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# *ADH* single nucleotide polymorphism associations with alcohol metabolism *in vivo*

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**We have previously found that variation in alcohol metabolism in Europeans is linked to the chromosome 4q region containing the *ADH* gene family. We have now typed 103 single nucleotide polymorphisms (SNPs) across this region to test for allelic associations with variation in blood and breath alcohol concentrations after an alcohol challenge. *In vivo* alcohol metabolism was modelled with three parameters that identified the absorption and rise of alcohol concentration following ingestion, and the rate of elimination. Alleles of *ADH7* SNPs were associated with the early stages of alcohol metabolism, with additional effects in the *ADH1A*, *ADH1B* and *ADH4* regions. Rate of elimination was associated with SNPs in the intragenic region between *ADH7* and *ADH1C*, and across *ADH1C* and *ADH1B*. SNPs affecting alcohol metabolism did not correspond to those reported to affect alcohol dependence or alcohol-related disease. The combined SNP associations with early- and late-stage metabolism only account for approximately 20% of the total genetic variance linked to the *ADH* region, and most of the variance for *in vivo* alcohol metabolism linked to this region is yet to be explained.**

## INTRODUCTION

After consumption of an alcoholic drink or after an experimental oral alcohol challenge, the blood alcohol concentration rises during the absorption phase, reaches a peak, and then decreases at a near-linear rate. There is considerable between-individual variation in the blood alcohol concentrations achieved, even after a standard weight-adjusted dose of alcohol (1,2). Such variation affects both the peak concentration, which will depend on body composition and on pre-absorptive or first-pass metabolism, and the rate of elimination after the peak concentration is reached, which reflects hepatic alcohol metabolism. This pharmacokinetic variation affects both the degree and duration of intoxication. These differences between people are reproducible (2–5), heritable (2,3,6), and show linkage to the alcohol dehydrogenase (*ADH*) locus on chromosome 4 (7).

The conversion of alcohols to the corresponding aldehydes is catalysed by alcohol dehydrogenases, and this is the rate-limiting step in the elimination of ethanol in humans or experimental animals. Seven *ADH* genes are located as a cluster on chromosome 4q22-23 (8,9). The Class 1 enzymes (coded by *ADH1A*, *ADH1B* and *ADH1C* in humans) have high affinity for ethanol and contribute most to its conversion to acetaldehyde, particularly during the elimination phase. ADH7 acts early in the time course of alcohol metabolism in the stomach mucosa (10) which is exposed to high concentrations of alcohol. Several association studies for gene polymorphisms potentially affecting alcohol elimination have been reported, concentrating on *Arg48His* and *Arg370Cys* in *ADH1B*. They have substantial effects on the *in vitro* kinetic properties of the enzyme (11) but paradoxically small effects *in vivo*. We have recently shown that variation in *ADH7* is associated with

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variation in early (pre-absorptive or first-pass) alcohol metabolism (12).

In addition to effects on blood alcohol concentrations and therefore on the extent and duration of intoxication, variation in alcohol metabolism is implicated in the genetics of alcohol dependence and other alcohol-related disease. *ADH1B* and *ADH1C* polymorphisms have been studied extensively in relation to alcohol dependence. The main focus has been candidate gene association studies based on *ADH1B Arg48His* (13–17) and *ADH1B Arg370Cys* (18,19). Apparent effects of *ADH1C Ile350Val* or the accompanying *Arg272Gln* have been attributed to linkage disequilibrium (LD) with the *ADH1B Arg48His* site (16). Five reports on associations between a wider range of *ADH* SNPs and alcohol dependence (20–24), and one on upper aerodigestive cancers (25), are now available.

The rate at which alcohol is oxidized to acetaldehyde is believed to be an important aetiological factor for the genetic risk of alcohol dependence, mainly because the alleles of non-synonymous *ADH1B* polymorphisms associated with high $V_{max}$ *in vitro* are the ones which confer protection against alcohol dependence. The hypothesis that high rates of *in vivo* ethanol metabolism (leading to higher acetaldehyde concentrations) are negatively associated with risk of alcohol dependence was generated from studies of East Asian populations where the *ADH1B 48His* allele is at intermediate frequency, and has been extended to Europeans in whom it is less common. These studies are summarized by Whitfield and Eng *et al*. (26,27). Moreover, the risk of alcohol dependence is higher, at least in Europeans, for those with a lower rate of *in vivo* alcohol metabolism (28). Three variants, *ADH1B Arg48His, ADH1C Arg272Gln/Ile350Val* and *ADH1B Arg370Cys*, are known to affect enzyme activity *in vitro*; but we previously found that the first two only accounted for approximately 1% of the genetic variance for *in vivo* metabolism because of linkage with the *ADH* region (7). This small contribution may be because the *ADH1B Arg48His* polymorphism has low minor allele frequency in Europeans, in contrast to East Asians; or because factors unrelated to the kinetic properties of this ADH enzyme determine the *in vivo* rate of alcohol metabolism. The *in vivo* studies in Asians also suggest that the contribution of *ADH1B Arg48His* to variation in alcohol metabolism is minor (29,30). *ADH1B Arg370Cys* is monomorphic in European populations, but it has been shown to have a small effect on the rate of alcohol elimination in African-Americans (31).

