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

STATEMENT ON THE MUTAGENICITY OF ALCOHOL (ETHANOL) AND ITS METABOLITE ACETALDEHYDE: UPDATE ON INFORMATION PUBLISHED BETWEEN 2000-2014 (Second Draft)

BACKGROUND

1. In 1995, the COM provided a statement on the mutagenicity of alcoholic beverages (COM, 1995) for the Interdepartmental Working Group 1995 Report on Sensible Drinking (DH, 1995). At that time, the Committee concluded that the consumption of alcoholic beverages was of no concern in relation to their mutagenic potential. In 2000, the Committee on Carcinogenicity (COC) requested an update of the COM's 1995 opinion, following the COC's statement in 2000 on alcohol consumption and breast cancer; there being reported evidence of a possible association and hypotheses proposed on the potential etiological role of reactive oxygen species (ROS) (COC, 2000). The COM evaluated studies published between 1995 and 2000 to update their review (COM, 2000a) and concluded that there was insufficient evidence to support the hypothesis that alcohol induces breast cancer via the formation of ROS. The COM also reaffirmed its previous conclusion with respect to the lack of concern regarding the mutagenic potential of alcoholic beverages (COM, 2000b).

2. In 2013, the Committee on Carcinogenicity of chemicals in Food, Consumer Products and the Environment (COC) started to reassess the relationship between alcohol consumption and cancer, and requested a further update of the COM opinion, to provide insight into potential mechanisms.

3. This statement details the conclusions reached by the COM with regard to the published evidence on the genotoxicity and mutagenicity of ethanol, acetaldehyde and alcoholic beverages from January 2000 to May 2014, including the modifications made to the conclusions drawn in 2000.

4. A discussion on the role of reactive oxygen species and other metabolites in the genotoxicity and mutagenicity of alcohol is also included.

Mutagenicity of ethanol

5. The Committee noted that seven new in vitro genotoxicity studies on ethanol have been published since January 2000, mostly in human cell lines and also primary cells collected from humans or rats. Effects suggestive of genotoxicity were generally reported, however, the Committee were unable to draw any clear

conclusions due to the poor quality or relevance of the evidence. The four-fold increase in levels of DNA strand breaks detected in primary human gastric mucosa cells was associated with exposure to a very high concentration of ethanol (1M) that would most likely cause secondary effects such as irritation, dehydration and cell tissue damage (Blasiak et al., 2000). The Committee felt that the damage caused to the genome from such effects would be difficult to distinguish from any primary effects that ethanol might have.

6. Although three of the studies observed statistically significant increases in genotoxic endpoints in cells exposed to ethanol at concentrations within a range realistically achievable in alcohol drinkers, no clear conclusions could be reached (Lamarche et al., 2003, Benassi-Evans & Fenech., 2011, Kayani & Parry., 2010). DNA strand breaks in acutely exposed primary rat astrocytes were associated with only a very marginal increase in the percentage of DNA in the tail of exposed cells (Lamarche et al., 2003) or no effect (Signorini-Allibe 2005). Chromosome damage and genome instability observed in a poorly characterised human lymphoblast cell line (WIL2-NS) could not be attributed with confidence to a proposed aneugenic effect of ethanol in the absence of any studies to rule out acetaldehyde as the causative agent (Benassi-Evans & Fenech., 2011).

7. Members agreed that Kayani & Parry (2010) provided an interesting observation that the induction of micronuclei (MN) by ethanol appeared to be by an aneugenic mechanism. Members suggested that the increase in kinetochore positive (K^+) MN in ethanol treated cells might also be explained by: (i) spindle damage via an oxidative effect (although, it was noted that in vivo studies provide evidence that contradicts such a direct acting MOA); (ii) an artefact due to the acetaldehyde-protein cross-links which may disrupt the kinetochore protein leading to a false kinetochore negative (K^-) responses. Members felt the data were difficult to rationalise, but the findings could not be ignored. Further investigations would be required before definite conclusions could be drawn by the Committee.

