Human Molecular Genetics, 2009, Vol. 18, No. 3 **580–593**
doi:10.1093/hmg/ddn372
Advance Access published on November 7, 2008

Associations of *ADH* and *ALDH2* gene variation with self report alcohol reactions, consumption and dependence: an integrated analysis

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Received August 31, 2008; Revised and Accepted November 4, 2008

Alcohol dependence (AD) is a complex disorder with environmental and genetic origins. The role of two genetic variants in *ALDH2* and *ADH1B* in AD risk has been extensively investigated. This study tested for associations between nine polymorphisms in *ALDH2* and 41 in the seven *ADH* genes, and alcohol-related flushing, alcohol use and dependence symptom scores in 4597 Australian twins. The vast majority (4296) had consumed alcohol in the previous year, with 547 meeting DSM-IIIR criteria for AD. There were study-wide significant associations ($P < 2.3 \times 10^{-4}$) between *ADH1B-Arg48His* (rs1229984) and flushing and consumption, but only nominally significant associations ($P < 0.01$) with dependence. Individuals carrying the rs1229984 G-allele (*48Arg*) reported a lower prevalence of flushing after alcohol ($P = 8.2 \times 10^{-7}$), consumed alcohol on more occasions ($P = 2.7 \times 10^{-6}$), had a higher maximum number of alcoholic drinks in a single day ($P = 2.7 \times 10^{-6}$) and a higher overall alcohol consumption ($P = 8.9 \times 10^{-8}$) in the previous year than those with the less common A-allele (48His). After controlling for rs1229984, an independent association was observed between rs1042026 (*ADH1B*) and alcohol intake ($P = 4.7 \times 10^{-5}$) and suggestive associations ($P < 0.001$) between alcohol consumption phenotypes and rs1693482 (*ADH1C*), rs1230165 (*ADH5*) and rs3762894 (*ADH4*). *ALDH2* variation was not associated with flushing or alcohol consumption, but was weakly associated with AD measures. These results bridge the gap between DNA sequence variation and alcohol-related behavior, confirming that the *ADH1B-Arg48His* polymorphism affects both alcohol-related flushing in Europeans and alcohol intake. The absence of study-wide significant effects on AD results from the low *P*-value required when testing multiple single nucleotide polymorphisms and phenotypes.

INTRODUCTION

Alcohol dependence (AD) is a complex behavioral disorder with both social and biological origins (1–3), characterized by a syndrome of serious problems related to alcohol use. Diagnostic criteria include withdrawal symptoms after cessation of heavy intake, tolerance to alcohol's effects, continued use despite evidence of physical or psychological problems due to drinking, persistent desire to quit drinking or inability to do so, and spending substantial amounts of time drinking or recovering from drinking. Family, twin and adoption studies support the view that genes contribute between 40 and 60% of the variance in AD risk (4–8). Environment also contributes a substantial proportion to the risk of AD, and different phenotypic characteristics add to the AD risk in different families (9–13). The genetic variance in AD risk is likely to reflect gene polymorphism effects on multiple characteristics, which collectively impact on the probability of hazardous or harmful drinking and on the progression to dependence. Some of these characteristics seem to be related

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to alcohol's metabolism or to its acute effects, while others relate to other types of psychopathology or to personality.

The roles of the biologically relevant alcohol-metabolizing genes, alcohol dehydrogenases (particularly the Class I, low-K_m ADHs *ADH1A*, *ADH1B* and *ADH1C*) and aldehyde dehydrogenases (particularly the low-K_m mitochondrial *ALDH2*), in AD risk have been extensively investigated, initially in Asians (14–16) and subsequently in European (17–22) and African (23,24) populations. The significant associations between both *ALDH2* and *ADH1B* variants and AD risk have been explained on the hypothesis that any increase in acetaldehyde production, or reduction in its subsequent elimination, will reduce an individual's vulnerability to alcohol abuse and alcoholism because of the aversive effects associated with elevated blood and tissue acetaldehyde levels (2,25). This has been demonstrated by the measurement of acetaldehyde in the blood after alcohol consumption in people with ALDH2 deficiency, but not in relation to *ADH* variants.

ALDH2

Acetaldehyde, generated by ethanol oxidation in the liver, is further metabolized to acetate by aldehyde dehydrogenases (ALDH) (26,27). The high-affinity mitochondrial ALDH2 is mainly responsible, but ALDH2 deficiency is common in parts of Asia. The effect of the inactive ALDH2*2 enzyme was first investigated by Wolff (28) who observed racial differences in the facial flushing response during alcohol consumption. The *ALDH2*1* (504Glu) allele encodes an active subunit while *ALDH2*2* (504Lys) encodes a subunit that is essentially inactive (consequently causing a build-up of acetaldehyde in the blood and other tissues). Hybridization of an ALDH2*2 enzyme subunit with an ALDH2*1 subunit results in the inactivation of the isozyme and an ALDH2-deficient phenotype (1,29,30). Individuals homozygous for the *ALDH2*2*, or heterozygous, are therefore deficient in the conversion of acetaldehyde to acetate, have high blood acetaldehyde levels after alcohol consumption and suffer from adverse reactions to alcohol, such as severe facial flushing, nausea, headache and tachycardia. The importance of *ALDH2* genetic variation in risk for AD has been well established among Asian populations where the flushing response is observed in 57–80% of individuals, and the *ALDH2*2* allele frequency ranges from 0.25 to 0.35. Heterozygotes are at reduced risk of AD compared to *ALDH2*1* homozygotes, while individuals homozygous for *ALDH2*2* have a very low risk for AD (14,15,31). This polymorphism (rs671) is confined to populations from North-East Asia and groups with ancestry from that region.

ADH1B

Both *ADH1B* and *ADH1C* exhibit single nucleotide polymorphisms (SNPs) resulting in critical single amino acid exchanges at different sites in the NAD^+ coenzyme-binding domain. Accordingly, the enzymes encoded by polymorphic forms of *ADH1B* and *ADH1C* display different maximal activities (V_{max}) and affinities (K_m) for ethanol (1,32,33). This feature has been widely cited as the explanation for the well-validated effects of *ADH1B* variation (34–36) on alcohol use and dependence.

The $\beta_2\beta_2$ isozyme resulting from the 48His form of *ADH1B Arg48His* has a significantly higher K_m value for ethanol, and a maximal activity 40-fold that of $\beta_1\beta_1$ (27). Therefore, *ADH1B 48His* homozygotes are predicted to produce higher levels of acetaldehyde than any other *ADH1B* genotype. While facial flushing has been observed in Japanese drinkers either hetero- or homozygous for *ADH1B*2* after controlling for *ALDH2* genotype (37–39), other reports are negative or equivocal (40–43).

The *ADH1C*-encoded γ_1 and γ_2 homodimers also show a difference in their V_{max} activities. Since the V_{max} of the $\gamma_1\gamma_1$ isozyme is 2.5-fold higher than $\gamma_2\gamma_2$, people carrying *ADH1C 349Ile* have a faster predicted elimination rate for alcohol. Conflicting reports have been published on the association between *ADH1C Ile349Val* and AD. Although strong LD exists between *ADH1B Arg48His* and *ADH1C Ile349Val* (16,18), the genetic polymorphisms of *ADH1B*, rather than *ADH1C*, have the stronger association with the development of alcoholism.

Most early studies on the effects of *ADH1B* variation were performed in China or Japan, where the frequency of the *ADH1B 48His* allele is higher than elsewhere (44). Turning to studies on population groups outside East Asia, a study on Israeli Jews by Neumark *et al*. (45) indicated that the *ADH1B* polymorphism accounted for 20–30% of variation in alcohol intake, and the less common *ADH1B 48His* served as a protective factor against AD. Similar effects on alcohol consumption have been reported in young (age <33), but not older, Israeli Jews (46). We also found an association between *ADH1B 48His* and alcohol intake and dependence in our earlier study of Australians of European descent (17). Borras *et al*. (18) found an association between *ADH1B* but not *ADH1C* genotype and AD in Caucasian people. Other studies have observed a small protective function for *ADH1C 369Ile* in Caucasians (2,47). Some recent studies indicate that both *ADH1C* and *ADH1B* independently affect drinking habits in European (48) and Japanese (49) people.