The current state of knowledge is that variation in the *ADH* gene region is thought to contribute substantially to variation in alcohol metabolism, but the relevant polymorphisms and the size of the allelic effects are still undefined. There is an expectation that *ADH* polymorphisms which affect alcohol dependence or alcohol intake should do so by affecting alcohol metabolism, but the available evidence tends to contradict this. More extensive SNP association studies may resolve these questions. We now present results for association testing between *in vivo* alcohol metabolism and SNPs across the entire *ADH* region. This represents an extension of earlier results for 25 SNPs in the *ADH7* gene, to 103 SNPs covering all seven *ADH* genes, and includes some coverage of intergenic regions and the two nearest flanking genes, *C4orf17* and *METAP1*. Our study had three main aims: to test (a) whether *ADH* SNPs show associations with alcohol metabolism, distinguishing between the early and late pharmacokinetic phases; (b) whether such associations can account for the linkage of alcohol metabolism to this chromosomal region; and (c) how far the SNPs associated with alcohol metabolism correspond to those associated with other alcohol-related phenotypes.

## RESULTS

### Single nucleotide polymorphism coverage

The 103 SNPs (Table 1) covered 497 kb on chromosome 4 in the *ADH* gene region. Totally, 75 SNPs had a minor allele frequency $\geq 0.2$, and 88 $\geq 0.1$. Most were within or immediately flanking the coding regions of the seven *ADH* genes. Of the 103 SNPs, 76 are included in HapMap, and these tag (at $r^2 \geq 0.8$) a further 215 of the 534 non-monomorphic CEU HapMap SNPs in this region. However, we did not impute genotypes for these additional SNPs. The coverage of HapMap SNPs was 54% overall (291/534), 48% for intergenic SNPs (177/366), 66% for intronic, 3′-UTR or 5′-UTR SNPs (104/157), and 91% for exonic SNPs (10/11). The number of SNPs per kilobase in coding regions was *ADH1C* 1.11; *ADH7* 0.56; *ADH1B* 0.55; *ADH4* 0.39; *ADH1A* 0.34; *ADH6* 0.18; *ADH5* 0.11. Intergenic regions averaged about 0.13 SNPs/kb. Non-synonymous coding SNPs included rs1229984 in exon 3 of *ADH1B* (*Arg48His)* and rs698 in exon 8 of *ADH1C (Ile350Val)*. SNP rs1693482 in exon 6 of *ADH1C* also results in a non-synonymous substitution (*Arg272Gln*); it is in near-complete LD with rs698 (*ADH1C Ile350Val)*.

### Single nucleotide polymorphism effects on alcohol metabolism

Following our previous approach (12), the effects of SNPs in the *ADH* region on blood and breath alcohol concentrations were assessed using a kinetic model of *in vivo* alcohol metabolism [see Eq. (1) in Materials and Methods]. This distinguished between SNPs that act early in the time course of *in vivo* metabolism (the joint effect of the two parameters $A_0$ and $k_1$ for 2 df) and later effects (the rate of elimination $k_2$ for 1 df). Results are shown in Table 1.

The early effects were strongest for the SNPs within or close to the *ADH7* gene. There are also significant early effects ($P < 0.05$) across *ADH1A* and *ADH1B* (but not *ADH1C*), and in *ADH4* and *ADH5*. For the later effects on $k_2$, reflecting post-absorptive alcohol elimination, significant associations were seen for a range of SNPs across the region between *ADH7* and *ADH1C*, through *ADH1C* and into *ADH1B* (but not *ADH1A*). Associations are also seen for two SNPs in or near *ADH4*.

### Linkage disequilibrium in the ADH region

The distribution of pairwise LD ($D'$) values (Supplementary Material, Fig. S1) shows several blocks where $D'$ is close to 1. In contrast, most values $>0.5$ of the genetic correlation coefficient $r^2$ are confined to blocks of about 100 kb or less.

**Table 1.** Positions, polymorphisms and minor allele frequencies (MAF) of typed single nucleotide polymorphisms (SNPs) within and flanking the *ADH* gene family; and significant associations with early or late alcohol metabolism, tested on blood or breath alcohol concentrations