8. A number of new in vivo studies were available in which DNA adduction, and the formation of MN, chromosome aberrations (CAs) and DSBs, had been investigated in rodents exposed to ethanol orally at concentrations of up to 20% v/v in the drinking water. The Committee found that the studies evaluating genotoxic endpoints were unclear, and yielded mixed results that made them difficult to interpret. A study investigating MN in rats chronically exposed to 0 to 15% v/v ethanol (Ellahueñe et al., 2012) found no alcohol-related increase in MN frequency after accounting for the confounding effect of age-dependent increases in MN. This contrasted with the findings of two other studies in rodents exposed subchronically to 10% ethanol, in which there were increases in MN (Kotova et al., 2013; Cebral et al., 2011). Inconsistent results were also obtained in studies of the types of acetaldehyde-specific DNA adducts detected in the liver and stomach of aldehyde dehydrogenase-type 2 knockout mice chronically exposed to 20% ethanol v/v (Matsuda et al., 2007; Nagayoshi et al., 2009). N^2-ethylidene-dG adduct levels were increased by up to 40-fold in ALDH2-knockout mice compared to untreated wild-type mice, whereas no N^2-ethyl-dG or α-Me-γ-OH-PdG adducts could be detected.

9. Members emphasised the importance of considering the capacity of a tissue to metabolise ethanol via the microsomal P450 2E1 (CYP2E1) monooxygenase

pathway in light of growing evidence of the involvement of this enzyme in the genotoxicity of alcohol from experiments conducted in animals (and in vitro) (Seitz and Stickel 2006; Lindhart et al 2014).

10. Members considered that the in vivo studies evaluating effects of ethanol in germ cells did not provide any data of relevance to its mutagenicity (Cebral et al., 2011; Talebi et al 2011; Ellahueñe et al., 2012; Rahimipour et al 2013).

11. The Committee updates its previous conclusion with regard to the mutagenicity data on ethanol: namely that although some new in vitro studies reported evidence of genotoxicity, the mixed findings from animal experiments in vivo and the poor quality of the studies for ethanol in general, prevent any definite conclusions from being drawn.

Mutagenicity of acetaldehyde

12. Acetaldehyde is widely accepted as being genotoxic in vitro and in vivo, when administered directly. However, there is uncertainty whether such effects occur when it is produced in vivo following metabolism of ethanol.

13. The Committee agreed that the recent in vitro data on acetaldehyde added further strong evidence for the genotoxicity of this compound. These studies, described below, all reported that acute exposure of human and mammalian cells resulted in the induction of MN, sister chromatid exchanges (SCEs), and DSBs. However, as documented in previous statements, many of these effects were observed only with high concentrations of acetaldehyde, well above those that would be experienced in human saliva, blood or tissues after drinking alcohol.

14. Members noted the number of recent studies that have helped characterise some of the key lesions thought to play a role in the mutagenicity of acetaldehyde; namely interstrand DNA cross-links (Blasiak et al., 2000), 1,N^2-propano-dG or PdG (Wang et al., 2000, Sako et al., 2003), and N^2-ethylidene-dG (Hori et al., 2012), albeit using very high concentrations of acetaldehyde. Studies conducted in bacterial and in human cell lines transfected with either synthetically-derived N^2-ethyl-dG or PdG adducts (Stein et al., 2006; Upton et al., 2006;) or exposed to high levels of acetaldehyde (Noori & Hou., 2001) suggested guanine nucleotides were the primary targets for point mutations, particularly G to T transversions.

15. Concentration-dependent increases in PdG adducts were observed in a study in which pig liver DNA was exposed directly to acetaldehyde at a concentration range realistically achievable in saliva (Theruvathu et al., 2005). Detectable amounts of PdG adducts were formed in the presence of polyamines at concentrations as low as 100µM acetaldehyde. Mammalian cells exposed to biologically relevant concentrations of acetaldehyde also exhibited concentration-dependent increases in MN (Kayani & Parry., 2010); Speit et al., 2008), SCEs (Speit et al., 2008) and DSBs (Signorini-Allibe et al., 2005).

16. The Committee considered the suggestion that acetaldehyde induction of MN is via a clastogenic mechanism (Kayani & Parry, 2010). Members felt that despite

the concentration-dependent increase in kinetochore negative (K^-) cells and the decrease in kinetochore positive (K^+) cells there was no evidence of acentric fragments. However this may not represent a clastogenic response because acetaldehyde is thought to interact with kinetochore proteins, due to the artefactual binding described previously (see paragraph 7) The Committee suggested the proposed clastogenic effect could be considered plausible, until there was evidence to the contrary.

17. Members considered Kotova et al (2013) to be a very sound study, which provided a plausible hypothesis for the mechanism of induction of MN by acetaldehyde: namely via formation of replication-associated DSBs in dividing cells. However, given that this was just one study in cells exposed to very high concentrations of acetaldehyde, further investigation would be required before conclusions could be reached by the Committee.

18. A single study evaluated the possible in vivo genotoxicity of acetaldehyde (Torres et al., 2002). The implications of the observed dose-dependent increase in levels of SCEs in treated mice were limited by the choice of route of exposure (i.p.) and the use of unrealistically high doses of acetaldehyde.

