003
ADH1B Arg370His	0.990	0.990	0.010	0.010	0.979	0.981	0.021	0.020	0.000	0.000
ADH1B Arg370Cys	1.000	0.999	0.000	0.001	1.000	0.997	0.000	0.003	0.000	0.000
ADH1C Arg272 Gln/Ile350Val	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

For all mutations, we have examined that if they were in Hardy-Weinberg equilibrium. According to the results, none of the polymorphisms showed significant deviation from the Hardy-Weinberg equilibrium.

For each single polymorphism, odds ratios were calculated by using data obtained on samples for which genotype data of all the polymorphisms were available. The results of the single SNP analyses can be seen in Table 14.

Table 14.

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 Arg48His	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 Arg370His	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 Arg272 Gln/Ile350Val	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.

The presence of the ADH1B*2 allele was associated with a significantly lower probability of chronic liver disease (OR=0.47; p=0.003; CI 0.287-0.781), the frequency of drinking (OR=0.63; p=0.004; CI 0.453-0.864) and with the probability of alcohol dependence, according to CAGE status (OR=0.53; p=0.009; CI 0.330-0.853), e.g. heavy drinkers are less likely to carry ADH1B*2 allele, moreover the carrier status of the mutant allele has a protective effect against being a heavy drinker. Similar strong relationships were found both with the number of positive CAGE answers (OR=0.52; p=0.002; CI 0.346-0.788). However, the allele was not associated with the summary drinking data (p=0.38).

To determine whether the genotype acts directly on chronic liver disease or through drinking behavior, models were created with and without adjustment for alcohol consumption. The clear association between genotypes and disease development disappeared after controlling the number of positive answers given in the CAGE questionnaire and CAGE status, and frequency of drinking (OR=0.63; p=0.123; OR=0.62; p=0.101; OR=0.60; p=0.061, respectively), but remained the same after adjustment for the summary drinking measure.

To determine whether the association between ADH1B*2 allele and the risk of the development of CLDs varies by alcohol exposure, further models were fitted with interaction between genotype and alcohol exposure indicators. In CAGE negatives, the presence of the ADH1B*2 allele was associated with significantly lower odds of CLDs (OR=0.27; p=0.014; CI 0.093-0.761), while in CAGE positives, a non-significant effect in the opposite direction was observed (OR=1.21; p=0.500; CI 0.537-2.721). The effect modification was significant (p=0.026). However, no interaction was detected when using the exposure defined as number of positive CAGE answers (p=0.205), frequency of drinking (p=0.8819) or summary drinking data (p=0.624) as measures of alcohol exposure.

The ADH1B 370His allele was not associated with any of the examined outcomes, although a trend can be observed in cases of alcohol drinking frequency and summarized drinking data.

The ADH1B Arg370Cys was excluded from further analysis as a consequence of its low frequency.

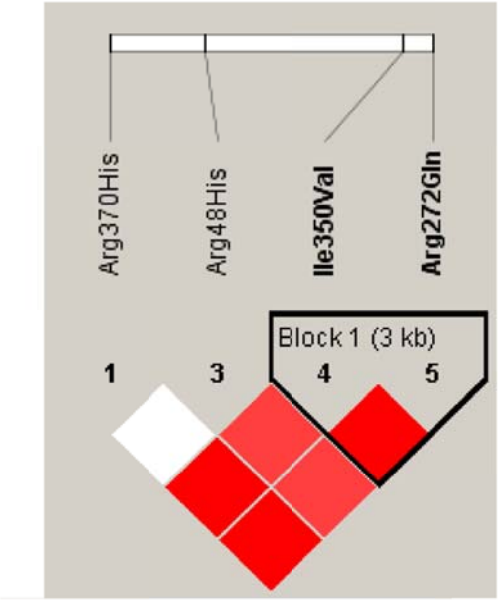
The ADH1C 272Gln and the ADH1C 350Val showed no association with chronic liver diseases, but they were associated with three of the variables describing drinking pattern, namely an increased odds ratio for frequency of drinking, summary drinking data and CAGE status could be observed. However, these associations were only significant when the mutations were homozygous. The effect of ADHC*2 allele on chronic liver diseases in alcoholics was also investigated, but no significant effect was found.

Linkage analysis

As expected, there is a very high linkage between the two ADH1C mutations. The high D' combined with the low r^2 values in case of ADH1C Arg272Gln and Ile350Val versus ADH1B Arg48His 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. The results of the linkage analysis and the frequency of the main haplotypes are shown in Figure 12.

Figure 12.

The results of the linkage analysis



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

Multivariate SNP analysis

The results of the multivariate analysis are shown in Table 15.

Table 15.

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*1, and ADH1C*1(wild/wild) were regarded as reference types.*
The first part of the group's 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.

Among drinking habits, only the CAGE status and the frequency of drinking showed significant associations. The latter was significantly associated with the heterozygous/wild group, with an odds ratio of 0.550 (p=0.011, CI 0.346-0.874). Both wild/heterozygous and wild/mutant group 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, respectively). Interestingly, neither the heterozygous/wild, nor the heterozygous/heterozygous showed difference for CAGE status.