| | SNP | Position (bp)[a] | Polymorphism[b] | Functionality/placement | MAF | *P* $A_0$ and $k_1$, Blood[c] | *P* $A_0$ and $k_1$, Breath[c] | *P* $k_2$, Blood[c] | *P* $k_2$, Breath[c] |
|---|---|---|---|---|---|---|---|---|---|
| | *METAP1* | *100135043* | | | | | | | |
| 1 | rs1020624 | 100163877 | T→C | Intron 7 | 0.296 | | | | |
| 2 | rs1230210 | 100186714 | G→A | Intron 5 | 0.298 | | | | |
| | *METAP1 5′* | *100202983* | | | | | | | |
| 3 | rs1230165 | 100205396 | A→G | Intergenic | 0.190 | | | | |
| 4 | rs1230155 | 100208282 | A→G | Intergenic | 0.334 | | | | |
| | *ADH5 mRNA* | *100211152* | | | | | | | |
| 5 | rs896992 | 100221395 | T→C | Intron 4 | 0.333 | | | | |
| 6 | rs1154409 | 100226893 | C→A | Intron 1 | 0.100 | | | | |
| | *ADH5 mRNA 5′* | *100228954* | | | | | | | |
| 7 | rs1154400 | 100229033 | T→C | Intergenic | 0.321 | | | | |
| 8 | rs1377689 | 100240810 | G→A | Intergenic | 0.266 | | | | |
| 9 | rs2602859 | 100249606 | A→G | Intergenic | 0.270 | | | | |
| 10 | rs2602877 | 100258870 | A→T | Intergenic | 0.268 | | | | |
| 11 | rs2602878 | 100258976 | G→T | Intergenic | 0.266 | | | | |
| 12 | rs2602891 | 100262307 | T→C | Intergenic | 0.280 | | | | |
| | *ADH4 mRNA* | *100263855* | | | | | | | |
| 13 | rs1042364 | 100264597 | G→A | Exon 9 3′-UTR | 0.277 | | | | |
| 14 | rs1126673 | 100264639 | A→G | Exon 9 Non-syn (Ile→Val) Splice site | 0.298 | | | | |
| 15 | rs1573495 | 100265708 | G→A | Intron 8 | 0.017 | | | | 0.013 |
| 16 | Hpy 1881 | 100265848 | T→C | Intron 8 | 0.264 | | | | |
| 17 | rs1126672 | 100266835 | C→T | Exon 8 Non-syn (Ala→Gln) | 0.278 | | | | |
| 18 | rs1126671 | 100267437 | G→A | Exon 7 Non-syn (Val→Ile) | 0.304 | | | | |
| 19 | rs1126670 | 100271756 | T→G | Exon 6 Syn (Pro→Pro) | 0.301 | | | | |
| 20 | rs2032349 | 100281842 | C→T | Exon 3 Syn (Ser→Ser) | 0.022 | | | | 0.045 |
| | *ADH4 mRNA 5′* | *100284472* | | | | | | | |
| 21 | rs1800759 | 100284532 | C→T | Intergenic | 0.378 | 0.004 | | | |
| 22 | rs4140388 | 100284757 | G→C | Intergenic | 0.441 | | | | |
| 23 | rs3762894 | 100285107 | T→C | Intergenic | 0.138 | 0.039 | | | |
| 24 | rs1984364 | 100289806 | T→G | Intergenic | 0.280 | | | | |
| 25 | rs1984362 | 100289996 | C→T | Intergenic | 0.280 | | | | |
| 26 | rs1540053 | 100301177 | A→G | intergenic | 0.238 | | | | |
| 27 | rs2051428 | 100342209 | T→C | Intergenic | 0.207 | | | | |
| 28 | rs2000864 | 100342799 | G→T | Intergenic | 0.465 | | | | |
| | *ADH6 mRNA* | *100342818* | | | | | | | |
| 29 | rs4147545 | 100347776 | A→G | Intron 6 | 0.292 | | | | |
| 30 | rs3857224 | 100348708 | C→T | Intron 6 | 0.284 | | 0.032 | | |
| 31 | rs4147544 | 100353537 | C→A | Intron 3 | 0.472 | | | | |
| | *ADH6 mRNA 5′* | *100359426* | | | | | | | |
| 32 | rs1230021 | 100395030 | C→A | Intergenic | 0.022 | | | | 0.046 |
| 33 | rs1497379 | 100395093 | T→C | Intergenic | 0.424 | | | | |
| 34 | rs1230025 | 100405399 | T→A | Intergenic | 0.229 | | 0.026 | | |
| 35 | rs1618572 | 100414144 | C→G | Intergenic | 0.228 | | 0.033 | | |
| | *ADH1A mRNA* | *100416547* | | | | | | | |
| 36 | rs3819197 | 100419532 | C→T | Intron 8 | 0.206 | | | | |
| 37 | rs1229976 | 100421101 | T→C | Intron 6 | 0.228 | | 0.033 | | |
| 38 | rs2276332 | 100422470 | A→C | Intron 6 | 0.075 | | 0.048 | | |
| 39 | rs1229967 | 100426601 | G→C | Intron 3 | 0.227 | | 0.041 | | |
| 40 | rs931635 | 100429870 | C→T | Intron 1 | 0.226 | | 0.041 | | |
| | *ADH1A mRNA 5′* | *100431165* | | | | | | | |
| 41 | rs904092 | 100433187 | G→A | Intergenic | 0.173 | | | | |
| 42 | rs1789877 | 100445081 | A→G | Intergenic | 0.027 | | | 0.042 | 0.005 |
| | *ADH1B mRNA* | *100445157* | | | | | | | |
| 43 | rs1042026 | 100447489 | A→G | Exon 9 3′-UTR | 0.258 | | | | |
| 44 | rs17033 | 100447968 | A→G | Exon 9 3′-UTR | 0.067 | 0.029 | | | |
| 45 | rs1229985 | 100451901 | T→C | Intron 6 | 0.027 | | | 0.034 | 0.003 |
| 46 | rs1789882 | 100454076 | G→A | Intron 6 | 0.175 | | | | |
| 47 | rs2018417 | 100454163 | G→T | Exon 6 Syn (Ala→Ala) | 0.041 | 0.015 | | | |
| 48 | rs2075633 | 100458021 | A→G | Intron 3 | 0.257 | | | | |
| 49 | rs4147536 | 100458135 | G→T | Intron 3 | 0.204 | | | | |
| 50 | rs1229984 | 100458342 | G→A | Exon 3 Non-syn (Arg→His) | 0.035 | | | | |
| 51 | rs1353621 | 100460598 | A→G | Intron 1 | 0.417 | | | | |
| | *ADH1B mRNA 5′* | *100461581* | | | | | | | |
| 52 | rs1159918 | 100462032 | G→T | intergenic | 0.333 | | | | |