19. The Committee updates its previous conclusion and notes that the weight of evidence for the in vitro mutagenicity of acetaldehyde has been further strengthened, particularly with regard to generation of specific DNA adducts and induction of MN in mammalian cells at concentrations of acetaldehyde realistically achievable from alcoholic beverage consumption.

Mutagenicity of alcoholic beverages

20. The Committee noted a number of recent studies investigating genotoxic and mutagenic effects arising from the consumption of alcoholic beverages in humans. No new studies were identified in experimental animals or in vitro. A potential for publication bias was highlighted by the fact that the majority of studies reported positive findings for all of the mutagenic and genotoxic endpoints assessed.

21. Members considered the extent of exposure to sources of ethanol other than from alcoholic beverage consumption, and to other alcohols including the lower volatile alcohols such as propanol and methanol. The Committee considered that with the exception of sanitizers, mouthwashes, and personal care products, exposure to these alcohols generally occurs from endogenous production (production by gut bacteria, and the fermentation of yeast and fruit) or from the diet, and only in minute quantities compared to consumption of alcoholic beverages. Members were informed that these additional sources are not being considered by the COC. Members agreed that there is no evidence to suggest that exposure to these additional sources of ethanol/alcohols would significantly increase the frequency of genotoxic events above background levels in non-drinking individuals.

22. In one study, no change in basal levels of the N^2-ethylidene-deoxyguanosine (N^2-ethyl-dG) adduct were observed in peripheral blood white cells taken from healthy non-smoking Polish volunteers, 48h after exposure to 150ml of vodka in an

experimental setting (Singh et al., 2012). This contrasted with the dose-dependent increase in adduct levels observed in peripheral blood white cells and oral epithelial cells of healthy non-smoking US University students/staff who achieved up to 0.07% blood alcohol concentrations within three weeks under controlled exposure conditions (Balbo et al., 2012ab). Exposure to alcohol increased adduct levels in oral epithelial cells by up to 15-fold compared to background levels. However, substantial intra-individual variation in baseline adduct levels was apparent in both studies, the US study did not account for body weight index (BMI), a known carcinogenic risk factor, and furthermore, the human DNA obtained from mouthwash samples was potentially contaminated with bacterial DNA. Members advised caution in interpreting the results of studies of DNA adduction due to the different approaches used in the studies. For example, different sensitivities of the study methods, duration of exposure and account for confounding factors affecting background adduct levels (e.g. BMI, diet or nutritional status)

23. Members considered that the N^2-ethyl-dG adduct was a good biomarker of acetaldehyde exposure, although its specificity for ethanol exposure was questioned given that the adduct is also generated endogenously (present in normal animal and human liver DNA at levels in the range of 0.1 lesion/10^6 normal nucleotides, IARC 2010). Exposure to acetaldehyde also arises from endogenous processes, and can occur through diet/lifestyle and occupation.

24. Few of the observational investigations reviewed had stratified drinking categories into levels that would enable evaluation of a quantitative dose-response relationship. The changes observed in mutagenic endpoints assessed in studies that provided two or more drinking categories either were not dose-dependent (Ishikawa et al., 2007, Lu & Morimoto., 2009), or the strength of evidence that the effects observed were due to alcohol consumption (e.g. increased DNA adduction) was weakened by use of relatively unreliable estimates of exposure (questionnaire) and/or lack of consideration of potential confounders (Balbo et al., 2008).

25. Members considered the reported evidence of chromosomal damage and MN induction across studies. Evidence for increased levels of MN in binucleated peripheral blood lymphocytes of clinically diagnosed alcoholic subjects was limited either by the small size of the studies high control MN frequencies (Maffei et al., 2000; 2002) or the lack of account of smoking differences between subjects, in addition to other factors already discussed (Ramirez & Saldanha., 2002). Members cautioned that the evaluation of binucleate MN was a complicating factor as MN in binucleated cells could be considered to be representative of ex-vivo formation. Furthermore, only two of the eight studies reporting on MN induction provided data on the range of background levels of MN in controls; most reported only mean and standard deviation. The Committee recalled that background levels of MN and chromosomal aberrations in human peripheral blood lymphocytes were previously considered by COM (with respect to pesticide exposure) when it was concluded that the large variability in background levels complicates interpretation of human genotoxicity studies (Battershill et al 2008).

