Polymorphisms of alcohol-metabolizing enzymes and the risk for alcoholism and alcoholic liver disease in Caucasian Spanish women

Alfons Lorenzo a,1, Teresa Auguet a,1, Francesc Vidal a,∗,1, Montserrat Broch b,1, Montserrat Olona c, Cristina Gutiérrez b, Miguel López-Dupla a, Joan-Josep Sirvent d, Joan-Carles Quer e, Mauro Santos f, Cristóbal Richart a

a Department of Internal Medicine, Hospital Universitari de Tarragona Joan XXIII, Institut d’Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, C/ Dr. Mallafré Guasch, 4, 43007 Tarragona, Spain

b Research Unit, Hospital Universitari de Tarragona Joan XXIII, Institut d’Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, 43077 Tarragona, Spain

c Epidemiology and Preventive Medicine Unit, Hospital Universitari de Tarragona Joan XXIII, Institut d’Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, 43007 Tarragona, Spain

d Department of Pathology, Hospital Universitari de Tarragona Joan XXIII, Institut d’Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, 43007 Tarragona, Spain

e Section of Digestive Diseases, Hospital Universitari de Tarragona Joan XXIII, Institut d’Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, 43007 Tarragona, Spain

f Department of Genetics and Microbiology, Universitat Autònoma de Barcelona, Bellaterra, Spain

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Abstract

Background: The relationship of polymorphisms of the genes that encode for alcohol-metabolizing enzymes and individual vulnerability to alcoholism and alcoholic liver disease (ALD) in women is unclear. We determined the genotypes of ADH1B, ADH1C, CYP2E1 (Dra-I and Pst-I) and ALDH2 in a group of Caucasian Spanish women.

Methods: We performed a cross-sectional case–control study. The study group was made of 220 women. Of these, 85 were alcoholic (27 without liver disease and 58 with alcoholic liver disease) and 135 were non-alcoholic (42 healthy controls and 93 with liver disease unrelated to alcohol). Genotyping of alcohol-metabolizing enzymes was performed using PCR-RFLP methods.

Results: The distribution of the allelic variants (alleles 1 and 2) in the whole subjects analyzed was: ADH1B 91.6% and 8.4%; ADH1C 58.4% and 41.6%; CYP2E1 Dra-I 15% and 85%; CYP2E1 Pst-I 96.8% and 3.2%; and ALDH2 100% and 0%, respectively. Carriage of genotypes containing the ADH1B*2 mutant allele significantly protected against alcoholism [odds-ratio (OR) = 0.00; 95% confidence interval (95% CI): 0.00–0.94; p = 0.02] but was associated with an increased risk for alcoholic liver disease among alcohol-dependent women [OR = 0.43; 95% CI: 0.18–0.41; p = 0.004]. Analysis of the remaining loci showed no significant associations.

Conclusions: In Caucasian Spanish women the ADH1B*2 allele modulates the risk for alcohol dependence and for alcoholic liver disease. Given the small number of alcoholic women analyzed here, these data need further validation in larger cohorts.

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Keywords: Women; Alcoholism; Alcoholic liver disease; Alcohol dehydrogenase; Cytochrome P4502E1; Aldehyde dehydrogenase

1. Introduction

Women are less likely to drink and less likely to drink greater amounts of alcohol than men. Despite this, women appear to have greater vulnerability than men to some of the adverse effects of long-term alcohol abuse. For example, liver injury appears more frequently and develops more readily in women than in men with comparable levels of alcohol abuse (Morgan and Sherlock, 1977). Although this has been known for a long time, the mechanisms underlying this gender difference are just becoming elucidated. Women’s smaller body size (Kwo et al., 1998), estrogens (Järveläinen et al., 1990; Yin et al., 2000), and lower alcohol dehydrogenase (ADH) activity in the gastric...
mucosa, which means that for a given amount of alcohol consumed blood levels are higher in women than in men (Frezza et al., 1990; Seitz et al., 1993), are some of the mechanisms proposed to explain the greater vulnerability of women to the toxic effects of alcohol abuse.