*Continued*

**Table 1.** *Continued*

| | SNP | Position (bp)[a] | Polymorphism[b] | Functionality/placement | MAF | $P$ $A_0$ and $k_1$, Blood[c] | $P$ $A_0$ and $k_1$, Breath[c] | $P$ $k_2$, Blood[c] | $P$ $k_2$, Breath[c] |
|---|---|---|---|---|---|---|---|---|---|
| 53 | rs2866152 | 100470090 | C→G | intergenic | 0.287 | | | | |
| 54 | rs1789895 | 100475752 | C→G | intergenic | 0.353 | | | | |
| 55 | rs1662031 | 100475816 | A→G | intergenic | 0.454 | | | | |
| 56 | rs3098808 | 100475847 | A→G | intergenic | 0.078 | | | | |
| | *ADH1C 3′* | *100476672* | | | | | | | |
| 57 | rs2298753 | 100476930 | T→C | exon-9 3′utr | 0.100 | | | | |
| 58 | rs1612735 | 100477030 | T→C | intron-8 | 0.454 | | | | |
| 59 | rs1662060 | 100478864 | A→G | intron-8 | 0.453 | | | 0.003 | |
| 60 | rs698 | 100479812 | A→G | exon-8 Non Syn (Ile→Val) | 0.452 | | | | |
| 61 | rs1693481 | 100482857 | C→T | intron-6 | 0.450 | | | | |
| 62 | rs1789912 | 100482965 | C→T | intron-6 | 0.445 | | | | |
| 63 | rs1693482 | 100482988 | C→T | exon-6 Non Syn (Arg→Gln) | 0.450 | | | | |
| 64 | rs1693424 | 100484259 | C→G | intron-5 | 0.453 | | | | |
| 65 | rs1625439 | 100484348 | G→T | intron-5 | 0.453 | | | | |
| 66 | rs283411 | 100484980 | G→T | intron-5 | 0.052 | | | | 0.015 |
| 67 | rs1693425 | 100485135 | C→T | exon-5 Syn (Val→Val) | 0.452 | | | | |
| 68 | rs2241894 | 100485156 | A→G | exon-5 Syn (Val→Val) | 0.216 | | | | |
| 69 | rs1693426 | 100485353 | A→G | intron-4 | 0.454 | | | | |
| 70 | rs1662053 | 100485606 | T→G | intron-3 | 0.454 | | | | |
| 71 | rs1662052 | 100485658 | T→C | intron-3 | 0.454 | | | | |
| 72 | rs1693431 | 100487072 | T→G | intron-3 | 0.454 | | | | |
| 73 | rs1629270 | 100490329 | A→G | intron-1 | 0.453 | | | | |
| 74 | rs283416 | 100490379 | C→T | intron-1 | 0.052 | | | | 0.015 |
| | *ADH1C 5′* | *100492940* | | | | | | | |
| 75 | rs1789924 | 100493309 | C→T | intergenic | 0.457 | | | | |
| 76 | rs980972 | 100498270 | C→A | intergenic | 0.457 | | | | |
| 77 | rs1596179 | 100506872 | G→A | intergenic | 0.454 | | | | |
| 78 | rs1583973 | 100506906 | G→A | intergenic | 0.103 | | | 0.007 | |
| 79 | rs283406 | 100517494 | C→T | intergenic | 0.052 | | | | 0.016 |
| 80 | rs1826906 | 100520071 | T→C | intergenic | 0.314 | | | | |
| 81 | rs1442484 | 100525199 | T→C | intergenic | 0.200 | | | | |
| 82 | rs2032350 | 100533085 | C→T | intergenic | 0.197 | | | | |
| 83 | rs1348276 | 100544653 | T→G | intergenic | 0.394 | | | | |
| 84 | rs994772 | 100546687 | G→A | intergenic | 0.118 | 0.016 | | | |
| 85 | rs969804 | 100548716 | T→A | intergenic | 0.392 | | | | |
| 86 | rs729147 | 100552290 | A→G | intergenic | 0.228 | 0.023 | | | |
| | *ADH7 mRNA* | *100552441* | | | | | | | |
| 87 | rs284787 | 100552579 | C→T | exon9-3′utr | 0.236 | | | | |
| 88 | rs3805329 | 100552635 | T→C | exon9-3′utr | 0.074 | | | | |
| 89 | rs894369 | 100552869 | C→G | exon9-3′utr | 0.229 | | 0.014 | | |
| 90 | rs3805331 | 100552955 | A→G | exon9-3′utr | 0.073 | | | | |
| 91 | rs284786 | 100553000 | A→T | exon9-3′utr | 0.309 | | 0.049 | | |
| 92 | rs284784 | 100554897 | G→T | intron-8 | 0.236 | | | | |
| 93 | rs1154454 | 100557365 | T→C | intron-7 | 0.182 | | | | |
| 94 | rs1154458 | 100559545 | C→G | intron-6 | 0.413 | | | | |
| 95 | rs971074 | 100560884 | G→A | exon 6 Syn (Arg→Arg) | 0.110 | | | | |
| 96 | rs1154461 | 100561925 | G→C | intron-5 | 0.336 | 0.0003 | 0.010 | | |
| 97 | rs1573496 | 100568692 | C→G | exon-3 Non Syn (Ala→Gly | 0.100 | | | | |
| 98 | rs1154468 | 100573280 | A→T | intron-1 | 0.335 | 0.0006 | 0.016 | | |
| 99 | rs1154470 | 100575360 | G→A | intron-1 | 0.335 | 0.0008 | 0.012 | | |
| | *ADH7 mRNA 5′* | *100575548* | | | | | | | |
| 100 | rs894363 | 100595869 | C→T | intergenic | 0.403 | 0.0007 | 0.0049 | | |
| 101 | rs2851024 | 100610127 | T→G | intergenic | 0.480 | | | | |
| 102 | rs1583971 | 100626045 | A→T | intergenic | 0.107 | | | | |
| | *C4orf17 mRNA 5′* | *100651197* | | | | | | | |
| 103 | rs1354368 | 100660909 | G→A | intron-2 | 0.350 | | | | |
| | *C4orf17 mRNA* | 100682483 | | | | | | | |

[a]NCBI Build 36.1.
[b]More common→Less common nucleotides.
[c]Blank cells indicate $P > 0.05$.