26. The Committee noted that several recent studies on the mutagenicity of alcoholic beverages had evaluated the influence of polymorphisms in alcohol and

Comment [K1]: Detection range 2-400fmol – results expressed as Level of N2-ethyl-dG/10^8 nucleosides – background level range 14.3-116 (mean approx 30)

Comment [K2]: Detection range 0.1-100fmol –results expressed as level of N2-ethyl-dG fmol/umol of dG) –Study a background range 520-12,550 (most 7-9ooo) – study b range 244-4892

Comment [BG3]: Is there evidence that BMI is a confounder for genotoxicity?

Comment [BG4]: As above – is there any evidence that BMI directly affects background adduct levels?

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aldehyde dehydrogenases (ADH, ALDH[1]) and P450 2E1 (CYP2E1), all in Japanese subjects. It was agreed that data in ALDH2-deficient individuals (genotype ALDH2*2 – conferring slow activity) was particularly noteworthy, with consistent evidence of higher adduct levels and MN in individuals bearing the ALDH2-deficient allele (ref). A single study, of hospitalised alcoholics, assessed alcohol exposure using both a questionnaire and measurement of alcohol concentrations in the blood and saliva (Yukawa et al., 2012). The average alcohol intake measured over a 24h period was 100ml ethanol (equivalent to 80 g/day). Levels of N^2-ethyl-dG adduct were highest in subjects with ALDH2*2 genotype who were also hetero/homozygotic for ADH1B*2 genotype (confers faster activity) compared to wild-type. Elevated levels of N^2-ethyl-dG and α-Me-γ-OH-PdG DNA adducts were also detected in a similar cohort of Japanese patients who reported consuming an average of 105 grams alcohol per day in the year proceeding admission (Matsuda et al., 2006). However, both these studies were relatively small in size and did not account for the nutritional status of individuals. Deficiency in micronutrients i.e. key vitamins and minerals such as selenium, vitamin E, folate and impaired glutathione levels are common in heavy drinkers, and can lead to impairment of DNA defence mechanisms and thus higher levels of genotoxicity.

27. The remaining studies on polymorphisms based their exposure assessment either solely on self-completed questionnaires (Ishikawa et al., 2003; 2006; 2007; Lu & Morimoto., 2009; Wu et al., 2010) or provided no details on the exposure method used (Weng et al 2010). Higher levels of MN or DSBs in ALDH2-deficient subjects were detected, although the robustness of the data reported in three studies was weakened by the use of drinking frequency and not alcohol intake as the exposure metric (Ishikawa et al., 2006; Weng et al., 2010; Wu et al., 2010). One study that accounted for both BMI and nutrition observed a negative association between DSBs and drinking frequency in ALDH2-deficient subjects (Lu & Morimoto., 2009). In considering the author's suggestion that alcohol consumption might induce DNA crosslinks, it is important to note that the homozygous ALDH2-deficient group smoked significantly more cigarettes than the other groups, which confounds the interpretation of these data.

Comment [K5]: only n=9 compared to the n=67 of the heterozygous deficient who smoked less. Is this of importance?

28. Data from studies looking at combinations of ADH and ALDH polymorphisms were inconsistent. Several studies reported increased genotoxicity when ALDH2*2 genotype was associated with the ADH1B*2 genotype (confers faster ADH activity – Weng et al., 2010; Wu et al., 2010); whilst Ishikawa et al (2007) observed increased genotoxicity when ALDH2*2 was associated with ADH1B*1/*1 or ADH1B*1/*2 genotypes (confers slower activity – Ishikawa et al., 2007);

29. Overall, Members concluded that most of the studies investigating genotoxicity of alcoholic beverage consumption did not account for the confounding effects of BMI, or nutritional intake. Members also considered other quality issues limited the reliability of the study findings e.g. use of small sample sizes, and poor exposure assessments based solely on self-completed or interview-led

Comment [BG6]: See previous comments

[1] Polymorphisms in *ADH1B* result in variants that code for isozymes that show a faster rate of alcohol metabolism, while the *ALDH2*2* polymorphism results in a "deficient" form of *ALDH2* that causes an accumulation of acetaldehyde and its associated physiological effects.

questionnaires. A handful of studies did not assess alcohol intake or account for smoking as a confounding exposure.

30. In view of these limitations, the Committee updates its previous conclusion on the genotoxicity of alcoholic beverages to acknowledge the emergence of additional studies on DNA adduct formation in humans, and studies reporting the influence of polymorphisms in enzymes involved in alcohol metabolism, particularly in relation to induction of MN. However, the poor quality of most of these studies prevents any useful conclusions from being drawn.