As occurs in men, not all women who drink alcohol in excess develop alcoholic liver disease (ALD). To explain the variable interindividual vulnerability to alcohol dependence and to its damaging end-organ consequences, both environmental and genetic factors have been advocated (Bellentani et al., 2000). Among host genetic factors, attention has recently been paid to the alcohol-metabolizing enzymes (Day and Bassendine, 1992; Goedde et al., 1992; Lumeng and Crabb, 1994; Shibuya and Yoshida, 1988). Several single nucleotide (SNP) polymorphisms with functional translation at the protein level have been detected and studied at the alcohol dehydrogenase (ADH), cytochrome P4502E1 (CYP2E1), and aldehyde dehydrogenase (ALDH) loci, often with controversial or non-conclusive results, especially in whites (Chao et al., 1994; Day and Bassendine, 1992; Goedde et al., 1992; Lumeng and Crabb, 1994; Shibuya and Yoshida, 1988; Thomasson et al., 1991; Vidal et al., 2004).

Noteworthy is that most genetic studies of polymorphism of alcohol-metabolizing enzymes and its relation with alcoholism and ALD have been done in men (Chao et al., 1994; Thomasson et al., 1991; Vidal et al., 2004), and data from women are to date scarce (Whittlefield et al., 1998; Borrás et al., 2000). The rationale for studying the role of these genetic host factors separately in women and in men is that, if studied together, the higher vulnerability of women to alcohol probably overshadows the effect of any predisposing or protective genetic factor. Recently, we reported a lack of association between the allelic variants of alcohol-metabolizing enzymes with alcoholism and ALD in Caucasian Spanish men (Vidal et al., 2004). Now, we provide here a study on the relationship of ADH1B (formerly ADH2), ADH1C (formerly ADH3), CYP2E1 Dra-I and Pst-I, and ALDH2, polymorphisms and their relation to alcoholism and ALD in Caucasian Spanish women.

2. Patients and methods

2.1. Participants

We studied 220 Spanish alcoholic and non-alcoholic women, either with or without liver disease, at the Hospital Universitari de Tarragona Joan XXIII. Immigrants from other countries and their descendants, including those from European countries, were excluded. As a control group, we studied a group of women, comparable by age and by number and type of liver disease, randomly selected from our database.

Patients were categorized as alcoholics according to the result of the CAGE (acronym of Cut, Annoyed, Guilty, Eye opener) questionnaire (Ewing, 1984) and data were obtained by face-to-face interview which included a detailed drinking history (age of starting of alcohol consumption, type and amount of alcohol ingested in beverages, age of first subsequent visits at the alcohol outpatient clinic, hospitalizations due to alcohol-related problems). In unclear cases, relatives were also interviewed. Almost all alcoholic women met the DSM-IV diagnostic criteria for alcoholism (Grant et al., 1992). Alcoholic women included patients with alcohol-induced cirrhosis, non-cirrhotic ALD (steatosis plus chronic hepatitis) and heavy drinkers without liver disease. The different types of ALD were diagnosed by means of examination of a liver biopsy. In alcoholic women with no liver disease, it was usually done because of the presence of an enlarged liver and/or abnormalities in the liver enzyme levels, or during elective abdominal surgery. Histologic examination of these samples indicated that the liver was normal. Non-alcoholic patients were women who drank less than 10 g/day of alcohol. They included healthy controls and patients with non-alcoholic chronic liver disease (in most cases chronic hepatitis or cirrhosis due to HCV infection, diagnosed by liver biopsy examination). Healthy controls were people with no history of alcoholism or chronic disease, no evidence of liver disease at physical examination and normal liver function test. Information about toxic and medical history was collected through a short standardized questionnaire. Informed consent was obtained from each patient included in the study. The study was approved by the institution’s Human Research Committee.

All 220 women studied were Caucasian Spaniards. Main demographic data are reported in Table 1. There were 85 alcoholic women with a mean age of 51.2 years. Of these, 27 (31.8%) showed no evidence of ALD, and 58 (68.2%) had ALD: 30 (35.3%) cirrhosis of the liver and 28 (32.9%) non-cirrhotic liver disease. These women were heavy alcohol consumers. The mean ± S.D. daily drink of alcohol was 114 ± 41 g, during a mean period of time of 13.3 ± 4.8 years, and these data were similar in patients with ALD and in those without. The non-alcoholic group was made of 135 women with a mean age of 54.6 years. Of these, 42 (31.1%) were healthy controls, and 93 (68.9%) had non-alcoholic liver disease: 49 (36.3%) non-alcoholic non-cirrhotic liver disease, and 44 (32.6%) viral cirrhosis.