The main gap in our understanding lies between the effects of *ADH* polymorphisms on enzyme activity and the differences in alcohol use associated with these polymorphisms. Although *in vitro* ADH activity is affected, the evidence that alcohol metabolism *in vivo* is affected is sparse and largely (50,51) but not entirely (52) negative. Also, there is no direct evidence that *ADH* variation produces differences in acetaldehyde generation during alcohol metabolism, nor that the *ADH* variants lead to differences in the subjective responses (positive or negative) to alcohol use.

To date, the majority of association studies investigating the role of alcohol metabolizing genes in risk for AD have focused on the well-characterized coding variants within *ADH1B, ADH1C* and *ALDH2* and on the phenotype of AD. Three recent studies, however, have systematically analyzed polymorphisms within the seven *ADH* cluster genes (53) as well as *ALDH1A1* and *ALDH2* (21) in non-Asian samples. Edenberg *et al.* (19) observed evidence of association between AD and multiple *ADH4* SNPs spanning a single haplotype block within the Collaborative Study on the Genetics of

Table 1. Summary of the sample demographics and alcohol-related traits, by sex

	Male ($n = 1478$)	Female ($n = 2818$)
Age at time of interview (years)	42.5 ± 10.9	44.1 ± 11.6
Age range (years)	26–89	28–84
Has consumed alcohol in the previous 12 months, *n* (%)	1407 (95.2)	2624 (93.1)
Drinking frequency during the past 12 months (days)	124.6 ± 112.8	82.6 ± 105.3
Percentage who drink daily (%)	8	6
Percentage who drink at least weekly (%)	69	46
Number of drinks typically consumed on days when they drank alcohol in the past 12 months	2.9 ± 2.3	1.9 ± 1.5
Percentage of men who consume four or more drinks and women who consume three or more drinks (%)	28	24
Quantity of drinks consumed in previous 12 months (Freq × TypDy)	406.9 ± 537.8	180.7 ± 277.0
Most drinks consumed in a single day in the previous 12 months	7.6 ± 6.6	3.7 ± 3.2
Most drinks ever consumed in a single day	16.7 ± 11.5	7.1 ± 6.0
Has ever flushed or blushed while consuming 1 or 2 drinks of alcohol, *n* (%)	241 (16.6)	887 (31.6)
Percentage who always experience flushing (%)	2.3	8.1
Has ever had a negative reaction (flushing, nausea, headaches, palpitations, hives, extreme sleepiness) while consuming one or two drinks of alcohol, *n* (%)	575 (39.6)	1452 (52.0)
Percentage who always experience negative reaction (%)	7.9	15.5
DSM-IIIR AD score (number of criteria met)	2.15 ± 2.12	0.80 ± 1.47
Percentage with no symptoms (%)	34	69
Diagnosed with DSM-IIIR AD, *n* (%)	375 (25.4)	172 (6.1)

Lifetime abstainers are excluded.

Alcoholism (COGA) cohort of United States families enriched for alcoholism. They did not report association with either the *ADH1B Arg48His* or *ADH1C Ile369Val* variants. Luo *et al.* (20) typed 20 SNPs in ALDHs and ADHs and found evidence for effects of the major *ADH1B* variants in European- and African-Americans. They also found evidence for differences in *ADH4* SNP genotype (but not allele) frequencies between controls and patients with alcohol or cocaine dependence (54). Kuo *et al*. (21) reported the association between one SNP each in *ADH5* and *ADH1B* and AD in the Irish Affected Sib Pair Study of Alcohol Dependence (IASPSAD) sample composed of severely affected cases. No association was observed with any *ALDH1A1* or *ALDH2* marker, nor with the *ADH1C*1* variant.

We have now genotyped 50 SNPs, spanning the seven *ADH* genes and *ALDH2*, in 4597 individuals representative of the general European-Australian population. SNPs were chosen to provide comprehensive coverage of the *ADH* gene cluster, including the atypical *ADH1B*2* (His48) marker, and also of *ALDH2*. We explore the relationships between variation in the alcohol metabolizing genes and self-reported reactions to alcohol, as well as quantitative alcohol consumption measures and both quantitative and binary AD measures within our Caucasian population.

RESULTS

A total of 4597 twin participants provided blood samples and completed the SSAGA-OZ interview. Two thousand six hundred and eighteen families were included in the analysis. Since parents were not genotyped, only families containing a non-identical sibling pair contributed to the 'within-family' tests of association. One thousand one hundred and seventy-seven families included a dizygotic (DZ) pair and hence were potentially informative for both the 'within' and the 'total' tests of association. Eight hundred and fourteen families included a monozygotic (MZ) pair and hence contributed information to only the 'total' test. Similarly, 627 families had just a single offspring and these contributed information to only the 'total' test. The mean age of the study population was 43.8 ± 11.5 years, ranging from 26 to 89 years. The mean age of female participants was significantly higher (44.1 versus 42.5) than for males ($P = 0.001$) (Table 1). While a small percentage (<3%) of the sample had always abstained from alcohol, the majority (~93%) had consumed alcohol in the previous year, with 7.6 and 5.5% of male and females, respectively, reporting that they drank alcohol on a daily basis. With respect to AD, male participants were approximately four times more likely to meet a lifetime diagnosis of DSM-IIIR dependence than females (25.4 versus 6.1%). However, ~36% of males and ~70% of females did not report any symptoms of AD.

Marker information including chromosomal position and minor allele frequencies for the 50 SNPs genotyped in *ALDH2* and the seven *ADH* genes are given in Table 2. Call rates of ≥98% were achieved for all SNPs except rs441 (97.9%), rs3098808 (97.7%), rs737280 (97.3%) and rs968529 (95.4%). The average genotyping rate among all SNPs was 98.8% with samples successfully genotyped on an average for 49 out of 50 SNPs. Two markers (rs1154409 in *ADH5* and rs671 in *ALDH2*) were non-polymorphic in our sample and two SNPs had a minor allele frequency (MAF) <0.05. Discordant genotypes between MZ twins were identified using PEDSTATS and made up 0.01% of the MZ data. Non-polymorphic markers and SNPs that showed significant deviations from Hardy–Weinberg equilibrium (HWE) at a $P < 0.001$ level in the overall sample (rs284784 in *ADH7* and rs3098808 in *ADH1C*) were excluded from further analysis. The physical locations of and linkage disequilibrium (LD) between the remaining 8 *ALDH2* SNPs and 38 *ADH* SNPs are presented schematically in Figure 1. Substantial LD ($|D'| > 0.8$) was observed between eight *ALDH2* SNPs which are consistent with LD data (on a smaller number of people)

