MUT/2014/05 – Updated 2015/03/05

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD CONSUMER PRODUCTS AND THE ENVIRONMENT

DRAFT UPDATE REVIEW ON THE MUTAGENICITY OF ALCOHOL (ETHANOL) AND ITS METABOLITE ACETALDEHYDE

INTRODUCTION

1. Following their recent discussions on alcohol and cancer , COC members requested COM to consider the genotoxicity of alcohol (ethanol) and acetaldehyde (as the major metabolic product of alcohol), to provide evidence that would feed into the COC's ongoing update review/deliberations on possible mechanisms for cancers causally associated with the consumption of alcoholic beverages. The intention is for COM to produce a statement to serve as an information/ reference paper.

2. The current paper provides an update of studies published since the previous COM statement in 2000 (COM Statement, 2000).

BACKGROUND

Previous Committee Papers

I. COM Statement on alcoholic beverages (1995)

3. The COM conducted a detailed review on the mutagenicity of ethanol, acetaldehyde and alcoholic beverages in 1995, as part of their contribution to the Inter-departmental Working Group's (IDWG) Report on Sensible Drinking (published in Dec 1995) (DH, 1995). In a statement, the COM concluded that the consumption of alcoholic beverages did not present any significant concern with respect to their mutagenic potential (COM Statement, 1995).

II. COM Statement on alcoholic beverages (2000) – COM00S5 (In response to the COC Statement on alcohol consumption and breast cancer (2000))

4. In 2000, the COC conducted an update review of new studies that investigated the association between consumption of alcoholic beverages and breast cancer[1]. The COC made a number of interim conclusions and requested that the COM update their opinion on the mutagenicity data to assist with ongoing COC deliberations on possible mechanisms (COC Statement, 2000). In May 2000, the DH Toxicology Unit (Imperial College London) prepared a review of in vitro and in vivo studies published from 1995 to February 2000 for the COM (MUT/2000/04). Following an evaluation of the published evidence, the COM reaffirmed their 1995 conclusion in a statement

[1] The DH Tox Unit had previously produced papers on potential mechanisms (DH Tox Unit, 1999ab). Subsequent papers fed into the COC statement on alcohol consumption and breast cancer (2004). http://www.iacoc.org.uk/papers/documents/alco04full.pdf

published in November 2000, and concluded (overall) that alcoholic beverages did not present any significant concern with respect to their mutagenic potential (COM Statement 2000).

IARC monographs (2010; 2012)

5. IARC has produced two critical reviews on the in vitro and in vivo evidence for carcinogenicity following exposure to alcoholic beverages, ethanol and acetaldehyde: IARC Monographs Volumes 96 and 100E (IARC, 2010; 2012 respectively)[2]. IARC 2010 reviewed studies published until 2007. The most recent monograph, IARC (2012), updates IARC 2010 and incorporates new data published from 2007 to 2011.

6. Both publications report on changes occurring at the molecular level and provide an overview discussion of selected genotoxicity/ mutagenicity studies of alcohol/ethanol and acetaldehyde. The monographs also briefly report on potential mechanisms of alcohol-induced carcinogenesis and consider the genotoxic, mutagenic and clastogenic effects of acetaldehyde as supportive evidence for its role in alcohol-induced carcinogenesis.

UPDATE REVIEW

7. The current review is divided into sections according to compound of interest i.e. (1) alcoholic beverages; (2) ethanol; and (3) acetaldehyde. The literature search strategy used to identify studies is summarised in Annex 1. Tables with further study details are provided in Annex 2. The final section considers key discussion points arising from the current lines of evidence and compares it to the previous COM conclusions to ascertain whether any changes need to be made. Annex 3 contains COM papers and key publications for members' consideration.

8. A total of 46 studies providing data on either classic mutagenic endpoints or acetaldehyde-specific DNA adducts were identified and selected for evaluation in the current paper. NB. Other related endpoints e.g. oxidative mechanisms, i.e. reactive oxygen species/free radicals, epigenetics and synergism will be discussed in future papers. Tables (1.1 to 1.10), and (2.1 & 2.2), and (3.1 & 3.2), provide additional data for studies conducted in vivo in either humans or animals, and in vitro (human and animal cells) respectively. Tables (4.1 & 4.2) list key findings for all studies evaluated.

1. Alcoholic Beverages

9. Nineteen studies were identified that investigated genetic and related effects of exposure to alcoholic beverages in humans. There were no in vitro studies or experimental studies performed in animals on these substances. (See Tables 1.1 to 1.10 for further details)

[2] The subject was previously considered in 1987 (IARC, 1988, Volume 44).

a. DNA adducts

10. DNA adduct formation in alcohol drinkers was examined in six studies, and all (except one) detected higher levels of specific acetaldehyde-DNA adducts in alcohol-exposed individuals. Balbo et al (2008) used LC-ESI-MS/MS to determine levels of N2-ethyl-deoxyguanosine[3] (N2-ethyl-dG) in the leukocytes of alcoholic drinkers selected from controls of two European case control studies. Drinkers (n=119) had significantly higher levels of adducts cf. non-drinkers (n=58) (p=0.04). A dose response relationship for levels of N2-ethyl-dG was observed across categories of alcohol intake 0-199g/day (in subjects taken from ARCAGE study) which ranged from mean 3010 ± SD 3580 (fmol/umol dG) to 9090 ± 11800 (p=0.02) P=0.02 for trend). The effect of cigarette smoking on adduct levels was non-significant. It was suggested that the unexpectedly higher adduct levels found in younger than older drinkers (p=0.01) could be due to underreporting, and/or binge drinking

11. The same author investigated the levels of these adducts in the granulocytes and lymphocytes (Balbo et al 2012a) and oral epithelial cells (Balbo et al 2012b) of 10 healthy non-smoking male and female volunteer US students and employees of the University of Minnesota, under controlled experimental conditions. Subjects received increased doses of alcohol for three weeks to achieve blood concentrations of 0.03, 0.05 and 0.07%. Peak baseline ratios[4] were significant for all samples tested, with alcohol exposure causing up to 15-fold increase in adduct levels relative to background levels present in oral epithelial cells (p=0.001). Both studies also investigated the effect of alcohol consumption on time course of DNA adduct formation. Peak levels were apparent within 40 hours in blood cells compared to 4 hours in oral epithelial cells. However, although the volunteers minimised acetaldehyde exposure from food sources, there was substantial variation in baseline adduct levels. Other limitations included the small study size and the potential for contamination of human DNA with bacterial DNA from mouthwash samples.

12. In contrast to the positive findings reported above, Singh et al (2012) found no significant association between alcohol consumption encountered under social drinking conditions (150ml vodka) and levels of N2-ethyl-dG in the leukocyte DNA of 30 healthy male non-smoking Polish subjects. The authors processed different DNA samples from the same individual to minimise analytical variation, and observed no significant increase in average adduct levels per 10^8 2'-deoxynucleotides over a 48h period (box and whiskers plot). Significant inter-individual differences in adduct levels were also apparent.

13. The influence of aldehyde dehydrogenase (ALDH2) polymorphism on the formation of acetaldehyde-specific DNA adducts was examined in two studies. Matsuda et al (2006) used PCR-RFLP to genotype 44 male alcoholic Japanese patients due to receive alcoholism treatment as either ALDH2*1/2*1

[3] Sodium borohydride reduction product of N2-ethylidene-dG

[4] Peak baseline ratio = ratio between the average max adduct level reached after each dose and the average baseline level for that dose (with 95CIs)

(proficient) or 2*1/2*2 (deficient). Significantly higher levels of N2-ethyl-dG and S and R isomers of Me-γ-OH-PdG were detected in ALDH2-deficient patients compared to ALDH2-proficient patients ($p<0.003$). This was particularly pronounced for N2-ethyl-dG in which a 7-fold difference in levels was detected between genotypes.

Yukawa et al (2012) examined the relationship between N2-ethylidene-dG adduct levels in leukocytes of 66 intoxicated alcoholic Japanese men and polymorphisms of ADH1B and ALDH2 genes. Subjects drank an average of at least 100ml of ethanol in the last 24h and were admitted to hospital for alcoholism treatment. Patients were stratified into Groups 1 to 4 depending on the type of allele they carried. No patients were identified with the ALDH2*2/*2 genotype. The levels of N2-ethylidene-dG adducts were significantly elevated in individuals with a combination of ADH1B*2 and ALDH2*2 alleles. Patients with the ALDH2*1/*2 genotype (n=16) who were also heterozygous or homozygous for the ADH1B*2 allele (Group 4) had 4-fold higher levels of adducts than WT (ADH1B*1) (Group 3) ($p<0.01$; n=8 respectively). Group 4 patients had approximately 9-fold higher levels of adducts compared to the proportion of ADH1B*2 patients that were homozygous for the ALDH2*1 allele (Group 2). There was no significant difference between adduct levels of Group 1 (ADH1B and ALDH2 WT) and Group 2 patients. However, these findings are limited by use of an acute exposure assessment that comprised of alcohol intake measured within a 24h period. Furthermore, mean body weight differences were apparent between Groups1 and 2. Group 2 individuals had significantly lower body weights ($p<0.01$), which could implicate potential nutrition deficiencies, which is known to impact on genome stability.

b. Micronuclei

14. Eight studies investigated the induction of micronuclei (MN) in alcohol-exposed individuals. All reported a positive association.

15. Maffei et al (2000) observed significantly higher frequencies of MN per 1000 binucleates in binucleated peripheral blood lymphocytes (PBLs) of 20 male and female Italian alcoholics who consumed more than 120g ethanol per day compared to 20 healthy age-, sex- and smoking-matched controls who drank 8-13g ethanol/day ($P<0.05$). MN were further analysed via pancetromeric fluorescent in-situ hybridisation (FISH) analysis to delineate the mechanism underlying MN formation. The authors suggested that the higher frequencies of centromere-positive (C+) MN in alcoholics cf. controls (8.2 ± 4.8 and 3.4 ± 1.4, respectively) demonstrated that alcohol induced MN via a possible aneugenic mechanism ($P<0.05$).

16. Maffei and colleagues also observed a positive association with MN frequency in a related analysis conducted in alcoholics stratified according to whether they were former or current drinkers ($P=0.001$; n=20 respectively) (Maffei et al 2002). Abstinence ranged from 12-60 months and resulted in mean MN frequencies comparable to levels reported in 20 matched non-drinking controls (7.6 ± SD 1.6). A 4-fold increase in MN levels in buccal cells of the tongue was found in a study of 40 non-smoking Brazilian drug addict alcoholics when compared to the levels measured in 20 abstinent individuals

(P<0.01) (Reis et al 2002). However, no information was provided in relation to the type of drugs consumed, and data acquisition was limited as the study was published in Portuguese (only a basic translation was available).

17. Buccal cells were also sampled in an investigation of MN and meta-nucleated anomaly induction in 30 smoking Brazilian alcoholics diagnosed with oral / oro-pharyngeal carcinomas (Ramirez & Saldana, 2002). Epithelium was obtained from three distinct regions in the mouth assigned letters A (side opposite the lesion), B (around lesion) and C (upper gingival-labial gutter). Respective areas in 30 non-drinking non-smoking controls were assigned letters D (right cheek); E (left cheek); F (upper gingival-labial gutter). Both MN and most meta-nucleated anomalies were significantly increased in patients compared to controls, with a gradient effect apparent in areas closest to lesion; B region: 7-fold increase; A region: 3.4-fold; C: non-significant increase. However, the strength of these findings is limited by an apparent lack of account of smoking differences between groups and the relevance of the study is questioned by the use of samples closely associated with a tumorigenic endpoint.

18. The effect of ALDH2 polymorphisms on MN frequency in humans were evaluated in four studies. Ishiwaka and colleagues conducted a series of investigations in PBLs of healthy Japanese men stratified according to their habitual or non-habitual drinking habits and genotyped via PCR-RFLP as being either ALDH2-proficient (*1/*1) or ALDH2-deficient (*1/*2 or *2/*2). In all three studies, the average MN frequency was significantly increased in habitual drinkers carrying the ALDH2-deficient genotype compared to those without the mutation (p-values ranged from <0.01 to <0.05) (Ishiwaka et al 2003; 2006; 2007). Ishiwaka also examined the combined effects of polymorphisms in genes for other enzymes involved in alcohol metabolism i.e. alcohol dehydrogenase (ADH1B) and the P450 CYP2E1. Highest MN levels were observed in drinkers carrying at least one ADH1B*1 allele ($p \leq 0.004$) (Ishiwaka et al 2007) or CYP2E1*1/*1 ($p<0.05$) (Ishiwaka et al 2006) in addition to an ALDH2-deficient genotype when compared to their respective ADH1B*2 and CYP2E1*3 variants.

19. Comparable findings on ALDH2 were also apparent in a similar investigation carried out in reticulocytes of 156 male Japanese employees of a hard metal tooling factory (Wu et al 2010). Higher MN-RET frequencies were observed in habitual drinkers cf. non-habitual drinkers ($p<0.015$), with presence of the mutant ALDH2 allele increasing MN-REF frequency by 45% ($p< 0.047$). However, covariate analysis revealed a significant association w.r.t the influence of drinking on the MN-RET frequency for the ADH1B*2-ALDH2*2 haplotype P=0.012. It is unclear why there is an apparent lack of consistency between this study and Ishiwaka et al (2007) in relation to the influence of ADH1B genotype on MN levels. Wu and colleagues argue that reticulocytes present a much more direct biomarker compared to lymphocytes that are affected by several environmental and genetic factors. The study however did not provide an assessment of alcohol intake and was also of a smaller size cf. to Ishiwaka et al 2007 (248 subjects).

c. Chromosome aberrations

20. Two[5] studies explored the relationship between alcohol drinking and chromosome aberrations (CAs) and both observed significantly higher levels in alcoholics vs. non-drinkers. The mean frequency of aberrant cells, chromatid and chromosome type damage in PBLs of 40 alcoholics (current and abstinent) increased more than 2.3-fold in alcoholics drinking more than 120g/day compared to 20 non-drinking subjects ($p<0.001$) (Maffei et al., 2002). As reported for MN, abstinence appeared to normalise CA frequency. However, abstinence had no effect in a study of 40 Brazilian alcoholics who consumed more than 60g ethanol per day (levels in former alcoholics remained significantly >2-fold higher than controls) (Burim et al 2004). Current alcoholics exhibited three-fold higher CAs and 4-fold higher levels of chromosome translocations (assessed via fluorescence in situ hybridisation (FISH) analysis) relative to 20 non-drinking controls ($p<0.001$ and <0.05 respectively). Although all subjects smoked, further analysis did not find any significant interaction effect between smoking and alcohol consumption.

d. Comet assay

21. DNA strand breaks were evaluated in three studies using the Comet assay. Two studies reported an inverse association with alcohol exposure and DNA migration rates. Pool-Zobel et al (2004) performed their analysis in intact biopsied rectal cells and PBLs. Percentage of fluorescence in comet tail was significantly lower in the 10 male German alcoholics relative to the nine male and female social drinking referents in both samples ($p<0.001$), a somewhat unexpected finding as this suggests less DNA damage in alcoholics. However, the authors did not assess the smoking status of the subjects, and a repeat study in a larger sample would be required due to the very small numbers studied. A similar inverse association was observed by Lu & Morimoto (2009) in their analysis of 150 Japanese smoking males stratified according to ALDH2 genotype and their alcohol intake and drinking frequency. Electrophoretic DNA migration in ALDH2-deficient male Japanese subjects was negatively correlated with both alcohol drinking frequency (Spearman's correlation) and the total amount of pure alcohol consumed (Pearson's correlation). Stepwise multiple linear regression analysis showed that both alcohol drinking and levels of daily exposure to cigarette tar were significant predictors for electrophoretic DNA migration.

22. Possible explanations suggested for these inverse findings include enhancement of endogenous defences in alcoholics (i.e. upregulation of DNA repair in response to damage) and the likelihood that AA-induced protein cross-links were formed that typically migrate less and result in reduced DNA in comet tails.

[5] A study by Lopez et al 2001 was excluded from the analysis due to inaccessibility of the full paper.

23. The only study reporting a positive association was by Weng et al (2010), between DNA strand break induction and alcohol consumption in mononuclear cells of 122 Japanese men (mean age of 47 years) stratified according to their ALDH2 and ADH1B genotype. The authors did not provide any information on alcohol intake but used drinking behaviour as the exposure metric. There was a statistically significant elevation in habitual drinkers carrying ADH1B*2 (fast) and ALDH2*2 (slow) variant alleles of mean TM value (1.60 ± 0.50; n=12) cf. corresponding genotypes (1.09 ± 0.20; n=20) (p=0.012, Kruskal-Wallis H-test). A significant difference was also seen when subjects were stratified according to age. Cigarette smoking was also found to be have a significant effect on mean TM value (P<0.001). However, when stratified according to ALDH2 or ADH1B genotype this was only significant for ADH1B as the numbers of *2 allele increased (P=0.048). Multiple regressions analysis suggested that ALDH2, but not ADH1B, polymorphism had a significant effect on basal TM value.

e. Sister chromatid exchange

24. Karaoguz et al (2005) was the only study in which sister chromatid exchange (SCE) was assessed, in PBLs of 15 Turkish male alcoholics who were both heavy drinkers and smokers. Positive (n=10) and negative (n=10) age-matched non-drinking controls were used (i.e. cigarette smokers and non-smokers respectively). Mean SCE frequencies were significantly increased 1.4-fold (alcoholics vs. smoker controls) and 1.6-fold (alcoholics vs. non smoker controls) (p<0.05). The significant 1.4-fold difference between the alcoholics and smoking controls suggested an interactive effect between smoking and alcohol consumption.

2. Ethanol

25. Seventeen studies were identified that investigated genetic and related effects of exposure to ethanol in seven in vitro (human and animal) and ten in vivo (animal) studies. (See Tables 2.1 and 3.1 for further details on in vivo and in vitro studies respectively)

a. DNA adducts

(i) In vivo studies

26. The effects of ALDH2 on ethanol-induced DNA damage was examined in C57BL/6 ALDH2 knockout mice in three studies. Levels of N2-ethylidene-dG[6] adducts quantified by LC/MS/MS in DNA isolated from liver of mice sub-chronically exposed to 20% ethanol were significantly higher than in untreated, WT i.e. Aldh2+/+ mice (P<0.01) (Matsuda et al., 2007). Adduct levels were increased in the absence of partial absence of Aldh2: 40-fold (Aldh2-/-, n=2), 10-fold (Aldh2-/+, n=5) and 4-fold (Aldh2+/+, n=6) higher than untreated, WT mice (1.9 ± 0.7 adducts/10^7 bases, n=5). Other AA-specific DNA adducts were either not detected or did not show any alcohol or ALDH2-

[6] DNA was purified from tissue using sodium borohydride to detect N2-ethylidene-dG

dependent differences. The authors considered these findings suggest N2-ethylidene-dG is a sensitive biomarker for AA exposure in vivo. Almost identical results were obtained in mouse stomach, with higher levels of ALDH2-dependent N2-ethylidene-dG in treated animals; baseline adduct levels were slightly higher than in liver – 3.1 adducts/10^7 bases (Nagayoshi et al 2009).

27. Ogawa et al (2007) found little difference between levels of DNA adducts in the liver, kidney and stomach of the same strain of mice acutely exposed to radiolabelled ethanol (6-24h). However, the radioactivity of DNA (dpm/mg/DNA) in all organs of knockout mice (n=10 per time point) was significantly higher than in WT mice (n=10 per time point) after 24h oral administration of [1-3H] ethanol ($p<0.05$). Carcinogenic potency calculations based on covalent binding index (CBI) suggested that ethanol was only moderately carcinogenic to the liver.

28. No in vitro studies were identified that investigated the formation of AA-specific DNA adducts in ethanol-treated cells.

b. Micronuclei

(i) In vivo studies

29. Sub-chronic exposure to 10% w/v ethanol (n=17) in male and female CF1 mice resulted in significantly increased mean MN frequencies in bone marrow erythrocytes cf. the respective control group ($p<0.01$) (Cebral et al., 2011). A 2.4-fold increase relative to control was apparent for males with a mean plasma alcohol concentration of 28.1 mg/dL (mean MN frequency =13.91 ± SD 1.9; n=6). In contrast to this, Ellahueñe et al (2012) did not find any increase in MN frequency in CF1 males fed 5-15% v/v ethanol for 32 weeks compared to 5 week-old controls. A significant, weak inverse relationship was observed when treated animals were compared with 38-week old controls ($p<0.05$). The authors suggested this was due to a protective effect of ethanol against age related MN induction.

30. As part of an in vivo and in vitro investigation to delineate mechanisms of genotoxicity induced by acetaldehyde, Kotova et al (2013) subchronically exposed 8 week-old Wistar rats (n=3) to 10% ethanol and observed a statistically significant, 3.5-fold increase in the frequency of micronucleated polychromatic erythrocytes (MPCEs) cf. untreated controls (n=4) $P<0.05$. Together with their in vitro data (discussed below) the authors concluded that alcohol consumption in rats is directly genotoxic.

(ii) In vitro studies

31. Four studies examined the effect of in vitro exposure of human cells to ethanol and all reported positive results. Majer et al (2004) used two hepatoma cell lines (HepG2 and less characterised Hep3B cells). Ethanol (≥

17mM) caused significant increases in MN frequency only in HepG2 cells ($p<0.05$), although this was not more than 2-fold above background.

32. Teo & Fenech (2008) examined the interactive effects of folic acid (FA) and ethanol on genome stability in a human B-lymphoblastoid cell line (WIL2-NS). Although the ability of these cells to metabolise ethanol is unknown, the authors observed small increments in the frequency of binucleated cells containing MN at ethanol concentrations achievable via binge drinking i.e. 0.09-0.36%, $p<0.0001$. The significant induction at day 15 of MN, nucleoplasmic bridges (NPB) and nuclear buds (NBud) with increasing [ethanol] and decreasing [FA] (2000-20nM) (p values ≤ 0.002), led the authors to conclude that FA deficiency in the physiological range does impact on genome stability, a finding that supports consideration of nutritional status/ FA deficiency in alcohol mutagenicity studies.