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

Significant association with CLD was found only in case of groups containing the mutant allele for Arg48His. Both appeared to be protective, as it was expected on the basis of the single SNP analysis, 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 further logistic regression models. In these models, we used either the presence of chronic liver disease or the CAGE status as an outcome variable, as we used them in previous analyses, except that in this analysis we controlled for the other variables. It means that if CAGE status was the outcome, the controlling was made to the presence of CLDs, and vice versa. If 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 the presence of CLDs 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 if there is any difference in the effects of these polymorphism in drinkers and non-drinkers, additional models were used. When the logistic regression model were restricted 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). Including only the samples with CAGE positive status, none of the groups showed significant result. We investigated whether the effect of these polymorphisms differs

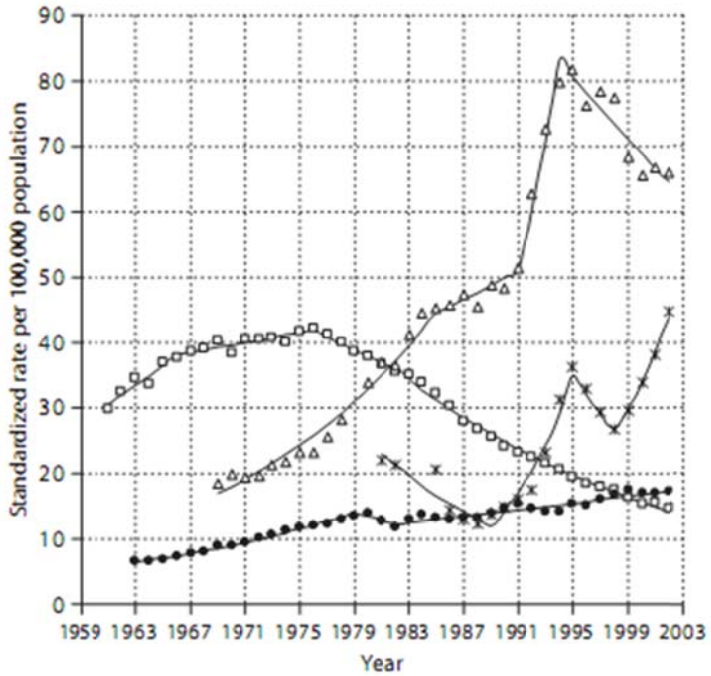
between alcoholics and non-alcoholics, but no significant interaction was seen with either of the alcohol consumption variables.

Discussion

Although there have been recent declines, death rates from chronic liver diseases and cirrhosis remain much higher in the majority of the CEE countries – including Hungary - than in the countries of western Europe. In Europe there is a clear East-to-West gradient in the mortality, but among the eastern countries, two groups can be identified according to the cirrhosis death rates. Higher mortality can be observed in the South-eastern European countries, among them, in Hungary [60]. The difference is even higher among males (Figure 13.).

Figure 13.

Trends in liver cirrhosis mortality, among males aged 20–64 years



□ *Mediterranean countries: 1961–2002 France, Greece, Italy, Portugal, Spain.*
Δ *South-eastern European countries: 1959–1963 Hungary; 1964–1968 + Bulgaria; 1969–1970 + Romania; 1971–1984 + Slovenia; 1985–1990 + Croatia; 1991–2002 + Moldova.*
* *North-eastern European countries: 1981–2002 Belarus, Estonia, Latvia, Lithuania, Russia, Ukraine.*
● *Other countries: 1959–1962 Austria, Belgium, Denmark, Finland, Iceland, Ireland, the Netherlands, Norway, Sweden, Switzerland, UK, Czechoslovakia; 1963–1964 + Poland; 1965–1966 + Malta; 1967–1989 + Luxembourg; 1990–2002 + Germany [60]*

There is a general agreement that the high rates cannot simply be attributed to the high level of alcohol consumption, as aggregate consumption levels are not markedly higher than in many other European countries. However, the very high mortality from cirrhosis has been the subject of intense speculation but rather less research. Previous studies have examined the potential role of the quality of alcohol consumed in this region [8, 61], and they concluded that in homemade spirits or in spirits from illegal sources, the concentration of short-chain aliphatic alcohols (methanol, isobutanol, 1-propanol, 1-butanol, 2-butanol and isoamyl alcohol) was significantly higher. These alcohols are highly hepatotoxic. These contaminants in the alcoholic beverages combined with the considerable rates of unrecorded consumption in the CEE countries can contribute to the high cirrhosis prevalence.

Apart from the alcohol consumption patterns, potential genetic factors have received less attention, however, in the genetic background the East-West difference can also be observed. Although this bias is between Asia and Europe, it may be the source of the differences in Europe. Although research has been conducted all over Western Europe [44-46, 62-66], in Eastern Europe, beside our study, data are available only from Poland [67, 68]. Therefore, in the present study we would like to clarify whether the possible genetic differences play a role in the East-West differences in cirrhosis mortality.