Table 2. *ADH* and *ALDH2* gene marker information

Gene	Chr	SNP	Chromosome location[a]	Gene location[b]	Alleles Minor[c]	Major	MAF	pHWE[d]	Call rate (%)
METAP1	4	rs1230210	100 186 714	Intron 5	T	C	0.273	0.39	99.2
ADH5	4	rs1230165	100 205 396	3′-UTR	C	T	0.181	0.79	99.1
ADH5	4	rs896992	100 221 395	Intron 4	G	A	0.329	1.00	99.1
ADH5	4	rs1154409	100 226 893	Intron 1	T	G	0[e]	1.00	99.3
	4	rs2602877	100 258 870	Intergenic	A	T	0.271	0.59	99.1
	4	rs2602878	100 258 976	Intergenic	A	C	0.270	0.57	99.1
ADH4	4	rs1042364	100 264 597	3′-UTR	A	G	0.277	1.00	99.1
ADH4	4	rs1126672	100 266 835	Exon 8	T	C	0.278	0.65	99.4
ADH4	4	rs1800759	100 284 532	Promoter	A	C	0.385	0.18	98.6
ADH4	4	rs4140388	100 284 757	Upstream	C	G	0.459	0.69	98.4
	4	rs3762894	100 285 107	Intergenic	G	A	0.160	0.98	98.7
	4	rs1984364	100 289 806	Intergenic	G	T	0.275	0.95	99.2
	4	rs1540053	100 301 177	Intergenic	C	T	0.235	0.95	98.6
ADH6	4	rs2000864	100 342 799	Downstream	T	G	0.487	0.43	98.9
ADH6	4	rs4147545	100 347 776	Intron 6	G	A	0.320	0.24	99.4
	4	rs1230025	100 405 399	Intergenic	T	A	0.230	0.51	98.0
ADH1A	4	rs3819197	100 419 532	Intron 8	A	G	0.231	0.05	98.6
ADH1A	4	rs2276332	100 422 470	Intron 6	G	T	0.086	0.68	98.9
ADH1B	4	rs1042026	100 447 489	3′-UTR	G	A	0.279	0.92	98.6
ADH1B	4	rs17033	100 447 968	3′-UTR	G	A	0.086	0.52	99.5
ADH1B	4	rs6850217	100 452 643	Intron 6	A	G	0.390	0.72	99.2
ADH1B	4	rs2018417	100 454 163	Exon 6	T	G	0.040	1.00	98.9
ADH1B	4	rs4147536	100 458 135	Intron 3	T	G	0.213	0.89	99.0
ADH1B	4	rs1229984	100 458 342	Exon 3	A	G	0.035	0.11	99.0
ADH1B	4	rs1159918	100 462 032	Upstream	T	G	0.361	0.89	98.9
	4	rs2866152	100 470 090	Intergenic	G	C	0.244	0.98	99.5
ADH1C	4	rs1789895	100 475 752	Downstream	C	G	0.323	0.60	98.9
ADH1C	4	rs3098808	100 475 847	Downstream	C	T	0.114	<0.0001	97.7
ADH1C	4	rs1693482	100 482 988	Exon 6	A	G	0.410	0.84	98.7
ADH1C	4	rs283416	100 490 379	Intron 1	T	C	0.065	1.00	98.6
	4	rs1583973	100 506 906	Intergenic	T	C	0.099	0.95	98.8
	4	rs1826906	100 520 071	Intergenic	C	T	0.326	0.45	99.1
	4	rs2032350	100 533 085	Intergenic	T	C	0.198	0.08	98.5
	4	rs1348276	100 544 653	Intergenic	G	T	0.410	0.48	98.5
	4	rs994772	100 546 687	Intergenic	A	G	0.106	0.87	98.8
ADH7	4	rs284784	100 554 897	Intron 8	T	G	0.270	<0.0001	98.4
ADH7	4	rs1154454	100 557 365	Intron 7	C	T	0.163	1.00	99.3
ADH7	4	rs1154458	100 559 545	Intron 6	G	C	0.408	0.23	98.8
ADH7	4	rs971074	100 560 884	Exon 6	A	G	0.113	0.97	99.4
ADH7	4	rs1154461	100 561 925	Intron 5	C	G	0.351	0.45	98.8
	4	rs1583971	100 626 045	Intergenic	A	T	0.111	0.08	99.7
ALDH2	12	rs737280	110 657 696	Upstream	C	T	0.272	0.04	97.3
ALDH2	12	rs886205	110 667 147	5′-UTR	G	A	0.170	0.20	99.0
ALDH2	12	rs2238151	110 674 553	Intron 1	C	T	0.322	0.07	99.0
ALDH2	12	rs4648328	110 685 508	Intron 3	T	C	0.165	0.10	99.1
ALDH2	12	rs441	110 691 569	Intron 6	C	C	0.184	0.68	97.9
ALDH2	12	rs968529	110 697 088	Intron 9	T	C	0.069	0.19	95.4
ALDH2	12	rs4646778	110 698 503	Intron 9	A	C	0.165	0.17	99.1
ALDH2	12	rs671	110 704 486	Exon 12	A	G	0[e]	1.00	99.3
ALDH2	12	rs16941667	110 707 133	Intron 12	T	C	0.079	0.23	99.4

Chr, chromosome; HWE, Hardy–Weinberg equilibrium; MAF, minor allele frequency; SNP, single-nucleotide polymorphism; UTR, untranslated region.

[a]Position in nucleotides as estimated in dbSNP (Build 127).

[b]Position within or near gene.

[c]The allele with the lowest frequency.

[d]pHWE = *P*-value for the Hardy–Weinberg equilibrium test run in PEDSTATS (71).

[e]rs671 and rs1154409 are non-polymorphic.

from the HapMap database for CEPH families of European origin. A gene-centric pattern of LD was observed among the *ADH* gene markers in which high LD was observed within each gene and lower LD between genes.

Results of the association testing are summarized in Figure 2 for ADH, and given in full in Table 3.

Alcohol reactions

None of the tested SNPs in *ALDH2* were associated with flushing or with the more broadly defined reactions to alcohol. As expected, we found no occurrences of *ALDH2**2 among our subjects.