2.2. Measurements

2.2.1. Blood samples. Blood samples were drawn in an EDTA vacutainer from an antecubital vein. Whole blood was used for DNA isolation, by using MagNa Pure LC Instrument and the MagNa Pure LC DNA isolation Kit I (Roche Diagnostics, USA).

2.2.2. Analytical methods: genotype determination. Restriction fragment length polymorphisms (RFLP) in the ADH1B, ADH1C, CYP4502E1 and ALDH2 genes were detected by digesting polymerase chain reaction-amplified DNA. For each PCR analysis, 100 ng of DNA was used. PCR analyses were performed with

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Alcoholism and liver disease in groups of women used for association studies with polymorphism of alcohol-metabolizing enzymes</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Alcohols (n=85)</td>
</tr>
<tr>
<td></td>
<td>No liver disease</td>
</tr>
<tr>
<td>Population studied, n (%)</td>
<td>27 (31.8)</td>
</tr>
<tr>
<td>Alcohol consumption (mean ± S.D.)</td>
<td>107 ± 36 g/day</td>
</tr>
<tr>
<td>Years</td>
<td>13.1 ± 3.3</td>
</tr>
<tr>
<td>Age (mean ± S.D.)</td>
<td>53.8 ± 16</td>
</tr>
</tbody>
</table>

a N: negligible.
2.2.2.1. ADH1B and ADH1C genotypes. The ADH1B SNP studied is a G to A transition in exon 3 which translates histidine instead of arginine at the residue 47 (Arg47Gly). The ADH1C SNP studied is an A to G transition in exon 8 which translates valine instead of isoleucine at the residue 349 (Ile349Val). The amplification reactions were carried out in a final volume of 15 μl containing 1.5 mM of MgCl₂, 0.2 mM of each nucleotide (Boehringer Mannheim, Germany), 0.2 μM of each primer and 2 units of Thermus aquaticus (Taq) DNA polymerase (Gibco BRL). DNA was amplified for 35 cycles. Each cycle consisted of 1 min denaturation at 94 °C, 45 s annealing at 55 °C and 5 min extension at 72 °C. The primers used were: 5′-ATCTTTTCTCTGAAATGACA-3′ and 5′-GAAGGGGGCTACCAAGTTG-3′ for ADH1B genotypes and 5′-GCTTTAAGGATTAATACCTGTC-3′ and 5′-AATCTACC-TCTTTCAGAGC-3′ for ADH1C genotypes (Gibco BRL). For allele detection, aliquots of the amplified DNA products were digested with Msel at 55 °C for ADH1B, or with Sphl at 37 °C (Roche Molecular Biochemicals) for ADH1C. Digestion products were run on 2.5% resolution agarose gels and stained with ethidium bromide. The genotypes identified were named according to the presence or absence of the enzyme restriction sites. So MselI GG/G = *1/*1, GA/A = *1/*2 and AA/A = *2/*2 are homozygotes for the absence of site (95 bp), heterozygotes (60/35/95 bp), and homozygotes for the presence of site (60/35/95 bp), Sphl A/A = *1/*1, AG/G = *1/*2 and GG/G = *2/*2 are homozygotes for the absence of site (130 bp), heterozygotes (67/63/130 bp), and homozygotes for the presence of site (67/63 bp).

2.2.2.2. CYP4502E1 genotypes. We studied two SNPs. The Pst-I SNP is a G to C transversion in the 5′ flanking region of the gene. The Dra-I SNP is a T to A transversion in intron 6. The amplification reactions were conducted in a final volume of 50 μl containing 1.5 mM of MgCl₂, 0.2 mM of each nucleotide (Boehringer Mannheim, Germany), 0.2 μM of each primer and 2 units of Taq DNA polymerase (Gibco BRL). DNA was amplified for 35 cycles. Each cycle consisted of 30 s denaturation at 95 °C, 30 s annealing at 63 °C for the Dra-I polymorphism and at 54 °C for the Pst-I polymorphism, and 45 s extension at 72 °C. The primers used were 5′-AGTCGACATGTGATGGATCCA-3′ and 5′-TCTTTCCAGAGC-3′ for Dra-I, 5′-ATCTTTCTCTGAAATGACA-3′ and 5′-GAAGGGGGCTACCAAGTTG-3′ for Pst-I (Gibco BRL). For allele detection, aliquots of the amplified DNA products were digested with Dra-I and Pst-I, both at 37 °C for each polymorphism (Roche Molecular Biochemicals). Digestion products were run on 4% agarose gels and stained with ethidium bromide. The genotypes identified were named according to the presence or absence of the enzyme restriction sites. So, Dra-I *1/*1, *1/*2 and *2/*2 were homozygotes for the absence of site (375 bp), heterozygotes (249/126/375 bp), and homozygotes for the presence of site (249/126 bp). Pst-I *1/*1, *1/*2 and *2/*2 were homozygotes for the absence of site (410 bp), heterozygotes (290/120/410 bp), and homozygotes for the presence of site (290/120 bp).