Beside this, our goal was to examine the important ADH polymorphisms' 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. Although the diagnosis of alcoholic liver disease contains the excessive amount of alcohol intake for at least 10 years [69], but, according to our hypothesis, alternate operation of alcohol metabolizing enzymes, such as ADH, may lead to liver diseases among those who does not meet the criteria of AD, but regularly consume alcohol. The results of this complex analysis may 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 we assessed its frequency. This polymorphic sequence has already been submitted into the PubMed's SNP database, but

without any information about the population studied, frequency or possible effects. Because of its close proximity to ADH1B Arg370Cys, it cannot be detected with either the original hybridization probe assay [70] or with newer methods such as Illumina GoldenGate or Sequenom MassArray techniques and restriction fragment length polymorphism (RFLP). A highly specific melting point analysis as used in this study is required to detect it. According to these results it would worth to re-genotype or sequence the samples that were previously found to be carrier of ADH1B Arg370Cys, to validate those results. Although we did not find any significant association with alcohol drinking habits and liver diseases, but its low prevalence means that there may be possible associations that could not be identified in the present study. Consequently, the effect of this SNP needs further investigation, including studies in other populations.

The most relevant polymorphisms affecting the development of liver diseases were found in the ADH1B and ADH1C genes. Among European and Asian populations a strong relationship was found between alcoholic liver disease or alcoholism and the polymorphism of genes coding ADH enzyme [44-47, 52, 67, 68, 71-76]. The effect is stronger and more obvious in case of ADH1B 48His polymorphism. These studies indicate that the allelic variations of ADH1B and ADH1C act through the accumulation of acetaldehyde, as a consequence of fast ethanol degradation caused by ADH1B*2 and ADH1C*1 alleles. Both the physiological and behavioral effects (e.g. increased heart and respiratory rates, nausea, headache, sedation, ataxia, amnesia and, in lower doses, stimulation and reinforcement) are results of the action of acetaldehyde on several signal molecules (e.g. neurotransmitters, endogenous opioids, molecules involved in the activity of calcium channels, epinephrine, nor-epinephrine, histamine, bradykinin), in some cases it is also interacting with other peptides, too. These effects have led to the hypothesis that if individuals carrying the ADH1B*2 allele and/or the ADHC*1 allele persist in drinking, it may lead to development of liver disease [21, 24].

In European populations the average allele frequency of the ADH1B*2 is about 5 percent, varying by ethnicity [47]. This allele was found in 8.31% of our control group, almost two-fold higher than reported previously in Western European populations. This may, however, be explained by genetic admixture from neighboring Slavic populations, given the much higher prevalence observed in the Russian population [77].

According to our results, carriage of ADH1B 48His allele reduces the risk of developing liver disease (OR=0.47), but this is no longer significant after adjustment for most of the measures of alcohol consumption. In the light of this finding, it seems that this allele acts directly on alcohol consumption, and its effect on liver diseases is only indirect. By lowering the alcohol consumption level this variant can prevent the development of liver diseases. Despite the previously mentioned hypothesis, we did not find significant increase in the odds of liver diseases among patients with AD. However, in alcoholics, higher, but not significantly increased odds were observed and the interaction between the presence of the alcoholism and the allele proved to be significant. This can also means that although this protective polymorphism has high prevalence among Hungarians, the benefits of carrying it can be neutralized or even inverted by carrying on drinking. In a population, where there is a deeply embedded culture of drinking, with an easy availability of alcohol at low cost, this may be sufficient to overcome the adverse physical effects of drinking, leading to an increase in both morbidity and mortality rates.

The two main polymorphisms of ADH1C (Arg272Gln and Ile350Val) exhibit an almost complete linkage, as it was expected from previous studies. However, the results regarding the effect of these mutations are not so obvious among Caucasians. Although previous meta-analyses and other studies [37, 38, 45, 46] did not find an association between ADH1C alleles and alcoholism or liver diseases, some researchers have described associations with alcohol drinking habits and/or AD among Europeans [63, 66, 78]. 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*2homozygous patients. This ambiguity may be due to the differences in categorizing drinking habits in different studies and the relatively minor effect of this genotype. Unlike in case of ADH1B*2 allele, we did not find any alteration in the effect of ADH1C*2 alleles when investigated only alcoholics or non-alcoholics. Our results of the linkage disequilibrium completely concur with previous studies [43, 46, 63], which showed that ADH1B*2 allele is associated with the ADH1C*1 allele. This means that polymorphisms with fast ethanol degradation ability are likely to occur together.

To clarify the independent effect of the main polymorphisms of ADH1B and ADH1C, a multivariate analysis was conducted. When CLD or alcohol use habits were used as an outcome variable, the results were in concordance with the expectations on the basis of the single SNP analysis, except that being heterozygous for the ADH1B*2 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*1 remained significant. When CLD was the outcome variable and CAGE status was controlled for, the association with carriage of ADH1B*2 and homozygous for ADH1C*1 remained significant. These results suggest that the polymorphisms of ADH1C impact directly only on drinking habits. The outcome of the single SNP analyses also supports this hypothesis. On the contrary, while the ADH1B*2 allele seemed to impact only on drinking habits when analysed individually, the results of the multivariate analysis suggest that in the absence of the controversial ADH1C*2 allele it is 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*2 allele and homozygous for ADH1C*1 allele 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.