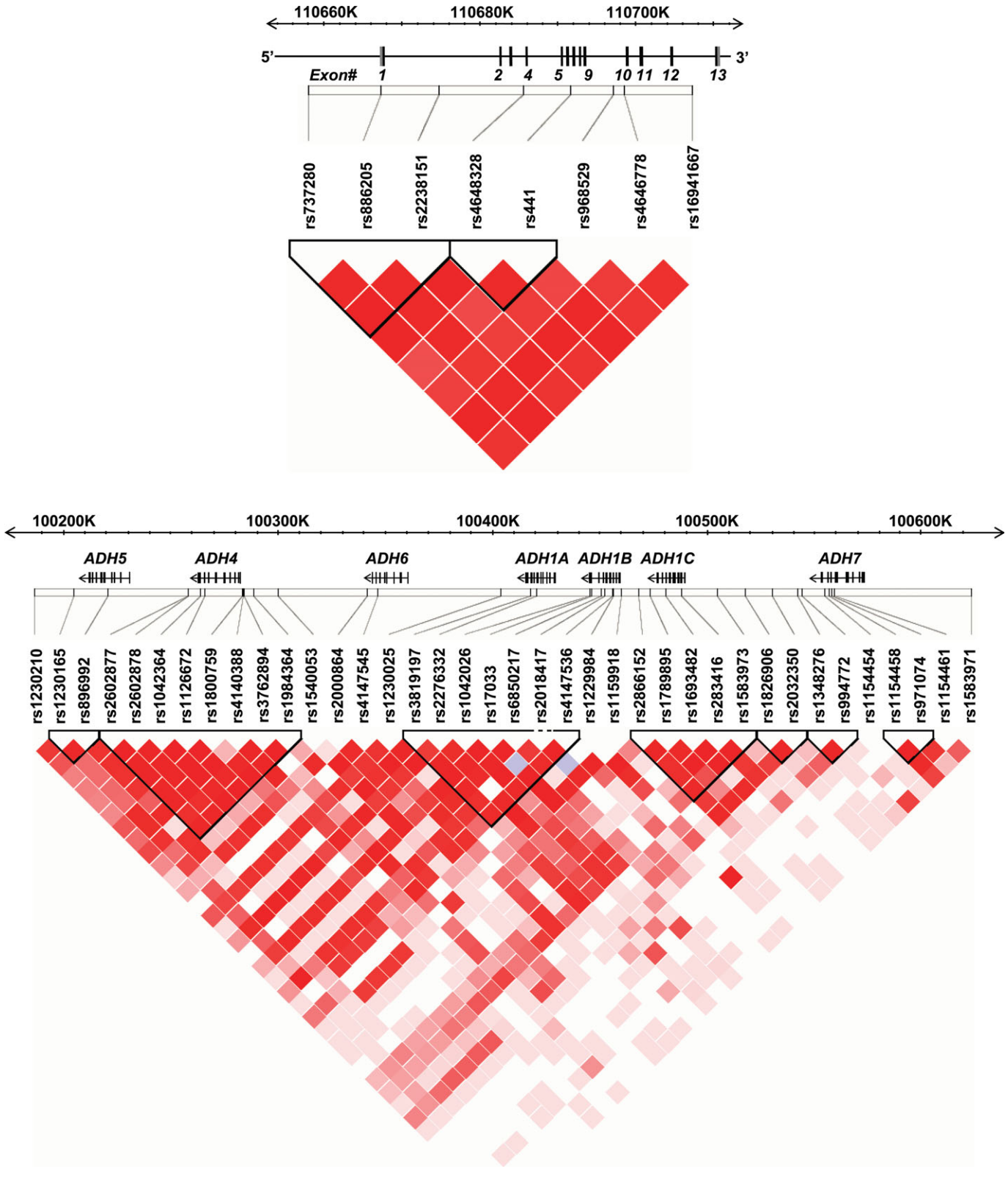

Figure 1. LD between the SNPs genotyped in *ALDH2* and seven *ADH* genes: (**A**) the gene structure of *ALDH2* is shown with exons numbered (1–13) and relative exon size denoted by the width of the vertical bars. Pairwise marker–marker LD (shown below the gene structure) was generated using Haploview 4.0 (75). Regions of low to high LD (D') are represented by white to red shading, respectively; (**B**) gene location, structure and LD (D') between the SNPs genotyped in the *ADH* cluster.

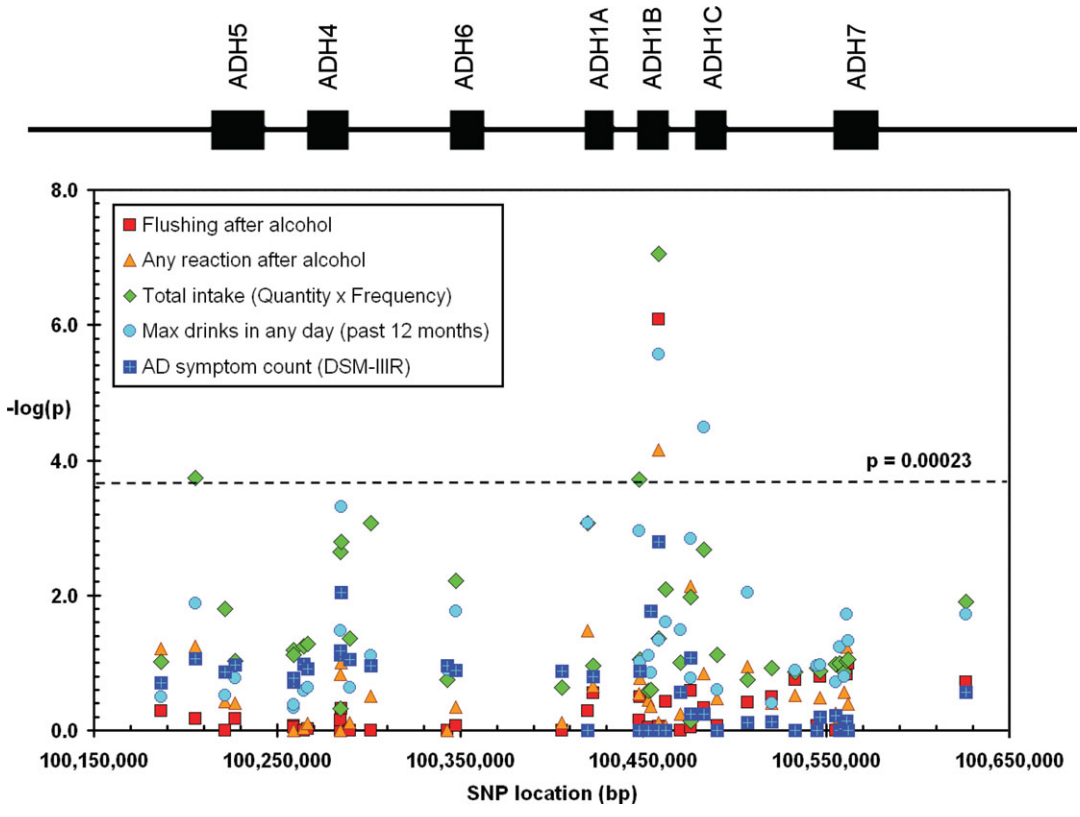

Figure 2. Total association of SNPs across the *ADH* gene cluster with quantitative AD variables, alcohol consumption variables and self-reported alcohol reactions. The location of the seven *ADH* genes is shown at the top of the figure. Results are plotted as $-\log_{10}(P\text{-value})$ against the physical location of each SNP on chromosome 4. See Table 1 for further description of the variables.

Self-reported flushing after consumption of small amounts of alcohol was significantly associated ($P = 8.2 \times 10^{-7}$) with the A-allele of rs1229984 (*ADH1B Arg48His*). This allele also conferred a higher likelihood of experiencing any negative reaction ($P = 6.9 \times 10^{-5}$). The direction of effect is consistent with published findings that the *ADH1B His48* (A-allele) variant is protective against AD. The prevalence of flushing or other reactions by rs122984 genotype is shown in Table 4.

Alcohol consumption

No significant linkage findings were observed across the polymorphisms in the *ADH* gene cluster or *ALDH2*. Total association results for quantitative measures of alcohol consumption are presented in Figure 2 and Table 3.

For *ALDH2*, we only detected nominal association ($P = 0.006$) with maximum drinks, where individuals carrying two T-alleles of rs2238151 (located in intron 1) reported consuming a higher maximum number of drinks in a single day (5.24 drinks) than heterozygous T/C (4.98) or homozygous C (4.49) individuals. However, this signal was not significant after taking account of multiple testing (with a threshold level of significance set at 2.3×10^{-4}, see Materials and Methods).

For *ADH*, the strongest evidence of association was found for the non-synonymous SNP rs1229984 located in exon 3 of *ADH1B (ADH1B Arg48His)*. Within the past 12 months, individuals carrying the rs1229984 G-allele (the more common arginine 48 variant, Arg48) reported consuming higher quantities of alcohol on a typical day when drinking ($P = 0.0005$), drank alcohol on more occasions ($P = 2.7 \times 10^{-6}$), reported a higher maximum number of alcoholic beverages consumed in a single day in the past year ($P = 2.7 \times 10^{-6}$) and had a higher overall consumption of alcohol ($P = 8.9 \times 10^{-8}$) than individuals with the A-allele (His48). The frequency distributions of overall alcohol intake by rs1229984 genotype in men and women are shown in Figure 3, and the medians are shown in Table 4.

Study participants with 0, 1 and 2 A-alleles for rs1229984 reported consuming, on average, a maximum of 5.1, 4.1 and 1.9 drinks in a single day in the previous year, respectively, and this trait has previously been shown to be highly correlated with alcohol use disorders (55). Similarly, the number of DSM-IIIR AD symptoms met were 1.25, 1.08 and 0.18 symptoms for 0, 1 and 2 A-alleles, respectively.