ADH2 genotype. The ADH2 SNP studied is a G to A transition in exon 12 which translates lysine instead of glutamic acid at the residue 487 (Glu487Lys). An amplification created restriction site was performed by introducing a single base mismatch into the 3′ end of an antisense primer, which is immediately adjacent to the mutation site and can create an MboII recognition site in the wild-type nucleotide sequences after amplification. In accordance with the principle mentioned above the primers used in the PCR were 5′-CCACACTCAAGTTTCTCTT-3′ and 5′-CCACACTCAAGTTTCTCTT-3′. Amplification was performed in a final volume of 50 μl containing 0.2 mM of each nucleotide (Boehringer Mannheim), 1 mM MgCl₂, 1 μM of each oligonucleotide and 1 U of Taq polymerase (Gibco BRL). The reaction was carried out using 30 thermal cycles in the following conditions: an initial denaturation of 5 min at 94 °C and a final extension of 10 min at 72 °C. The cycle program consisted of a 1 min denaturation at 94 °C, 3 min annealing at 53 °C and a 1 min extension at 72 °C. PCR products were digested with MboII restriction enzyme at 37 °C overnight and electrophoresed on a 4% agarose gel. A 135 bp band corresponded to the mutant allele (A = *2) and a set of 125 and 10 bp bands corresponded to the wild-type allele (G = *1).

2.2.3. Statistical analysis. We performed descriptive analyses of the variables using absolute and relative frequencies for categoric variables, and mean and standard deviation (S.D.) for continuous variables. The variation in the allele frequencies of different groups was analyzed by means of the population genetics software GENEPOP (Raymond and Rousset, 1995). Differences between groups were analyzed through the Pearson χ²-test or Fisher’s exact test for categoric variables. Odds-ratio (OR) and 95% confidence interval (95% CI) were used to evaluate associations. Assessment of 95% CI was performed according to exact method (Mehta et al., 2004). χ² goodness-of-fit tests were used to study agreement with Hardy–Weinberg equilibrium (HWE). Linkage disequilibrium between ADH1B and ADH1C was estimated by means of the complete dipheric disequilibrium coefficient ΔAB (Weir and Cockerman, 1989).

3. Results

The genotype distribution of the control group fit the expected HWE. The genotype distribution and allelic frequencies in the whole population studied and in the groups defined for the loci examined are shown in Tables 2–5.

3.1. ADH1B genotype

Data with respect to ADH1B are shown in Table 2. Overall, genotype and allele distribution showed no difference between alcoholics and non-alcoholics. For alcoholics with no liver disease, a G to A transition in exon 8 which translates glutamic acid instead of glutamic acid at the residue 487 (Glu487Lys).
locations when all alcoholic versus all non-alcoholic women were compared. To overlap the confusion created by the powerful effect of the mutant ADH1B*2 allele of patients with alcoholic liver disease (see below), we compared the patients without liver disease, alcoholics (n = 85) versus non-alcoholics (n = 42). Genotypewise comparisons indicated that heterozygous ADH1B*2 carriage (homozygous mutants were not detected in this sample) was higher in non-alcoholic women (0% versus 15%) and this difference was near the limit of significance or just-significant [OR = 0.00; 95% CI: 0.00–1.25; p = 0.07]. When we compared people without liver disease, alcoholics (n = 27) versus all non-alcoholics (n = 135), genotypes containing the ADH1B*2 mutant allele were significantly higher in non-alcoholics [OR = 0.00; 95% CI: 0.00–0.94; p = 0.02], indicating that carriage of this allele protects against alcoholism. Moreover, genotypes containing the ADH1B*2 allele were also significantly more frequent in alcoholics with liver disease than in alcoholics without liver disease [OR = 0.43; 95% CI: 0.18–0.41; p = 0.004], suggesting that carriage of the mutant ADH1B*2 allele increases the risk of alcoholic liver disease in women who are alcohol-dependent.