However, 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.

This study reinforces the observation that the CAGE questionnaire is a more accurate measure of problem drinking than questions on consumption. One well-known issue is that many drinkers under-estimate their consumption (for reasons ranging from social acceptability to forgetfulness). Table 12 shows that the odds ratios are lower among rare and moderate drinkers than among abstainers, with a similar relationship

seen with the frequency of drinking. This is probably a reflection of the inclusion, among the abstainers, of some individuals who have stopped drinking after being diagnosed with liver disease.

Nevertheless, our work represents an important step in determining the genetic background of alcoholism and chronic liver diseases. This study is not only the part of the mapping of ADH polymorphisms in Caucasians, but this is one of the most complex studies conducted in Europe, involving a remarkably high number of cases and controls. The novel finding of our investigation is that it worth to involve not only alcoholic cases and controls but non-alcoholics as well. With this study set-up we were able to yield novel and interesting findings, including new interactions between ADH SNPs and alcohol dependence or CLDs.

Summary

Although standardized death rates have recently declined, premature mortality from liver cirrhosis remains markedly higher in Hungary than in Western Europe, especially among males. Although the level of the alcohol consumption is the main determinant of chronic liver diseases, its risk is also affected by genetic factors. The aim of this study was to analyze the combined effect of the most frequent alcohol dehydrogenase (ADH) 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) 666 randomly selected controls without CLD were analyzed for all four polymorphisms. Associations between the polymorphisms, individually, and in combination, and excessive or problem drinking and CLD, were assessed using logistic regression.

In this study we have identified a novel mutation, called ADH1B Arg370His. The ADH1B*2 allele was associated with significantly lower odds ratio for variables describing drinking habits (frequency of drinking, alcoholism according to CAGE questionnaire). There was a significant association between ADH1B*2 and CLDs (OR=0.47; p=0.003), but it disappeared after adjusting for variables of the drinking pattern. Among heavy drinkers the presence of ADH1B*2 did not increase the risk of cirrhosis. The ADH1C Arg272Gln and Ile350Val showed almost complete linkage. The 272Gln/350Val allele increased the risk of frequent and problem drinking in homozygous form (OR=1.51, p=0.028, OR=1.780, p=0.016, respectively). The combined analysis showed that ADH1B 48His is protective against CLD but only when combined with the wild type ADH1C Arg272/Ile350 allele (OR=0.368, p=0.019).

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 chronic liver diseases. Our study helps to map the frequency and effect of allelic variants which lead to better understanding of the genetic composition of the Hungarian population. It can help in evolving public health programs more suitable to local specialties to lower the high level of cirrhosis and liver disease.

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Publications

The thesis is based on the following publications:

Toth R, Pocsai Z, Fiatal S, Szeles G, Kardos L, Petrovski B, McKee M, Adany R. ADH1B*2 allele is protective against alcoholism but not chronic liver disease in the Hungarian population. Addiction 2010 May; 105(5):891-6.

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Toth R, Fiatal S, Petrovski B, McKee M, Adany R. Combined effect of ADH1B rs1229984, rs2066702 and ADH1C rs1693482/ rs698 alleles on alcoholism and chronic liver diseases. Disease markers (accepted for publication)

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Other publications

Fiatal S, Szigethy E, Szeles G, **Toth R**, Adany R. Insertion/deletion polymorphism of angiotensin-1 converting enzyme is associated with metabolic syndrome in Hungarian adults. J Renin Angiotensin Aldosterone Syst 2011 Feb 17.

IF: 1.6

Lazar V, Ecsedi S, Szollosi AG, **Toth R**, Vizkeleti L, Rakosy Z, Begany A, Adany R, Balazs M. Characterization of candidate gene copy number alterations in the 11q13 region along with BRAF and NRAS mutations in human melanoma. Mod Pathol 2009 Oct; 22(10):1367-78.

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Keywords

Alcohol, chronic liver disease, alcoholism, genetic, genetic epidemiology, alcohol dehydrogenase, polymorphism

Kulcsszavak

Alkohol, krónikus májbetegségek, alkoholizmus, genetika, genetikai epidemiológia, alkohol dehidrogenáz, polimorfizmus

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Appendix

Addiction
RESEARCH REPORT
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ADH1B*2 allele is protective against alcoholism but not chronic liver disease in the Hungarian population

Reka Toth[1], Zsuzsa Pocsai[1], Szilvia Fiatal[1,2], Gyorgy Szeles[1], Laszlo Kardos[1], Beata Petrovski[1], Martin McKee[3] & Roza Adany[1,2]

Department of Preventive Medicine,[1] Public Health Research Group of the Hungarian Academy of Sciences, Faculty of Public Health, Medical and Health Science Centre, University of Debrecen, Debrecen, Hungary[2] and London School of Hygiene and Tropical Medicine, London, UK[3]