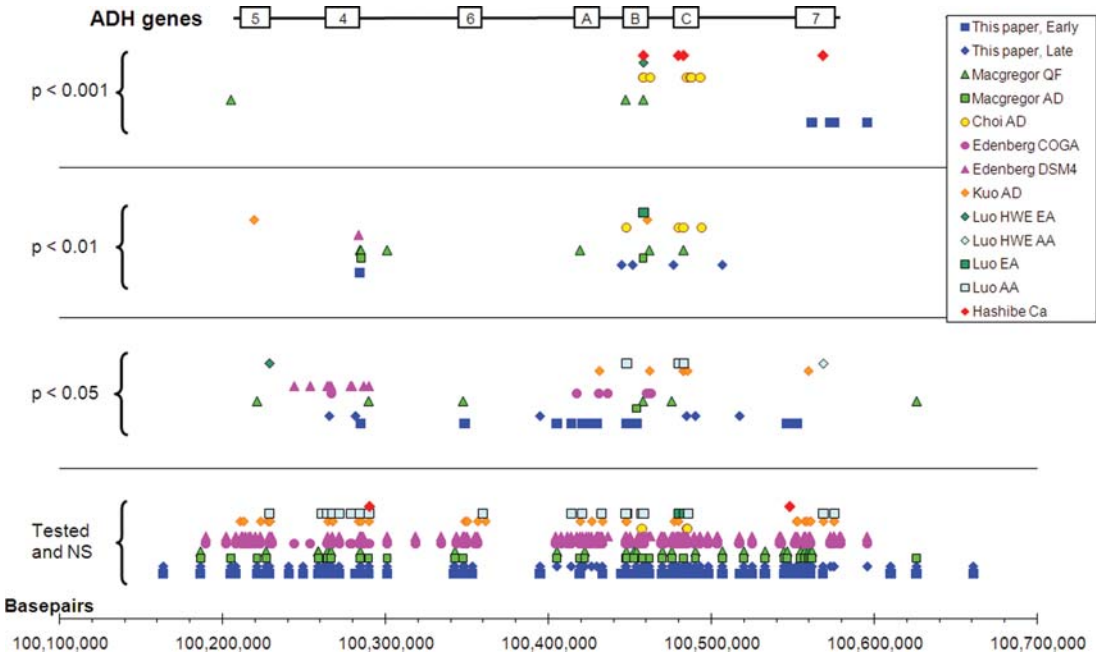


**Figure 1.** Comparison of single nucleotide polymorphism (SNP) associations across the *ADH* gene region. All data points are plotted as $-\log(p)$ ($y$-axis) against SNP location ($x$-axis, converted where necessary from the original publication to NCBI Build 36). For alcohol metabolism, the effects are estimated from the kinetic model of the time course of alcohol absorption and elimination. They are presented as the joint effect of parameters $A_0$ and $k_1$ (for early stages of alcohol absorption and metabolism), and parameter $k_2$, the rate of elimination of alcohol (for later stages of metabolism). Results are also shown for alcohol dependence (8,21–24), alcohol consumption (24) and alcohol-related upper aerodigestive cancers (25). The position of *ADH* genes is indicated by the boxes: A = *ADH1A*, B = *ADH1B*, C = *ADH1C*, 4 = *ADH4*, 5 = *ADH5*, 6 = *ADH6*, 7 = *ADH7*. Studies are distinguished as follows: this paper, early = effects on the absorption/distribution phase of alcohol pharmacokinetics, $A_0$ and $k_1$; this paper, late = effects on the elimination phase of alcohol pharmacokinetics, $k_2$; Macgregor (24) QF = alcohol intake from quantity and frequency data; Macgregor AD = alcohol dependence symptom count; Choi (21) AD = alcohol dependence diagnosis; Edenberg (22) COGA = alcohol dependence by COGA criteria; Edenberg DSM4 = alcohol dependence by DSM-IV criteria; Kuo (23) AD = alcohol dependence diagnosis; Luo (8) HWE EA and AA = alcohol dependence diagnosis using Hardy–Weinberg disequilibrium testing in European-Americans or African-Americans, respectively; Luo EA and AA = alcohol dependence diagnosis using allelic association test in European-Americans or African-Americans, respectively; Hashibe (25) Ca = case–control study of upper aerodigestive cancers.

(Although $r^2$ and $D'$ are equivalent when both SNP allele frequencies are equal, $D'$ exceeds $r^2$ when they are not.)

The pattern of LD for SNPs in the *ADH1C* region (Supplementary Material, Fig. S2) is of a major LD block (rs2032350 to rs2866152) although there is an apparent discontinuity at rs1826906, a SNP with intermediate frequency. The LD block in the *ADH1C* region flanks the transcribed sequences by 40.15 kb in the 5′ direction and 6.58 kb in the 3′ direction, although the highest allelic correlations are within the *ADH1C* transcriptional unit. SNPs rs1693482 (Arg272Gln in exon 6) and rs698 (Ile350Val in exon 8), the only two genotyped non-synonymous substitutions in the region, are in near-total LD. Although they are associated with a two-fold difference in ADH $V_{max}$ *in vitro* there was no evidence of association with *in vivo* alcohol oxidation, consistent with our earlier analyses of the same sample.