Table 4
Genotype distribution and allele frequencies of CYP4502E1 Dra-I in all women, and according to drinking habits and presence of liver disease

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Genotype (n)</th>
<th>Allele (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>*1/*1</td>
<td>*1/*2</td>
</tr>
<tr>
<td>All</td>
<td>220</td>
<td>4</td>
<td>58</td>
</tr>
<tr>
<td>Alcoholics</td>
<td>85</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>No liver disease</td>
<td>27</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Liver disease</td>
<td>58</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>Non-alcoholics</td>
<td>135</td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td>No liver disease</td>
<td>42</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Liver disease</td>
<td>93</td>
<td>4</td>
<td>18</td>
</tr>
</tbody>
</table>

Differences in genotype distribution and allele frequencies between the groups defined were not significant.

3.2. ADH1C genotype

For ADH1C, when we compared alcoholics versus non-alcoholics, as well as the different types of liver disease, the differences in genotype distribution and allele frequencies were non-significant (Table 3).

3.3. Linkage disequilibrium between ADH1B and ADH1C loci

No significant linkage disequilibrium could be demonstrated. The digenic disequilibrium coefficients were $\Delta_{AB} = -0.0141; p = 0.24$, for the non-alcoholic group and $\Delta_{AB} = -0.0068; p = 0.46$, for the alcoholic group.

3.4. CYP2E1 Dra-I genotype

Table 4 shows that there were no significant differences in the distribution of the rare d1 and common d2 allele between alcoholics and non-alcoholics, either with or without liver disease.

3.5. CYP2E1 Pst-I genotype

For the CYP2E1 Pst-I locus data are shown in Table 5. Genotype and allele distribution was similar between alcoholics and non-alcoholics. With regard to the risk of liver disease, carriers of the rare c2 allele were overrepresented in alcoholics with liver disease with respect to alcoholics without liver disease although comparison of numbers gave a just-significant difference [OR = 0.00; 95% CI: 0.00–2.31; p = 0.06]. Comparison of the distribution of the common c1 and rare c2 alleles in the remaining groups defined did not find differences.

3.6. Analysis of ADH and CYP2E1 polymorphism associations

When the polymorphic ADH and CYP2E1 systems were analyzed together, there was no evidence of gene–gene interaction in relation to alcoholism or the development of ALD.

3.7. ALDH2 genotype

ALDH2 gene polymorphism was evaluated in 100 women (50 alcoholics and 50 non-alcoholics) with and without liver disease. All carried the 1/1 wild-type allele.

4. Discussion

The present report shows that carriage of the mutant ADH1B*2 allele modulates risk of alcohol dependence in Caucasian Spanish women. In contrast, no relationship between ADH1C and CYP2E1 Dra-I and Pst-I polymorphisms and the risk of alcoholism could be established. We report also that the mutant ADH1B*2 allele is overrepresented in alcohol-dependent women with ALD. On the contrary, ADH1C, and CYP2E1 Dra-I and Pst-I genotypes are not related to the risk of developing...
alcoholic liver disease. As men, women have a monomorphic \textit{ALDH2} locus, since they lack the \textit{ALDH2*2} inactive isoform.