ABSTRACT

Background Standardized death rates from chronic liver diseases (CLDs) in Hungary are much higher than the European Union average. Carrying the alcohol dehydrogenase 1B 48His allele (rs1229984 or ADH1B*2) could decrease the risk of alcoholism, but with persistent drinking may confer a greater risk of CLDs. The aim of this study was to assess the prevalence of this polymorphism in the Hungarian population and its association with alcohol consumption and with CLDs. **Methods and results** A total of 278 cases with diagnosed CLDs and 752 controls without any alterations in liver function, all males aged 45–64, were screened for ADH1B Arg48His polymorphism. ADH1B*2 allele frequencies in controls and cases were 8.31% and 4.50%, respectively ($\chi^2 = 9.2$; $P = 0.01$). Carrying the ADH1B*2 allele was associated with significantly lower odds ratio (OR) for drinking frequency (OR = 0.63; $P = 0.003$), the number of positive answers on CAGE (Cut-down, Annoyed, Guilt, Eye-opener) assessment (OR = 0.58; $P = 0.005$) and a positive CAGE status (OR = 0.55; $P = 0.007$). There was a significant association between ADH1B*2 and CLDs (OR = 0.50; $P = 0.003$), but it disappeared after adjusting for CAGE status and scores (OR = 0.67 $P = 0.134$; OR = 0.67 $P = 0.148$, respectively) and weakened after adjusting for drinking frequency (OR = 0.61; $P = 0.045$). Among heavy drinkers the presence of ADH1B*2 did not increase the risk of cirrhosis but there was a significant interaction between genotype and CAGE status ($P = 0.003$, $P = 0.042$), with ADH1B*2 conferring reduced risk of CLDs in CAGE negatives. **Conclusion** In Hungarians, the ADH1B 48His allele reduces the risk of alcoholism, but not the risk of chronic liver disease among heavy drinkers.

Keywords Alcohol, alcohol dehydrogenase, case control study, chronic liver diseases, genetic, genetic epidemiology.

Correspondence to: Roza Adany, Department of Preventive Medicine, Faculty of Public Health, Medical and Health Science Centre, University of Debrecen, PO Box 9, Debrecen, H-4012 Hungary. E-mail: adany@dote.hu
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INTRODUCTION

Although death rates have declined recently, premature mortality from chronic liver diseases (CLDs) remains markedly higher in the countries of central and eastern Europe than in western Europe. Other factors besides high alcohol consumption may contribute, such as the nature of alcohol consumed and the genetic composition of the population. Locally consumed alcohol beverages are known to be rich in hepatotoxic aliphatic alcohols [1] but there has, however, been no research so far on the contribution of genetic factors.

Ethanol is first oxidized to acetaldehyde by alcohol dehydrogenases (ADHs). Enzymes encoded by class I ADH genes (ADH1A, ADH1B, ADH1C) are very abundant in the liver, playing a significant role in alcohol metabolism [2,3]. Among them, the ADH1B subclass has attracted recent attention with evidence on the role of the Arg48His polymorphism. The presence of the His allele (called ADH1B*2) results in a highly active β subunit, with approximately 40-fold higher V_{max} than the wild-type [3,4].

The elevated acetaldehyde levels induced by rapid ethanol oxidation in ADH1B*2 carriers explain the crucial role of this polymorphism. Accumulation of this toxic intermediate leads to unpleasant effects which deter those affected from drinking heavily, resulting in a lower prevalence of alcoholism in carriers [3,5]. Among heavy

drinkers the liver can be at greater risk [3], due to the toxic effects of acetaldehyde.

Several studies have investigated the varying prevalence of the ADH1B*2 allele in Caucasian and Asian populations. There is evidence of an East–West geographical spectrum. While its frequency ranges from 70% to 90% in Oriental populations [6–9], it varies from 0% to 20% in European populations [6,10–14], while in the Russian population it was found to be 41% [15].

Several meta-analyses [16–18] describe a higher prevalence of the ADH1B*2 allele in non-alcoholics in both Asians and Caucasian populations [10], but the association is stronger in Asian populations, perhaps because of different haplotypes [2].

It is plausible that the shift in the allele frequency could contribute to the ethnic differences in CLDs mortality. Populations with a higher prevalence of ADH1B*2 experience reduced mortality but it is plausible that, where cultural norms overcome the adverse physical effects of drinking, mortality may increase.

To explore whether this mechanism could play a role in the high level of alcoholic liver disease in Hungary, a population-based case–control study was undertaken, first to assess the prevalence of the ADH1B Arg48His polymorphism in the Hungarian population and, secondly, to assess whether the presence or absence of this major polymorphism potentially linked to its the high prevalence of cirrhosis.

MATERIALS AND METHODS

Sample recruitment

The General Practitioners' Morbidity Sentinel Stations Program [19] is a country-wide monitoring network for chronic non-communicable diseases in Hungary. In the present study, restricted to men aged 45–64 years from 57 general practices in four participating counties (Győr-Moson-Sopron, Zala, Hajdú-Bihar and Szabolcs-Szatmár-Bereg), 752 control subjects with no diagnosed CLDs were selected randomly, while all subjects identified as having CLDs ($n = 278$) were selected. Controls were representative for the overall population by age ($P = 0.975$). Case definition has been described previously [20]. Data on alcohol consumption were collected by self-completed questionnaires. Ethylenediamine tetraacetic acid (EDTA) anti-coagulated blood samples were taken by general practitioners. Written informed consent was obtained from each patient. The study was approved by the Regional and Institutional Ethics Committee, Medical and Health Science Centre, University of Debrecen.

