MUT/2015/02

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

The Potential Role of Oxidative Damage in Alcohol's Mutagenic and Carcinogenic Mode of Action – An Overview

1. The COC is currently undertaking a substantial review of the carcinogenicity of alcohol and to support this review, COM evaluated recent information on the mutagenicity of alcohol and its principle metabolite, acetaldehyde at the meeting in October 2014. Whilst reviewing the literature it became apparent that there is a significant body of work which suggests that oxidative damage caused by alcohol may contribute to its mutagenic and carcinogenic mode of action (MOA). Members showed interest in considering this aspect of alcohol's MOA as part of their review.

2. This paper provides an overview of the hypothesis that alcohol induces CYP2E1, generates reactive oxygen species and carcinogenic etheno-DNA adducts and this is important in alcohols carcinogenic MOA. A systematic review of the literature was not undertaken as the research area is vast and wide-ranging, but it is hoped that this is a good representation of the current evidence, including studies assessing oxidative endpoints in humans following alcohol consumption. However it is not always possible to assess what is hard evidence and what is optimistic speculation. A recent review article, presenting the hypothesis, is appended (Linhart et al 2014) for Members consideration.

3. Members are invited to review the paper with view to generating a section to be included in the statement on mutagenicity of alcohol (MUT/2015/01). A number of questions are given to guide discussion.

Secretariat/PHE Toxicology Unit

February 2015

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MUT/2015/02

The Potential Role of Oxidative Damage in Alcohol's Mutagenic and Carcinogenic Mode of Action – An Overview

Introduction

1. As an adjunct to the ongoing review of alcohol consumption and cancer by COC, the COM reviewed a paper summarising studies since 2000 investigating the genotoxicity of alcohol, with a view to updating the current statement (COM 2000). The paper focussed on the genotoxicity of alcoholic beverages in consumers, mutagenicity tests of ethanol (EtOH) and acetaldehyde (AA), its primary metabolite and on the generation of DNA adducts by EtOH. In the review of the literature, a number of reports which examined the potential for alcohol to cause oxidative damage to DNA and proteins were retrieved. At the meeting in October 2014, COM were presented with brief summaries of these studies. It was decided that a detailed or systematic review of this topic was not necessary. However it was suggested that an overview of the potential role of oxidative damage, and the role of CYP2E1 in particular, would provide useful insight into the overall mode of action of alcohol-induced mutagenesis and carcinogenesis. It is considered that it might be helpful to include a short section on this topic in the COM's statement on alcohol and mutagenicity.

Oxidative damage mode of action in [hepato]carcinogenesis

2. There is an extensive body of research which associates the toxicity of alcohol in alcoholic liver disease (ALD) with the generation of reactive oxygen species (ROS), the resultant lipid peroxidation and free-radical mediated damage (Albano 2006). In animal models, particularly in animals fed diets high in polyunsaturated fatty acids, pathological liver damage correlated with markers of lipid peroxidation such as manganese superoxide dismutase (MnSOD), glutathione peroxidise and catalase levels (Nanji et al 1994; Polavarapu et al 1998). Chronic alcohol consumption is associated with inflammation, which is considered to be a major factor in the development of alcoholic hepatitis (Seitz and Stickel 2006).

3. The relationship between inflammation, oxidative stress, generation of ROS and carcinogenesis is well established for a number of cancers including lung, liver and pancreas (Nair et al 2007; Filaire et al 2013; Valavanadis et al 2013; Seitz and Stickel 2006; Ling et al 2014). Inflammatory reactions can generate ROS and reactive nitrogen species via macrophage activation, which are believed to cause DNA damage directly (via deamination or guanine oxidation), or indirectly via the decomposition of lipid peroxidation products such as 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) (Lonkar and Dedon 2010). These can result in the production of DNA reactive electrophiles and subsequent generation of hydroxyethyl radicals (HER) and exocyclic DNA etheno adducts (e.g. N^6-etheno-2'-deoxyadenosine, εdA; N^4-etheno-2'deoxycytidine, εdC) (Bartsch and Nair 2004; Blair 2008).