33. This same cell line was exposed to slightly higher concentrations of ethanol (still within physiologically relevant levels) for six weeks, which resulted in more than 2-fold increase in MN and NPBs relative to control cells ($P<0.0001$ n=2) (Benassi-Evans & Fenech., 2011). Chromogenic in-situ hybridisation indicated that ethanol treatment for 6 weeks induced aneuploidy (measured in chromosome 17)[7] as shown by the significant increase in the number of cells displaying polysomic signals ($p<0.001$, n=2). The frequencies of MN, NPB and NBuds were signicantly increased in the BRCA1 mutant lymphoblast cell line GM1705 exposed to ethanol for 6 weeks.

34. Kayani & Parry (2010) performed a cytokinesis-blocked MN assay together with kinetochore staining in a lymphoblastoid cell line (MCL-5) to determine and differentiate between the mechanism of ethanol and acetaldehyde genotoxicity. Statistically significant, dose-dependent increases in the frequency of MN (up to 5-fold) were observed in cells exposed for 22h to 0.1 to 2% v/v ethanol (or lower concentrations of acetaldehyde) cf. controls ($p<0.05$). Kinetochore analysis revealed significant dose-dependent increases in kinetochore-positive (K+) MN in cells exposed to ethanol. In contrast, the majority of MN in acetaldehyde-exposed cells were K-. K+ staining indicates that MN contain mostly whole chromosomes. This therefore suggested that ethanol acted via an aneugenic mechanism, whereas acetaldehyde acted predominantly by a clastogenic mechanism. Given that the two compounds caused genotoxicity by different mechanisms, the authors concluded that these findings confirmed the ability of ethanol per se to produce DNA damage, at least in vitro.

c. Chromosome aberrations

(i) In vivo studies

35. A single study examined the effect of ethanol on in vivo levels of chromosome damage and did not find any significant increase in CAs in the bone marrow cells of rats treated with 20% v/v ethanol (n=10) for 30 days compared to water-fed controls (n=10) (Tavares et al., 2001).

[7] Aneuploidy in chromosome 17 is commonly detected in breast cancer

36. No in vitro studies were identified that investigated the production of chromosome aberrations in ethanol-treated cells.

d. Comet assay

(i) In vivo studies

37. A single study examined the effect of ethanol on in vivo levels of DNA strand breaks (Fedeli et al., 2003). Sub-chronic oral exposure to 10% ethanol induced DNA damage in the lymphocytes of nine alcohol-preferring Marchigan-Sardinian rats as evidenced by a 3-fold increase in mean Comet tail length cf. water fed controls ($P<0.01$). In view of negative findings obtained in cells taken from the liver, the authors suggested that DNA strand breaks occurred in lymphocytes before any such (eventual) damage occurs in hepatocytes in this strain.

(ii) In vitro studies

38. Two studies explored the induction of DNA strand breaks in primary Sprague Dawley rat neurones/astrocytes following acute and chronic exposure to 20 mM ethanol and both reported positive results albeit with limitations. Lamarche et al (2003) observed significant dose-dependent increases in percentage of DNA in Comet tail of cells exposed to 20, 50 and 100mM for 6h respectively cf. to control ($p<0.0001$). Longer exposure, from 3 to 9 days, at 20mM ethanol proved too toxic for meaningful observations. The same group found that acute exposure to ethanol had no effect on cells exposed to 100mM for 3h (Signorini-Allibe 2005). However, exposure to 20mM for 3 to 6 days resulted in a time-dependent incease in the percentage of DNA in Comet tails (up to 2.5-fold) compared to control cells ($n=3$, $P<0.001$ Fisher PLSD) although this was associated with a dose-dependent decrease in cell viability (~40% after 6 days).

39. Blasiak et al (2000) investigated DNA damage induced by ethanol and acetaldehyde in PBLs obtained from healthy non-smoking donors () or in biopsies (gastric mucosa –GM; colonic mucosa – CM). NB. Acetaldehyde results are reported separately in the acetaldehyde section below. Cells were exposed for 1h to concentrations of ethanol that mimic the level these cells would potentially be exposed to in vivo. GM cells exposed to 1M ethanol yielded the highest fold increase in mean comet tail moment (4-fold). This concentration is high but the authors considered it is reachable in the stomach of alcoholics. Cell sensitivity analysis showed that the CM had the highest sensitivity to ethanol (at 10mM), Comet tail length increasing by 50% compared to controls ($n=100$, $p<0.01$). The authors concluded that these results suggest that ethanol itself is genotoxic.

e. Germ cell DNA damage

(i) In vivo studies

40. Four studies examined the effects of in vivo ethanol exposure on either germ cell DNA integrity, for which the findings were positive (Cebral et al., 2011; Talebi et al., 2011; Rahimipour et al., 2013) or germ cell DNA mutation, for which the findings were negative (Ellahueñe et al., 2012). The Talebi and Rahimipour investigations used either cytochemical methods or sperm chromatin dispersion test (SCD) to evaluate the nuclear effects of up to 10% ethanol in drinking water on sperm chromatin integrity or DNA fragmentation respectively. Both studies observed significant, 4.5-fold or greater, increased changes in the percentage of positively stained cells in treated animals cf. controls ($p \leq 0.03$) suggesting that alcohol consumption increases sperm chromatin and DNA damage in experimental animals. Morphological abnormalities were also assessed in sperm (Cebral et al., 2011; Talebi et al 2011; Rahimipour et al., 2013) and oocytes (Cebral et al., 2011). Alcohol had significant detrimental effects on these germ cell parameters ($p \leq 0.034$).

41. The above findings were not confirmed in an assessment of germ cell mutagenicity conducted in CF1 mice exposed to 5-30% ethanol for 20 weeks (Ellahueñe et al., 2012). Negative findings for dominant lethal mutation test (and MN assay as described earlier) led the authors to suggest that chronic treatment with ethanol does not induce genotoxic damage in germinal mouse cells evaluated by the DLM assay (or in somatic mouse cells evaluated by the MN assay – see above).

42. No in vitro studies were identified that investigated the germ cell DNA damage/mutations in ethanol-treated cells.

3. Acetaldehyde

43. Fifteen studies were identified that investigated genetic and related effects of exposure to acetaldehyde in 14 in vitro (human, mammalian and bacterial) and one in vivo animal system(s). (See Tables 2.2 and 3.2 for further details on in vivo and in vitro studies respectively)

a. DNA adducts

44. No in vivo studies were identified that investigated DNA adduct formation in acetaldehyde-treated animals.

(i) In vitro studies

45. Wang et al (2000) provided the first structural characterisation of a DNA cross-link resulting from the reaction of acetaldehyde with calf-thymus DNA. Nuclear material received acute exposure to a range of acetaldehyde concentrations that resulted in the identification of three previously uncharacterised adducts detectable only after 96h of exposure to 40mM acetaldehyde: these were 1,N2-propano-dG (PdG), N2-dimethyldioxane-dG (N2-Dio-dG), and an interstrand cross-link.

46. These adducts have since been detected in studies using much lower concentrations of acetaldehyde and two studies sought to further understand

the key reactions underpinning their formation. In a short communication paper, Sako et al (2003) observed that the reaction of acetaldehyde with dG or calf thymus DNA resulted in the formation of PdG (also referred to as Me-α-OH-PdG or crotonaldehyde adducts (Cr-PdG)) which was accelerated in the presence of histones (i.e. basic amino acids arginine or lysine). However, the acetaldehyde concentrations used were very high, which prompted Theruvathu et al (2005) to expose pig liver DNA to concentrations of acetaldehyde that overlap the physiological range found in saliva (25 uM – 4 mM) in the presence of polyamines – highly basic molecules similar to histones implicated in a range of cellular and nuclear functions. The authors found that detectable amounts of PdG adducts were formed at concentrations as low as 100uM acetaldehyde. It has been suggested these adducts give rise to DNA-protein or interstrand crosslinks that have been directly linked to the formation of subsequent point mutations (Brooks & Zakhari., 2014).

47. Hori et al (2012) sought to analyse the stability of the N2-ethylidene-dG adduct in vitro to further understanding of its biochemical properties. Adduct levels in HL60 cells acutely exposed to a high concentration of 1.8mM acetaldehyde were approximately 4 to 7-fold higher than in controls when measured immediately after 1 or 2h exposure respectively (p values not reported). The authors also determined that the adduct has a half-life of approximately 35h in vitro. However several limitations were associated with this study, which reduce the significance of the findings.

b. Micronuclei

48. No in vivo studies were identified that investigated MN formation in acetaldehyde-treated animals.

(i) In vitro studies

49. Five studies assessed MN induction in human and animal cells exposed to acetaldehyde in vitro and all reported positive results. Speit et al (2008) performed a comparative study between various genotoxic assays with the dual aim of further characterising the Comet assay as a tool to detect crosslinks and to establish whether the induction of crosslinks is related to other mutagenic endpoints. The assessment of MN induction is reported here. In V79 Chinese hamster ovary cells exposed for 1h to 0.5 to 10mM acetaldehyde the mean frequency of MN cf. untreated controls was increased between 2.5 to 18-fold ($n=3$ $p<0.01$).

50. Kotova et al (2013) sought to study the mechanism of genotoxicity induced by acetaldehyde by assessing the in vitro (and in vivo – see ethanol section) induction of genome instability (i.e. MN and catastrophic mitosis) and in vitro induction of DNA strand breaks in Chinese hamster ovary (CHO) cells (XRCC3-deficient AA8 and irs1SF cell lines) and V79 Chinese hamster lung fibroblasts. Cells were exposed acutely to acetaldehyde (1.2 or 60 mM) for a period of 30 min to 24h depending on the endpoint being measured and subsequently assessed for MN induction. This paper is extremely comprehensive and complex, and other tests were performed using confocal microscopy, with measures of cell cycle progression, homologous

recombination and replication to determine key events involved in the genotoxic pathway of acetaldehyde. Only a high level summary of the genotoxic effects is provided here. Exposure of V79 cells to 1.2mM acetaldehyde for 24h resulted in an approximate 5-fold increase in the frequency of both MN and catastrophic mitosis (P ranged from <0.001 to < 0.05) n=3. The authors suggested that their findings showed that acetaldehyde-treated cells experienced lesions that interacted with the replication process. This damage occurred mostly in replicating cells and triggered a DNA damage response that was not immediate. The authors proposed that acetaldehyde induces MN via the formation of replication-associated DNA strand breaks (as a late response) which also results in catastrophic mitosis.

51. Majer et al (2004) used two human hepatoma cell lines (HepG2 and less characterised Hep3B). Unlike ethanol, acetaldehyde (≥ 0.9mM) caused significant increases in MN frequency in both cell lines ($p<0.05$ n=3), although this was not more than 2-fold above background. The authors suggested that the negative findings with ethanol, and positive response with acetaldehyde in the 3B cell line is indicative of a lack of alcohol dehydrogenase in these cells.

52. Kim et al (2005) examined MN induction by acetaldehyde in primary PBLs collected from healthy Korean graduate students genotyped for their ALDH2 status. After adjusting the data for potential confounders, the authors found significant ALDH2*2 allele-dependent increases in MN frequency in cells acutely exposed to 1.5mM acetaldehyde. Highest fold increase of 3.5 was observed in exposed cells from subjects carrying the ALDH2*2/*2 cf. control P=0.004. Alcohol-induced facial flushing (assessed via questionnaire) was also associated with an increased frequency of MN when lymphocytes from such subjects were treated with acetaldehyde.

53. Kayani & Parry (2010) performed a cytokinesis-blocked MN assay together with kinetochore staining in the human lymphoblastoid cell line (MCL-5) to determine and differentiate between the mechanism of ethanol and acetaldehyde genotoxicity. Results of cells acutely exposed to 0-0.250 (% v/v) acetaldehyde showed a statistically significant dose-dependent increase in MN induction cf. controls at much lower doses cf. ethanol $p<0.05$; (maximum increase ~ 4-fold). A dose-dependent increase in cells with kinetochore negative MN indicated MN harboured chromosomal fragments and further suggested that in contrast to ethanol, acetaldehyde acted via a clastogenic mechanism.

c. Comet assay

(i) In vivo studies

54. No in vivo studies were identified that investigated induction of DNA strand breaks in acetaldehyde-treated animals.

(ii) In vitro studies

55. In contrast to the results obtained for low dose acute ethanol exposure, rat primary astrocytes exposed for 3h to 0.25, 0.5 or 1 mM acetaldehyde yielded a dose-dependent increase in the frequency of single and double DNA strand breaks and alkali labile sites cf. control; n=3 P<0.001 Anova test; maximum increase 1.5-fold (Signorini-Allibe 2005).

56. Speit et al (2008) performed a comparative study of various genotoxic assays in V79 Chinese hamster ovary cells to further characterise the Comet assay for detecting crosslinks and determine whether their induction is associated with other mutagenic endpoints. In contrast to the MN assay, no positive associations were observed on exposure to acetaldehyde. The Comet assay was insensitive towards acetaldehyde-induced DNA damage (including cross-links evidenced by post-treatment of slides with Proteinase K producing no clear effect on DNA migration in either controls or exposed cells).

57. The findings for the various human tissue samples obtained from healthy non-smoking donors or from biopsies exposed to acetaldehyde for 1h suggest that it may form crosslinks with DNA (Blasiak et al., 2000). Significant dose-dependent decreases in mean comet tail moment were observed in lymphocytes, gastric and colonic mucosa (n=100 cells per treatment), the largest fold decrease (5-fold reduction) arising in GM exposed to 100mM acetaldehyde. This was comparable to the effect of the well-established cross-linking agent formaldehyde.

d. SCE

(i) In vivo studies

58. Positive findings were reported in a study of C3A mice treated with an intraperitoneal injection of a range of acetaldehyde doses (Torres-Bezauri et al., 2002). The two highest doses (40 and 400 mg/kg) yielded a mean SCE increase of 69% and 123% respectively cf. controls (2.10± SD 0.26 based on 30 metaphases in cells/mouse) p<0.01 n=5. The authors considered that their findings suggest that lower doses of acetaldehyde are insufficient to produce in vivo damage (explain may possibly be due to high efficiency of repair and detoxification systems in mammals).

(ii) In vitro studies

59. Speit et al (2008) exposed V79 Chinese hamster ovary cells to acetaldehyde 0.2 – 20 mM for 1 h, and assessed SCE. In contrast to results with the Comet assay, but similar to those with the MN assay, significant genotoxicity was observed, with a concentration-dependent increase in the mean frequency of SCEs; the highest dose yielding ~10-fold increase in the number of SCEs per metaphase, n=3 p<0.01.

e. Gene mutation/ Mutation spectra

60. No in vivo studies were identified that investigated gene mutation spectra of acetaldehyde-treated animals.

(i) In vitro studies

61. Two studies examined the mutagenicity of synthetically formed acetaldehyde-type adducts transfected into host cells using a single or double stranded DNA vector containing the adduct located within a reporter gene. Upton et al (2006) adopted this approach in their study using the reporter plasmid pLSX containing the N2-ethyl-dG adduct in the *supF* gene. Following transfection of *E-coli* cells the mean mutant fraction (calculated by dividing the no. of white colonies by no. of black colonies) showed that the adduct was only minimally mutagenic resulting in a non-significant, 2-fold increase in mutant fraction relative to lesion free controls (P=0.09). However, this increase became significant (p=0.04) when the thymidines (that normally flank the adducted positions) were replaced with dU on the complementary strand at 5' and 3' positions flanking the adduct. After harvesting and sequencing the plasmid DNA containing dU on the complementary strand, the authors reported the presence of mainly single base deletions downstream of the adduct (61%), and G to T transversions at the adduct site (20%).

62. Stein et al (2006) inserted both S and R forms of the Me-α-OH-PdG adduct into the double stranded pTBE shuttle vector (confers antibiotic resistance) and transfected them into human XPA cells. After counting number of antibiotic resistant cells, the blocking potency of PdG adduct was determined from the ratio of progeny from each strand. The authors noted that blocking the formation of cross-links (by incorporating the adducts into a mismatched region) was necessary to enable their genotoxicity to be assessed. This was found to be similar between each isomer. Miscoding frequencies for the S and R isomers were 10% and 5% respectively. The predominant mutagenic event observed was G to T transversion.

63. Noori & Hou (2001) removed pre-existing in vivo HPRT mutants from human peripheral T lymphocytes before exposing them to 2.4 mM acetaldehyde for 24h. This resulted in an approximate 3-fold induction in mutant frequency for 6TG resistant mutants compared to untreated cultures. Base substitutions were identified in 55 induced mutants and 26 control mutants. G to A transitions were the most predominant changes (40% cf 15.4%, respectively p=0.04).

DISCUSSION

64. The Committee last considered the mutagenicity of ethanol in a statement produced in December 2000 (see COM 2000[8]). The following conclusions reached are presented in italics and discussed in turn in relation to the current evidence which is summarised:

(1). Mutagenicity of alcoholic beverages:

65. *"The Committee recalled that in 1995, considerable weight had been attached to one study from the Medical Research Council's Cell Mutation Unit, who has examined hprt mutant*

[8] This was preceded by the discussion paper MUT/2000/04.

frequency in circulating T-lymphocytes of normal adults and the relationship with alcohol intake. The study showed that alcohol intake in 143 people over the range of 0-56 units/week (1 unit - 8g ethanol) had no effects on hprt mutant frequency. Less weight had been placed on studies which examined the mutagenicity of concentrated extracts of wines and spirits in bacteria, and the significance of such data was felt to be questionable. There were no adequate in-vivo mutagenicity studies of alcoholic beverages available in 1995 or for the current review.

Since 1995 two further studies of the relationship between hprt mutant frequency in lymphocytes obtained from individuals for whom information on drinking patterns were available. There was no association between hprt mutant frequency and alcohol ingestion in these studies, thus confirming the results of the earlier MRC investigation."

66. No new studies were identified on the genotoxicity testing of alcoholic beverages i.e. following in vitro exposure of human, mammalian or bacterial cells to alcoholic beverages or in vivo exposure of experimental animals). Since COM last reviewed this topic, the range of genotoxic endpoints assessed in humans consuming alcoholic beverages has expanded from the HPRT mutant frequency in PBLs. Studies have included assessment of induction of MN, chromosome aberrations, DNA strand breaks and sister chromatid exchanges in a range of tissues and also evaluated the influence of polymorphisms in key alcohol metabolising enzymes.

67. Peripheral blood lymphocytes (PBLs) were the most widely used tissue and most studies assessing the frequency of MN reported significant increases, with approximately half the studies reporting a 2-fold or greater increase in the frequency of MN in alcoholics relative to non-drinkers. Maffei et al (2000) used the FISH technique to investigate the mechanism underlying MN formation and their findings support an aneugenic mechanism in alcoholics drinking more than 120g alcohol per day. In a later study the same authors showed that abstinence from alcohol (ranging from 12 to 60 months) normalises MN frequency (Maffei et al 2002). Studies on the influence of the ALDH2 genotype on MN induction consistently showed significantly higher levels in habitual drinkers carrying the deficient allele i.e. (ALDH2*1/*2 or ALDH2*2/*2). Ishiwaki et al (2006) examined the influence of CYP2E1 polymorphism on MN levels and reported a positive association particularly for individuals carrying the CYP2E1 WT allele (i.e. CYP2E1*1) which confers slower enzyme activity. The two studies reporting on ADH1B genotype-dependent changes provided mixed results. Ishiwaki et al (2007) observed the highest MN levels in drinkers carrying at least one ADH1B*1 allele (confers slow activity), while Wu
et al (2010) found the haplotype including the ADH1B*2 allele was associated with a significant effect of drinking on MN-RET frequency. However, Wu et al (2010) based alcohol exposure on drinking frequency without any data on alcohol intake. It has been suggested that less active forms of both ADH1B and ALDH2 would expose individuals to increased levels of ethanol and acetaldehyde, which would linger in the body for longer. Furthermore, it has been postulated that slow ADH activity could increase alcohol metabolism through non-ADH pathways

68. Studies examining MN induction in buccal cells obtained from otherwise healthy alcoholics (Reis et al 2002) or those with oral/ oro-pharyngeal carcinomas (Ramirez & Saldanha 2002) reported 4 to 5-fold increased frequencies cf. abstaining individuals. However, the latter study was

limited by an apparent lack of matching of subjects for their smoking status. The amount of data retrieved from Reis et al was limited due to the study being published in Portuguese.

69. Mixed results were reported for studies investigating the formation of DNA strand breaks in alcoholic drinkers. Pool-Zobel et al (2004) observed an inverse association between percentage fluorescence in tails of comet images in PBLs and rectal cells of alcoholics drinking more than 100g ethanol per day compared to social drinkers. Significantly reduced rates of DNA strand breaks in PBLs of ALDH2-deficient drinkers cf. controls were also reported by Lu & Morimoto (2009). However, Weng et al (2010) observed a positive association between mean tail moment in mononuclear cells and drinking, and reported that highest levels were found in individuals older than 47 years of age and ADH1B*2/*2 or ALDH2*1/*2 genotype.

70. Burim et al (2004) and Maffei et al (2002) reported significant, greater than 2-fold, increases in the frequency of chromosome aberrations in PBLs of alcoholics consuming more than 60g ethanol per day compared to non-drinkers. However, the effects of abstinence differed between the studies: either having no effect when abstinent for 3 to 48 months or reducing levels to background when abstinent for 12 to 60 months, respectively.