Questionnaire-based data collection

Detailed information on alcohol consumption included the following variables:

- Frequency of drinking, with seven possible outcomes: (i) has never drunk alcohol; (ii) did not drink in the last 12 months; (iii) drinks less than once a month; (iv) drinks one to three times a month; (v) one to two times a week; (vi) three to four times a week; and (vii) at least five times a week.
- Summary drinking data contain four categories: (i) non-drinker; (ii) infrequent drinker; (iii) moderate drinker; and (iv) heavy drinker [21].
- CAGE (Cut-down, Annoyed, Guilt, Eye-opener) score, from 0–4, according to the number of positive answers on the CAGE questionnaire, a widely used tool to detect alcoholism [22].
- CAGE status is negative with zero or one positive answers, and positive with two or more [22].

Alcohol use in the study group is shown in Table 1.

Two variables were formed to describe educational and financial status:

- Education was split into four categories, from 8 or less years to higher education.
- Financial status has three possible values, from 0 (bad/ very bad) and 1 (adequate) to 2 (good/ very good).

Genotyping

DNA was isolated from leucocytes using the MagNA Pure LC DNA Isolation Kit–Large Volume (Roche Diagnostics, GmbH, Mannheim, Germany), according to the manufacturer's instructions. Genotyping was performed on a LightCycler real-time polymerase chain reaction (PCR) system (Roche Diagnostics) by melting curve analysis. The PCR mix contained 1× LightCycler DNA Master HybProbe (Roche Diagnostics, cat. no. 12015102001), 5% dimethylsulphoxide (DMSO), 2 mM $MgCl_2$ and 0.5 μM of each primer (5′-CAAAACCCTCAAATACATTTTAGAAA-3′; 5′-GGCCTAAAATCACAGGAAGG-3′). Two probes were applied in the melting curve analysis in 0.2 μM concentrations (5′-TTCTCTTTATTCTGTAGATGGTGGCTGTAGG-FL, LC640-ATCTGTCACACAGATGACCACG-PH). The PCR reactions were carried out following the manufacturer's instructions. Both controls and cases were in Hardy–Weinberg equilibrium ($P = 0.13$, $P = 0.43$, respectively).

Duplicate analyses were carried out in a random selection of 10% of the samples and no difference between pairs was found from the two series.

Statistical analysis

Logistic regression was applied to test the association between the ADH1B genotype as an explanatory variable and the outcomes. Given the small number of homozygous mutant samples, they were analysed those carrying 48Arg/48His alleles.

The logistic regression was either bi-factorial (containing only one of the explanatory variables) or multi-

Table 1 Composition and alcohol use habits of the study group.

	Controls		*Cases*	
	n	%	*n*	%
Frequency of drinking				
Data availability	727		271	
Never	98	13.48	19	7.01
Did not drink in the last 12 months	21	2.89	10	3.69
Less than 1 time in a month	51	7.02	7	2.58
1–3 times in a month	91	12.52	11	4.06
1–2 times in a week	165	22.70	23	8.49
3–4 times in a week	122	16.78	47	17.34
At least 5 times a week	179	24.62	154	56.83
Summarized drinking data				
Data availability	702		245	
Non-drinker	139	19.80	33	13.47
Infrequent drinker (<3 times/month)	80	11.40	12	4.90
Moderate drinker (at least weekly, weekly total ≤14 units, and the daily amount never >5 units)	160	22.79	38	15.51
Heavy drinker (>14 units weekly or >5 units on at least one day of week)	323	46.01	162	66.12
Number of positive CAGE answers given				
Data availability	607		232	
0	330	54.37	36	15.52
1	103	16.97	41	17.67
2	67	11.04	44	18.97
3	62	10.21	62	26.72
4	45	7.41	49	21.12
CAGE status				
Data availability, *n*	607		232	
Negative	433	71.33	77	33.19
Positive	174	28.67	155	66.81

CAGE: Cut-down, Annoyed, Guilt, Eye-opener.

factorial (besides the ADH1B genotype, taking into account one of the variables describing alcohol consumption habits), including interactions where applicable. Ordered logistic regression was used with non-binary outcome variables.

Odds ratios (ORs) were calculated to estimate the association between risk factors and outcome variables. ORs were adjusted for age, education and financial status. Results are expressed with both 95% confidence intervals (CIs) and *P*-values. Data were analysed using STATA 9.0 statistical software (StataCorp LP, College Station, TX, USA).

RESULTS

Allele frequencies

Altogether, 1030 subjects were genotyped for ADH1B Arg48His. The genotype and allele frequencies are presented in Table 2. The difference in allele frequencies between controls and cases was statistically significant ($\chi^2 = 9.2$; $P = 0.01$).

Table 2 Genotype and allele frequencies obtained in the control and case groups.