Present knowledge suggests that alcoholism is at least partially genetically mediated. This is highlighted by data from adoption and twin studies, which situate the influence of genetics in about half of the risk for alcohol dependence (Schuckit, 2000), a finding that has already been shown both in men and in women (Heath et al., 1994, 1997). Information available on the relationship between alcoholism and candidate genes involved, such as those that encode for alcohol-metabolizing enzymes, is extensive in men but data in women are scarce. In this regard, our results suggest a relationship between the highly active atypical \textit{ADH}, which is encoded by the \textit{ADH1B*2}, and alcohol dependence in a very homogenous sample of Spanish women. These data are in disagreement with a study performed in Chinese women (Cheng et al., 2004) and with two previous studies who involved a subset of European women one of which was participated by us (Whitfield et al., 1998; Borràs et al., 2000). No data have been reported in the literature regarding the effect of \textit{ADH1C} and \textit{CYP2E1} genetic variants and alcoholism in women. Although to a lesser extent than \textit{ADH1B}, \textit{ADH1C} and \textit{CYP2E1} oxidize a significant portion of the alcohol consumed, particularly \textit{CYP2E1} which is induced in chronic alcohol abusers (Oneta et al., 2002). The data presented here suggest that polymorphisms at the \textit{ADH1C} and at the \textit{CYP2E1} Dra-I and \textit{Pst-I} loci do not modulate the risk for alcohol dependence. Evidence of allele linkage between \textit{ADH1B} and \textit{ADH1C} genes has been found in Asians (Thomasson et al., 1991; Osier et al., 1999) and also in Europeans (Borràs et al., 2000). Our results show that the \textit{ADH1B*2} and \textit{ADH1C*1} alleles do not demonstrate a significant linkage disequilibrium in our female population, similar to that occurred in our male population (Vidal et al., 2004). The low \textit{ADH1B*2} frequency in Caucasians means that the effect of the allele linkage on the \textit{ADH1C} distribution must be smaller than in Asians. The role of \textit{ALDH2} has not been assessed because is monomorphic in Caucasians, a fact also confirmed here.

If information regarding the association of carriage of the allelic variants of the genes that encode for alcohol-metabolizing enzymes with alcoholism in women is scarce, virtually no data are available regarding the risk for developing alcoholic liver disease. In this regard, we report a positive relationship between the \textit{ADH1B*2} mutant allele and the presence of alcoholic liver disease in women, although these data should be interpreted with great caution (see below). On the contrary, carriage of allelic variants of \textit{ADH1C}, and \textit{CYP2E1} Dra-I and \textit{Pst-I} do not appear to influence the risk for alcoholic liver disease in the population examined, a finding consistent with our results in males (Vidal et al., 2004) and a meta-analysis (Wong et al., 2000). As the \textit{ADH1B*2} allele encodes a very active enzyme, which is 10–40 fold more active than encoded by \textit{ADH1B*1}, our results are consistent with the hypothesis that the amount of generation of acetaldehyde from ethanol, a step mainly done by ADH, is a significant determinant for the risk of alcoholic liver disease, because the most active ADH isoenzyme is associated with a greater risk for alcoholic liver disease. A question arises whether this genotype does not play a similar role in Spanish women (Vidal et al., 2004), and plausible explanations should be searched in that the higher amount of ethanol that arrives at the hepatocyte after the first-pass metabolism in women modulates the relevance of the genetic variants of \textit{ADH1B}.

The data presented here also point out that the overall distribution of the genotypes of alcohol-metabolizing enzymes in Caucasian Spanish women is similar to that in Caucasian Spanish men, but their effects on alcohol dependence and ALD are somewhat different. While in men we found no association between allelic variants of genes that encode for alcohol-metabolizing enzymes (Vidal et al., 2004), in women this association exists and is particularly robust for \textit{ADH1B} and the risk for ALD. This means that gender could be a determinant factor of the modulator effect of the genetic variants of \textit{ADH1B} over the risk for alcoholism and for ALD. To validate this finding, we performed a test for heterogeneity of our data for men (Vidal et al., 2004): OR = 0.80; 95% CI: 0.26–2.31, and for women: OR = 0.00; 95% CI: 0.00–1.14 (p = NS, when comparing both OR). With these results, we cannot exclude an association between the two genders, hence the fact that our findings in women might be due to chance, given the low numbers of women studied, cannot be ruled out. We acknowledge that the small size of the groups defined is the main limitation of our study, because some of the polymorphisms assessed are so infrequent that assessment of large cohorts will be needed to offer reproducible results. This, however, will be difficult to achieve since alcoholism is uncommon in women and, often, a hidden conduct. This is highlighted by the fact that we have spent more than 10 years for collecting a series of 85 exquisitely defined alcoholic women. Multicentre studies are therefore needed to collect appropriate numbers.

We can conclude that, in contrast to what occurs in Caucasian Spanish men (Vidal et al., 2004), carriage of \textit{ADH1B*2} protect against alcohol dependence but increases the risk for ALD in Caucasian Spanish women. This may be a complementary explanation for the women’s greater vulnerability to liver damage induced by alcohol. However, given the small number of women presented here studies involving larger numbers of alcoholic women are warranted to confirm these data.

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