Among the remaining *ADH* markers analyzed, rs1693482 (the non-synonymous *Arg272Gln* polymorphism in *ADH1C*) was the only SNP significantly associated with alcohol consumption after correcting for multiple testing ($P = 3.2 \times 10^{-5}$, with the maximum number of drinks consumed in a single day in the past year). SNPs in *ADH1A* (rs3819197), *ADH1B* (rs1042026), *ADH4* (rs3762894) and *ADH5* (rs1230165) were associated to a smaller degree ($P < 0.001$) with one or more consumption variables tested (Table 3). To determine whether LD with rs1229984 accounted for the association signals across this region, we analyzed multiple

Table 3. Allelic associations between SNPs in ADH and ALDH2 genes, and phenotypes associated with alcohol reactions, alcohol use and AD

Genes and SNPs		Phenotypes (*P*-values)							
SNP	Gene(s)	Usual quantity (no. of drinks)	Max drinks in any day past 12 months	Max drinks on any day lifetime	Frequency of alcohol use	Total intake (Quantity × frequency)	Flushing after alcohol	Any reaction after alcohol	AD symptom score (DSM-IIIR)
ADH (chromosome 4)									
rs1230210	METAP1	0.24	0.32	0.79	0.26	0.097	0.51	0.061	0.2
rs1230165	ADH5	0.061	0.0133	0.047	0.00035	0.00018	0.68	0.056	0.086
rs896992	ADH5	0.53	0.3	1	0.03	0.016	1	0.37	0.137
rs2602877	ADH4	1	0.46	0.64	0.071	0.064	0.85	1	0.19
rs2602878	ADH4	0.75	0.42	0.72	0.075	0.076	0.89	1	0.17
rs1042364	ADH4	0.57	0.26	0.44	0.098	0.056	1	0.93	0.105
rs1126672	ADH4	0.5	0.23	0.59	0.08	0.052	0.94	0.79	0.124
rs1800759	ADH4	0.15	0.033	0.0075	0.0055	0.0023	1	0.148	0.075
rs4140388	ADH4	0.62	0.072	1	0.53	0.48	0.71	1	0.067
rs3762894	ADH4	0.00078	0.00048	0.0139	0.02	0.0016	0.47	0.1	0.009
rs1984364	ADH4	0.46	0.23	0.48	0.069	0.044	1	0.76	0.089
rs1540053	NA	0.126	0.077	0.61	0.0049	0.00084	1	0.31	0.109
rs2000864	ADH6	0.19	0.125	1	0.33	0.18	1	1	0.11
rs4147545	ADH6	0.004	0.017	0.49	0.066	0.006	0.86	0.45	0.129
rs1230025	NA	0.21	0.133	0.72	0.35	0.23	1	0.76	0.132
rs3819197	ADH1A	0.00033	0.00085	0.076	0.0069	0.00086	0.52	0.033	1
rs2276332	ADH1A	0.38	0.16	0.01	0.075	0.11	0.28	0.22	0.16
rs1042026	ADH1B	0.00067	0.00111	0.046	0.0023	0.00019	0.7	0.29	1
rs17033	ADH1B	0.33	0.096	0.0041	0.079	0.088	0.32	0.17	0.132
rs6850217	ADH1B	0.17	0.078	0.24	1	0.26	0.91	0.35	1
rs2018417	ADH1B	0.43	0.139	0.031	0.28	0.25	1	0.44	0.017
rs4147536	ADH1B	0.066	0.046	0.46	0.133	0.043	0.88	0.76	1
rs1229984	ADH1B	0.0005	2.7E−06	0.022	2.7E−06	8.9E−08	8.2E−07	0.000069	0.0016
rs1159918	ADH1B	0.084	0.025	0.26	0.025	0.0081	0.37	0.86	1
rs2866152	ADH1C	0.18	0.032	0.16	0.19	0.1	1	0.57	0.27
rs1789895	ADH1C	0.0058	0.00143	0.0132	0.114	0.0106	0.26	0.0074	0.57
rs1693482	ADH1C	0.00045	0.000032	0.0062	0.098	0.0021	0.46	0.142	0.57
rs283416	ADH1C	0.63	0.25	0.28	0.11	0.075	0.85	0.34	1
rs1583973	NA	0.057	0.0091	0.32	0.31	0.18	0.38	0.113	0.77
rs1826906	NA	1	0.39	0.28	0.097	0.119	0.32	0.39	0.74
rs2032350	NA	0.16	0.128	0.39	0.25	0.136	0.18	0.3	1
rs1348276	ADH7	0.28	0.109	0.34	0.18	0.119	0.86	1	1
rs994772	ADH7	0.7	0.106	0.46	0.27	0.133	0.16	0.33	0.64
rs1154454	ADH7	1	0.058	1	0.24	0.102	0.91	0.63	1
rs1154458	ADH7	0.39	0.16	0.34	0.6	0.137	1	0.27	0.96
rs971074	ADH7	0.077	0.019	0.22	0.36	0.093	0.149	0.058	0.73
rs1154461	ADH7	0.26	0.047	0.18	0.097	0.09	0.101	0.4	1
rs1583971	NA	0.09	0.019	0.22	0.043	0.0124	0.19	0.25	0.27
ALDH2 (chromosome 12)									
rs737280	NA	0.23	0.112	0.021	0.15	0.21	0.81	0.7	0.0033
rs886205	ALDH2	0.49	0.8	0.45	0.11	0.47	0.35	0.85	0.22
rs2238151	ALDH2	0.15	0.05	0.0061	0.2	0.096	0.8	0.42	0.00095
rs4648328	ALDH2	0.45	1	0.38	0.23	1	0.54	1	0.138
rs441	ALDH2	0.44	0.38	0.041	0.47	0.61	0.9	0.39	0.079
rs968529	ALDH2	0.4	1	0.5	0.126	0.23	0.9	1	0.19
rs4646778	ALDH2	0.48	1	0.44	0.17	0.62	0.43	0.95	0.17
rs16941667	ALDH2	0.28	0.69	1	0.18	0.66	0.84	1	1

NA, SNP in intragenic region.

Table 4. Effects of rs1229984 (*ADH1B Arg48His*) genotype on proportions of participants reporting flushing or other alcohol reactions, alcohol consumption (number of drinks in the past year), symptom score for DSM-IIIR AD and DSM-IIIR AD

	Male			Female		
	GG (RR)	AG (RH)	AA (HH)	GG (RR)	AG (RH)	AA (HH)
Genotypes, *n* (%)	1381 (93)	107 (7)	2 (0.1)	2699 (94)	157 (6)	9 (0.3)
Flushing, *n* (%)						
Never	1123 (84)	83 (78)	1 (50)	1782 (69)	81 (53)	2 (25)
Sometimes	188 (14)	19 (18)	1 (50)	600 (23)	53 (35)	3 (38)
Always	28 (2)	5 (5)	0 (0)	206 (8)	18 (12)	3 (38)
Any negative reaction, *n* (%)						
Never	815 (61)	60 (56)	0 (0)	1251 (48)	52 (34)	2 (25)
Sometimes	423 (32)	38 (36)	1 (50)	944 (37)	67 (44)	3 (38)
Always	103 (8)	9 (8)	1 (50)	396 (15)	35 (23)	3 (38)
Yearly drinks (median)	208	150	12	60	30	3
DSM-IIIR symptom count, *n* (% of column total)						
0	446 (33)	48 (45)	1 (50)	1791 (69)	119 (77)	8 (100)
1	144 (11)	10 (9)	0 (0)	170 (6)	10 (6)	0 (0)
2	205 (15)	12 (11)	1 (50)	307 (12)	11 (7)	0 (0)
3	202 (15)	19 (18)	0 (0)	183 (7)	9 (6)	0 (0)
4	144 (11)	7 (7)	0 (0)	89 (3)	3 (2)	0 (0)
5	106 (8)	4 (4)	0 (0)	38 (1)	1 (1)	0 (0)
>5	99 (7)	7 (7)	0 (0)	36 (1)	2 (1)	0 (0)
DSM-IIIR AD (%)						
Negative	989 (74)	89 (83)	2 (100)	2442 (94)	150 (97)	8 (100)
Positive	352 (26)	18 (17)	0 (0)	166 (6)	5 (3)	0 (0)

Genotypes are shown as the base on the reverse strand of DNA (G or A) and as the corresponding amino acid (R = Arginine, H = Histidine). Note that some phenotypic information was missing so that the number of subjects cross-tabulated by phenotype and genotype may not sum to the total of subjects with valid genotypes.

SNPs jointly for four variables (total quantity, frequency, maximum number of drinks in past 12 months and flushing) by regression analysis with rs1229984 genotype included. There was evidence for residual association at rs1042026 (which is in moderate LD with rs1229984; $D' = 0.67$; $r^2 = 0.01$) for the total quantity variable ($P = 4.7 \times 10^{-5}$) with the A-allele conferring higher alcohol intake over the previous 12 months, indicating there may be two independent signals in *ADH1B*. Alternatively, both SNPs may be in incomplete LD with a single causal variant. The significant association observed between rs1693482 (*ADH1C Arg272Gln*) and maximum number of drinks in past 12 months was mostly explained by rs1229984 ($D' = 0.84$; $r^2 = 0.02$), but with nominally significant residual association detected ($P = 0.0007$) after the effect of rs1229984 was accounted for. Smaller residual associations were also observed between rs1230165 in *ADH5* and frequency ($P = 0.001$) and rs37262894 in *ADH4* ($P = 0.0004$) and maximum number of drinks in the past 12 months. For the flushing trait, the large effect at rs1229984 explained all of the other signals in the *ADH* region.