71. Karaoguz et al (2005) reported a significant increase in the mean frequency of SCE with high alcohol consumption.

72. Studies reporting on the formation and detection of acetaldehyde-specific DNA adducts are discussed in the acetaldehyde section.

73. Few studies in human subjects provided data on the nutritional status of alcoholics.

(2). Mutagenicity of ethanol:

74. *"The Committee noted that there were no new in-vitro mutagenicity studies with ethanol. No conclusions could be drawn regarding the in-vitro investigations of effects of ethanol in the pre-implantation development of mouse oocytes injected with spermatozoa stored in 70% ethanol.*
The Committee reaffirmed its previous conclusions with regard to the mutagenicity data on ethanol, namely: negative results have been obtained in a wide range of in-vitro tests and in in-vivo tests including those for effects on germ cells; it was concluded that there was no evidence that ethanol induces germ cell mutation in-vivo."

i. In vitro

75. Seven new in vitro studies were identified that investigated the mutagenicity of ethanol in mainly human cells/ cell lines and reported mostly positive results. Chromosome damage evidenced by increased frequencies of MN, nucleoplasmic bridges (NPB) and nuclear buds (NBud) was observed in cells exposed to ethanol concentrations within the physiological range achievable during binge drinking. These studies also provided additional data demonstrating the ability of folic acid deficiency to elevate MN levels (Teo & Fenech., 2008) and the ability of ethanol to act independently of acetaldehyde

via an aneugenic mechanism (Benassi-Evans & Fenech.,2011); a finding also supported from the results of kinetochore analysis performed by Kayani & Parry (2010). Blasiak et al (2000) provided some of the early in vitro evidence to suggest that ethanol itself was genotoxic in primary human cells obtained from biopsies or blood sample exposed to ethanol concentrations that mimic in vivo conditions.

76. Two related studies using primary rodent cells reported significant increased percentage of DNA in Comet tails of rat neurones exposed either acutely (Lamarche et al 2003) or chronically (Signorini-Allibe et al 2005) to concentrations of ethanol within the range of levels achieved in the blood of intoxicated humans compared to controls, although the latter study was associated with a 40% reduction in cell viability by Day 6.

ii. In vivo

77. Ten new in vivo studies were identified, in which animals were exposed to ethanol in drinking water at concentrations ranging from 5% to 30% v/v. An increased frequency of MN or micronucleated polychromatic erythrocyte (MPCE) in RBCs was observed in two studies in which CF1 mice or Wister rats were exposed subchronically to 10% ethanol in their drinking water (Cebral et al 2011; Kotova et al 2013 respectively). The latter study claimed to provide in vivo evidence of the direct genotoxicity of alcohol in rats. This was not supported in a study that sought to mimic chronic alcoholism in CF1 mice, by exposing them for 32 weeks to 5 or 15% ethanol in the drinking water, which reported potentially protective effects (Ellahueñe et al., 2012).

78. One study evaluated chromosome aberrations in rats sub-chronically exposed to 20% ethanol but found no difference in CA frequency (Tavares et al 2001). It is possible that the concentration used contributed to the reduced body weight gain apparent in treated animals.

79. One study examined the formation of DNA strand breaks in rats genetically selected to prefer alcohol sub-chronically exposed to 10% ethanol (Fedeli et al 2003). An increased frequency of DNA strand breaks in lymphocytes but not hepatocytes, support the value of the former measure as a biomarker.

80. Studies reporting on the formation of acetaldehyde-specific DNA adducts are discussed in the acetaldehyde section. One study reported increased levels of DNA adducts in organs of ALDH2-knockout C57BL mice acutely exposed to radiolabelled ethanol cf. WT (Ogawa et al 2007).

81. Mixed results were obtained for studies reporting on germ cell DNA damage/mutation with negative results obtained in the dominant lethal mutation assay from the above study of chronically exposed CF1 mice (Ellahueñe et al., 2012), and positive findings reported in two studies assessing sperm chromatin condensation and DNA integrity/fragmentation in chronically exposed Wistar rats (5% ethanol) (Talebi et al 2011) and Balb/c mice (up to 10% ethanol) (Rahimipour 2013). The significance of the findings of the latter study were limited by the use of saccharin, which itself displayed

detrimental germinal effects. Morphological alterations were also observed in sperm, as well as in oocytes, in these studies (Cebral et al 2011).

82. No in vitro or in vivo animal studies were identified that investigated the formation of SCEs, gene mutations or mutation spectra in ethanol treated animals or cells.

(3). Mutagenicity of acetaldehyde.

83. *"The committee agreed that the most recent experiments using human lymphoma cells had confirmed earlier studies that acetaldehyde induces protein-DNA cross links, but only at concentrations which resulted in cell death. In addition acetaldehyde induced HPRT mutations in human T cells. Members agreed that no conclusions could be drawn from the finding of acetaldehyde DNA adducts in peripheral white blood cells of alcoholics in view of lack of control for the effects of smoking by alcoholics in the study group and the well known abnormalities in metabolism in alcoholics.*

The Committee reaffirmed its previous conclusions with regard to acetaldehyde. The available data show that acetaldehyde induced chromosome aberrations in mammalian cells in the absence of an exogenous metabolising fraction. There is some evidence to show covalent binding (DNA-protein cross links) in the nasal mucosa of rats exposed to high levels of acetaldehyde by inhalation.

The mutagenic profile of acetaldehyde is very similar to that of formaldehyde. The compound has direct acting mutagenic potential in-vitro, but would only be expected to have the potential of in vivo activity at sites where it is not rapidly metabolised to acetic acid. The COC has concluded that the observation of tumours in animals exposed to high inhalation doses of acetaldehyde is not relevant to drinking alcohol."

i. In vitro

84. Two new studies provide data on DNA cross-links. Wang et al (2000) detected an interstrand cross-link and two acetaldehyde-specific adducts after exposing calf thymus DNA to 0.01-40mM acetaldehyde, however, in agreement with the previous COM conclusion these were only detectable at the highest concentrations (40 mM). In contrast, Speit et al (2008) found no positive association with level of DNA strand breaks or DNA crosslinks (protein or DNA) in V79 cells exposed to 0.2 – 20 mM acetaldehyde – the latter concentration being cytotoxic to cells. The findings for the various human tissue samples obtained from healthy non-smoking donors or from biopsies exposed to acetaldehyde for 1h suggest that it may form crosslinks with DNA (Blasiak et al., 2000). The reduction in mean Comet tail moment of lymphocytes exposed to 3 mM acetaldehyde was comparable to the effect of the well-established cross-linking agent formaldehyde.

85. Since the last COM statement the number of mutagenic endpoints showing positive findings has expanded. In addition to induction of chromosome aberrations in mammalian cells, the new studies report that acetaldehyde also induces MN in both human (Majer et al 2004; Kim et al 2005; Kayani & Parry 2010) and mammalian cells (Speit et al 2008; Kotova et al 2013) at concentrations within the range formed in saliva of human volunteers who drank alcohol in a lab setting i.e. up to 4mM. Only a few of these studies were without their limitations e.g. lack of data on cell viability, not accounting for the high volatility of acetaldehyde, etc. Sister chromatid exchange was another mutagenic endpoint reported (Speit et al 2008).

86. Two notable studies proposed potential mechanisms behind acetaldehyde's ability to cause chromosome damage: Kayani & Parry (2010) suggested that acetaldehdye acts via a clastogenic mechanism, distinct from ethanol's apparent ability to cause aneugenicity; while Kotova et al (2013) suggested that acetaldehyde induces lesions such as DNA strand breaks (as a late response) that interact with the replication process resulting in the formation of MN and catastrophic mitosis. Further support for the role of ALDH2*-deficient alleles increasing susceptibility to genetic toxicity was provided by Kim et al (2005).

87. Although the N2-ethylidene-dG adduct is unstable in nucleoside form, Hori et al (2012) provide preliminary data on its stability in vitro with the finding that it has an in vitro half-life of approximately 35h. Findings from Sako et al (2003) and Theruvathu et al (2005) provide additional upstream mechanistic clues regarding the formation of Me-α-OH-PdG adducts (also referred to as crotonaldehyde adducts (Cr-PdG) or simply PdG), which were found to be accelerated in the presence of basic amino compounds such as histones or polyamines. PdG adduct is believed to be a precursor lesion for DNA-protein or interstrand crosslinks, which themselves have been directly linked to the formation of subsequent point mutations (Brooks & Zakhari 2014). Three studies provide further information regarding downstream mechanistic events. DNA of human T cells exposed to 2.4mM acetaldehyde contained base substitutions, with guanine bases as the predominant target (Noori & Hou 2001), and G to T transversions as the predominant mutagenic event irrespective of the initial DNA adduct lesion being N2-ethy-dG (Upton et al 2006) or Me-α-OH-PdG (Stein et al 2006).

ii. In vivo

88. Five out of six studies provide positive evidence of higher levels of acetaldehyde-specific DNA adducts measured in peripheral white blood cells and oral epithelial cells of alcohol exposed individuals. The main adduct detected and/or measured was the reduced form of N2-ethylidene-dG (N2-ethyl-dG). In contrast to the previous COM conclusion, the effects of smoking were controlled for either through matching, statistical adjustment, or use of non-smoking subjects. Few studies provided data on the nutritional status of alcoholics.

89. Significantly higher levels of N2-ethyl-dG adducts were apparent in blood cells and oral epithelial cells of healthy volunteers exposed for up to 120h to alcohol in a controlled experimental setting compared to controls (Balbo et al 2012ab respectively). However, Singh et al (2012) found no statistical difference in adduct levels in volunteers exposed to alcohol encountered under normal social drinking conditions for up to 48h cf. controls. These studies were limited by the substantial variation in baseline adduct levels.

90. Matsuda et al (2006) confirmed that alcoholics carrying the ALDH2-deficient genotype had significantly higher levels of both N2-ethyl-dG and Me-γ-OH-PdG adducts. The results of this study are consistent with the findings of another investigation performed by the same group i.e. Yukawa et al (2012)

who extend this with the finding that adduct levels increase in subjects who also carry the ADH1B*2 (faster) genotype. However, use of an acute exposure assessment and the lower body weight of subjects in one of the groups containing ADH1B*2 variants, does limit the strength of these findings. Higher levels of the N2-ethyl-dG adduct in subjects harbouring the ALDH2-deficient allele was supported by rodent in vivo studies that detected higher levels of the adduct in both the liver and stomach of ALDH2 knockout rats sub-chronically exposed to 20% ethanol (Matsuda et al., 2007 and Nagayoshi et al 2009 respectively).

91. Positive results for induction of SCEs were also reported in mouse bone marrow cells exposed intraperitoneally but only at high levels of acetaldehyde (Torres-Bezauri et al 2002)

92. No in vitro or in vivo animal studies were identified that investigated the formation of chromosome aberrations or germ cell DNA damage/mutagenicity in acetaldehyde treated animals or cells.

Ethyl sulphate

93. A brief communication by Mitchell et al (2014) considered Phase II conjugation products of ethanol metabolism and highlighted how sulphonation, which is generally a detoxification reaction, can bioactivate xenobiotics into toxic forms particularly in chronic situations. The authors noted that ethyl sulphate, was a recently confirmed human metabolite of ethanol, and suggested that even with a small amount of alcohol passing through this pathway, chronic alcoholism would result in a steady stream of ethyl sulphate molecules being continually available.

94. Although its stability is controversial, it has been associated with inducing toxicity in various organ systems. However, more importantly, its physicochemical properties (i.e. energy rich and kinetic resistance to hydrolysis) suggest that it could potentially alkylate various biological macromolecules particularly in the gut where it is produced and hydrolysed in greatest concentration.

95. The authors argue that given the uncertainty over the importance of current proposed mechanisms of cancer induction by alcohol, it would be unwise to exclude ethyl sulphate as a potential mechanism. The authors concede that although biological evidence for its chemical reactivity and induction of adduct formation in vivo is currently lacking, its physicochemical properties and relatively high exposure over long periods of time (as would occur in alcohol abuse) suggest further work should be done to delineate its metabolic fate and whether such reactions do occur in biological systems.

QUESTIONS FOR THE COMMITTEE

1. What are the views of Members on the genotoxicity of alcoholic beverages, ethanol and acetaldehyde on the basis of the available evidence?

2. What are members' views on the genotoxic mechanisms of action proposed by Kotova et al (2013) and Kayani & Parry (2010) for ethanol and acetaldehyde?

3. What are members' views on the relative contribution of the different compounds to any genotoxicity of alcohol?

4. Do members consider low levels of ethyl sulphate (if shown to be a potent mutagen) to be of greater mechanistic importance than high levels of a weak mutagen such as acetaldehyde?

5. Is the evidence presented in this review convincing enough to warrant changes to the previous COM conclusions?

Imperial PHE Toxicology Unit

September 2014

ANNEXES

Annex 1 Literature search strategy

The following strategy was used to ensure maximal acquisition of relevant studies.

		Notes
Timeframe	January 2000 to May 2014	Previous COM papers reviewed studies published until February 2000 (MUT/2000/04)
Search engine	Pubmed	Basic search was required
Search terms	(alcohol OR alcoholic*) AND (ethanol OR acetaldehyde) AND (mutagen* OR genotoxic* OR DNA OR chromosom*) AND (consumption OR consume)	Other permutations yielded either too many or too few results.
Inclusion criteria	Exposure to alcohol, ethanol or acetaldehyde	Or synthetically formed acetaldehyde adducts
	Provides data on classic mutagenic endpoints e.g. point mutations, chromosome aberrations, micronuclei, DNA strand breaks, sister chromatid exchanges, dominant lethal mutations, etc	Other endpoints considered include DNA adduction, germ cell DNA damage, polymorphisms of enzymes involved in alcohol metabolism
Exclusion criteria	Data on oxidative DNA mechanisms; synergisms, epigenetics	Although relevant, it was decided that these topics would not be considered in the current paper but reviewed in upcoming future papers
	Provides access to abstract only	Full papers must be accessible
	Published in a foreign language	Translatable papers accepted
Citational searches	Papers were also retrieved using PubMed's Related Citations search (which allows users to find papers related to a particular relevant paper – either because an article cited the study, or they share similar topic areas/MeSH Terms)	Citational searches were conducted on the following references: Obe & Anderson (1987); Kayani & Parry (2010); Kotova et al (2013).
Technical reports	IARC	Genotoxicity chapters of the IARC 2010 and 2012 monographs
Cross-referencing	Further relevant studies were identified by cross-referencing original studies and relevant reviews.	This included screening studies cited in the IARC reviews, the review by Phillips & Jenkinson 2001 and recommendations from experts.

After screening papers for relevance (with duplicates omitted) a total of 52 papers were selected for inclusion in this review paper (46 provided original study data; 6 were identified as relevant reviews/communications).

Annex 2 Tables of study data

1. Tables 1.1 to 1.10 of human in vivo DNA adduct and Mutagenicity data
2. Tables 2.1 to 2.2 of animal in vivo DNA adduct and Mutagenicity data
3. Tables 3.1 to 3.2 of in vitro DNA adduct and Mutagenicity data
4. Summary tables 4.1 and 4.2 of all DNA adduct and Mutagenicity data

Annex 3. Appended items

1. Previous COM papers and statement on alcohol mutagenicity

a. MUT/2000/04. Update on the mutagenicity of ethanol. DH Tox Unit discussion paper presented to the COM on 25/05/2000

b. COM Statement (2000). COM Statement on alcohol beverages (Nov 2000) COM00S5. http://www.iacom.org.uk/statements/COM00S5.htm

2. Key Papers

a. Kayani & Parry (2010). The in vitro genotoxicity of ethanol and acetaldehyde. Toxicol In Vitro. 2010 Feb;24(1):56-60.

b. Kotova et al (2013). Genotoxicity of alcohol is linked to DNA replication-associated damage and homologous recombination repair. Carcinogenesis. 2013 Feb;34(2):325-30

c. Mitchell et al (2014). Ethyl sulphate, a chemically reactive human metabolite of ethanol? Xenobiotica. 2014 Jul 18:1-4.

REFERENCES

1. Balbo S, Hashibe M, Gundy S, Brennan P, Canova C, Simonato L, Merletti F, Richiardi L, Agudo A, Castellsagué X, Znaor A, Talamini R, Bencko V, Holcátová I, Wang M, Hecht SS, Boffetta P. N2-ethyldeoxyguanosine as a potential biomarker for assessing effects of alcohol consumption on DNA. Cancer Epidemiol Biomarkers Prev. 2008 Nov;17(11):3026-32.

2. Balbo S, Meng L, Bliss RL, Jensen JA, Hatsukami DK, Hecht SS. Time course of DNA adduct formation in peripheral blood granulocytes and lymphocytes after drinking alcohol. Mutagenesis. 2012a Jul;27(4):485-90.

3. Balbo S, Meng L, Bliss RL, Jensen JA, Hatsukami DK, Hecht SS. Kinetics of DNA adduct formation in the oral cavity after drinking alcohol. Cancer Epidemiol Biomarkers Prev. 2012b Apr;21(4):601-8.

4. Benassi-Evans B, Fenech M. Chronic alcohol exposure induces genome damage measured using the cytokinesis-block micronucleus cytome assay and aneuploidy in human B lymphoblastoid cell lines. Mutagenesis. 2011 May;26(3):421-9.

5. Blasiak J, Trzeciak A, Malecka-Panas E, Drzewoski J, Wojewódzka M. In vitro genotoxicity of ethanol and acetaldehyde in human lymphocytes and the gastrointestinal tract mucosa cells. Toxicol In Vitro. 2000 Aug;14(4):287-95.

6. Brooks PJ, Zakhari S. Acetaldehyde and the genome: beyond nuclear DNA adducts and carcinogenesis. Environ Mol Mutagen. 2014 Mar;55(2):77-91. doi: 10.1002/em.21824. Epub 2013 Nov 27. Review. PubMed PMID: 24282063.

7. Burim RV, Canalle R, Takahashi CS, Tavares DC, Martinelli Ad Ade L, Sakamoto-Hojo ET. Clastogenic effect of ethanol in chronic and abstinent alcoholics. Mutat Res. 2004 Jun 13;560(2):187-98.

8. Cebral E, Abrevaya XC, Mudry MD. Male and female reproductive toxicity induced by sub-chronic ethanol exposure in CF-1 mice. Cell Biol Toxicol. 2011 Aug;27(4):237-48.

9. COC Statement (2000). COC statement on breast cancer and alcohol (April 2000) http://www.iacoc.org.uk/statements/alcbrst.pdf

10. COM (1995). COM Statement on alcoholic beverages (1995).

11. COM Statement (2000). COM Statement on alcohol beverages (Nov 2000) COM00S5. http://www.iacom.org.uk/statements/COM00S5.htm

12. Department of Health (1995). Sensible Drinking. The report of an Interdepartmental Working Group.

13. Ellahueñe MF, Pérez-Alzola LP, Olmedo MI. Chronic ethanol consumption in mice does not induce DNA damage in somatic or germ cells, evaluated by the bone marrowmicronucleous assay and the dominant lethal mutation assay. Biol Res. 2012;45(1):27-31.

14. Fedeli D, Fedeli A, Luciani F, Massi M, Falcioni G, Polidori C. Lymphocyte DNA alteration by sub-chronic ethanol intake in alcohol-preferring rats. Clin Chim Acta. 2003 Nov;337(1-2):43-8.

15. Hori K, Miyamoto S, Yukawa Y, Muto M, Chiba T, Matsuda T. Stability of acetaldehyde-derived DNA adduct in vitro. Biochem Biophys Res Commun. 2012 Jul 13;423(4):642-6.

16. IARC (2010). IARC Monograph Vol 96. Section 4.7/4.8; Page 1183-1206: http://monographs.iarc.fr/ENG/Monographs/vol96/mono96-6D.pdf

17. IARC (2012). IARC Monograph Vol 100e. Section 4.2/4.3; Page 464-472: http://monographs.iarc.fr/ENG/Monographs/vol100E/mono100E-11.pdf

18. Ishikawa H, Yamamoto H, Tian Y, Kawano M, Yamauchi T, Yokoyama K. Effects of ALDH2 gene polymorphisms and alcohol-drinking behavior on micronuclei frequency in non-smokers. Mutat Res. 2003 Nov 10;541(1-2):71-80.

19. Ishikawa H, Miyatsu Y, Kurihara K, Yokoyama K. Gene-environmental interactions between alcohol-drinking behavior and ALDH2 and CYP2E1 polymorphisms and their impact on micronuclei frequency in human lymphocytes. Mutat Res. 2006 Feb 22;594(1-2):1-9.

20. Ishikawa H, Ishikawa T, Yamamoto H, Fukao A, Yokoyama K. Genotoxic effects of alcohol in human peripheral lymphocytes modulated by ADH1B and ALDH2 gene polymorphisms. Mutat Res. 2007 Feb 3;615(1-2):134-42.

21. Karaoğuz MY, Coşar B, Arikan Z, Başaran F, Menevşe A, Menevşe S. Increased frequency of sister chromatid exchanges in peripheral lymphocytes of alcoholics and cigarette smokers. Cell Biol Int. 2005 Feb;29(2):165-8.

22. Kayani MA, Parry JM. The in vitro genotoxicity of ethanol and acetaldehyde. Toxicol In Vitro. 2010 Feb;24(1):56-60.

23. Kim JS, Kim YJ, Kim TY, Song JY, Cho YH, Park YC, Chung HW. Association of ALDH2 polymorphism with sensitivity to acetaldehyde-induced micronuclei and facial flushing after alcohol intake. Toxicology. 2005 Jun 1;210(2-3):169-74.

24. Kotova N, Vare D, Schultz N, Gradecka Meesters D, Stepnik M, Grawé J, Helleday T, Jenssen D. Genotoxicity of alcohol is linked to DNA replication-associated damage and homologous recombination repair. Carcinogenesis. 2013 Feb;34(2):325-30.