An extensive but weak LD block (170.6 kb) from *ADH1B*, across *ADH1A* and *ADH6* to the *ADH6–ADH4* intergenic region (Supplementary Material, Fig. S3) contains three instances where effects on blood or breath alcohol concentration reach a statistical significance level of $P < 0.01$, and there is a cluster of 12 SNPs for which $0.01 < P < 0.05$ (Table 1, Fig. 1). This block extends beyond the three corresponding transcribed sequences. The significant effects in this region are for both the early and late stages of alcohol metabolism and are only associated with lower-minor allele frequency (MAF) alleles in the *ADH1B* haplotype block: rs2018417 (with an early-increasing effect on alcohol oxidation), rs1229985 (late-increasing effect), rs17033 (early-decreasing) and rs1789877 (late-increasing). The low-MAF SNP rs1229984 is the *ADH1B Arg48His* polymorphism, which was not associated with alcohol metabolism. In contrast, effects on early stage metabolism in the *ADH1A* region are associated with four common alleles (rs931635, rs1229967, rs1618572, rs1230025), all with an increasing effect, and one less common allele at rs2276332 (with a decreasing effect).

In the *ADH6–ADH4* intergenic region two SNPs at intermediate frequency (rs3857224 and rs3762894) are related to early stages of metabolism. One SNP (rs1800759), located in the 5′ flanking region of the *ADH4* coding sequence, is at intermediate frequency and associated ($P < 0.01$) with early stage metabolism. Two out of three less common SNPs (rs2032349, rs1573495) fall within the *ADH4* transcribed region and are associated ($0.05 \geq P \geq 0.01$) with rate of elimination.

Overall, the significantly lower MAF SNPs (MAF $< 0.11$) in the *ADH1C–ADH5* region were generally related to rate of elimination (10 out of 13), and all seven significant SNPs at intermediate frequency (MAF $> 0.11$) were associated with early stages of metabolism (Table 1). MAF was significantly associated with early/late phase effects ($P = 0.001$, Mann–Whitney test) and even after taking haplotype sharing

into account, the association between SNP frequency (low/intermediate) and physiological effect (early/late) in this region remained significant ($P = 0.035$ by Fisher's exact test).

In the *ADH7* region, two LD blocks were found (see Supplementary Material, Fig. S4). Our previous analysis of this region (12) showed that variation in early alcohol metabolism is associated with the *ADH7* 5′ LD block, and that this is most likely associated with rs1154461, rs1154468, rs1154470 or rs894363. These SNPs show significant associations with early alcohol metabolism, assessed by either blood or breath alcohol results (Table 1), but because of the strong LD it is not possible to assign the effect to any individual SNP.

## DISCUSSION

Oxidation of ethanol to acetaldehyde, catalysed by the high-affinity forms of ADH, is the initial and rate-limiting step in alcohol metabolism. It follows that variation in *ADH* genes, or in the control of their expression, may explain the genetic component of variation in human alcohol metabolism. Linkage between the family of ADH-encoding genes, located at 4q21-23, and both post-challenge alcohol concentrations (7) and alcohol dependence risk (32–34) strengthens this hypothesis. Associations between variation in at least one *ADH* polymorphism (*ADH1B Arg48His*, rs1229984) and alcohol dependence risk have repeatedly been found [summarized in (26,27)]. The effects of this polymorphism on *in vitro* enzyme activity are consistent with the theory that high ADH activity, leading to high rates of acetaldehyde generation, is protective against alcohol dependence.

Based on this prior information, we expect that polymorphic variation within the *ADH* gene cluster will affect a series of events: the rate of alcohol metabolism, the rate of formation and hence the steady-state concentration of acetaldehyde, the subjective effects of alcohol consumption, the quantity of alcohol consumed and the risk of alcohol dependence. Our recent work on these phenotypes related to alcohol dependence (24) has strengthened the evidence for this. There may also be effects of *ADH* variation on alcohol-induced liver damage [(35), but see (36)] and carcinogenesis (25). In this paper, we have examined associations between *ADH* SNP variation and alcohol metabolism *in vivo*, and we compare these associations with the previous findings on *ADH* variation and other alcohol-related phenotypes, including alcohol dependence.

### Allelic association with alcohol metabolism

Multiple significant allelic associations between the tested SNPs and either the absorption or elimination phase of alcohol pharmacokinetics were found. The strongest were in the *ADH7* region, as previously reported, and with the early or absorption phase. At first sight, it may seem surprising that the early phase of alcohol pharmacokinetics (captured by the volume of distribution $A_0$ and the absorption rate constant $k_1$) is associated with variation in *ADH*. However, there is significant ADH-dependent alcohol metabolism in the stomach, before absorption, and in the liver before alcohol reaches the systemic circulation. We have argued previously (12) that ADH7 is well-suited by its location and kinetic properties to participate in this 'first-pass' metabolism. Variation in first-pass metabolism will lead to variation in the apparent volume of distribution of alcohol.

Another noteworthy aspect of our results is that the detected SNP effects on early alcohol metabolism are mainly for SNPs at intermediate to high MAF. This may be a function of power to detect the effects, which will be greater for common polymorphisms, but several less common SNPs had detectable effects on the post-absorption or elimination phase and therefore their effect sizes must be substantial.

Compared with the effect size implied by our linkage analysis, the SNP associations we find in the *ADH* region have comparatively small effects on alcohol metabolism. The effect of variation in the extensively studied *ADH1B Arg48His* variant (rs1229984) on post-challenge alcohol concentrations is minimal; we estimated it to contribute $<1\%$ of variance in our previous analysis of these data. Others have presented relevant data on this polymorphism from East Asian subjects, where no significant *in vivo* effects were found (29,30); and from Israelis (37) in whom a significant effect accounting for 8% of the population variation was found. The largest effect in the *ADH* region on blood or breath alcohol concentration was located in an LD block in the 5′ part of the *ADH7* gene, and only accounts for approximately 18% of the genetic variance (12).

Variation which is not tagged by the SNPs typed here, where we mainly targeted coding regions, must be contributing to the linkage effect. Such linkage in the absence of SNP association could be observed for several reasons; multiple rare variants, each arising on a different haplotype background and associated with different alleles at the tagging SNPs, or by polymorphisms not in LD with any of our typed SNPs that modify or regulate ADH levels. *Cis*-acting regulatory polymorphisms can be located within or outside a gene cluster and can result in coordinated control of a set of genes.