Analysis of the subset of the data known to have European ancestry (defined as having all four grandparents with known European ancestry) gave results which were consistent with those seen in the full sample—restricting analysis to this subsample halved the sample size but the effect size estimates were similar (data not shown). Similarly, family based 'within' association tests yielded results consistent with those seen in the main analysis (Supplementary Material, Table S)—for these 'within' analyses, the effective sample size was substantially smaller because only families with two DZ individuals contribute (since parents were not genotyped, singleton and MZ families contribute no information to the 'within' test). These analyses rejected a role for the population stratification in the effects observed. A small proportion of subjects had reported their religion as 'Jewish' in a previous questionnaire, and in view of the reported higher prevalence of the rs1229984 *48His* allele in this group the allele frequency was estimated. Out of 33 such subjects, 4 were *48His* homozygotes and 16 were heterozygotes, giving a MAF of 0.36. However, further analysis of the alcohol-related phenotypes controlling for Jewish ancestry left the strength of the associations unchanged.

Alcohol dependence

Total association results for the quantitative measure of AD are presented in Figure 2 and Table 3. For *ALDH2*, the most significant findings ($P < 0.001$) in the total association analysis were detected with the *ALDH2* SNP rs2238151 (intron 1) where T-allele conferred a higher DSM-IIIR score. The T-allele of rs737280 (*ALDH2*) also conferred higher DSM-IIIR AD scores, but the effect was less significant ($P \approx 0.003$). No study-wide significant association was noted between the AD measures and the SNPs genotyped in the *ADH* gene cluster. However, rs1229984 did show nominal association with the DSM-IIIR score ($P = 0.0016$).

Subsequent binary analyses of DSM-IIIR AD diagnosis were run, both on the overall data and in DZ families to permit within-family tests of association to be performed. The purpose of these analyses was to be able to directly compare our findings with those of other groups who only analysed the dichotomous diagnoses of AD. The number of affected (case) individuals in the entire sample was 959,

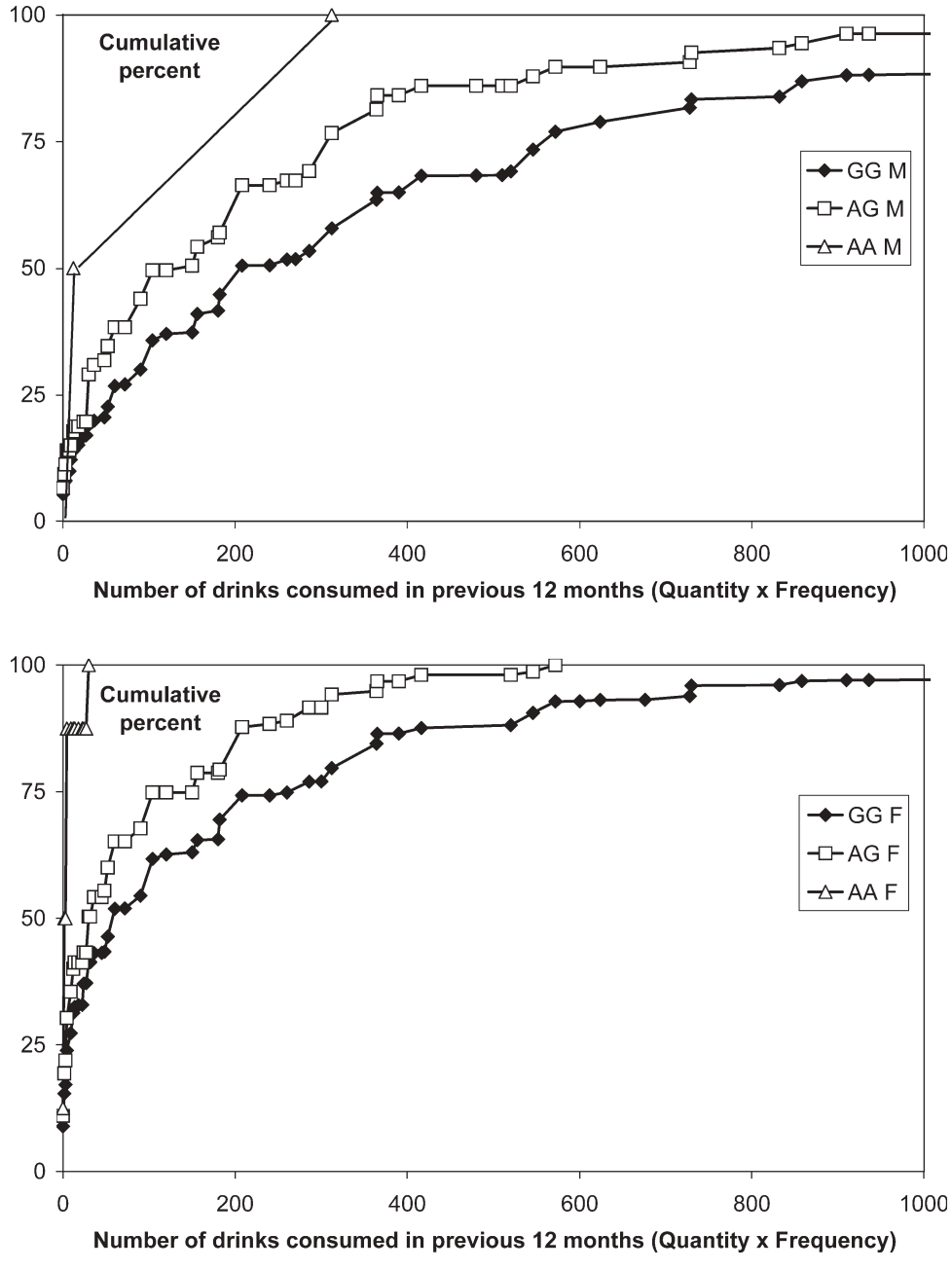

Figure 3. Cumulative frequency distribution plots for number of drinks in the previous 12 months (estimated from self-reported frequency and quantity) by rs1229984 (*ADH1B Arg48His*) genotypes, in (**A**) men and (**B**) women (GG = *48Arg* homozygotes, AG = heterozygotes, AA = *48His* homozygotes).

with a reduced number of cases (540) within DZ families only. No study-wide significant association was observed with AD diagnosis in the DZ family or MZ case–control analyses (smallest *P*-value 0.01 before correction for multiple testing).

DISCUSSION

The object of this study was to examine a connected series of phenotypes on a pathway to alcoholism, and to test for allelic associations with multiple polymorphisms across alcohol dehydrogenases (*ADH1A, ADH1B, ADH1C, ADH4, ADH5, ADH6* and *ADH7*) and the high-affinity aldehyde dehydrogenase (*ALDH2*). Although there have been many genetic association studies on these genes, they have either concentrated on a single phenotype (usually AD), or on a narrow range of genetic markers, or both. There have been suggestions that the polymorphisms studied have not been causative, or that they account for only part of the effects located within these genes; there have been questions about the applicability of results across populations; and a greater integration of information about gene and enzyme variation, the subjective effects of alcohol, habits of alcohol use and symptoms of dependence have been needed. We have found that the

effects of the much-studied *ADH1B Arg48His* polymorphism can be traced through these alcohol-related phenotypes, and that the most easily detected effects are on the phenotypes near the start of the proposed sequence of events. On the whole, our results confirm the prevailing theory, but with some additions and with some features which require further consideration.