4. Etheno adducts are considered to be highly mutagenic (Barbin 2000). Evidence for this includes, the detection of persistent adducts which arise during

vinyl chloride and urethane carcinogenesis (Barbin et al 2000; Swenberg et al 1992). The mutations identified following alcohol exposure include: AT→GC transitions and AT→TA and AT→CG transversions induced by εdA and CG-AT transversions and CG→TA transitions induced by εdC (summarised in Linhart et al 2014- ANNEX 1). It is also suggested that etheno adducts are preferentially formed in codon 249 of TP53 (Hu et al 2002).

5. There is a large database of studies which have investigated exocyclic DNA adducts, such as those generated by MDA and HNE, as biomarkers of oxidative stress in both rodent and human studies. There are strong associations between the generation of these adducts and human cancers considered to have an inflammatory component in their aetiology (Nair et al 2007). As well as etheno adducts, there are many adducts considered to arise from an oxidative mode of action (MOA) of alcohol or acetaldehyde exposure – these include N^2-ethyl-2'-deoxyguanosine (Singh et al 2012) and 8-oxoguanine (8-hydroxyguanine) (Hirano 2011).

6. 8-Hydroxy-2'-deoxyguanosine (8-OH-dG) adducts are repaired by by 8-Oxoguanine glycosylase (OGG1) and there are examples of both being used as biomarkers to estimate ROS-induced DNA damage in studies investigating alcohol induced toxicity (Hirano et al 2009; Guo et al 2008). However as repair of 8-OH-dG is efficient, it may not be a good marker of oxidative damage.

7. Studies in both animals and humans support the hypothesis that free-radical oxidative damage is of importance in alcohol-induced hepatotoxicity generally and that chronic inflammation is likely to contribute to the responses observed (Albano 2006; Bartsch and Nair 2006; Seitz and Stickel 2006).

Alcohol and Cytochrome P450 2E1

8. Cytochrome P450 2E1 (CYP2E1) is widespread amongst mammalian species and is considered to be of particular importance in toxicology as it is known to metabolise a wide variety of xenobiotics, including many which result in the production of toxic metabolites (Novak and Woodcroft 2000). It is transcriptionally and post-transcriptionally regulated, and induced by a wide range of substances including ethanol. CYP2E1 is primarily localised in the liver. Increased expression, up to 10-20-fold, has been demonstrated in rats following treatment with ethanol (Ingelman-Sundberg et al 1993; Albano et al 1996) and it is elevated in humans following high, chronic alcohol consumption (166 ± 10.8 g/day) by up to 10-fold (Dupont et al 1998) in the liver. It is also induced in some extrahepatic tissues, including the oesophagus and intestines (Seitz and Wang 2013). It is estimated to have a turnover rate of approximately 2.5 days in humans – this makes it possible to monitor changes during alcoholic withdrawal with relative accuracy.

9. CYP2E1 has a high rate of NADPH oxidase activity which results in enhanced production of ROS (O_2^- and H_2O_2) from O_2 and consequent generation of HER and lipid peroxidation products (Seitz and Stickel 2007). This is evidenced *in vitro* and *in vivo,* in both animal models and in humans. CYP2E1 metabolises alcohol to acetaldehyde, with concomitant generation of ROS (Seitz and Wang 2013).

10. A role for CYP2E1 in alcohol-induced liver damage is well established in animal models. For example, key studies in rats showed that the administration of CYP2E1 inhibitors (diallyl sulphide, DAS and phenyliocyanate PIC) reduced lipid peroxidation, liver damage and hydroxyethyl radicals induced by ethanol treatment (Morimoto et al 1995; Albano et al 1996). Oxidative damage, as measured by oxidative DNA adducts, mutagenic apurinic/pyrimidinic sites, and expression of base excision DNA repair genes, was not seen in the livers of CYP2E1 null mice compared to wild-type mice and rats given EtOH orally in liquid diet (Bradford et al 2005). In mice, transgenic for human CYP2E1, liver damage following administration of an alcohol rich diet (30% of calories) is more pronounced than in non-transgenic mice (Morgan et al 2002). In a rat model of alcoholic liver disease (genetically obese Zucker (fa/fa) rats and their lean littermates (Fa +/-), a correlation between adducts (εdA, εdC) and CYP2E1 localisation was demonstrated in the liver following alcohol treatment. However there was no significant difference in hepatic 4-HNE protein adducts in rats (Wang et al 2009).