The nature and locations of regulatory elements for *ADH* gene expression in the *ADH* region have been studied by several groups (38). The regulatory elements are not necessarily gene-specific; for example a HNF1-binding site controlling Class 1 *ADH* expression, located upstream of *ADH1C*, interacted with promoter sequences for all Class 1 *ADH* genes (39). Polymorphisms in sites with regulatory functions have an obvious potential to affect alcohol metabolism; a promoter polymorphism affects *ADH4* expression in transfected hepatoma cells (40). Haplotypes which included a 66-bp insertion/deletion polymorphism upstream of *ADH1C*-affected promoter activity in a transfection assay (41), accounting for a two-fold change in transcription activity. We can therefore expect to find polymorphisms both for the coordinated regulation of several members of the *ADH* gene family, and regulation of specific *ADH* genes.

Genome-wide association and expression array methods allow detection of SNP-associated levels of gene expression (transcript abundance). This approach may be useful for relating *ADH* gene expression, and by implication of ADH enzyme activity, to rate of metabolism after alcohol challenge. Studies on gene expression in lymphocytes have shown

linkage of *ADH1B* expression to loci elsewhere on 4q (42). A comprehensive genome-wide study of SNPs affecting gene expression in transformed lymphocytes (http://www.sph.umich.edu/csg/liang/asthma/mRNA_bySNP_browser_v1.0.1.exe) (43) showed effects of SNPs between *ADH1A* and *ADH6* on *ADH1A* expression. However, a similar study on gene expression in the liver (44), which is more relevant to post-absorptive alcohol metabolism, did not report SNPs which significantly affected transcript abundance of any of the *ADHs*.

### Single nucleotide polymorphism effects, alcohol dependence and alcohol-related disease

Many studies have shown associations between *ADH* polymorphisms and the probability of alcohol dependence. The assumption is that this association is mediated by variation in alcohol metabolism. Therefore, it is useful to consider how far the *ADH* polymorphisms reported to affect alcohol dependence show associations with either early or late alcohol metabolism in our data. To facilitate this comparison, our SNP association results for the early and late phases of alcohol metabolism are plotted in Figure 1, together with those from five other studies which tested multiple SNPs in *ADH* genes for association with alcohol dependence (20–24), and one (25) which tested for associations with alcohol-related cancers. Although the methods and the SNPs tested varied, some general conclusions about the genes or regions showing significant associations can be drawn.

As described above, variation in the early phase of alcohol metabolism was associated with SNPs in *ADH7* and to a lesser extent with SNPs across *ADH1B* and *ADH1A*, in *ADH4*, and possibly *ADH6*. Variation in the elimination phase was associated with SNPs in Class 1 *ADHs*, particularly *ADH1B* and *ADH1C*. The SNPs associated with alcohol dependence in other studies may be compared with these. Although the specific SNPs typed vary between studies, the existence of substantial LD leads us to expect that the regions showing significant effects should be identifiable. Figure 1 shows that significant associations for alcohol dependence or alcohol intake cluster in *ADH1B* and *ADH1C*, with some in *ADH1A*. There are also some in or near *ADH4* and *ADH5*, but associations are not usually found in *ADH6* or *ADH7*. There are some features of individual studies which deserve comment: the study by the COGA consortium (22) found different SNP associations (near *ADH4*, or across *ADH1A–1B*) with different definitions of the alcohol dependence phenotype. Another US study (20) stratified the analysis by race and found that the different non-synonymous coding SNPs found in *ADH1B* in European-Americans and African-Americans (*Arg48His* and *Arg370Cys*) both affected dependence risk. Their conventional allelic association analysis identified SNPs in *ADH1B* and *ADH1C*, but a test based on deviation from Hardy–Weinberg equilibrium also suggested effects in *ADH5* and *ADH7*. It is also notable that all studies, including ours, report a mixture of significant and non-significant SNPs in regions of substantial LD, where one might expect a high degree of concordance for either positive or negative results.

In addition to the studies which focused on *ADH* variants affecting alcohol dependence risk, a large multicentre study on upper aerodigestive cancers (25) found that rs1229984 (in *ADH1B*) and rs1573496 (in *ADH7*) had highly significant effects on risk for this group of cancers. However, these associations are also at variance with our results on alcohol metabolism, because rs1229984 is the *Arg48His* polymorphism discussed above and has no significant effect on blood or breath alcohol concentrations. We did find [(12) and Table 1] that variation within *ADH7* affects post-challenge alcohol concentrations but rs1573496 has no significant effect. This reinforces the paradox of well-documented *ADH* effects on disease risk, but with no evidence that the proposed causal mechanism of variation in the rate of conversion of ethanol to acetaldehyde is involved.

### Conclusions

A number of SNPs which show significant allelic associations with alcohol metabolism have been identified. The early or 'first-pass' phase of alcohol metabolism is affected by variation in or near *ADH7*, *ADH1A* and *ADH1B*, and possibly in *ADH4*. Post-absorptive alcohol metabolism is affected by variation across *ADH1A*, *ADH1B*, *ADH1C* and the intergenic region between *ADH1C* and *ADH7*, and again possibly in *ADH4*. Because of multiple testing, any of these associations could have arisen by chance but it is unlikely that they are all false-positives. There is evidence that variation in the Class 1 *ADHs*, *ADH1A*, *ADH1B* and *ADH1C*, affects alcohol dependence risk as well as alcohol metabolism. In contrast, *ADH7* affects alcohol metabolism but not dependence. The combined effects of the tested SNPs in the *ADH* region account for only approximately 20% of the total genetic variance for alcohol metabolism, in contrast to the approximately 60% because of the linked quantitative trait loci, implying that other large effects on *in vivo* alcohol metabolism in the region, not in LD with the SNPs we tested, are still to be discovered. Given the importance of the *ADH* gene family in alcohol detoxification and risk of alcoholism, a search for rare single-nucleotide or copy-number polymorphisms in regulatory elements close to the *ADH* gene cluster may provide the key to the unexplained genetic variation in alcohol metabolism.