	Controls		*Cases*	
	n	%	*n*	%
Genotype frequency				
ADH1B*1/ADH1B *1	629	83.64	253	91.01
ADH1B*1/ADH1B *2	121	16.09	25	8.99
ADH1B*2/ADH1B *2	2	0.27	0	0.00
Allele frequency				
ADH1B*1		91.69		95.50
ADH1B*2		8.31		4.50

ADH: alcohol dehydrogenase.

Association between genetic status, alcohol consumption and disease

The presence of the ADH1B*2 allele was associated with a significantly lower probability of CLDs (OR = 0.5; $P = 0.003$; CI 0.314–0.790) and also with the frequency

of drinking (OR = 0.63; $P = 0.003$; CI 0.463–0.858), positive CAGE status (OR = 0.55; $P = 0.007$; CI 0.351–0.849) and number of positive CAGE answers (OR = 0.58; $P = 0.005$; CI 0.392–0.848). However, the allele was not associated with the summary drinking measure ($P = 0.265$).

To determine whether the genotype acts directly on cirrhosis or through drinking behaviour, models were created with and without adjustment for alcohol consumption. The clear association between genotypes and disease development disappeared or weakened after controlling CAGE score, CAGE status and frequency of drinking (OR = 0.67; $P = 0.148$; OR = 0.67; $P = 0.134$; OR = 0.61; $P = 0.045$, respectively), but remained the same after adjustment for the summary drinking measure.

To determine whether the association between ADH1B*2 allele and the risk of the development of CLDs varies by alcohol exposure, further models were fitted with interaction between genotype and alcohol exposure indicators. In CAGE negatives, the presence of the ADH1B*2 allele was associated with significantly lower odds of CLDs (OR = 0.23; $P = 0.006$; CI 0.080–0.650), while in CAGE positives, a non-significant effect in the opposite direction was observed (OR = 1.57; $P = 0.239$; CI 0.741–3.325). The effect modification was strongly significant ($P = 0.003$). Similarly contrasting effects were found, with exposure defined as number of positive CAGE answers (interaction $P = 0.042$). However, no interaction was detected when using frequency of drinking ($P = 0.977$) or summary drinking data ($P = 0.375$) as measures of alcohol exposure.

DISCUSSION

The very high morbidity and mortality from cirrhosis in a cluster of central–eastern European countries has been the subject of intense speculation but rather less research. Because consumption is not markedly higher than in other European countries, this cannot be explained only by alcohol consumption. In previous studies it was shown that the widely available illegal alcohol beverages in Hungary contained toxic aliphatic alcohols [1,23].

In other European and Asian populations a strong relationship was found between alcoholism and the polymorphism of genes coding the ADH enzyme [7–15,24–26]. These studies indicate that the ADH1B 48His polymorphism can be protective against alcoholism, acting through the accumulation of acetaldehyde. Nevertheless, given the effect of acetaldehyde, it has been hypothesized that ADH1B*2 carriers persisting in drinking may increase their risk of liver disease [3,27].

In European populations the average allele frequency of the ADH1B*2 is approximately 5%, varying by ethnicity [25]. This allele was found in 8.31% of our control group, almost twofold higher than reported previously in western European populations. This may, however, be explained by genetic admixture from neighbouring Slavic populations, given the much higher prevalence observed in the Russian population [28].

ADH1B*2 carriage reduces the risk of developing liver disease (OR = 0.50), but this is no longer significant after adjustment for most of the measures of alcohol consumption. Hence, the polymorphism is likely to be associated with drinking behaviour, the main determinant of the disease.

Our results do not prove that the ADH1B*2 allele is implicated in the high prevalence of CLDs in the Hungarian population. We found a high prevalence of the protective ADH1B*2 allele, which reduces the risk of heavy drinking, but we did not demonstrate an increased risk of CLDs among carriers who drink heavily, although with CAGE status a significant effect modification was seen. In CAGE negatives, the allele's effect on cirrhosis is likely to manifest through changing drinking habits, but there is no effective tool to measure, and thus control for, the exact quantity of alcohol consumed. Overall, the CAGE questionnaire proved to be the most accurate measure of problem drinking.

However, it is important to recognize that our study may have been underpowered to capture a true effect due to the possibility that, among abstainers, some individuals have stopped drinking after being diagnosed with liver disease. Although, according to recent studies [29–31], there are several other polymorphisms affecting the alcohol metabolism, we have included only the main candidate to determine whether there is a possibility that genetic factors influence mortality rates and to provide a basis for further studies on this topic.

This is the first study to describe the allele frequency of ADH1B*2 in the general Hungarian population and in the subgroup with CLDs. There was an unexpectedly high prevalence of this polymorphism identified in the study, which should stimulate further work on identifying genetic influences on drinking problems among Hungarians.

Declarations of interest

None.