25. Lamarche F, Gonthier B, Signorini N, Eysseric H, Barret L. Acute exposure of cultured neurones to ethanol results in reversible DNA single-strand breaks; whereas chronic exposure causes loss of cell viability. Alcohol Alcohol. 2003 Nov-Dec;38(6):550-8.

26. Lu Y, Morimoto K. Is habitual alcohol drinking associated with reduced electrophoretic DNA migration in peripheral blood leukocytes from ALDH2-deficient male Japanese? Mutagenesis. 2009 Jul;24(4):303-8.

27. Maffei F, Fimognari C, Castelli E, Stefanini GF, Forti GC, Hrelia P. Increased cytogenetic damage detected by FISH analysis on micronuclei in peripheral lymphocytes from alcoholics. Mutagenesis. 2000 Nov;15(6):517-23.

28. Maffei F, Forti GC, Castelli E, Stefanini GF, Mattioli S, Hrelia P. Biomarkers to assess the genetic damage induced by alcohol abuse in human lymphocytes. Mutat Res. 2002 Feb 15;514(1-2):49-58.

29. Majer BJ, Mersch-Sundermann V, Darroudi F, Laky B, de Wit K, Knasmüller S. Genotoxic effects of dietary and lifestyle related carcinogens in human derived hepatoma (HepG2, Hep3B) cells. Mutat Res. 2004 Jul 13;551(1-2):153-66.

30. Matsuda T, Yabushita H, Kanaly RA, Shibutani S, Yokoyama A. Increased DNA damage in ALDH2-deficient alcoholics. Chem Res Toxicol. 2006 Oct;19(10):1374-8.

31. Matsuda T, Matsumoto A, Uchida M, Kanaly RA, Misaki K, Shibutani S, Kawamoto T, Kitagawa K, Nakayama KI, Tomokuni K, Ichiba M. Increased formation of hepatic N2-ethylidene-2'-deoxyguanosine DNA adducts in aldehyde dehydrogenase 2-knockout mice treated with ethanol. Carcinogenesis. 2007 Nov;28(11):2363-6.

32. Mitchell SC, Waring RH, Wilson ID. Ethyl sulphate, a chemically reactive human metabolite of ethanol? Xenobiotica. 2014 Jul 18:1-4.

33. MUT/2000/04. Update on the mutagenicity of ethanol. DH Tox Unit discussion paper presented to the COM on 25/05/2000

34. Nagayoshi H, Matsumoto A, Nishi R, Kawamoto T, Ichiba M, Matsuda T. Increased formation of gastric N(2)-ethylidene-2'-deoxyguanosine DNA adducts in aldehyde dehydrogenase-2 knockout mice treated with ethanol. Mutat Res. 2009 Feb 19;673(1):74-7.

35. Noori P, Hou SM. Mutational spectrum induced by acetaldehyde in the HPRT gene of human T lymphocytes resembles that in the p53 gene of esophageal cancers. Carcinogenesis. 2001 Nov;22(11):1825-30.

36. Obe G & Anderson D (1987). International Commission for Protection against Environmental Mutagens and Carcinogens. ICPEMC Working Paper No. 15/1. Genetic effects of ethanol. Mutat Res, 186: 177–200.

37. Ogawa M, Oyama T, Isse T, Saito K, Tomigahara Y, Endo Y, Kawamoto T. A comparison of covalent binding of ethanol metabolites to DNA according to Aldh2 genotype. Toxicol Lett. 2007 Jan 30;168(2):148-54.

38. Phillips BJ, Jenkinson P. Is ethanol genotoxic? A review of the published data. Mutagenesis. 2001 Mar;16(2):91-101.

39. Pool-Zobel BL, Dornacher I, Lambertz R, Knoll M, Seitz HK. Genetic damage and repair in human rectal cells for biomonitoring: sex differences, effects of alcohol exposure, and susceptibilities in comparison to peripheral blood lymphocytes. Mutat Res. 2004 Jul 13;551(1-2):127-34.

40. Rahimipour M, Talebi AR, Anvari M, Sarcheshmeh AA, Omidi M. Effects of different doses of ethanol on sperm parameters, chromatin structure and apoptosis in adult mice. Eur J Obstet Gynecol Reprod Biol. 2013 Oct;170(2):423-8.

41. Ramirez A, Saldanha PH. Micronucleus investigation of alcoholic patients with oral carcinomas. Genet Mol Res. 2002 Sep 30;1(3):246-60

42. Reis SR, Sadigursky M, Andrade MG, Soares LP, Espirito Santo AR, Vilas Boas DS. [Genotoxic effect of ethanol on oral mucosa cells]. Pesqui Odontol Bras. 2002 Jul-Sep;16(3):221-5.

43. Sako M, Inagaki S, Esaka Y, Deyashiki Y. Histones accelerate the cyclic 1,N2-propanoguanine adduct-formation of DNA by the primary metabolite of alcohol and carcinogenic crotonaldehyde. Bioorg Med Chem Lett. 2003 Oct 20;13(20):3497-8.

44. Signorini-Allibe N, Gonthier B, Lamarche F, Eysseric H, Barret L. Chronic consumption of ethanol leads to substantial cell damage in cultured rat astrocytes in conditions promoting acetaldehyde accumulation. Alcohol Alcohol. 2005 May-Jun;40(3):163-71.

45. Singh R, Gromadzinska J, Mistry Y, Cordell R, Juren T, Segerbäck D, Farmer PB. Detection of acetaldehyde derived N(2)-ethyl-2'-deoxyguanosine in human leukocyte DNA following alcohol consumption. Mutat Res. 2012 Sep 1;737(1-2):8-11.

46. Speit G, Fröhler-Keller M, Schütz P, Neuss S. Low sensitivity of the comet assay to detect acetaldehyde-induced genotoxicity. Mutat Res. 2008 Dec 8;657(2):93-7.

47. Stein S, Lao Y, Yang IY, Hecht SS, Moriya M. Genotoxicity of acetaldehyde- and crotonaldehyde-induced 1,N2-propanodeoxyguanosine DNA adducts in human cells. Mutat Res. 2006 Sep 19;608(1):1-7.

48. Talebi AR, Sarcheshmeh AA, Khalili MA, Tabibnejad N. Effects of ethanol consumption on chromatin condensation and DNA integrity of epididymal spermatozoa in rat. Alcohol. 2011 Jun;45(4):403-9.

49. Tavares DC, Cecchi AO, Jordão AA Jr, Vannucchi H, Takahashi CS. Cytogenetic study of chronic ethanol consumption in rats. Teratog Carcinog Mutagen. 2001;21(5):361-8.

50. Teo T, Fenech M. The interactive effect of alcohol and folic acid on genome stability in human WIL2-NS cells measured using the cytokinesis-block micronucleus cytome assay. Mutat Res. 2008 Nov 17;657(1):32-8.

51. Theruvathu JA, Jaruga P, Nath RG, Dizdaroglu M, Brooks PJ. Polyamines stimulate the formation of mutagenic 1,N2-propanodeoxyguanosine adducts from acetaldehyde. Nucleic Acids Res. 2005 Jun 21;33(11):3513-20.

52. Torres-Bezauri R, Madrigal-Bujaidar E, Alvarez-González RI, Zepeda G, Chamorro G. Effects of chlorophyllin on acetaldehyde: lack of modulation of the rate of sister-chromatid exchanges in mouse bone marrow, and of complex formation in aqueous solution. Food Chem Toxicol. 2002 Oct;40(10):1507-13.

53. Upton DC, Wang X, Blans P, Perrino FW, Fishbein JC, Akman SA. Mutagenesis by exocyclic alkylamino purine adducts in Escherichia coli. Mutat Res. 2006 Jul 25;599(1-2):1-10.

54. Wang M, McIntee EJ, Cheng G, Shi Y, Villalta PW, Hecht SS. Identification of DNA adducts of acetaldehyde. Chem Res Toxicol. 2000 Nov;13(11):1149-57.

55. Weng H, Weng Z, Lu Y, Nakayama K, Morimoto K. Effects of alcohol-drinking behaviour and ADH1B and ALDH2 polymorphisms on basal DNA damage in human mononuclear cells as determined by the comet assay. Mutat Res. 2010 Aug 30;701(2):132-6.

56. Wu C, Lu Y, Morimoto K. Effect of gene polymorphisms and ethanol consumption on micronucleus frequency in human reticulocytes: a preliminary study. Environ Health Prev Med. 2010 May;15(3):188-93.

57. Yukawa Y, Muto M, Hori K, Nagayoshi H, Yokoyama A, Chiba T, Matsuda T. Combination of ADH1B*2/ALDH2*2 polymorphisms alters acetaldehyde-derived DNA damage in the blood of Japanese alcoholics. Cancer Sci. 2012.;103(9):1651-5.

MUT/14/05 – updated 2015/03/05

DNA ADDUCT DATA (Studies on enzyme polymorphisms are considered separately)

Table 1.1

Author/Country	Study info	Controls	Alcohol drinkers	Notes/ Comments on study design and/or findings
Balbo 2008 (Europe)	**No. of subjects** - **Hungary** - **ARCAGE**	-- 30m/28f (Total=58)	25m/25f 43m/26f (Total=119)	Healthy alcoholic abstainers used as controls; all subjects selected from controls of two case control studies [Hungary]: entering hospital to treat alcohol abuse [ARCAGE]:diagnosed with disease unrelated to tobacco or alcohol drinking (hospital based controls) No data on BMI, diet or nutritional status or calorific intake of subjects
	Mean age (±SD)	50.9 ± 12.4y (m, n=98); 53.9 ± 11y (f, n=79)		Mean adduct levels higher in younger than older drinkers (accounting for dose) ranged from 10,700 ± 11700 n=12 (<35 y) to 3360 ± 5030 n=73 (>55 y) p=0.01for trend
	Alcohol consumption	[Hungary]: -- [ARCAGE]: (abstainers)	<10g/d to 416g/d [Hungary]: high alcohol intake (i.e. >36 g/d in males, >20g/d in females) for minimum of 5y [ARCAGE]: mean 61.7g/d ±51.1;	Exposure assessed via interview/structured questionnaire No data on duration of alcohol exposure Dose response relationship observed for drinking categories in ARCAGE study 0-199g/d (P=0.02 for trend) (NB. In blood samples taken within 4 days after hospitalisation)
	Smoking - Non-smokers - Smokers - Amount (cig/day)	N=53 N=124 Range (<10 to >20)		Smokers had a slightly higher level of DNA adducts compared to non-smokers (p=0.65) which augmented with increase in no of cigarettes/day (p for trend=0.17), although none were statistically significant (even after adjusting for alcohol intake and other variables)
	DNA adduct measurements (mean, fmol/umol dG)			
	Cell type	Leukocytes		
	N^2-Ethyl-dG*	2690 ± SD 3040 n=38	5270 ± SD 8770 n=41(p=0.04 test for trend)	[ARCAGE study] Data adjusted for age, sex and number of cigarettes smoked
Balbo 2012a[x] (US) **Balbo 2012b[xy]**	**No. of subjects**	None	10 (5m/5f)	Healthy volunteers (students or employees) of the University of Minnesota; subjects acted as own controls Small study size NB. Authors did not assess the significance of the different weight values between genders and within genders;
	Mean age (range)	-	25 ± 3 SD y	
	Alcohol consumption	-	(1) 0.03; (2)0.05 and (3)0.07%	Exposure assessed via questionnaire, and measurement of blood alcohol concentration

Author/Country	Study info	Controls	Alcohol drinkers	Notes/ Comments on study design and/or findings
				Subjects received measured doses of alcohol once a week for 3 weeks to achieve blood alcohol levels (mg alcohol in 100ml expressed as %); asked to abstain from alcohol; used vodka has less congeners cf. other spirits. Weight used to determine the amount of alcohol served. Participants followed a regime of light breakfast (cereal, milk, coffee) before treatment and abstinence from food/drink during dosing and sampling. Light meal was provided during 4-6h sampling
	Smoking	-	None	
	DNA adduct measurements			
	Cell type	Granulocytes (G) Lymphocytes (L)		Collected at several time points before and after each dose i.e. baseline , 1, 2, 4, 6, 24, 48 and 120h (and 1 week before study)
		Epithelial cells (E)[y]		Collected by mouthwash with saline solution (after subjects brushed teeth) at same timepoints as Balbo 2012a before and after each dose Potential contamination of human DNA with bacterial DNA from mouthwash samples
	N^2-Ethyl-dG* (mean peak/ baseline ratio (95%CI) – initially calculated on a log scale	G: (1)1.5 (1.2-2.0); (2)1.7 (1.2-2.4); (3)2.7 (1.7-4.2) p<0.001 (for trend) L: (1)1.8 (1.0-2.7); (2) 1.6 (1.1-2.5); (3)3.0 (2.2-4.1); p=0.06		Average background adduct levels in subjects ~7500 fmol/umol dG (15 adducts per 10^7 nucleotides Effect was significant within 40h in both granulocytes and lymphocytes NB. Substantial variation in baseline adduct levels
	N^2-Ethyl-dG* (mean, fmol/umol dG ± SD)	Pre-exposure E(1): 2510 ± 1372; E(2):1768 ± 554; E(3): 1296 ± 638	Post exposure (peak levels) E(1):7549 ± 4233; E(2):17490 ± 28720; E(3):20450 ± 14640 (p=0.001)	Clear peaks were observed within 2-4 h from each dose Mean peak/baseline ratio (95% CI) initially calculated on a log scale: (1): 3.8 (2.4-5.9) (2): 8.6(3.8-19.3) (3): 17.5(8.5-35.9) P=0.001 for trend
Singh 2012[x] (Poland)	**No. of subjects**	**None**	**30 men**	Served as own controls Small study size No data on diet or nutritional status or calorific intake of subjects
	Mean age (range)	-	29 (range 21-44) years	NB. Mean BMI = 25.2 (ranging from 18.3 to 33); Authors do not conduct any further analyses wrt BMI
	Alcohol consumption	-	150 ml vodka - contained 42% pure ethanol	Exposure assessed via direct exposure to alcohol and measurement of BAC
	Smoking	-	None	

Author/Country	Study info	Controls	Alcohol drinkers	Notes/ Comments on study design and/or findings
	DNA adduct measurements (mean ± SD/10^8 2'-deoxynucleotides)			
	Cell type	Leukocytes		Collected before alcohol and 3-5h and 24 and 48h post consumption
	N^2-Ethyl-dG*	Pre-exposure 34.6 ± 21.9	Post-exposure [3-5h]=35.1 ± 21.0, [24h]=36.8 ± 20.7 [48h]=35.6 ± 21.1	Calculated using the Wilcoxon rank signed test NB. Significant differences in interindividual adduct levels

*obtained from reduction of DNA samples (containing N^2-ethylidene-dG) with sodium borohydride (NaBH3CN); ^{x} controlled exposure ; NR= not reported;

Table 1.2 DNA adduct formation in alcohol drinkers with different ALDH2 genotypes

Matsuda 2006 (Japan)	**Enzyme polymorphism**	**ALDH2*1/2*1**	**ALDH2*1/2*2**	**Notes/Comments on study design and/or findings**
	No. of subjects	19 men	25 men	Small study size No data on BMI, diet or nutritional status or calorific intake of subjects
	Average age (range)	52±11y;	51±11y;	Comparable
	Alcohol consumption/ Duration	130±54g/day (910g/w);	105±59g/day (735g/w)	Exposure assessed based on interview/structured questionnaire administered on admission of patients to hospital clinically diagnosed with alcohol dependence
	Smoking (cig/day)/Duration	22±13 cigs/day (26y)	24±16 cigs/day (24y)	Comparable
	DNA adduct levels (fmol/umol dG)			
	N^2-Et-dG;	17.8±15.9 (2/19 samples)	130±52 (p=0.003); (14/25 samples)	Wilcoxon-Mann-Whitney;
	Me-γ-OH-PdG: S);	42.9±6.0	92.4±12.9 (p=0.001);	t-test
	Me-γ-OH-PdG: R);	61.3±6.4	114±15 (p=0.002);	t-test

Based on IARC 2010

Yukawa 2012 (Japan)	**Enzyme polymorphism**	**Group 1**	**Group 2**	**Group 3**	**Group 4**	Subjects were genotyped via PCR/RFLP
	ADH1B	***1/*1**	***1/*2 & *2/*2**	***1/*1**	***1/*2 & *2/*2**	
	ALDH2	***1/*1**	***1/*1**	***1/*2**	***1/*2**	
	No. of subjects	13 men	37 men	8 men	8 men	All were alcoholics being treated at the Kurihama Alcoholism Centre of the National Hospital Organisation No data on diet or nutritional status or calorific intake of subjects
	Median age, years (25^{th}, 75^{th} percentile)	50 (40, 57)	56 (43, 63)	55 (44, 57)	54 (51, 64)	Comparable

	Weight (kg)	67±16*	57±8*	59±10	60±9	Mean body weight differences apparent between Groups 1 and 2 *P=0.05
	Median amount ethanol consumed in previous 24h (mls) (25^{th}, 75^{th} percentile)	120 (110, 180)	100 (80, 160)	120 (105, 140)	150 (95, 205)	Comparable Exposure assessment based on questionnaire and measurement of alcohol concentration in blood and saliva
	Interval from last drink (h) (25^{th}, 75^{th} percentile)	12 (8, 14)	13 (10, 24)	9.5 (8,14)	10.5 (7.5, 14)	Comparable
	Mean no. cig smoked in 24h	21±11	16±13	21±12	121±11	Comparable
	DNA adduct levels (10^7 bases)					
	N^2-Ethylidine-dG - Median (25^{th}, 75^{th} percentile);	2.14 (0.97, 2.37)	2.38 (1.18, 2.98)	5.38 (3.19, 6.52)	21.04 (12.75, 34.80)	Group 4 vs. Group 3: p<0.01; Group 3 vs Group 1: p<0.05; Group 4 vs Group 2: p<0.01 (Mann-Whitney; Holm's method)

MUTAGENICITY DATA (Studies on enzyme polymorphisms are considered separately below)

Table 1.3 Studies of MN in alcohol drinkers

Reference, study location	Characteristics of subjects	Characteristics of controls	Matching factors	Alcohol consumption/ duration	Tissue and genetic biomarker	Results	Comments
Maffei 2000 (Italy)	20 alcoholics (7f, 13m); mean age 49.1±9.9y Smoking 28±9.1y	20 (7f, 13m) Mean age 47.7 ± 10.2y Smoking 25.1±7.2y	Age, smoking. NB. None of subjects had a deficient diet or change in dietary habits during alcohol dependence. Assessed to be in a fair state of general nutrition; Assessment via measurement of triceps skinfold, midarm muscle and fat area, urinary creatinine/height ratio and	Control: 8-13g/day Alcoholics: >120g/day; 19.6± 8.8 y Exposure based on interview/structured questionnaire/ alcoholics clinically diagnosed as being alcohol-dependent	PBLs/MN - Cytochalasin B technique; May Grunwald-Giemsa staining NB. 2000 binucleate lymphocytes analysed per subject. (MN classified as centromere-positive	MN frequency in BN lymphocytes significantly higher in alcoholics cf. controls (12.0 ± 5.4 and 7.6 ± 1.6; P<0.05, Student's t test). Controls levels ranged from 6 to 12 Higher frequencies of C+ MN in alcoholics cf.	Small study size Accounted for exposure to occupational, diet nutrition, lifestyle and medicinal agents that may affect MN frequency

Reference, study location	Characteristics of subjects	Characteristics of controls	Matching factors	Alcohol consumption/ duration	Tissue and genetic biomarker	Results	Comments
			haemoglobin values		(C+) if showed a fluorescent signal (=> whole chromosome) and centromere-negative is no signal (=> fragment))	controls (8.2 ± 4.8 and 3.4 ± 1.4 respectively; P<0.05, Student's t test)	
Maffei 2002 (Italy)	20 alcoholics (7f, 13m); mean age 49.9 ± SD 9.9y; Smoking years mean 28.0±9.1 20 abstinent alcoholics (7f, 13m); mean age 52.2 ± SD 10.6y; Smoking years mean 28.5±7.1 NB. Clinical tests conducted on alcoholics to rule out malnutrition	20 controls (7f, 13m); mean age 47.5 ± SD 10.2y; Smoking years mean 25.1±7.2	Age, sex, smoking Subjects were assessed to be in a fair state of nutrition. Assessment via measurement of triceps skinfold, midarm muscle and fat area, urinary creatinine/height ratio and haemoglobin values	Controls: None Alcoholics: >120g/day; mean 19.5±8.8y (range:4-40y) Abstinent alcoholics: >120g/day for 5y before quitting; abstinent for mean 32.5±15.5m (range: 12-60m) Exposure based on interview/structured questionnaire/ alcoholic dependence scored according to specific clinical criteria (Psychiatric Association DSM IV)	PBLs/MN (CAs - see below)	Significantly higher levels of mean MN (12.05 ± SD 5.4) in alcoholics cf. controls; P=0.001, Kruskal-Wallis test. Esp. alcoholics drinking > 120g/day (cf. controls/abstinent drinkers) Abstinence appears to normalise frequency of MN (mean 7.2 ± SD 2.6) p>0.05, Kruskal-Wallis test	Small study size NB. Control and alcoholic MN data taken from Maffei 2000
Ramirez & Saldanha 2002 (Brazil)	30 alcoholics with oral / oro-pharyngeal carcinomas; average age 52.9±1.6y. Smoking habit (mean 36.9y ± 2.09y) (Samples taken from 3 distinct regions in the mouth A, B and C)	30 abstinent healthy individuals, average age 38.4±1.5y (significantly different cf. alcoholics P<0.0001). Non-smokers	SES	Control: None (religious grounds) Alcoholics: Mean duration =32.0y±2.4) Exposure based on interview (anamnestic – patients own recollection). Alcohol dependence in alcoholics assessed via CAGE questionnaire	Buccal cells (non-tumour)/ MN and Metanucleated anomalies (i.e. binucleated cells (BI), karyorrhexis (KR), karyolysis (KL) and broken eggs (BE)) Fuelgen nuclear reaction plus fast green stain (1%) Total number of MN (TMN) and number of cells with MN (CMN) per individual per 2000 cells per	Gradient of MN frequencies towards carcinogenesis: C→A→B; Control levels ranged from 0 to 2 in all regions B region: 1.97 ± 0.39 vs. 0.27 ± 0.09; P=0.03, Mann Whitney U test); A region: 0.93 ± 0.21 vs 0.27 ± 0.09; P=0.0004); C region: 0.60 ± 0.23 vs 0.23 ± 0.09; P=0.4409) Highly significant (mostly) differences between patients and controls for	Samples closely associated with tumuorigenic endpoint Younger patients had more TMN cf. older ones, although this was not significant (regression analysis) Sub-analyses of smoking influence in patients on TMN levels unclear; study

Reference, study location	Characteristics of subjects	Characteristics of controls	Matching factors	Alcohol consumption/ duration	Tissue and genetic biomarker	Results	Comments
					individual	metanucleated anomalies P values ranged from <0.0001 to 0.005 for BI, KR and KL in region. Non-significant for BE (p>0.71).	failed to match on smoking No data on BMI, diet or nutritional status or calorific intake of subjects
Reis 2002 (Brazil)	40 male alcoholics; aged 42y (average) nonsmokers; drug addicts Status obtained via questionnaire	20 abstinent individuals (incl. from cigarettes) aged 42y (average)	Age, smoking status	Controls: None Alcoholics: 2555ml ethanol/w; 25.5y	Buccal cells (tongue and jugal mucosa)/ MN	Significant increase in MN frequency of buccal cells of tongue of alcoholics cf. controls (p<0.01, Mann Whitney U test) Non-significant increase in MN frequency of buccal cells of jugal mucosa of alcoholics cf. controls (p>0.05, Mann Whitney U test).	Study published in Portuguese. Poor English translation available. MN data (figures) in Portuguese.