ALDH2

None of the *ALDH2* SNP-phenotype associations reached the study-wide level of significance of 2.3×10^{-4}. However, it would be unwise to dismiss the possibility of a true effect of variation in *ALDH2* in Europeans; precautions against false-positive results can lead to false negatives even with large sample size. A number of phenotypes related to AD, particularly the symptom score for dependence, were associated with rs2238151 at $P \approx 0.001$ and with rs737280 at $P < 0.01$ (Table 3). In a previous study, on a smaller group who had participated in our Alcohol Challenge Twin Study, we found that these two SNPs were associated with significant differences in alcohol metabolism (22). We postulated that this could be due to a variant which decreased the activity of ALDH2, to a lesser extent than the Asian rs671 polymorphism, and predicted that people homozygous for C at rs2238151 and/or for G at rs737280 should have a lower prevalence of AD and a lower symptom score. That was not shown among the 376 people typed for *ALDH2* SNPs in our previous study, but with the larger number of subjects typed in this study we note that the T allele at rs2238151 showed higher AD symptom scores, with *P*-values around 0.001 and in the predicted direction. Further studies or meta-analysis on *ALDH2* variants in Europeans seem justified. However, neither of these two SNPs showed associations with self-reported flushing or with alcohol consumption so the mechanism of any effect on dependence is unclear.

ADH

Our main finding is that the variation at rs1229984, the well-known *ADH1B Arg48His* polymorphism, is associated with flushing or other reactions to alcohol as well as with alcohol consumption in Europeans. Self-reported unpleasant reactions to alcohol are known to occur in a substantial proportion of people, particularly women [see Table 1, and (56)], but our earlier attempts to test whether *ADH1B* variation affected these reactions (57) were inconclusive. With larger numbers in the present study, the *ADH1B 48His* allele, which has a frequency of around 5% in this group, is highly significantly associated with more frequent alcohol reactions and lower alcohol intake. The effect of this polymorphism on reported alcohol intake is summarized in Figure 3 and Table 4; for both men and women, the median alcohol intake was notably lower in heterozygotes than in *48Arg* homozygotes, and in the small number of *48His* homozygotes alcohol consumption was very low.

However, this polymorphism is not significantly associated, at least at our study-wide significance level, with AD or AD symptom count. Practically, all previous studies, in Asia and in Europe, have found that the *48His* allele is protective against AD. For Europeans, the relative risk of AD for heterozygotes against *48Arg* homozygotes has been estimated at 0.47 with 95% confidence intervals of 0.29 to 0.76 (36). Our estimated relative risk for DSM-IIIR AD in heterozygotes compared to 48Arg homozygotes, from the current data, is 0.57 for men and 0.49 for women. There is no evidence of heterogeneity and the pooled odds ratio estimate is 0.53 with 95% confidence intervals 0.32–0.88. The point to note is that our results are compatible with the expected size of the effect on AD risk. As noted above in relation to *ALDH2* variation, failure to meet a stringent significance level does not exclude an effect; and the AD symptom score phenotype showed association with rs1229984 at the $P < 0.002$ level. On balance, we consider that rs1229984 does affect AD risk but the nature of our study (with subjects drawn from the general population), the low MAF for this polymorphism, and the stringent study-wide significance level arising from the examination of multiple SNPs and phenotypes, contribute to our inability to demonstrate this unequivocally.

A number of previous reports have considered whether variation at *ADH1B Arg48His* affects the alcohol flush reaction or alcohol reactions in general, both in Asians (38–43,58) and in Europeans. The results are very mixed; in Europeans a measure of 'level of response' to alcohol was affected by this polymorphism (59) but alcohol-induced flushing was not (60). In Asians also there are about equal numbers of positive and negative reports, and some suggestion of gene–gene interaction in that *ADH1B* genotype may only affect flushing in *ALDH2* heterozygotes. However, our results on this point are strong, and we conclude that *ADH1B Arg48His* does affect the immediate response to alcohol consumption.

No other polymorphisms in either *ADH* or *ALDH2* had significant or suggestive associations with alcohol reactions. However, many *ADH* SNPs showed associations with drinking behavior (Fig. 3 and Table 3), some because of LD with rs1229984. When the effect of rs1229984 was regressed out, there was an independent association between rs1042026 in *ADH1B* and the overall quantity phenotype ($P = 4.7 \times 10^{-5}$), with the A-allele conferring higher alcohol intake on average over the previous 12 months. Smaller associations between rs1230165 in *ADH5* and frequency of alcohol use, and between both rs37262894 (in *ADH4*) and rs1693482 (*ADH1C Arg272Gln*) and the maximum number of drinks consumed in 1 day in the previous 12 months were also observed. No SNPs producing stronger effects than rs1229984, or explaining the effects at rs1229984 through LD with a distinct causative variant, were found. However, there were indications of independent effects in *ADH1B, ADH1C, ADH4* and *ADH5* which did not reach our required significance level, but which still need to be confirmed or refuted.

Comparable data are available from three other studies with SNP coverage of the *ADH* region and one of *ADH4* only, which focused on associations with AD. Using a conventional test for allelic association, Luo *et al*. (20) found significant results for rs1229984 (*Arg48His*) in European-Americans and for rs2066702 (*Arg369Cys)* in African-Americans, both in *ADH1B*. Using an analysis based on deviation from HWE, there were indications that variation in *ADH1A, ADH1B, ADH4, ADH5* and *ADH7* affected dependence risk

(20,54). Significant associations between AD in European-Americans and SNPs in *ADH4, ADH1A* and *ADH1B* were found in another *ADH*-cluster-wide study by the Collaborative Study on the Genetics of Alcoholism (19). While no association was detected between rs1229984 and AD in European-Americans, in African-Americans rs2066702 was significantly ($P < 0.05$) associated with AD. The most significant associations were in the *ADH1A-ADH1B* region or in *ADH4*, depending on the AD criteria used. It is interesting to note that out of 110 SNPs tested on 2139 people, three were significant at the 0.01 level and a further 18 had *P*-values between 0.05 and 0.01; we can infer that the effects on AD risk are either small or they are associated (as we found) with polymorphisms with low minor allele frequencies. A further study with recruitment in Ireland (21) found associations at $P < 0.01$ between AD and SNPs in *ADH1B* and *ADH5*, and at $P < 0.05$ in *ADH1A, ADH1B, ADH1B* and *ADH7*. Significant association between AD and *ADH4* SNPs was also reported in Brazilian subjects (61). Overall, the genes coding for Class I ADHs with high affinity for ethanol are the ones which most frequently show significant effects on dependence risk, but *ADH4* has also been implicated in several studies.

From our results and the published literature, we can now propose that rs1229984, or *ADH1B Arg48His*, affects the subjective experiences associated with alcohol use, specifically through flushing or similar reactions, even in Europeans homozygous for the active form of *ALDH2*. This affects alcohol use, which in turn affects AD risk. Other polymorphisms in *ADH* genes may also affect AD through similar mechanisms, but no strong evidence of this is yet available. The main anomaly in this narrative is that rs1229984 probably does not affect the rate of alcohol metabolism, at least so far as it can be measured from changes in blood and breath alcohol concentrations (51). It still remains possible that this variant affects the steady-state concentration of acetaldehyde within the hepatocytes, which is where acetaldehyde is produced, and that this affects the release of vasoactive compounds into the circulation and causes alcohol-induced flushing. Measurement of acetaldehyde concentrations in the liver of humans seems beyond our reach, but measurements of histamine or other vasoactive molecules in the circulation might allow testing of this concept; and transgenic mice expressing rs1229984 might allow more invasive studies of the effects of this polymorphism on alcohol and acetaldehyde metabolism.