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COMBINED EFFECT OF ADH1B RS1229984, RS2066702 AND ADH1C RS1693482/ RS698 ALLELES ON ALCOHOLISM AND CHRONIC LIVER DISEASES

Réka Tóth[1,2], Szilvia Fiatal[1,2], Beáta Petrovski[1], Martin McKee[3], Róza Ádány [1,2]

[1] Department of Preventive Medicine, [2] Public Health Research Group of the Hungarian Academy of Sciences, Faculty of Public Health, Medical and Health Science Centre, University of Debrecen, Debrecen, Hungary, [3] London School of Hygiene and Tropical Medicine, London, UK

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/35Val 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 catalyzed 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 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 (Győr-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. Liver biopsy was not performed in the study group due its invasiveness. Patients with hepatitis B or hepatitis C infection were excluded from the analysis. Patients with Cytomegalovirus or Epstein-Barr virus infections or cholestatic diseases were included in the study. The final study population contained 666 controls and 241 cases. The age distribution of controls was representative of 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 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 LightCyler 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 ADB1B 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.

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).

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 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 Figure 2. As expected, there is a very high linkage between the two 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).

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

		Controls	Cases
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
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

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 labeled with fluorescein on their 3’ ends. The anchor probes are labeled 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

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

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 Ile350 Val (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.

Figure legend

Figure 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).

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

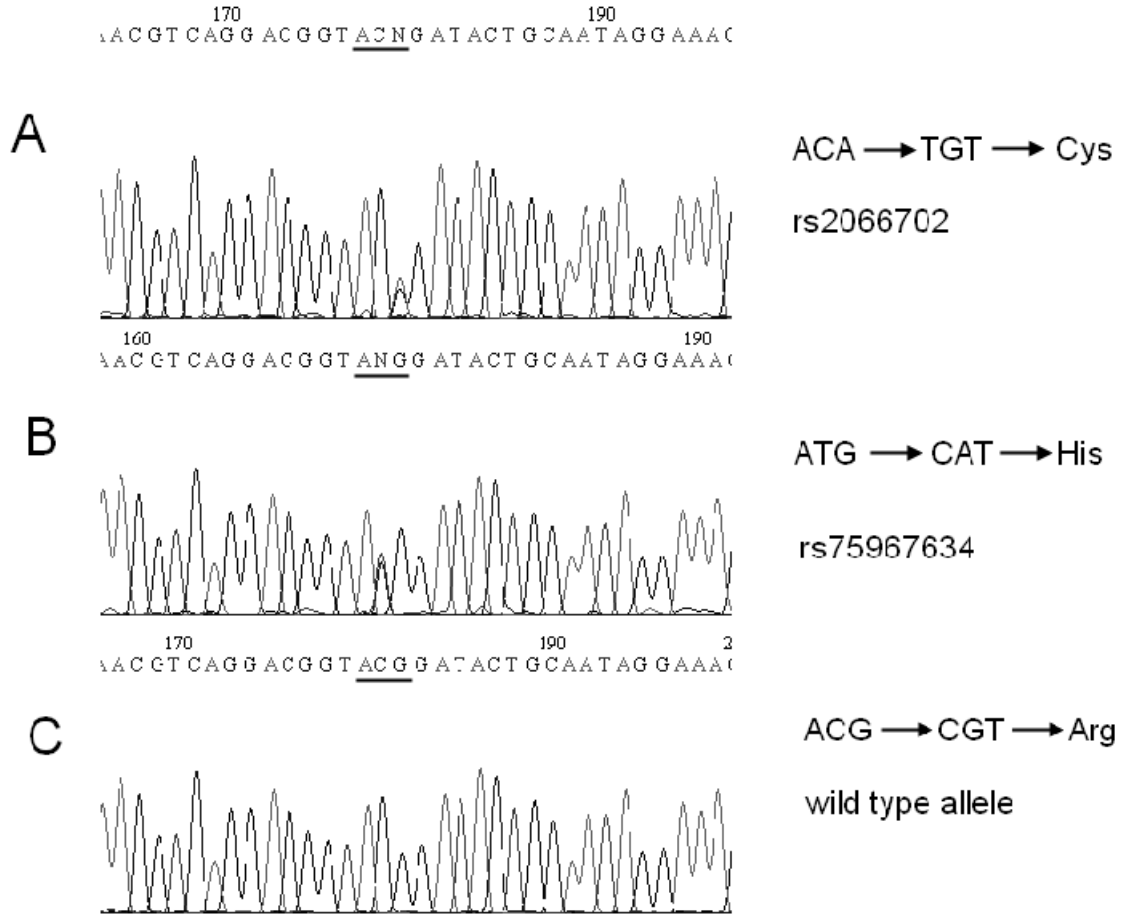
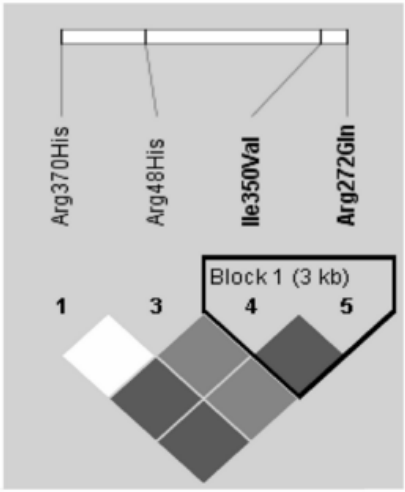

170
190
A
ACA → TGT → Cys
rs2066702
160
190
B
ATG → CAT → His
rs75967634
170
190
C
ACG → CGT → Arg
wild type allele

Figure 1.



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

Figure 2.