Table 1.4 Studies of Chromosomal aberrations in alcohol drinkers

Reference, study location	Characteristics of subjects	Characteristics of controls	Matching factors	Alcohol consumption	Tissue and genetic biomarker	Results	Comments
Maffei et al 2002 (Italy)	20 alcoholics (7f, 13m); mean age 49.9 ± SD 9.9y. Smoking years mean 28.0±9.1 20 abstinent alcoholics (7f, 13m); mean age 52.2 ± SD 10.6y Smoking years mean 28.5±7.1 NB. Clinical tests conducted on alcoholics to rule out	20 controls (7f, 13m); mean age 47.5 ± SD 10.2y; Smoking years mean 25.1±7.2	Age, sex, smoking	Controls: None Alcoholics: >120g/day; mean 19.5±8.8y (range:4-40y) Abstinent alcoholics: >120g/day for 5y before quitting; abstinent for mean 32.5±15.5m (range: 12-60m) Exposure based on interview/structured questionnaire/ alcoholic dependence scored according to specific clinical	PBLs/CAs (MN - see above)	Significantly higher in alcoholics cf. controls; esp. alcoholics drinking > 120g/day (chromatid/chromosome breaks, total CAs cf. controls/abstinent drinkers) Kruskal-Wallis test Active alcoholics exhibited significantly higher frequencies of CA in lymphocytes compared to both controls and abstinents Kruskal-Wallis test Abstinence appears to normalise frequency of CAs Kruskal-Wallis	Small study size NB. Control and alcoholic MN data taken from Maffei 2000

Reference, study location	Characteristics of subjects	Characteristics of controls	Matching factors	Alcohol consumption	Tissue and genetic biomarker	Results	Comments
	malnutrition			criteria (Psychiatric Association DSM IV)		test [Aberrant cells] Alcoholics – 4.1 ± 1.9; n=82 Control – 1.5 ± 0.83; n=29; P=0.001; Abstinent – 2.0 ± 1.1; n=39; P=0.001; [Chromatid-type] Alcoholics – 3.3 ± 1.5; n=65 Control – 1.20 ± 0.8; n=24; P=0.001; Abstinent – 1.8 ± 1.2; P=0.002; n=36 [Chromosome type] Alcoholics – 0.7 ± 1.0; n=13 Control – 0.3 ± 0.4; n=5; P=0.044; Abstinent – 0.1 ± 0.3; n=2	
Burim 2004 (Brazil)	29 chronic alcoholics (23m/6f) aged between 24-62y 11 abstinent alcoholics (9m/2f) aged between 44-73y Alcohol dependent subjects were not clinically verified	10 healthy volunteers (9m/1f) aged between	Smoking habit (smokers present in all groups =20 ± 5 cig/day). Subjects assessed for hazardous occupational exposures, diet, medicinal exposure, medical illness	Chronic]>60g ethanol/day ranged from 72-752g/day; duration ranged from 3-46y [Abstinent] >60g ethanol/day ranged from 80.4-801.6g/day; duration ranged from 6-48y; abstinence time ranged from 3m-4y Exposure assessed via interview	PBLs/ CA (analysed via conventional fluorescence and giemsa staining); chromosome translocations (analysed via FISH)	[CA – mean frequency%] Chronic alcoholics = 5.15 ± SEM 0.37 CA/100 cells (n=29) Abstinent alcoholics = 3.87 ± SEM 0.34 CA/100 cells (n=11) Controls = 1.72 ± SEM 0.52 CA/100 cells (n=10) (P<0.001, 2 proportions test) [Translocations – mean genomic frequency] Chronic alcoholics = 0.267 translocations /100 cells (n=12) Abstinent alcoholics = 0.167 translocations /100 cells) n=6 Controls = 0.067 translocations/100 cells) n=6 (P<0.05, 1-way ANOVA F-test) Abstinence did not reduce CA frequency cf. chronic alcoholics	Small study size Unclear if subjects were comparable wrt age or gender No significant difference in CA frequency between smoking and non-smoking alcoholics No data on BMI, diet or nutritional status or calorific intake of subjects

Table 1.5 Studies of DNA strand breaks in alcohol drinkers

Reference, study location	Characteristics of subjects	Characteristics of controls	Matching factors	Alcohol consumption	Tissue and genetic biomarker	Results	Comments
Pool-Zobel 2004 (Germany)	10 Male Alcoholics (mean age 46 ± sd. 11y)	9 m/f social drinkers mean age 40 ± sd. 10y (3 males); 35 ± sd. 14 (6 females)	Gender	Social drinkers = <60g/w Alcoholics = >100g/d; Exposure assessment method unclear. Alcoholism diagnosed via interview, clinical and laboratory parameters and medical examination (same method used for non-alcoholics?)	Rectal cells (sourced from biopsy/ diagnostic colonoscopy) PBLs (to assess systematic exposure loads) Strand breaks (% fluorescence in tails of comet images - 50 cells/slide, 3 slides per data point)	Male alcoholics had significantly less genetic damage cf. male controls [Rectal] mean (SEM) Controls: 10.43 (5.8) n=3; Alcoholics 6.31 (1.8) n=10;p<0.001, 2-way ANOVA unweighted means analysis and Bonferroni post test [PBLs] Controls: 6.17 (2.5) n=3; Alcoholics 2.33 (0.9) n=10; p<0.001	Small study size Used male subjects and mixture of male and female controls ↓DNA in comet trails possibly due to formation of crosslinks?? Did not account for confounding exposure to cigarette smoke No data on BMI, diet or nutritional status or calorific intake of subjects

Table 1.6 Studies of SCEs in alcohol drinkers

Reference, study location	Characteristics of subjects	Characteristics of controls	Matching factors	Alcohol consumption	Tissue and genetic biomarker	Results	Comments
Karaoğuz 2005 (Turkey)	15 type II male alcoholics (mean age 33.8y (range 24-44y) Also heavy smokers (mean duration 16.6y (range 8-35y); mean amount 27.7 cigs/day (range 10-50)	(+):10 male smokers (mean age 32.1y (range 27-41)/ smoked mean 22.6 cigs/day (range 13-60) for mean duration of 11.8y (range 4-20) (-):10 healthy male non-smokers (mean age 28.1y (range 23-33)	Smoking, gender	[Controls]: None [Alcoholics]: mean 223.65g/day (range 124.25-497)/ average alcohol dependency 15.3y (range 5-35y) Exposure assessment method unclear. Alcoholics diagnosed according to DSM-IV (American Psychiatric Association) by	PBLs/SCE	(Mean SCE frequency ± SD) Alcoholics = 10.89 ± 2.46 cf. positive control = 7.64 ± 1.0 (P<0.05 Student's t-test) and negative control = 6.96 ± 2.18 (P<0.05)	Small study size No data on BMI, diet or nutritional status or calorific intake of subjects Smoking interaction apparent

				psychiatrics. (same method used for non-alcoholics?)			

Polymorphisms

Table 1.7 MN formation in alcohol drinkers with different ALDH2 genotypes

Ishikawa 2003 (Japan)					**Notes /Comments on study design and/or findings**
	Enzyme polymorphism	**ALDH2*1/2*1**	**ALDH2*1/2*2**	**ALDH2*2/2*2**	ALDH2-deficient= (ALDH2*1/ ALDH2*2 and ALDH2*2/ ALDH2*2/) ALDH2 proficient= (ALDH2*1/2*1)
	No. of subjects	19	20	3	Total=42 male office workers Small study size No data on BMI, diet or nutritional status or calorific intake of subjects
	Mean age (range)	38.9 ± 12.7y			After adjusting for confounding effects of age: ORs for the MN frequency levels > 50th percentile value in ALDH2-deficient vs. ALDH2 proficient = 12.25(95%CI: 1.20-124.92) P<0.05, Multivariate logistic regression
	Alcohol consumption/ Duration				Alcohol exposure self-reported in questionnaire
	[Amount] >100g/w (n=20)	N=11	9		
	≤ 100g/w (n=22)	8	14		
	[Frequency]				
	Never drinkers: (n=8) Non-habitual: 0-3 times/week (n=16)	N=9	15		In subsequent analyses non-habitual drinkers included never drinkers (due to small numbers).
	Habitual drinkers: 4-7 times /week (n=18)	N=10	8		
	Smoking (cig/day)/Duration	None			Accounted for exposure to several agents/exposure that could affect the MN assay (e.g. X-rays, anti-cancer therapy)
	Tissue/genetic biomarker	PBLs /MN			Cytokinesis block method – cytochalasin B; Giemsa staining; 1000 T-lymphocyte binucleated cells
	MN frequency (average) % [Habitual drinkers]	3.20 ± SE 0.80; n=10	5.88 ± 0.58 (n=8) (p<0.05, Mann Whitney U test)		First report that habitual drinkers with the ALDH2 variant genotype have significantly increased MN frequency levels Lowest level observed in non-habitual drinkers 1.56 ± 0.41 (n=9)

Table 1.8 MN formation in alcohol drinkers with different ALDH2, ADH1B and/or CYP2E1 genotypes

Ishikawa 2006 (Japan)								**Notes /Comments on study design and/or findings**
	Enzyme polymorphism	**ALDH2*1/2*1**	**ALDH2*1/2*2**	**ALDH2*2/2*2**	**CYP2E1*1/*1**	**CYP2E1*1/*3**	**CYP2E1*3/*3**	ALDH2*1/ ALDH2*2 and ALDH2*2/ ALDH2*2/ grouped together CYP2E1*1/*3 and CYP2E1*3/*3 grouped together
	No. of subjects	126	105	17	149	88	11	Total = 248 healthy Japanese men No data on BMI, diet or nutritional status or calorific intake of subjects
	Mean age (±SD) years	(<42): n= 58 (≥ 42): n= 49	(<42): n=49 (≥ 42): n=56	(<42): n=8 (≥ 42): n=9	(<42): n=66 (≥ 42): n=83	(<42): n=44 (≥ 42): n= 44	(<42): n=5 (≥ 42): n= 6	Mean= 42y ± 12.5 Subjects ≥ 42y (n=133) had a higher mean MN frequency than subjects <42y (n-115 (4.14 vs. 2.88, p<0.001, Mann Whitney U test)
	Alcohol consumption/ Duration							Did not assess alcohol intake Exposure assessment based on self-completed questionnaire
	[Frequency] Non-habitual: 0-3 times/week (n=132)	N=44	88		74	58		Non-habitual drinkers included never drinkers (n=44) (due to small numbers).
	Habitual drinkers: 4-7 times /week (n=116)	N=82	34		75	41		
	Smoking (status)	Never (n=82); Former (n=50); Current (n=116) No data on the amount of cigarettes smoked or duration.						Observed no significant differences among smoking status and MN frequency Kruskal Wallis test p=0.33 (mean 3.13 ± 0.22 in never smokers)
	Tissue/genetic biomarker	PBLs /MN						Cytokinesis block method – cytochalasin B; Giemsa staining; 1000 T-lymphocyte binucleated cells
	MN frequency (average)%							Data was graphically presented – significant findings shown P values (habitual vs. non-habitual drinkers) Mann Whitney U test with Bonferroni correction NB. Highest mean frequency >4.7; n=26): observed in habitual drinkers who were of the combined genotype CYP2E1*1/*1 and (ALDH2*1/2*2 or ALDH2*2/2*2)
	[Habitual drinkers]		4.56; (n=34) p<0.01		4.15 (n=75)			
	[Non-habitual drinkers]	2.84; (n=44);				2.91; n=58); p<0.01		

Ishikawa 2007 (Japan)								**Notes /Comments on study design and/or findings**
	Enzyme polymorphism	**ALDH2*1/2*1**	**ALDH2*1/2*2**	**ALDH2*2/2*2**	**ADH1B*1/*1**	**ADH1B*1/*2**	**ADH1B*2/*2**	

Ishikawa 2007 (Japan)								**Notes /Comments on study design and/or findings**
	Enzyme polymorphism	**ALDH2*1/2*1**	**ALDH2*1/2*2**	**ALDH2*2/2*2**	**ADH1B*1/*1**	**ADH1B*1/*2**	**ADH1B*2/*2**	
	No. of subjects	151	118	17	25	80	181	No data on BMI, diet or nutritional status or calorific intake of subjects
	Median age (±SD) years	43y; ≤43y n=147; >43 n=139						Observed that subjects >43y (n=139- defined as elderly) had a higher mean MN frequency than subjects ≤43y (n-147) (4.04 vs. 2.97, p<0.001, Students t-test).
	Alcohol consumption/ Duration							Exposure assessment based on self-completed questionnaire
	[Amount] - Never (total=48) - Moderate (202) - Heavy (36)	N=9 112 30	26 86 6	13 4 0	3 16 6	16 55 9	29 131 21	Moderate (<60g/sitting), heavy (>60g/sitting)
	[Frequency]							
	Never drinkers: (total=48)	N=9	26	13	3	16	29	
	Non-habitual: ≤ 3 times/week (n=98)	N=41	54	3	6	34	58	
	Habitual drinkers: >3 times /week (n=140)	N=101	38	1	16	30	94	
	Smoking (status)	Non-smokers n=139; Smokers n=147						Non-smokers = never (n=84) and former (n=55)
	Amount (cig/day)	No exposure (n= 84); Most smoked 11-20 (n=70); > 30 (n=21)						No differences in mean MN frequencies observed among smoking subgroup
	Tissue/genetic biomarker	PBLs/MN						Cytokinesis block method – cytochalasin B; Giemsa staining; 1000 T-lymphocyte binucleated cells
	MN frequency (mean ± SE)%							
	[Habitual drinkers]	3.41 ± 0.31; n=46	4.83 ± 0.45; n=18	4.0; n=1 P=0.019	5.0 ± 0.93; n=8	4.46 ± 0.55; n=13	3.41 ± 0.3; n=44 P=0.039	P= for trends (1/1 to 2/2) – significant findings shown (ANOVA followed by Fisher PLSD multiple comparison) Combined analysis showed highest mean MN frequency (6.5, n=40) observed in (non-smoking and moderate drinkers) who were of the two genotypes ADH1B (1/1 or 1/2) and ALDH2 variant (deficient i.e. 1/2 or 2/2). Subjects with lowest mean MN frequency had a combined genotype of ALDH2(1/1) with ADH1B (2/2) (2.58 p=0.05, ANOVA followed by Fisher PLSD multiple comparison). Logistic regression revealed number of subjects with MN frequency > median value of 3 was significantly higher in subjects with ADH1B*1 allele OR=2.08, 95%CI=1.24-3.48 (when OR for subjects with ADH1B(2/2) genotype=1) . For
	[Moderate drinkers]	2.94 ± 0.26; n=64	3.85 ± 0.36; n=41	4.0; n=1 P=0.037	5.36 ± 0.66; n=11	3.29 ± 0.45; n=28	2.97 ± 0.24; n=67; P=0.029	

Ishikawa 2007 (Japan)								**Notes /Comments on study design and/or findings**
	Enzyme polymorphism	**ALDH2*1/2*1**	**ALDH2*1/2*2**	**ALDH2*2/2*2**	**ADH1B*1/*1**	**ADH1B*1/*2**	**ADH1B*2/*2**	
								ALDH2, the OR for ALDH2*2 allele was 1.79, 95%CI=1.04-3.11

Wu 2010 (Japan)	**Enzyme polymorphism**	**ALDH2*1/2*1**	**ALDH2*1/2*2**	**ALDH2*2/2*2**	**ADH1B*1/*1**	**ADH1B*1/*2**	**ADH1B*2/*2**	**Notes /Comments on study design and/or findings**
	No. of subjects	83	59	14	13	60	83	156 male employees of hard metal tooling factory NB. Enquired about the extent subjects were attentive to ensuring they had a balanced nutrition; and whether they regularly had breakfast
	Mean age years	45; ≤45y n=69; >45 n=87						
	Alcohol consumption/ Duration							No assessment of alcohol intake Significant increase in mean MN-RET frequency in habitual drinkers (0.067% ± SE 0.0052) cf. non-habitual drinkers (0.050% ± SE 0.0046); P=0.015, Students t test
	[Frequency]							
	Non-habitual: Habitual drinkers:	<3 times/week (n=82) ≥ 3 times /week (n=74)						
	Smoking (status)	Never (n=64); Ever (includes former smokers) n=92 No data on the amount of cigarettes smoked or duration.						Observed no sig difference in MN-RET frequency (P=0.153) – data not shown
	Tissue/genetic biomarker	Reticulocytes (transferrin +)/MN-Ret						7-aminoactinomycin staining and Flow cytometry (single laser) in transferrin-positive reticulocytes; Quadrant plot region analysis □ frequencies of MN-RET
	MN frequency (mean ± SE)%	0.055±0.0039	0.058±0.0064	0.080±0.016	0.043±0.0096	0.051±0.0053	0.065±0.0051	P value for (1-way ANOVA) for - ALDH2 genotypes = 0.047 - ADH1B genotypes = 0.071 - NB. lifestyle factors did not affect the MN-RET frequency

Table 1.9 DNA strand breaks in alcohol drinkers with different ALDH2 genotypes

Lu 2009 (Japan)					**Notes /Comments on study design and/or findings**
	Enzyme polymorphism	**ALDH2*1/2*1**	**ALDH2*1/2*2**	**ALDH2*2/2*2**	Expressed as NN, NM and MM respectively (latter two genotypes considered ALDH2 deficient)
	No. of subjects	74	67	9	Total = 150 No significant differences found between BMI values of subjects from different ALDH2 genotypes (p=0.5)
	Mean age (SD) years	45.6 (10.1)	45.0(9.7)	48.6(7)	Total = 45.5 (9.8)y
	Alcohol consumption				Exposure assessment based on self-completed questionnaire NB.Subjects asked about nutrition (nutritional balance, regularity of meals, breakfast habits, frequency of coffee and tea drinking and snack eating habits, consumption of fried and grilled

Lu 2009 (Japan)					**Notes /Comments on study design and/or findings**
	Enzyme polymorphism	**ALDH2*1/2*1**	**ALDH2*1/2*2**	**ALDH2*2/2*2**	Expressed as NN, NM and MM respectively (latter two genotypes considered ALDH2 deficient)
					meat.
	No. Drinkers **[Amount]** Mean ml pure alcohol (SD) per time	70 52.3 (32.8)	51 43.9 (41.1)	2 6.9 (13.9)	Total =123 ALDH2-NN drank significantly more times and greater amounts cf. ALDH2-MM (p<0.001, Kruskal-Wallis test)
	[Frequency] Mean times/month (SD)	 14.8(9.9)	 9.9(10.2)	 0.1(0.2}	
	Smoking				
	No. of subjects Mean amount (cig/day) (SD) Mean duration (years) (SD)	28 7.6 (11) 10 (14)	15 4.8 (10.6) 6.2 (11.9)	5 13.3 (14.8) 19.2 (18.2)	ALDH2MM smoked significantly more cigarettes cf. other groups (p<0.038, Kruskal-Wallis test)
	Tissue/genetic biomarker	PBLs/DNA strand breaks			Assessed as electrophoretic DNA migration (comet ratio)
	DNA migration - Comet ratio (%DNA in tail)				
	Correlation (r) with - Drinking frequency - Amount consumed	 Ns ns	 -0.321, p=0.005 (Spearman's) -0.257, p=0.025 (Pearsons)		NB. No significant variance in electrophoretic DNA migration (comet ratio ranged from 43.6 – 43.8) among ALDH2 genotypes p=0.634, Kruskal-Wallis test Stepwise multiple linear regression of DNA migration and lifestyle factors showed that nutritional balance was not predictive for DNA migration i.e. were not associated with DNA migration

Table 1.10 DNA strand breaks in alcohol drinkers with different ALDH2 and ADH1B genotypes