Hypotheses for the role of reactive oxygen species in the genotoxicity and mutagenicity of alcohol

31. The Committee considered a paper reviewing the theory which associates alcohol induced liver disease and carcinogenesis with the generation of reactive oxygen species (ROS) and the role of cytochrome P450 2E1 (CYP2E1) in this process [MUT/2015/02]. Whilst it was noted that the hypotheses were based on circumstantial associations generated principally from one group of researchers (Seitz and Stickel 2006, 2007, Wang et al 2009, Linhart et al 2014), Members agreed that there was sufficient evidence to support the hypotheses.

32. Alcohol consumption can result in the formation of reactive oxygen species either via inflammatory mediated processes or oxidative metabolism. Reactive oxygen species have the potential to generate lipid peroxidation products which in turn may yield mutagenic, exocyclic DNA adducts.

33. Ethanol consumption also results in the induction of CYP2E1, primarily in the liver, but also in certain extra-hepatic tissues such as the oesophagus and intestine. It is suggested that this induction enhances the metabolism of alcohol to acetaldehyde, the generation of ROS and accordingly the associated hazard of adduct formation. A correlation between CYP2E1 levels and DNA etheno adducts has been demonstrated in animal models and in humans. However an association between specific CYP2E1 polymorphisms and alcoholic liver damage or alcohol-induced carcinogenesis is not well defined.

34. Overall Members considered the hypothesis that alcohol-induced oxidative stress is of importance in the pathogenesis of alcohol-induced liver injury, including carcinogenesis, to be plausible and that there was evidence to support them. However, more work would be required in this complicated area before definitive conclusions could be drawn.

Hypothesis for the role of other metabolites in the genotoxicity and mutagenicity of alcohol

35. Members reviewed the recent publication by Mitchell et al (2014), which provided confirmation that ethyl sulfate is a metabolite of ethanol. The physicochemical properties of ethyl sulfate suggest an ability to alkylate biological macromolecules. The authors noted that in chronic alcoholism, ethyl sulfate would be continually available via phase II sulfonation. The authors cautioned that further

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research was necessary to delineate the metabolic fate of this compound and the extent to which the reaction occurs in vivo.

36. Members agreed that these findings were of interest, but required biological evidence of the chemical reactivity of ethyl sulfate and its ability to form DNA adducts in vivo. Members expressed concern about the difficulties of studying this in vitro, namely the absence of sulfonation metabolism in available cell models and the difficulty in testing exogenously applied sulfate compounds, which do not readily pass through cell membranes. Potential experimental strategies to overcome these issues would be possible and include use of sulfotransferase knock-out and knock-in cells.

Overall Conclusion

37. The Committee agreed that the conclusions reached in 2000 would need updating in view of the additional studies investigating the mutagenic and genotoxic potential of ethanol, acetaldehyde and consumption of alcoholic beverages in humans. The following overall conclusions were agreed:

a. The COM concluded that acetaldehyde remains the metabolite of most concern with respect to the genotoxic effects of alcohol.

b. The Committee noted that a number of studies have implicated the formation of acetaldehyde-specific DNA adducts and interstrand DNA crosslinks as upstream events in the genotoxicity of alcohol. However, the poor reliability of data available from studies on the genotoxicity of ethanol and alcoholic beverages (the latter being subject to a number of potentially confounding factors) in man prevent the Committee from drawing any clear conclusions on the genotoxicity of alcohol per se.

c. The Committee concluded that studies investigating polymorphisms in key enzymes involved in ethanol metabolism suggest that the ALDH2-deficient genotype is likely to contribute to the overall mutagenic and genotoxic potential of alcohol. Currently available data are inconsistent for polymorphisms in other enzymes in this respect.

d. The existing evidence is insufficient to support the suggestion that MN induced by ethanol occur via an aneugenic mechanism and by acetaldehyde via a clastogenic mechanism. Data suggest that multiple modes of action contribute to the overall genotoxicity.

e. There is currently emerging, but limited evidence to support the proposal that acetaldehyde induces micronuclei via formation of replication-associated double stranded breaks in dividing cells. However, the Committee acknowledges the plausibility of this hypothesis.

f. Further research is needed to determine whether the recently identified metabolite of ethanol, ethyl sulfate, contributes to the genotoxicity of ethanol and of alcoholic beverages.

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g. The Committee concluded that oxidative damage may also be an important mechanism of concern in the genotoxicity of alcohol. Studies on the capacity of target cells/tissues to metabolise ethanol via CYP2E1 and also consideration of the role of ADH in producing reactive oxygen species would further aid understanding of the role played by oxidative damage to DNA.

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Date 2015

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