## MATERIALS AND METHODS

The Alcohol Challenge Twin Study (ACTS) took place during 1979–1981 and details of the twins and procedures used to obtain the 10 breath and six blood alcohol readings are given elsewhere (2,7,12). The revised zygosity status of participants, based on genotyping since the original study, is 91 monozygotic (MZ) twin pairs (46 female and 45 male) and 115 dizygotic (DZ) twin pairs (41 female, 35 male and 39 opposite sex). Twin ancestry was 87% northern European and nearly all the remaining 13% was southern European/Mediterranean (15).

Genotyping was carried out on samples from 812 people. The median number of SNPs typed per person was 104 (range 89–104) and the median number of people typed per SNP was 808 (range 753–812). One SNP (rs1041969) was monomorphic, and the remaining 103 are listed in Table 1. Both twins were genotyped from 69 MZ pairs and 102 DZ pairs, and one twin was typed from 16 MZ and eight DZ pairs. In addition, 216 parents from 144 families and 226

non-twin siblings from 133 families were genotyped. The cross-tabulation of family members typed is shown in Supplementary Material, Table S1. Although non-twin relatives were not phenotyped, their genotypes provide information for within-family association tests.

### Genotyping and quality control

Alleles were assayed by primer extension on mass spectrometry (Sequenom MassArray system; Sequenom, San Diego, CA, USA). Historic polymorphisms previously analysed using restriction fragment length polymorphism-type assays were converted to the primer extension format. All known gene-coding regions including 3′- and 5′-untranslated region polymorphisms were included. Because of high homology among some of these gene family members, PCR primers were carefully chosen for single-locus specificity and verified on appropriate pedigrees.

No Mendelian errors were identified by inheritance checks [PEDSTATS version 0.4.6 (45)]. Of the 103 SNPs, four showed departures from Hardy–Weinberg equilibrium, within the expectations of sampling error. Genotyping errors were also identified as inconsistencies in the co-inheritance of SNPs [MERLIN version 1.0.1 (46)] identified 66 genotyping errors or 0.01% of all genotypes. These errors (14 SNP loci) were not attributable to location (map order provided by NCBI Build 36.1). Twelve of these SNPs gave error rates in the range of 0.14–0.95% while two were higher (1.9% and 1.5%). Inspection of the distribution of errors by family (34 families) did not provide any systematic indication of their cause and they were recoded as missing. Comparison of MAFs for the 98 SNPs also present in data for the CEU population [CEPH (Utah Residents with Northern and Western European Ancestry)], NCBI Build 36.1 (http://www.ncbi.nlm.nih.gov/projects/SNP/) or from ALFRED (http://alfred.med.yale.edu/alfred/index.asp) gave a very small mean difference ($0.00062 \pm 0.00423$) between allele frequencies genotyped in the present study and published MAFs.

### Linkage disequilibrium

Pairwise estimates of LD across the *ADH* region were obtained using HAPLOVIEW (version 4.1; http://www.broad.mit.edu.mpg/haploview/). The most likely haplotypes were identified following the examination of LD blocks with MERLIN 1.0.1 (46). Clusters of LD and haplotypes were resolved with the cluster option.

### Single nucleotide polymorphism associations with blood and breath alcohol levels—the pharmacokinetic model

Our approach to data analysis was described in a previous paper on the *ADH7* region (12). The SNP association analysis was based on deriving two sets of pharmacokinetic parameters from the observed blood or breath alcohol concentrations at six times (for blood) or 10 times (for breath) after consumption of the 0.75 g/kg dose of alcohol. The relationship between alcohol concentration and time follows Eq. (1):

$$C(t) = A_0(1 - e^{-k_1 t}) + k_2 t \quad (1)$$

Alcohol levels during the early absorptive phases are estimated by $A_0$ (the concentration of alcohol that would be achieved if all ingested alcohol were absorbed and evenly distributed throughout the whole body volume) and $k_1$ (the absorption rate constant). Because of substrate saturation, the rate of elimination of $k_2$ is independent of ethanol concentration; hence the concentration–time relationship in the elimination phase follows zero-order kinetics. The fit between this kinetic model and the observed data was examined in our previous paper [see Fig. 3 in (12)]; there was a good fit for the blood alcohol data, and although the difference was significant for breath alcohol its magnitude was small.

The three parameters $A_0$, $k_1$ and $k_2$ were estimated from the blood or breath alcohol concentrations $C$ at time $t$, as main effects and as allelic deviations. The maximum-likelihood estimates of effect sizes at alleles for the 103 SNPs were estimated in the means part of the multivariate normal equation using Mx (47). The model for the expected means (2,12,48) included adjustment for the covariates sex and age. The expected sib pair covariance matrices were modelled jointly with means and were parameterized as additive and specific environmental covariance by Cholesky decomposition taking into account the zygosity of twin pairs. Tests of significance for allelic deviations from the kinetic model were made from the corresponding change in log-likelihood when parameter values were set to zero. The change in log-likelihood follows a $\chi^2$ distribution and results are presented as $-\log_{10}(P)$.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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*Conflict of Interest statement*. None declared.

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