Weng 2010[†] (Japan)								**Notes /Comments on study design and/or findings**
	Enzyme polymorphism	**ALDH2*1/2*1**	**ALDH2*1/2*2**	**ALDH2*2/2*2**	**ADH1B*1/*1**	**ADH1B*1/*2**	**ADH1B*2/*2**	ADH1B*1/*1 + *1/*2 grouped together ALDH2*1/2*2 + *2/2*2 grouped together
	No. of subjects	68	43	11	7	43	72	122 males employed at a Japanese corporation; small sample size for multiple comparisons No data on BMI, diet or nutritional status or calorific intake of subjects
	Mean age (SD) years	47y; <47 n=58; ≥47 n=64						
	Alcohol consumption							No data on alcohol intake No details of exposure assessment method
	[Drinking frequency] Non-habitual: ≤ 3 times/w (n=66) Habitual: > 3 times/w (n=56)	 N=27 41	 39 15		 27 23		 39 33	Non-habitual drinkers included never drinkers (n=24) (due to small numbers). Drinking frequency had significant impact on mean TM value: Habitual drinkers – 1.27 ± SD 0.40; Non-habitual – 1.08 ± 0.27; Never drinkers – 1.04 ± 0.29; p=0.008, Kruskal-Wallis test
	Smoking	Nonsmokers (n=47); Former (n=30); Current (n=45)						Current smokers exhibited significantly higher mean TM (1.3) cf. non-smokers (TM=1.02) (P<0.001, Kruskal-Wallis

Weng 2010[†] (Japan)								**Notes /Comments on study design and/or findings**
	Enzyme polymorphism	**ALDH2*1/2*1**	**ALDH2*1/2*2**	**ALDH2*2/2*2**	**ADH1B*1/*1**	**ADH1B*1/*2**	**ADH1B*2/*2**	ADH1B*1/*1 + *1/*2 grouped together ALDH2*1/2*2 + *2/2*2 grouped together
								test).
	Tissue/genetic biomarker	Mononuclear cells/ DNA strand breaks						
	Tail Moment (mean ± SD)							
	- **Habitual (**≥47y)		1.65 ± 0.51 (n=9; p=0.021)				1.50 ± 0.49 (n=19; p=0.002)	P values refer to habitual vs non-habitual for either the ALDH2 or ADH1B genotypes (Mann Whitney U-test)) Older subjects who drank habitually and were of the (ADH1B*2 (fast) and ALDH2 deficient (slow) genotype) demonstrated significantly increased TM values cf corresponding genotypes.(Mann Whitney U-test) NB. Multiple regression analysis (stepwise) using log transformed TM values revealed that drinking frequency (p=0.003), smoking (p<0.001) and ALDH2 polymorphisms (0.037) significantly influenced the basal TM value; coefficient of determination (R2) was 0.25
	- **Non-habitual (**≥47y)	1.20 ± 0.35 (n=26)			1.08 ± 0..20 (n=16)			

Studies describing animal in vivo DNA adduct and Mutagenicity data following ethanol and acetaldehyde exposure

ETHANOL EXPOSURE

Table 2.1

Ref	Animals	Treatment regime	Biological sample	Endpoint	Detection/ Quantification method	Result	Comment
DNA adducts							
Matsuda 2007	C57BL/6 mice stratified according to ALDH2 genotype Treated: Aldh2+/+ (n=6) Aldh2+/- (n=5) Aldh2-/- knockout (n=2) Control: Aldh2+/+ (n=5); Aldh2+/- (n=7); Aldh2-/- knockout (n=5)	20% ethanol by oral gavage for 5 w Untreated fed water	Liver	N2-Ethyl-dG N2-ethylidene-DG PdG	LC/MS/MS	[N2-Ethyl-dG] Not detected in in any of the liver DNA samples analysed [N2-ethylidene-DG] Untreated: No sig difference among Aldh2 genotypes wrt average adduct levels (1.9±0.7 adducts/10^7 bases); p value not reported Treated: Significant differences in adduct levels wrt genotype Aldh2+/+ = (7.9±1.8); Aldh2+/- = (23.3±4); Aldh2-/- = (79.9±14.2); p<0.01 [PdG] Detected at 4.5-8.1 adducts per 10^8 nucleotides. No alcohol or Aldh2 genotype dependent increase was detected in any of the samples	Small number of animals. For N2-Ethyl-dG result the authors suggest this could be possibly due to: short exposure period preventing sufficient accumulation to detectable levels in liver; species-specific differences; tissue specific differences wrt endogenous reduction of N2ethylidenedG and DNA repair activity
Ogawa 2007	C57BL/6 ALDH2-knockout and WT (12w, males) Treated: Aldh2+/+ (n=10 per time point) Aldh2-/- (n=10 per time point) Control: Aldh2+/+ (n=10)	Animals administered (radiolabelled- [1-^{3}H] and [1-^{14}C]) ethanol at 2 x 10^7 Bq/kg BW of each by oral gavage for 6, 12 or 24h and sacrificed. Organs collected and radioactivity measured for DNA and organs Untreated fed water	Liver, stomach, kidney, serum	Unspecified (DNA radio activity) Organ radioactivity also evaluated NB. Carcinogenic potency also assessed via covalent binding index	Liquid scintillation counting	Radioactivity levels (dpm/ mg/DNA) graphically presented: 6h/12h – No significant change in radioactivity of DNA from any organs according to genotype 24h - significant differences in DNA radioactivity in all three organs according to genotype (p<0.05) ANOVA Aldh2-/- had slower decrease in DNA radioactivity cf. WT	Did not characterise DNA adducts Oesophagus was too small in mouse to examine DNA

Ref	Animals	Treatment regime	Biological sample	Endpoint	Detection/ Quantification method	Result	Comment
	Aldh2-/- (n=10)					Carcinogenic potency Knockout mice had significantly higher CBI cf. WT in all three organs at 24h (mean, SD; p<0.05) Liver: 18 ± 3.89 (WT), 27.9 ± 5.73 (KO); Kidney: 15.3 ± 2.36 (WT), 30.2 ± 8.28 (KO); Stomach: 395 ± 91.2 (WT), 825 ± 226 (KO)	
Nagayoshi 2009	Mice (10-11 wk males) backcrossed C57BL6 Treated: Aldh2+/+ (n=6) Aldh2+/- (n=5) Aldh2-/- knockout (n=4) Control: Aldh2+/+ (n=5) Aldh2+/- (n=6) Aldh2-/- knockout (n=5)	20% ethanol by oral Gavage for 5 w Untreated fed water	Stomach	N2-ethylidene-DG	LC/MS/MS	Untreated No sig difference among Aldh2 genotypes wrt average adduct levels (2.0-3.1 adducts/10^7 bases); p value not reported Treated Significant differences in adduct levels wrt genotype: Aldh2+/+ = (4.8±2.6); Aldh2+/- = (7.9±1.1); Aldh2-/- = (48.6±12); p<0.01	Small number of animals
Micronuclei							
Cebral 2011	CF-1 mice (sexually mature – 60d old) Treated: 6M/11F Control: 6M/12F (filtered tap water; also offered same food calorie amount as treated group)	Subchronic ethanol exposure 10% – both males females; Oral (ad libitum) for 27 (*or 17*)* days *Peri-gestational exposure (females) – mated after exposure and treated again with ethanol up to Day 10 of gestation*	Bone marrow ethryocytes (femur)	MN (35 animals)	MN assay (Schmid 1975) /Polychromatic erythrocytes (PCEs) were scored	Significant increase of mean MN frequencies in treated groups cf. each control group (ranged from 5.42(PF) to 6.70(F); n=6) (p<0.01, ANOVA 2-way) - M: 13.91 ± SD 1.9; n=6 (~ 2.4X higher); mean PAC=28.1 mg/dL - F: 10.96 ± SD 2.3; n=6 (~ 1.6X higher); mean PAC=19.8 mg/dL - PF: 11.83 ± SD 3.19; n=5 (~ 2.2X higher). NB. For blood concentrations between 19-28 mg/dL	Used low subchronic doses of ethanol resulting in low PAC and no ethanol denutrition. Attempted to account for possible ethanol related nutritional effects. Evaluated [ethanol]plasma (mg/dL) as well as intake
Ellahuene 2012	CF1 Mice (5-38wks old;	3 treated (5-30% ethanol (v/v)) and 2	Erythrocytes	MN	MN bone marrow assay	Treated group (5-15% ethanol) - No significant increase in MN	Attempted to simulate chronic ethanol exposure.

Ref	Animals	Treatment regime	Biological sample	Endpoint	Detection/ Quantification method	Result	Comment
	male) Treated: 38 Control: 16	control groups (water) were used; either 5 or 38 weeks of age ~reflect age of treated mice when treated and killed Oral (ad libitum) for 32w			/Scoring of polychromatic erythrocytes (PCEs)	induction compared to first (5 week age) control group (2.7 ± SD 1.2) n=6 per group p>0.05, Mann Whitney U test - Mean MN frequencies significantly lower (ranged from 2.5 to 4.0) than in 38w control group (6.5 ± SD 3.0; p<0.05); n=6 per group	Accounted for age related increases in MN frequency (via 2 different aged control groups)
Kotova 2013	Wistar rat (m) 8wk old Treated: 4 Control: 4	1 week acclimatisation; separation into groups; treatment with 10% ethanol in drinking water (ad libitum) for 4w	Erythrocytes/ reticulocytes	Genomic instability (MPCEs)	Flow cytometry MN assay	Graphically presented (dot plots and bar graph): Animals exposed to EtOH showed approx. 3.5 x significantly increased levels of MPCEs cf. untreated; P<0.05, Student's t-test two tailed (n=3)	Authors concluded that the findings suggest that alcohol consumption is directly genotoxic
Chromosome aberrations							
Tavares 2001	Wistar rat Treated: 10 Control: 10	20% v/v ethanol; Oral (aqueous solution ad libitum) for 30 days	Bone marrow	CA	CA test	Non-significant increase in CA frequency in treated vs control (p>0.05, ANOVA/Tukey test)	Reduced body weight gain apparent in treated animals (mean=3.16g/day) cf. controls (5.05g/day)
Comet assay							
Fedeli 2003	Genetically selected alcohol preferring rats (Marchigan-Sardinian) Treated: 2 groups:- n=3, received ethanol + water; n=6, received ethanol only Control: water; n=3	10% ethanol via oral (ad libitum) for 10 weeks	Blood Liver	DNA strand breaks (tail length, intensity and moment)	Single cell electro-phoresis (Comet assay) NB. Statistical tests used ANOVA/post-hoc t test, 1-way ANOVA	[Data graphically presented] Lymphocytes showed DNA damage (mean tail length) with differences among groups - Ethanol only (~18 uM: - Ethanol/Water (~11 uM) - Water only (~6 uM); P<0.01 Hepatocytes - No signs of DNA damage	
Germ cell DNA damage							
Talebi 2011	Wistar (albino males, 10 wks old)	Animals sacrificed after 50 days oral exposure to 5% ethanol ad libitum	Sperm	(i) Chromatin condensation	Cytochemical test (i) aniline blue (AB), chromo-mycin A3	Ethanol consumption disturbed DNA integrity of spermatozoa (cases n=10 vs control n=10)	No positive or negative controls used (wrt cytochemical tests)

Ref	Animals	Treatment regime	Biological sample	Endpoint	Detection/ Quantification method	Result	Comment
	Treated: n=10 Control:n=10 (water)	(to accommodate 45-day spermato-genesis cycle)		(ii) DNA integrity/ apop (sperm motility and morphology also tested)	(CMA3), (ii) toluidine blue (TB) acridine orange (AO) /% stain positive cells	CMA3+: 2.25 ± 2.30 vs. 0.5± 0.52; p=0.03 Mann-Whitney test & Kruskal-Wallis test AO+: 40.75 ± 24.81 vs. 3.50 ± 3.20; p=0.000 TB+: 59.66 ± 17.37 vs. 10.80 ± 6.89; p=0.000 Progressive sperm motility was significantly higher in control (18.57%) than treated rats (8.49%) p=0.034	
Rahimipour 2013	Balb/c mice (10 wks adult male) Treated: 2 groups: N=9 each Control: 2 groups: N=9 each; received either water or saccharin (0.2%)	5 or 10% ethanol (v/v); Oral (ad libitum) for 35 days (duration of mouse spermato-genesis) NB. Saccharin added to sweeten alcohol (facilitates oral administration)	Sperm	(i) Sperm chromatin condensation (ii) Sperm DNA integrity /apoptosis/ fragmentation Sperm motility/ morphorphology	Cytochemical tests: (i)CMA3, TB staining SDS (iii) TUNEL assay/SCD test /% stain positive cells Statistical analysis: ANOVA (differences with normal distributions) Post hoc tests (differences between each 2 groups)	Positive correlation between ethanol dose and mean sperm DNA fragmentation and sperm abnormalities (significantly elevated) cf. controls (mean, sd) TUNEL - Control: 6.57 ± 2.82 n=9 - 5%: 42.85 ± 6.76 (p=0.000) n=9 - 10%: 51.57 ± 7.45 (p=0.000) n=9 SCD - Control: 10.00 ± 3.74 n=9 - 5%: 47 ± 6.6 (p=0.000) n=9 - 10%: 54.85 ± 6.91 (p=0.000) n=9 Motility - Control: 73.85 ± 3.43 n=9 - 5%: 46.85 ± 5.81 (p=0.000) n=9 - 10%: 44.85 ± 5.01 (p=0.000) n=9 Normal Morphology - Control: 77 ± 6.4 n=9 - 5%: 57.14 ± 5.17 (p=0.000) n=9 - 10%: 51 ± 3.16 (p=0.000) n=9	Use of saccharin increased sperm DNA fragmentation (p=0.024) and reduced sperm with normal morphology, sperm viability (19%; p=0.002) (13%; p=0.001) and quick motility (10%; p=0.044);
Cebral	CF-1 mice	Subchronic ethanol	Sperm	Morphological	Sperm abnormality	Significantly higher mean % of	

Ref	Animals	Treatment regime	Biological sample	Endpoint	Detection/ Quantification method	Result	Comment
2011	(sexually mature – 60d old) Treated: 6M/11F Control: 6M/12F (filtered tap water; also offered same food calorie amount as treated group)	exposure 10% – both males females; Oral (ad libitum) 27 (*or 17*)* days *Peri-gestational exposure (females) – mated after exposure and treated again with ethanol up to Day 10 of gestation*	Oocytes	alterations (sperm: head, flagellum; oocyte: activated, degenerated, empty, immature)	test/ovulation rates, oocyte type	abnormal spermatozoa in treated males (31.1 ± SE 1.5; n=6) cf. controls 9.2. ± 0.8; n=6 (p<0.001, Student's t test) Significantly elevated parthenogenetic activated oocyte frequency in treated females (10% n=3) cf. controls (1.3%, n=6) (p<0.001, Chi-squared test) – oocyte quality was affected	
Ellahuene 2012	CF1 Mice (5-38wks old; male) Treated: 38 Control: 16 (water)	Treated exposed to 5-30% v/v ethanol Oral (ad libitum) for 20w	Embyros	Germ cell mutation /chromosomal damage	Dominant lethal mutation assay /% of induced DLM calculated	[Treated group (15 and 30% ethanol) - No of mean dead implants similar to control (0.74 ± SD 1.32; n=23)	Significant reduction in mean total implants (11.61 and 12.19 for 15 and 30% ethanol respectively) cf. control (14.04 ± SD 1.89; n=23; p<0.05) Authors say this suggest pre-implantation loss

PAC=plasma alcohol concentration; MPCE=micronucleated-polychromatic erythrocytes

ACETALDEHYDE EXPOSURE

Table 2.2

Ref	Animals	Treatment regime	Biological sample	Endpoint	Detection/ Quantification method	Result	Comment
Torres-Bezauri 2002	C3A mouse (m) Treated: n=5 Control: n=5	Animals received 30minutes exposure to 0.4, 4, 40 and 400 mg/kg AA via i.p. route.	Bone marrow cells	SCE	Microscopic analysis of cells in each mouse involved scoring 30 s-division metaphases to determine SCE rate	Two high doses (40 and 400 mg/kg) produced a mean SCE increase of 69% and 123% respectively cf. controls (2.10±0.26) 40=3.55±0.44 P=0.01 ANOVA & Student-Newman-Keuls test 400=4.70±0.18 P=0.01	Sample size unclear/not reported

Studies describing in vitro DNA adduct and Mutagenicity data following ethanol and acetaldehyde exposure

ETHANOL EXPOSURE

Table 3.1

Ref	Cell sample	Exposure regime	Endpoint	Detection/ Quantification method	Result (+ OR –)	Details	Comment
Micronuclei							
Majer 2004[1‡]	HepG2/3B cells; G2 contains Phase I&II enzymes	Cells exposed for 24h to:- G2 cells: 0, 8.5, 17, 85 and 170 mM; 3B cells: 0, 17, 85, 170 and 340 mM	MN Phase I and Phase II enzymes (present in either cytosol or microsomal fractions (incl. CYP450s)	MN assay (per 1000 binucleated cells) and C_D values (dose levels causing 2-fold increase of MN rate over background) Spectrofluorometry	+ (for HepG2 cells only)	Ethanol caused significant effects (increase in MN rate) at dose levels ≥ 17mM in HepG2 cells (mean & SDs graphically presented) n=3 ($p<0.05$, Dunnetts multiple comparisons test,) Ethanol was unable to increase MN rate 2-fold above background CYP2E1 not detectable	Did not determine ALDH2 or ADH activity No data on cell viability (possibly due to the fact the study was conducted on the basis of earlier studies) NB. 9 other carcinogens tested
Teo 2008[1]	WIL2-NS cells (Human B-lymphoblastoid cells) has mutation in p53 (p53 is inactivated) allows cells with DNA damage to survive due to inhibition of apoptosis	Cells exposed for 2 weeks to 0,0.09, 0.36, 1.34%, v/v ethanol Cells were cross-tested at different concentrations of ethanol and FA (20, 200, 2000nM)	Multiple: Chromosomal damage (MN, NPB, NBuds) NB. Cytotoxicity	Cytokinesis Blocked MN assay (validated in these particular cell lines)	+	Small increments in MNed BN frequency @ ethanol concentrations achievable via binge drinking (0.09-0.36%) – mean no MNed BN cells per 1000 BN cells graphically presented (n=6) Significant induction of MN, NPB and NBud with increasing [alcohol] and decreasing [FA] on day 15 – mean no BN cells with MN, NPB or NBud per 1000 BN cells graphically presented (p values ≤ 0.002, 2-Way ANOVA). (n=6) per endpoint With a baseline % of variance = 0.77 (viable cells); the interaction between FA and EtOH was significant for MN and NPB (i.e. 3.32; $p<0.001$, 2-Way ANOVA and 4.47; $p<0.01$ respectively)	Used ethanol concentrations achievable during binge drinking Ability of WIL2-NS cells to metabolise ethanol unknown
Kayani 2010[1‡]	Lymphoblastoid cells (MCL-5) Genetically engineered. (Constitutively express high levels of cytochrome P450 enzymes and	Cells exposed for 22h to 0 - 2 (% v/v) ethanol	MN	Cytokinesis Blocked MN assay / % Frequency Kinetochore staining (K^+/K^- MN) NB. Cell viability (% cytostasis –	+	Cell viability: 0.4 - 2.0% v/v (associated with CBPI of 1.4-1.14 cf 1.51 in controls) Mean MN freq (%) = 2.3 - 5.35; $p<0.01$; ~ 5-fold ↑ cf. negative cultures (0.99); No of cells scored ranged from 4538-5678 K^+/K^-: top dose (2% v/v) 92% K^+/8% K^-	Enabled genotoxic mechanisms of EtOH and AA to be differentiated

Ref	Cell sample	Exposure regime	Endpoint	Detection/ Quantification method	Result (+ OR –)	Details	Comment
	microsomal epoxide hydrolase)			reduction in cell proliferation expressed as CPBI)			
Benassi-Evans 2011[1]	WIL2-NS cells	Cells exposed for 6 weeks (describe as chronic) to physiologically relevant levels of ethanol: 0, 0.36%, 1.35% v/v NB. Cell samples taken from Week 6 used to test for chromosome 17 aneuploidy	Multiple: Chromosomal damage (MN, NPB, NBuds) Aneuploidy NB. Cell growth	Cytokinesis Blocked MN assay (validated in these particular cell lines) /1000 binucleated cells per slide scored for frequency of BN cells with each endpoint Chromogenic in situ hybridisation (using a chromosome 17 DNA probe) NB. Cells also scored for rate of necrosis, apoptosis and nuclear division cytotoxicity index (NDCI)	+	[WIL2-NS] – chromosome damage evident from week 1 MN– mean frequency significant increased wrt alcohol dose (0.36%=~35; 1.35%= ~50) cf. control (~15; P<0.0001 ANOVA n=2) and each other (0.36% vs 1.35%: P<0.05) – frequencies graphically presented; significant wrt linear trend (P<0.0001) ➔ MN frequency increased with alcohol dose NPB – mean frequency significant increased wrt alcohol dose (0.36%=~14; 1.35%= ~17) cf. control (~6; P<0.0001 ANOVA n=2); significant wrt linear trend (P<0.0001) ➔ NPB frequency is alcohol dependent increasing 2.5- to 3-fold relative to control NBuds – mean frequency non-significant wrt alcohol dose cf. control (P=0.06 ANOVA n=2); significant wrt linear trend (P=0.029) ➔ NBuds marginal association with alcohol; increase correlated positively with simultaneous increase in MN frequency Aneuploidy – chi-squared test significant for ch.17 aneuploidy (P<0001 n=2); no of cells with 2 signals (normal) and no of cells with 1 (monosomy), 3 (trisomy) or 4-7 (polysomy) signals increased 1.3-12-fold respectively	Did not measure AA levels to rule out possibility that chromosome damage was AA induced (although note that cultured lymphocytes have no AA activity) Aneuploidy assessed in one chromosome only
	GM13705 cells (Human B lymphoblast cell line: BRCA1 gene mutation)				+	[GM13705] – chromosome damage evident from week 3 (trend for alcohol-induced chromosomal damage less obvious) MN (mean MN frequency): control: ~3.6; 0.36%: ~6 (P<0.05); 1.35% P>0.05; - P for linear trend = 0.035 NPB: control: ~3; 0.36%: ~10 (P<0.01); 1.35% ~10 (P<0.01); P for linear trend = 0.002 NBuds: control: ~7; 0.36%: ~20 (P<0.05); 1.35% ~25 (P<0.05); P for linear trend = 0.009 Aneuploidy – also supported in this cell line	