MATERIALS AND METHODS

Participants

Participants were recruited for a 1992–1995 telephone interview based twin study conducted at the Queensland Institute of Medical Research (QIMR) (7,62). This interview was based upon an Australian modified version (SSAGA-OZ) of the Semi-Structured Assessment for the Genetics of Alcoholism instrument designed for genetic studies of alcoholism. The SSAGA is a psychiatric interview that retrospectively assesses physical, psychological and social manifestations of AD along with several other psychiatric disorders and has undergone both reliability and validity testing (63,64). It included questions on alcohol-related flushing or other unpleasant reactions to alcohol, quantity and frequency of alcohol use over the last 12 months, and about the maximum number of drinks in a single day in the last 12 months and ever. A total of 4597 subjects (34.6% males) from 2618 families, comprising 814 (583 female and 231 male) MZ pairs, 1177 (482 female, 198 male and 497 opposite sex) DZ pairs and 627 twins (38.8% male) whose co-twin did not participate, were included in genetic analysis. The participants were predominantly of Northern European ancestry, from information they provided on the place of birth and ethnicity of their grandparents. Fifty-one percent of the sample had ancestry information on all four grandparents. Of these, 88% had all European grandparents (with 89% of these Northern European, $N = 1873$ twins), a further 1% indicated 'adopted', and 8% indicated 'Australian' ancestry. Most of the 'Australian' ancestry individuals are thought to have fully European ancestry (all grandparents born in Australia but with European ancestry), whereas others have one or more aboriginal grandparents. The final 3% had one or more Asian or African grandparent. They were aged 26–89 years (mean age was 43.8 ± 11.5 years) at the time of testing. Subjects gave written informed consent and provided blood samples from which DNA was isolated using standard protocols. Genetic studies were approved by the QIMR Human Research Ethics committee. Zygosity of same-sex twin pairs was assessed using a combination of self-report only (32% of same-sex pairs), three blood groups (ABO, MNS and Rh), data from genome-wide microsatellite markers and a set of nine polymorphic DNA microsatellite markers (AmpF1STR Profiler Plus Amplification Kit, Applied Biosystems, Foster City, CA, USA). The zygosity assignment from the nine-marker set is highly accurate [probability of correct assignment $>99.99\%$ (65)] and we estimate that the overall accuracy of zygosity assignment in this cohort is $>99\%$.

Phenotypes

Assessment of current alcohol use, reactions to alcohol and lifetime history of AD were from the adapted SSAGA interview data. A small number of participants (2.3% of men and 3.1% of women) reported they had never had even one alcoholic drink, and their alcohol-related phenotypes were set to missing. Participants who reported never drinking more than three drinks in a single day were allowed to skip detailed questions from the SSAGA interview on drinking behavior. Reactions to alcohol use were self-reported (62). Alcohol consumption measures were frequency and quantity of alcohol consumption, maximum number of alcoholic drinks consumed in a single day ever and within the past 12 months. The total number of drinks taken in the past year was estimated from the reported quantity and frequency categories. AD was diagnosed by a computer algorithm based on the criteria of the Third Revised (DSM-IIIR) Diagnostic and Statistical Manual of the American Psychiatric Association (66). In addition to the dichotomous definition of AD, a quantitative measure based on reported AD symptoms was calculated (67).

Genotyping

Eleven SNPs flanking or within *ALDH2* locus were selected on the basis of (i) previous work completed by our group (68) in which six SNPs within or flanking *ALDH2* were genotyped in a partially overlapping Australian twin sample that completed an alcohol challenge test (69); and (ii) data available at the time from the International HapMap Project public database (http://www.hapmap.org/). Two SNPs failed during the assay design or provided unreliable genotype data and were excluded. rs671 was monomorphic. The locations of the eight remaining SNPs typed in the study are shown in Figure 1 and SNP information, including the observed MAF, is given in Table 2.

LD data from another study in which 104 SNPs across the *ADH* gene cluster were genotyped (Birley *et al*., submitted for publication), indicated that a reduced set of 43 haplotype-tagging SNPs would provide appropriate coverage ($r^2 \geq 0.80$) of the gene cluster. Additional tagging SNPs plus SNPs selected *a priori* based on existing research, specifically *ADH1B Arg48His* (rs1229984) and *ADH1C Ile350Val* (rs698), were typed giving a total of 51 SNPs. Ten SNPs provided unreliable genotype data and were excluded. One of these was rs698 (*ADH1C Ile350Val*) but it is in near-complete LD ($D' = 0.99$ (70)) with rs1693482 (*ADH1C Arg272Gln*). The locations of the 41 remaining SNPs typed are shown in Figure 1 and SNP information is described in Table 2.

Assays were designed using the Sequenom MassARRAY Assay Design (version 3.0) software (Sequenom Inc., San Diego, CA, USA). Genotyping was carried out in standard 384-well plates with 12.5 ng genomic DNA used per sample. We used a modified Sequenom protocol where half reaction volumes were used in each of the PCR, SAP and iPLEX stages giving a total reaction volume of 5.5 μl. The iPLEX reaction products were desalted by diluting samples with 18 μl of water and 3 μl SpectroCLEAN resin (Sequenom) and then were spotted on a SpectroChip (Sequenom), processed and analyzed on a Compact MALDI-TOF Mass Spectrometer by MassARRAY Workstation software (version 3.3) (Sequenom). Allele calls for each 384-well plate were reviewed using the cluster tool in the SpectroTyper software (Sequenom) to evaluate assay quality. Genotype error checking, sample identity and zygosity assessment and HWE analyses were completed in PEDSTATS (71).

Statistical analyses

Quantitative trait analysis was performed in Merlin (72) and QTDT (73,74), with eight traits examined. The alcohol-related quantitative traits were first transformed to normality using a piecewise normal transformation and were corrected for sex and age effects by fitting covariates in the regression model. MZ twin status was included in Merlin and QTDT analyses by adding zygosity status to the data file. Tests of total association modeling multipoint linkage were performed in Merlin; non-independent transmissions were corrected for by modeling multipoint linkage in a maximum likelihood framework. SNPs in high LD were clustered together for linkage modeling such that clusters included pairs of SNPs for which pairwise r^2 exceeded 0.05 (all intervening markers were included in the cluster). Secondary analyses robust to population stratification were conducted using the orthogonal 'within' test in QTDT. Since parents were not genotyped, this analysis only included families with two DZ offspring. To account for the fact that transmissions were not independent in the DZ families due to the presence of linkage in DZ pairs, a permutation procedure (10 000 permutations) was used to correct the *P*-values for the orthogonal test. The permutation procedure was also used to estimate the correlation within the set of phenotypes and within the set of genotypes (Lind *et al*., in preparation).

Binary trait analysis was performed for comparison with the above quantitative trait analysis and also for comparison with the work of other groups. Association of DSM-IIIR AD was examined using the UNPHASED statistical package in two stages. First, for DZ families, a pedigree disequilibrium (PDT) style test was used, in the PDTPHASE module, to test for over-transmission of allele to offspring (a 'within' family test that should be robust to any population stratification effects). Second, for MZ families, pairs with discordant phenotypes were set to phenotype unknown while pairs with concordant phenotypes were not changed. Subsequently, one MZ twin per family was included for each trait analyzed in COCAPHASE. Since no parents were genotyped, each MZ twin was treated as unrelated and a case–control test was implemented.

Pair-wise marker–marker LD was assessed using the D' and r^2 statistic in Haploview 3.31 (75). Many of the traits studied were correlated and there was substantial LD across *ALDH2* and the *ADH* gene cluster (see Fig. 1A and B). As a result, the effective number of statistical tests done was less than the actual number of tests. Using permutation to take into account the correlated phenotypes and correlated genotypes, a *P*-value less than 2.3×10^{-4} is required for study wide significance.

ACKNOWLEDGEMENTS

We would like to thank the twins for their long-term co-operation; Genetic Epidemiology Laboratory staff for sample processing and DNA extraction; Dixie Statham for coordinating the SSAGA Study; and the clinical staff and many research interviewers for data collection.

Conflict of Interest statement. None declared.

FUNDING

This research was funded by the National Institute on Alcohol Abuse and Alcoholism (NIAAA): sample ascertainment, phenotyping and blood collection by grants AA07535, AA07728, AA11998, AA13320 and AA13321, genotyping by grants (AA013326, AA013326 and AA014041). S.M. is the recipient of a Career Development Award from the Australian National Health and Medical Research Council (grant number 496674).

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