Ref	Cell sample	Exposure regime	Endpoint	Detection/ Quantification method	Result (+ OR –)	Details	Comment
Comet assay							
Lamarche 2003[2]	Primary cultures of rat neurones obtained from 18 day old rat embryos of Sprague Dawley female rats	Acute – 6h to 20, 50 or 100 mmol/l ethanol. Cells were evaluated either immediately after exposure or 24 h later. Chronic - 3, 6 or 9 days to 20 mmol/l ethanol; Controls: medium NB. Some cells also received chronic exposure followed by acute	DNA strand breaks (acute exposure) DNA cellular content / cell cycle and apoptosis dynamics (chronic exposure) NB. Cell viability main endpoint (MTT assay, neural red NR uptake assay and TBE)	Comet assay (Acute exposure) Flow cytometry (chronic exposure) /% DNA in tail (linearly related to DNA break frequency)	+	6h exposure – significant dose dependent increase in % of DNA in tail of exposed cells ~9%, ~14%, ~18% at 20, 50 and 100mM respectively cf. to control (~8%) (graphically presented) p<0.0001, Scheffe post test) n= 200 Cell viability = ~100% 24h recovery – near full recovery Chronic exposure was harmful to neurones: dose dependent decrease in cell viability in cells chronically exposed to ethanol	Used doses of ethanol within range of BALs in intoxicated humans
Signorini-Allibe 2005[2‡]	Primary cultures of astrocytes sourced from 1-2 day old Sprague-Dawley pubs	Acute – 3h to 0, 100mM ethanol Chronic – 3,6,9 days to 0, 20mM	DNA alterations NB. Cytotoxicity (MTT assay)	Comet (commenced immediately after exposure to avoid repair mechanisms taking effect) /% DNA in tail	+ (chronic exposure)	Acute exposure – No DNA damage (data not shown); No significant impact on cell viability Chronic exposure → DNA alterations (% tail DNA) after 3 and 6 days of treatment (graphically presented: ~27, 37.5 respectively cf. control ~15; n=3 P<0.001 Fisher PLSD test); Cell viability decreased with increasing duration of exposure – by 6 days ~40%; reduced to 30% after 9 days Levels reached steady state after 6 days ~ 35, n=3 P<0.001 Fisher PLSD test);	Dose dependent decrease in cell viability in cells chronically exposed to ethanol
Blasiak 2000[1‡]	Human PBLs (L) obtained from healthy non-smoking donors. Human colonic mucosa (CM) (biopsy samples) Human gastric mucosa (GM) (biopsy samples	Cells exposed to either ethanol or AA for 1h and analysed or exposed first to ethanol, washed and then exposed to AA before being analysed. [for combined exposure chemical concentrations	DNA strand breaks NB. DNA repair also evaluated Cell viability (Trypan blue exclusion)	Comet assay/ Tail Moment NB. Statistical tests used Snedecor-Fisher test/Students t test; Cochran Cox; 1-way ANOVA	+	Significant increase in mean comet tail moment (uM) (migration of DNA fragments) i.e. due to DNA strand breaks (n=100 cells per treatment) Damage repaired in 4h – graphically presented Cell viability= >70% for up to 1mM (CL): Control: 9.09 ± 0.88; EtOH: 11.08 ± 0.76 p<0.05); (GM)Control: 9.43 ± 2.01; EtOH: 40.25 ± 5.17 p<0.001; (CM)Control: 13.7 ± 1.62; EtOH: 20.4 ± 1.88 p<0.01	No data on smoking habits of patient donors for GM and CM sample, or drinking habits of all subjects

Ref	Cell sample	Exposure regime	Endpoint	Detection/ Quantification method	Result (+ OR –)	Details	Comment
	during gastroscopy in macroscopically healthy tissue)	varied] (L):30mM (CM):10mM (on the premise that these cells are exposed to much lower concs.) (GM):1M (on the premise that these cells are exposed to much higher concentrations)				Combined exposure (Ethanol → AA → - Significant decrease of mean comet tail moment (for GM and CM) cf. controls ($p<0.001$ and $p<0.01$ respectively)	

[1]Human cells; [2]Animal cells; ‡cells also separately exposed to acetaldehyde (see below)
BAL=Blood alcohol levels

ACETALDEHYDE EXPOSURE

Table 3.2

Ref	Cell sample	Exposure regime	Endpoint	Detection/ Quantification method	Result (+ OR -)	Details	Comment
DNA adducts							
Wang 2000[2]	Calf thymus DNA or dG	Sample exposed to 0.01-40mM AA for 20h-96h cultured at 37oC. NaBH3CN added	AA-derived adducts	HPLC, UV, NMR, MS	+	3 new adducts were characterised: 1,N2-propanodG (PdG) N2-dimethyldioxane-dG and interstrand cross-link adduct Found to be stable at nucleoside level	3 new adducts detectable only at high [AA]: 40mM 96h May reflect the relative insensitivity of the analytical methods
Sako 2003[2]	Calf thymus DNA	Cultured in presence of histones – 50ul, (0.89 mmol)	PdG		+		Short communication
Theruvathu 2005[2]	Pig liver DNA Or dG	37oC Cells exposed to 25uM-4mM AA for 36-48h (depending on DNA source). Polyamines present @ [spermidine]: 500uM – 5mM	CrPdG	HPLC L/GC-MS/ Isotope dilution LC-MS (novel)	+	dG + 100-450uM[AA] + 5mM[spermidine] → Adduct dG + uM[AA] → No adducts formed dG + spermidine→ No adducts formed	[AA] overlapping the range formed in saliva of human volunteers who drank alcohol in a lab setting (estimated to be up to 450uM) Proposed mechanism

Ref	Cell sample	Exposure regime	Endpoint	Detection/ Quantification method	Result (+ OR -)	Details	Comment
							on how polyamines → production mutagenic AA-derived adducts
Hori 2012[1]	HL60 cells (human promyelocytic leukaemia cell). Possess fully active ALDH2 activity (homozygous ALDH2*1)	Cells exposed for 1h or 2h to [Optimal AA]=0.01% (1.8mM) (n=4); Closed system during treatment step (due to AA volatility) Control: PBS (n=2) After AA exposure cells washed cultured and DNA isolated 24h or 48h after exposure (adduct levels also measured at these time points) Purified DNA digested for adduct analysis	N^2-ethylidene-dG Corrected adduct levels (n=4) calculated by subtracting mean control levels (n=2) from exposure group (n=4) for each time point (immediately, 24h and 48h after exposure)	LC/MS/MS	+	Levels (mean): [After 1h AA exposure]: Measured - immediately = 12.1 ± SD 1.28 adducts per 10^7 bases; control=3.26; {corrected=8.85} - 24h (after exposure) = 8.2 ± 0.64; control=3.31; {4.89} - 48h after exposure 6.7 ± 0.52; control=3.26; {3.45} [After 2h AA exposure]: Measured - immediately = 21.4 ± SD 7.5; control=3.19; {18.2} - 24h (after exposure) = 10.5 ± 3.61; control=2.44; {8.09} - 48h after exposure 9.83 ± 3.9; control=2.7; {7.13} t½ =35h for either 1h or 2h AA exposure	Used much higher [AA] than would occur in vivo. Necessary to enable adduct detection. Analysed only one type of AA adduct (may question accuracy of measurements). Authors justify this by studies showing this adduct is representative of most AA-derived DNA adducts Model did not mimic chronic alcohol consumption (cells received acute exposure); repeated exposure may extend t½
Micronuclei							
Speit 2008[2]	V79 Chinese hamster cells	Cells exposed for 1h to [0.5-10mM] defined from preliminary expts. Treatment in serum free media; assays performed immediately after treatment (or after necessary cultivation)	MN NB. Cytoxicity (population doubling)	MN assay	+	[Results are graphically presented] PD was significantly reduced at concentrations of 10 and 5mM; 20mM→ >50% reduction in PD Significant concentration dependent increase in mean frequency of - MN (@ [AA]=0.5mM (~25/1000 cells) – 10mM (180/1000 cells): cf. control (10/1000 cells)) n=3 p<0.01, 1-Way ANOVA & Dunnett post hoc test	
Kotova 2013[2]	CHO cell lines AA8 and irs1SF cells (deficient in XRCC3	Cells exposed to 0, 1.2mM or 60mM [AA] from 30min to	Genomic instability – MN, catastrophic mitosis; markers of	MN assay (flow cytometry) / % positive cells	+	Clonogenic survival (Graphically presented log of % of living cells) ~ 100% @ 1.2mM - CHO cells – irs1SF cells 50 X more	Complex study Proposes mechanism

Ref	Cell sample	Exposure regime	Endpoint	Detection/ Quantification method	Result (+ OR -)	Details	Comment
	protein involved in homologous recombination repair) Chinese hamster lung fibroblasts (V79) (produce truncated HPRT protein due to defective gene that can be reverted by Rad51 HR exchange)	24h depending on endpoint being measured	DNA strand breaks (53BP1, γH2AX); markers of homologous recombination (RAD51) NB. Other non-mutagenic endpoints e.g. replication, HR, cell cycle, Clonogenic survival (measured as colony outgrowth	Confocal microscopy		sensitive than wildtype - V79 cells - (prolonged treatment with AA → increased cytotoxicity. Treatments for 1, 4 and 24h at [AA] up to 60mM → dose dependent cell survival) Graphically presented - Treated vs. control: 1.2mM AA; 24h → ~5fold increase induction of both MN and catastrophic mitosis in V79 cells (P<0.001, Student's t-test, <0.05) n=3; - AA treatment delayed replication for progression in a dose dependent manner - AA treatment enhanced levels of RAD51 foci formation ~6-fold cf. untreated cells - After blocking replication AA significantly increased levels of γH2AX foci formation relative to control - AA induced a 6-fold increase in formation of 53BPI, other markers of DSBs	on AA-induced genotoxicity
Majer 2004[1‡]	HepG2/3B cells; G2 contains Phase I&II enzymes	Cells exposed for 24h to:- G2 cells: 0, 0.9 and 9 mM acetaldehyde 3B cells: 0, 0.9 and 1.8 mM acetaldehyde	MN NB. Phase I and Phase II enzymes (present in either cytosol or microsomal fractions (incl. CYP450s)	MN assay (per 1000 binucleated cells) and C_D values (dose levels causing 2-fold increase of MN rate over background) Spectro-fluorometry	+	AA caused significant effects at dose levels ≥ 0.9 mM in both cell lines; n=3 (P<0.05) AA was unable to increase MN rate 2-fold above background CYP2E1 not detectable	Did not determine ALDH2 or ADH activity No data on cell viability (possibly due to the fact the study was conducted on the basis of earlier studies) Unclear whether AA exposure procedure accounted for its volatility (i.e. being hermetically sealed or performed in an ice bath)
Kim 2005[1]	Peripheral blood lymphocytes (primary) Collected from 47 healthy Korean m/f subjects with mean age 29 and 25. Genotyped via PCR as	Cells incubated for 24h in culture medium prior to exposure to 0, 0.5 and 1.5mM AA for 20h Experiment performed in an ice bath (due to	MN	Cytokinesis block MN assay /MN Frequency per 1000 binucleated cells	+	[MN frequencies graphically presented] Dose dependent increase in MN frequency:- Significant finding @ Highest dose – 1.5mM: WT - 2-fold increase cf. control; av alc consumption 61g/sitting Hetero – 3-fold increase cf. control; av alc	Adjusted for drinking frequency, smoking, and age Limitations Samples also received in vivo ethanol exposure

Ref	Cell sample	Exposure regime	Endpoint	Detection/ Quantification method	Result (+ OR -)	Details	Comment
	ALDH2-WT, Heterozygote, Homozygote) Smokers=9; Non-smokers=38 Alcohol consumption/ smoking, facial flushing data obtained via questionnaire	AA volatility) NB. Drinking frequency of subjects: 9/16 m and 20/31 f subjects drank 1-4 days/month; followed by 5/16 and 3/31 > 4 days/month respectively				consumption 25g/sitting Homo – 3.5 fold increase cf. control P=0.004 (ANOVA; Wilcoxon rank sum test); av alc consumption 12g/sitting	Used graduate students with lower frequency and amount of alcohol consumed No data on the viability of cells following exposure to increasing [AA]
Kayani 2010[1‡]	Lympho-blastoid cells (MCL-5) Genetically engineered. (Constitutively express high levels of cytochrome P450 enzymes and microsomal epoxide hydrolase)	Cells exposed for 22h to 0-0.250 (% v/v) AA	MN	Cytokinesis Blocked MN assay Kinetochore staining (K^+/K^- MN) Cell viability (% cytostasis – reduction in cell proliferation expressed as CPBI). NB. Decrease in CBPI an indication of cytotoxicity	+	Cell viability: 0.005 - 0.025 % v/v (associated with CBPI of 1.22-1.19 cf. 1.55 in controls) Mean MN freq (%) = 1.86-3.73; p<0.01 Fischer's exact test; ~ 4-fold ↑ cf. negative cultures (0.85); No of cells scored ranged from 4036-5097 Dose-dependent increase in MN lacking kinetochore signals (K-)and concomitant decrease in MN with kinetochore signals (K+) (0.010%-0.025%v/v) K^+/K^-: top dose (0.025% v/v) 32%K^+/68% K^- (32% MN were kinetochore positive and 68% of MN were lacking kinetochore signals (K-)	
Comet assay							
Signorini-Allibe 2005[2‡]	Primary cultures of astrocytes sourced from 1-2 day old Sprague-Dawley pubs	Acute – 3h to 0, 0.25, 0.5, 1 mM AA Conducted in hermetically capped and completed filled flasks to avoid evaporation	DNA alterations NB. Cytotoxicity (MTT assay)	Comet (commenced immediately after exposure to avoid repair mechanisms taking effect) /% DNA in tail	+	Acute exposure→ dose dependent increase (0.25, 0.5 or 1 mM) in frequency of single and double DNA strand breaks and alkali liable sites (graphically presented: ~17, 19 and 21 respectively cf. control ~14; n=3 P<0.001, Anova test); cell viability not significantly affected with increasing dose	
Speit 2008[2]	V79 Chinese hamster cells	Cells exposed for 1h to [AA] defined from preliminary expts. Treatment in serum free media; assays performed	DNA protein crosslinks (DPX) NB. Cytoxicity (population doubling)	Comet assay (Proteinase K used to differentiate between crosslinks types – DNA-DNA or	-	[Results are graphically presented] PD was significantly reduced at concentrations of 10 and 5mM; 20mM→ >50% reduction in PD No induction of DNA strand breaks (regardless	

Ref	Cell sample	Exposure regime	Endpoint	Detection/ Quantification method	Result (+ OR -)	Details	Comment
		immediately after treatment (or after necessary cultivation); cells also exposed to Gamma radiation – 2Gy		DNA-protein)/ DNA migration (tail moment)		of [AA]: ranged from 0.2 – 20 mM); latter concentration is cytotoxic, p<0.05, 1-Way ANOVA & Dunnett post hoc test. Post-treatment of slides with PK produced no clear effect on DNA migration in either controls or exposed cells, p<0.05, 1-Way ANOVA & Dunnett post hoc test. Gamma radiation caused no significant induction of cross-links (i.e. no reduction of gamma ray-induced DNA migration) n=3;, p<0.05, 1-Way ANOVA & Dunnett post hoc test	
Blasiak 2000[1‡]	Human PBLs (L) obtained from healthy non-smoking donors. Human colonic mucosa (CM) (biopsy samples) Human gastric mucosa (GM) (biopsy samples during gastroscopy in macroscopically healthy tissue)	Cells exposed to either ethanol or AA for 1h and analysed or exposed first to ethanol, washed and then exposed to AA before being analysed. [for combined exposure chemical concentrations varied] (L):3mM (CM):100mM (GM):100mM	DNA strand breaks NB. DNA repair also evaluated Cell viability (Trypan blue exclusion)	Comet assay/ Tail Moment	+	Significant dose-dependent decrease in mean comet tail moment (n=100 cells per treatment) Due to possible formation of crosslinks (finding was comparable to FA) Cell viability= >70% for up to 200mM (typo in text says 200uM) (L) Control: 9.09 ± 0.88; AA: 7.11 ± 0.83 p<0.05) (GM) Control: 9.43 ± 2.01; AA: 2.45 ± 0.22 p<0.001) (CM) Control: 13.7 ± 1.62; AA: 2.49 ± 0.31 p<0.001) Combined exposure (Ethanol → AA) → - Significant decrease of mean comet tail moment (for GM and CM) cf. controls (p<0.001 and p<0.01 respectively)	No data on smoking habits of patient donors for GM and CM sample, or drinking habits of all subjects
SCE							
Speit 2008[2]	V79 Chinese hamster cells	Cells exposed for 1h to [AA] defined from preliminary expts. Treatment in serum free media; assays performed	SCE NB. Cytoxicity (population doubling)	SCE test	+	[Results are graphically presented] PD was significantly reduced at concentrations of 10 and 5mM; 20mM→ >50% reduction in PD Significant concentration dependent increase	

Ref	Cell sample	Exposure regime	Endpoint	Detection/ Quantification method	Result (+ OR -)	Details	Comment
		immediately after treatment (or after necessary cultivation)				in mean frequency of - SCE (@ [AA]=0.2mM (>5/metaphase) – 5mM (~50/met): cf. control (~5/met)), n=3, p<0.01, 1-Way ANOVA & Dunnett post hoc test	
Gene mutation/ Mutation spectra							
Upton 2006*	Gene: *supF* (contains DNA adduct). Reporter plasmid: pLSX (a derivative of pZ189; contains B-lactamase for ampicillin resistance; supF tRNA gene; and pBR32 origin of replication for replication in E.coli) Cells transfected: E-coli	Varied depending on experimental stage Control = plasmids without any adducts	Mutagenicity of N2-EtdG Mutation spectra	Mutation reporter vector/ mutant colonies determined by scoring WT supF (black) against the mutant supF (white) DNA sequencing / Mutant fraction (%)	+	Mean mutant fraction - [Adduct containing construct]: 0.9% ± 0.2% - [Lesion free control]: 0.4% ± 0.1% (p=0.09) + dU (5' and 3' of compl strand) - [Adduct containing construct]: 1.4% ± 0.5% - [Lesion free control]: 0.6% ± 4% (p=0.04) In order of prevalence (constructs contained dU on compl strand): - Single base deletion 3-5 nucleotides downstream of adduct (33/54, 61%) i.e. –G at d(pGGG) - G:C→ T:A transversions (11/54, 20%) (at site of adduct) - Single base deletion –G (5/54, 9%) (site of adduct) - Single base insertion (5/54, 9%) (downstream of adduct)	
Stein 2006*	Shuttle vector: pBTE inserted into cells (confers blastocidin resistance - driven by the SV40 promotor); Gene: *not specificed*; Cells transfected: human xeroderma pigmentosum complementation group A (XPA) cells (lack NER pathway)	Various conditions depending on stage of experiment Cells transfected with 1ug of plasmid containing adduct	Mutagenicity of Me-α-OH-PdG i.e. 1,N2-propano-dG (PdG) adducts i.e. (6R, 8R) and (6S, 8S) diastereomer monoadducts - Mutated cells (antibiotic resistant) - Miscoding	Mutagenicity: DNA synthesis blocking potency i.e. blocking the formation of cross-links by incorporating adducts into a mismatched region / Miscoding frequencies, % progeny derived from a modified strand	+	Miscoding frequencies: ~5% for 6R,8R (n=2?) and 10% for 6S, 8S (n=2?) Mutations (for both diastereomers): G→T transversion (most frequent) G→A G→C	Oligonucleotides containing adducts were synthesised and characterised according to previously described methods (not elaborated in paper)

Ref	Cell sample	Exposure regime	Endpoint	Detection/ Quantification method	Result (+ OR -)	Details	Comment
Noori 2001[1]	Human Peripheral T lymphocytes (purified from buffy coat of healthy blood donors)	Cells chilled 15min before exposure to 2.4mM AA for 24h (2h in fridge, 22h @ 37oC). NB. Prior 24h treatment of cells to hypoxanthine, aminopterin and thymidine supplement to remove pre-existing in vivo HPRT mutants. Cells grown in culture (8 days) → mutant expression; Independent mutants from different subcultures were selected for molecular analysis	Mutant cells (and cloning efficiency) MS	6-TG resistance /MF (%) Genomic analysis/ PCR/DNA sequencing	+	Average cloning efficiency (50%) of that of control cells Mutant frequency - Independent 6-TG resistant mutants selected from large numbers of subcultures showing a 3-fold induction of MF on average (13.5×10^{-6}) cf. 4.9×10^{-6} in untreated cultures Mutations identified: In 73 induced and 36 spontaneous mutants: missense, nonsense, frameshift, splice mutation. Base substitutions (coding or splicing sequences): (i) 55 induced mutants and (ii) 26 control mutants (i) Base changes: G→A transitions (22/55 (40%), G on non-transcribed strand); A→T transversions (8/55 (14.5%), A on non-transcribed strand) (ii) Base changes: G→A transitions (4/26 (15.4%), p=0.04 Fishers exact test); No A→T transversions	

*Cells were not exposed to AA but transfected with synthetically produced AA-DNA adducts; ‡cells also separately exposed to ethanol (see above)
[1]Human cells; [2]Animal cells;

Genetic and related effects of alcohol/ethanol and acetaldehyde – key findings from all studies

NB. Levels of ethanol associated with intoxication: >(40 to 60) g/day; 20 – 100 mM; 0.08 – 1.35% BAC

Table 4.1		Test system Endpoint/sample/system	Exposure duration	Dose (LED or HID)[b]	Result[a]	FC	Comments	Reference
IN VITRO	ANIMAL	DNA strand breaks/ Sprague Dawley rat neurones (primary)/ in vitro	6h	20 mM	+	1.13	Acute exposure; FC[100mM]=2.25; chronic exposure toxic	Lamarche 2003
		Comet/ Sprague Dawley rat astrocytes (primary)/ in vitro	3-9d	20 mM	+	1.8	Chronic exposure; FC @6d=2.5	Signorini-Allibe 2005
	HUMAN	Micronuclei/ Human HepG2 cells/ in vitro	24h	17 mM	+	<2	Unexpected	Majer 2004
		Micronuclei/ Human Hep3B cells/ in vitro	24h	340 mM	-	-	Ethanol insensitive	Majer 2004
		Micronuclei/ Human WIL2-NS lymphoblastoid cells/ in vitro	2w	15.4 mM (0.09% v/v)	+		Interactive effects with folic acid	Teo 2008
		Micronuclei/ Human MCL-5 lymphoblastoid cells/ in vitro	22h	68.6 mM (0.4% v/v)	+	2.3	FC[343.0 mM (2%)]=5; Kinetochore staining revealed aneugenic mechanism;	Kayani 2010
		Micronuclei/ Human WIL2-NS lymphoblastoid cells/ in vitro	6w	61.7 mM (0.36% v/v)	+	2.3	Physiologically relevant dose; FC[231.5 mM (1.35%)]=3.3	Benassi-Evans 2011
		Micronuclei/ Human GM13705 lymphoblast cells/ in vitro	6w	61.7 mM 0.36% v/v	+	1.7	For lower dose only. GM13705 contains BRAC1 gene mutation	Benassi-Evans 2011
		NPB/ Human WIL2-NS lymphoblastoid cells/ in vitro	2w	15.4 mM (0.09% v/v)	+		Interactive effects with folic acid	Teo 2008
		NPB/ Human WIL2-NS lymphoblastoid cells/ in vitro	6w	61.7 mM (0.36% v/v)	+	2.5	FC[231.5 mM (1.35%)]=3	Benassi-Evans 2011
		NPB/ Human GM13705 lymphoblast cells/ in vitro	6w	61.7 mM (0.36% v/v)	+	3.3	GM13705 contains BRAC1 gene mutation	Benassi-Evans 2011
		NBuds/ Human WIL2-NS lymphoblastoid cells/ in vitro	2w	15.4 mM (0.09% v/v)	+		Interactive effects with folic acid	Teo 2008
		NBuds/ Human WIL2-NS lymphoblastoid cells/ in vitro	6w	231.5 mM (1.35% v/v)	-	-	Non-significant increase (P<0.05)	Benassi-Evans 2011
		NBuds/ Human GM13705 lymphoblast cells/ in vitro	6w	61.7 mM (0.36% v/v)	+	2.85	GM13705 contains BRAC1 gene mutation	Benassi-Evans 2011
		Aneuploidy/ Human WIL2-NS lymphoblastoid cells/ in vitro	6w	61.7 mM (0.36% v/v)	+			Benassi-Evans 2011
		Aneuploidy/ Human GM13705 lymphoblast cells/ in vitro	6w	61.7 mM (0.36% v/v)	+		GM13705 contains BRAC1 gene mutation	Benassi-Evans 2011
		DNA strand breaks/ Primary Human lymphocytes/ in vitro	1h	30 mM	+	1.22		Blasiak 2000
		DNA strand breaks/ Primary Human gastric mucosa/ in vitro	1h	1 M	+	4.27		Blasiak 2000
		DNA strand breaks/ Primary Human colonic mucosa/ in vitro	1h	10 mM	+	1.49		Blasiak 2000
IN VIVO	ANIMAL	DNA adducts (N^2-Ethyl-dG) C57BL/6 ALDH2 knockout mouse liver/ in vivo	5w	20% (v/v) ethanol solution x o.g (~23g/day/kg b wt)	-	-	Not detected NB. Small study	Matsuda 2007
		DNA adducts (PdG)/ C57BL/6 ALDH2 knockout	5w	20% (v/v) ethanol	-	-	No alcohol or ALDH2 dependent increases	Matsuda 2007

Table 4.1		Test system Endpoint/sample/system	Exposure duration	Dose (LED or HID)[b]	Result[a]	FC	Comments	Reference
		mouse liver / in vivo		solution x o.g (~23g/day/kg b wt)			observed.	
		DNA adducts (N^2-ethylidene-dG)/ C57BL/6 ALDH2 knockout mouse liver / in vivo	5w	20% (v/v) ethanol solution x o.g (~23g/day/kg b wt)	+	10.1	Wrt (-/-) cf. treated WT(+/+); FC(-/+)=2.95;	Matsuda 2007
		DNA adducts (N^2-ethylidene-dG)/ C57BL/6 ALDH2 knockout mouse stomach/ in vivo	5w	20% (v/v) ethanol solution x o.g (~23g/day/kg b wt)	+	10.13	Wrt (-/-) cf. treated WT(+/+); FC(-/+)=1.64	Nagayoshi 2009
		DNA adducts (radiolabelled)/ C57BL/6 ALDH2 knockout mouse liver/ in vivo	6, 12 or 24h	2 x 10^7 Bq/kg BW x o.g	+	1.3	Radiolabelled ethanol: [1-^{3}H] and [1-^{14}C]	Ogawa 2007
		DNA adducts (radiolabelled)/ C57BL/6 ALDH2 knockout mouse kidney/ in vivo	6, 12 or 24h	2 x 10^7 Bq/kg BW x o.g	+	1.8	Radiolabelled ethanol: [1-^{3}H] and [1-^{14}C]	Ogawa 2007
		DNA adducts (radiolabelled)/ C57BL/6 ALDH2 knockout mouse stomach/ in vivo	6, 12 or 24h	2 x 10^7 Bq/kg BW x o.g	+	1.5	Radiolabelled ethanol: [1-^{3}H] and [1-^{14}C]	Ogawa 2007
		Micronuclei/ CF1 mouse bone marrow erythrocytes/ in vivo	27d, 17d	10% (w/v) x oral	+	2.4	For males. FC=1.6 for females. NB. Subchronic low exposure	Cebral 2011
		Micronuclei/ CF1 mouse erythrocytes/ in vivo	32w	15% (v/v) (& 5% (v/v)) x oral	- (&(~))	- (-1.6)	Result dependent on age of control: 5 or 38 weeks. Chronic exposure	Ellahueñe 2012
		Micronucleated polychromatic erythrocytes (MPCE)/ Wistar rat reticulocytes/ in vivo	4w	10% (v/v) sol x oral	+	3.5	Subchronic low exposure	Kotova 2013
		Chromosome aberrations/ Wistar rat bone marrow/ in vivo	30d	20% (v/v) x oral	-	-	Authors consider exposure to be chronic	Tavares 2001
		DNA strand breaks / Marchigan-Sardinian rat lymphocytes/ in vivo	10w	10% (v/v) sol x oral	+	3	Subchronic exposure; FC[alc+water]=1.83	Fedeli 2003
		DNA strand breaks / Marchigan-Sardinian rat hepatocytes/ in vivo	10w	10% (v/v) sol x oral	-	-	Subchronic exposure	Fedeli 2003
		Morphological abnormalities / CF1 mouse germ cells / in vivo	27d, 17d	10% (w/v) x oral	+		Subchronic low exposure	Cebral 2011
		Dominant lethal mutation/ CF1 mouse / in vivo	22w	30% (v/v) x oral	-	-	Chronic exposure	Ellahueñe 2012
		Chromatin integrity/ Wistar rat sperm/ in vivo	50d?	5% (v/v) x oral	+	4.5	Wrt CMA3+; FC[AO+]=11.6; FC[TB+]=5.5	Talebi 2011
		Sperm chromatin dispersion/ Balb/c mouse sperm/ in vivo	35d	5% (v/v) x oral	+	6.52	Wrt Tunel; FC[SCD]=4.7	Rahimipour 2013
	HUMAN	**Studies in humans**						
		N^2-Et-dG adducts/ Peripheral WBCs in ALDH2-genotyped alcoholics/ in vivo		105g/d (mean)	$+_c$	7.3		Matsuda 2006 (Japan)
		N^2-Et-dG adducts/ Leukocytes in ADH1B and ALDH2-genotyped alcoholics/ in vivo		100ml/d (median)	$+_c$	8.84	FC wrt Group 4 (150 ml ethanol/d) vs Group 2	Yukawa 2012 (Japan)
		Me-γ-OH-PdG: S(R)/ Peripheral WBCs in ALDH2-genotyped alcoholics/ in vivo		105g/d	$+_c$	2.15 (1.87)		Matsuda 2006 (Japan)
		N^2-Dio-dG adducts/ Peripheral WBCs in ALDH2-		105g/d	$-_c$	-	Undetected	Matsuda 2006

Table 4.1	Test system Endpoint/sample/system	Exposure duration	Dose (LED or HID)[b]	Result[a]	FC	Comments	Reference
	genotyped alcoholics/ in vivo						(Japan)
	N^2-Et-dG adducts/ Leukocytes of alcohol drinkers (vs. abstainers)/ in vivo		<10g/d	$+_c$	1.96	Wrt drinkers vs non-drinkers. NB. Subjects came from 2 studies with different designs and had differing alcohol consumptions	Balbo 2008 (Europe)
	N^2-Ethylidene-dG adducts/ Peripheral WBCS of healthy volunteers/ in vivo	1-120h	0.03% (BAC)	+	1.5[d]	Wrt granulocytes. FC[Dose3]=2.7; Controlled exposure; peak levels (40h);	Balbo 2012a (US)
	N^2-Ethylidene-dG adducts/ Oral epithelial cells of healthy volunteers/ in vivo	1-120h	0.03% (BAC)	+	3	FC[Dose3]=15; Controlled exposure; peak levels (4h)	Balbo 2012b (US)
	N^2-Ethylidene-dG adducts/ Peripheral WBCS of healthy volunteers/ in vivo	3-5h 24-48h	150ml of 42% ethanol	-	-	Controlled exposure	Singh 2012 (Poland)
	Micronuclei/ PBLs of alcoholics (vs. alcohol drinkers)/ in vivo		>120g/d	$+_c$	2.4	Wrt (C+ MN); FISH technique revealed aneugenic mechanism	Maffei 2000 (Italy)
	Micronuclei/ PBLs of alcoholics (incl. abstainers) vs. non-drinkers/ in vivo		>120g/d	$+_c$	1.58	Abstinence normalises MN frequency	Maffei 2002 (Italy)
	Micronuclei/ Buccal cells (non-tumour) of alcoholic cancer patients (vs. non-drinkers)/ in vivo		NR	$+_c$	2.6	C region. FC in B region=7.3. NB. Alcohol consumption (duration)	Ramirez & Saldanha 2002 (Brazil)
	Metanucleated anomalies (KL)/ Buccal cells (non-tumour) of alcoholic cancer patients (vs. non-drinkers)/ in vivo		NR	$+_c$	4.9	C region. FC in B region=13.01. NB. Alcohol consumption (duration)	Ramirez & Saldanha 2002 (Brazil)
	Micronuclei/ Buccal cells of alcoholics (vs. abstinents)/		2555ml ethanol/w	+	4.4	Data acquisition limitations due to publication in Portuguese	Reis 2002 (Brazil)
	Micronuclei/ PBLs in ALDH2-genotyped habitual drinkers (vs. non-habitual)/ in vivo		≤ 100g/w	+	1.84	Wrt ALDH2 proficient vs deficient; Highest MN in habitual drinkers with ALDH2*2 genotype	Ishikawa 2003 (Japan)
	Micronuclei/ PBLs in ALDH2- and CYP2E1-genotyped habitual drinkers (vs. non-habitual)/ in vivo		NR	$+_c$	1.88	Wrt MN in habitual drinkers with ALDH2*2 and WT CYP2E1 genotype cf. CYP2E1*3 and ALDH2 proficient	Ishikawa 2006 (Japan)
	Micronuclei/ PBLs in ADH1B- and ALDH2-genotyped habitual drinkers (vs. non-habitual)/ in vivo		<60g/sitting	$+_c$	1.46	Wrt ADH1B: (1/1) vs (2/2) (non-smokers). NB. FC slightly lower when comparing ALDH2 deficient cf. proficient	Ishikawa 2007 (Japan)
	Micronuclei/ Reticulocytes in ADH1B- and ALDH2-genotyped habitual drinkers (vs. non-habitual)/ in vivo		NR	$+_c$	1.45 1.51	Wrt ALDH2: (deficient vs. proficient) Wrt ADH1B: (2/2 vs. 1/1)	Wu 2010 (Japan)
	Chromosome aberrations/ PBLs of alcoholics (incl. abstainers) vs non-drinkers/ in vivo		>120g/day	$+_c$	2.73	Wrt aberrant cells; FC(chromatid)=2.75; ; FC(chromosome)=2.33; Abstinence normalises CA frequency	Maffei et al 2002 (Italy)
	Chromosome aberrations/ PBLs of alcoholics (incl. abstainers) vs non-drinkers/ in vivo		>60g/day	$+_c$	3.0	Wrt CAs; FC(translocation)=4.0; Abstinence had no effect	Burim 2004 (Brazil)
	DNA strand breaks/ Rectal cells of alcoholics (vs social drinkers)/ in vivo		>100g/d;	(~)	- 1.65	No data on subject smoking status	Pool-Zobel 2004 (Germany)
	DNA strand breaks/ PBLs of alcoholics (vs social drinkers)/ in vivo		>100g/d;	(~)	- 2.64	No data on subject smoking status	Pool-Zobel 2004 (Germany)
	DNA strand breaks/ PBLs in ALDH2-genotyped alcohol drinkers		6.9ml/time	$(\sim)_c$			Lu 2009 (Japan)

Table 4.1		Test system Endpoint/sample/system	Exposure duration	Dose (LED or HID)[b]	Result[a]	FC	Comments	Reference
		DNA strand breaks/ Mononuclear cells in ADH1B- and ALDH2-genotyped habitual drinkers (vs. non-habitual)/ in vivo		NR	$+_c$	1.38	Wrt ≥47y: AD1B (2/2) vs. (1/1, 1/2) and ALDH2 (1/2, 2/2) vs. (1/1)	Weng 2010 (Japan)
		Sister chromatid exchanges/ PBLs of alcoholics (vs. non-drinkers)/ in vivo		223.65 g/d (mean)	$+_c$	1.56	FC=1.43 in positive control ➔ interactive effect	Karaoğuz 2005 (Turkey)

[a] +, positive; –, negative; (~), inverse effect; [b] LED, lowest effective dose; HID, highest ineffective dose; [c] In these studies, people who consumed alcohol were also smokers.
[d] Peak baseline/ratio
BAC=Blood alcohol concentration; NR=not reported; o.g=oral gavage; FC= fold change relative to control (exposed value/unexposed (or referent) value) – calculated where possible; KL=karolysis

Genetic and related effects of acetaldehyde

NB. Acetaldehyde (AA) concentration up to 450 uM is estimated to be within the range formed in saliva after ethanol consumption (BAC = 44 mM) (Theruvathu 2005). Blood AA levels range between 1 to 5uM even after high ethanol dose. Kotova et al (2013) suggest alcoholics reach up to 119uM AA in saliva and 2 - 20uM in blood. IARC (2012): Alcoholics reach 14 uM in venous blood which can be as high as 200 uM in ALDH2-deficient subjects (IARC, 2012)

Table 4.2		**Test system** **Endpoint/sample/system**	**Exposure duration**	**Dose (LED or HID)[b]**	**Result[a]**	**FC**	**Comments**	**Reference**
IN VITRO (NO AA EXPOSURE)	BACTERIA	DNA adduct (N2-ethyl-dG) mutagenicity of *supF* gene / pLSX transformed *Escherichia coli*/ in vitro	None	1uLpLSX/plate(35uL)†	(+)*	2.3	2.3-fold increase in mutant fraction relative to background*	Upton 2006
		DNA adduct (N2-ethyl-dG) induced mutation spectra in *supF* gene / pLSX transformed *Escherichia coli*/ in vitro	None	1uLpLSX/plate(35uL)†	(+)*		Deletions, GC to TA transversions, insertions*	Upton 2006
	HUMAN	DNA adduct (Me-α-OH-PdG-S/R) mutagenicity / SV40 (pTBE) transformed human fibroblasts (XPA cells)/ in vitro	None	1ugpTBE/flask(25cm^2)†	+		Mutant fractions: 5-11%	Stein 2006
		DNA adduct (Me-α-OH-PdG-S/R) induced mutation spectra / SV40 (pTBE) transformed human fibroblasts (XPA cells)/ in vitro	None	1ugpTBE/flask(25cm^2)†	+		G→T transversions most prominent	Stein 2006
IN VITRO	ANIMAL	PdG adduct formation/ Calf thymus DNA (or dG)/ in vitro	20-96h	40 mM	+		Detectable only at high concentrations	Wang 2000
		N2-dimethyldioxane-dG adduct formation/ Calf thymus DNA (or dG)/ in vitro	20-96h	40 mM	+		Detectable only at high concentrations	Wang 2000
		DNA interstrand crosslink formation/ Calf thymus DNA (or dG)/ in vitro	20-96h	40 mM	+		Detectable only at high concentrations (NB. First study to characterise ICLs)	Wang 2000
		PdG adduct formation/ Calf thymus DNA/ in vitro		Unclear ~594mM	+		Accelerated in presence of histones	Sako 2003
		PdG adduct formation/ Pig liver DNA (or dG)/ in vitro	36-48h	100 uM (0.1mM)	+		Facilitated in presence of polyamines (i.e. spermidine (5mM)	Theruvathu 2005
		Micronuclei/ V79 Chinese hamster cells/ in vitro	1h	0.5 mM	+	2.5	FC[10mM]=18; Part of a study examining the validity of Comet assay;	Speit 2008
		Genomic instability (includes MN)/ V79 Chinese hamster cells/ in vitro	24h	1.2 mM	+	5	CHO cell lines (AA8 and irs1SF) also used	Kotova 2013
		DNA strand breaks / SD rat astrocytes (primary)/ in vitro	3h	0.25 mM	+	1.2	Acute exposure; FC[1mM]=1.5	Signorini-Allibe 2005
		DNA-DNA (or DNA protein) crosslinks - Comet/ V79 Chinese hamster cells/ in vitro	1h	20 mM	-	-	(+Proteinase K for crosslink differentiation)	Speit 2008
		Sister chromatid exchange/ V79 Chinese hamster cells/ in vitro	1h	0.2 mM	+	>1	FC[5mM]=~10 >sensitivity to AA-induced DNA damage cf. Comet	Speit 2008
	HU	N2-ethylidene-dG adduct stability/ Human HL60 cells/ in vitro	1 (or 2h)	1.8 mM	+	3.7 (6.7)	Wrt levels measured immediately after exposure; $t_{1/2}$=35h; [AA] higher than would	Hori 2012

Table 4.2		Test system Endpoint/sample/system	Exposure duration	Dose (LED or HID)[b]	Result[a]	FC	Comments	Reference
							occur in vivo	
		Micronuclei/ Human HepG2 cells/ in vitro	24h	0.9 mM	+	<2		Majer 2004
		Micronuclei/ Human Hep3B cells/ in vitro	24h	0.9 mM	+	<2		Majer 2004
		Micronuclei/ Primary Human lymphocytes from ALDH2-genotyped healthy subjects/ in vitro	20h	1.5 mM	+	3.5	Wrt FC in ALDH2*2/2*2;	Kim 2005
		Micronuceli/ Human MCL-5 lymphoblastoid cells/ in vitro	22h	0.9 mM (0.005% v/v)	+	2.19	Kinetochore staining revealed clastogenic mechanism (1.8 mM (0.010%v/v))	Kayani 2010
		DNA strand breaks/ Human lymphocytes/ in vitro	1h	3 mM	(~)	-1.3	Attributed to formation of cross-links (finding was comparable to FA)	Blasiak 2000
		DNA strand breaks/ Primary Human colonic mucosa/ in vitro	1h	100 mM	(~)	-3.85	Attributed to formation of cross-links (finding was comparable to FA)	Blasiak 2000
		DNA strand breaks/ Primary Human gastric mucosa/ in vitro	1h	100 mM	(~)	-5.5	Attributed to formation of cross-links (finding was comparable to FA)	Blasiak 2000
		Mutation spectra/ Primary Human Peripheral T lymphocytes/ in vitro	24h	2.4 mM	+	3	Wrt mutation frequency; MS: G→T transitions; A→T transversions (Comparable to p53 MS in oesophageal tumours)	Noori 2001
IN VIVO	ANIMAL	Sister chromatid exchange/ C3A mouse bone marrow cells/ in vivo	30min?	40 mg/kg x i.p.	+	1.69	Positive at @ higher doses only. FC (400 dose)=2.23	Torres-Bezauri 2002

[a] +, positive; (+), positive under certain conditions; –, negative; (~), inverse effect; [b] LED, lowest effective dose; HID, highest ineffective dose;
†no acetaldehyde exposure – cells transfected with synthetically-derived AA-type adducts; *when constructs contained dU on complementary strand; BAC=Blood alcohol concentration; FC= fold change relative to control (exposed value/unexposed (or referent) value) – calculated where possible;