11. Results of early long-term bioassays suggested that alcohol is not itself a carcinogen when administered under experimental conditions to animals but more recent studies report increased tumours in treated rats and mice (summarised in IARC 2010; Seitz and Stickel 2006). The ability of EtOH to enhance chemically-induced carcinogenesis such as that of nitrosamines is well documented and it is proposed that this co-carcinogenic activity is mediated via induction of oxidative metabolism by CYP2E1 which increases the generation of DNA reactive metabolites and exacerbation of DNA damage (IARC 2010; Seitz and Stickel 2006). There are a number of studies which have examined the potential of alcohol to enhance mutagenicity or carcinogenicity of nitrosamines in animal models. For example,

12. In a study examining the impact of EtOH administration on N-nitrosodiethylamine (NDMA) induced effects, rats were given EtOH as an acute dose (5g/kg) or for 4 weeks in liquid diet (5%w/v) with or without an acute dose of NDMA (4mg/kg). EtOH treatment increased CYP2E1 protein levels and NDMA demethylase activity. Enhanced formation of εdA and εdC were reported. NDMA increased O^6-Me-G levels and this was further increased by administration of EtOH (Navasumrit et 2001):

13. In rats treated with alcohol in drinking water with and without three daily doses of N-nitrosomethylbenzeylamine (NMBA 0.5 mg/kg) for 5 weeks, increased levels of CYP enzymes in the liver and oesophagus, including CYP2E1 (Tatematsu et al 2013). It is suggested that localised CYP2E1 induction contributes to nitrosamine activation in the oesophagus [Additionally, microsomes from alcohol treated rats significantly enhanced mutagenicity of a range of nitrosmaines (NPYR, DMN, DEN, NMBA) in the Ames test Mori et al 2002]

14. In humans, the synergistic carcinogenic effect of alcohol and tobacco may also be explained, to some extent, by the induction of CYP2E1 by alcohol, particularly in extra-hepatic tissues such as the oesophagus. This mechanism was examined by COC as part of their review of the effects of mixtures and co-administrations on cancer in man (CC/08/10 – ANNEX 2).

15. Linhart et al (2014, ANNEX 1) provides an overview of CYP2E1, the generation of ROS and etheno-adducts focussing on alcohol as an example. As well as describing the role of inflammatory processes and studies in which exocyclic DNA adducts are generated from lipid peroxidation as by-products from CYP2E1 induction, strong correlations between CYP2E1 induction and etheno-adduct formation in the liver are described. Furthermore, investigations which have assessed CYP2E1 in patients with oesophageal and colorectal tumours also point towards a role for CYP2E1 induction in their aetiology

Investigative studies in humans

16. There are a number of papers which have investigated the relationship between alcohol consumption in humans, CYP2E1 induction/expression and the generation of etheno adducts. CYP2E1 activity in man is often measured by chlorzoxazone (CHZ) metabolism, a substrate considered to be highly specific for this enzyme.

17. Oneta et al (2002) examined the kinetics of CYP2E1 in non-alcoholic and alcoholic males. Five healthy male volunteers were given 40 g ethanol/day for 4 weeks. CYP2E1 activity was increased, measured by CHZ metabolism after 1 week of ethanol consumption and continued to increase over the 4 week dosing period. In a second arm of the study, CYP2E1 activity was significantly reduced in alcoholics (n=5) 3 days after alcohol withdrawal, although the results were variable.

18. In a study examining CYP2E1 activity in alcoholics, 40/51 patients had elevated CYP2E1 (as measured by CHZ metabolism) – the remaining were within control range (Dupont et al 1998). It was also reported that alcohol increased IgG against protein-adducts of hydroxyethyl free radicals and a correlation with CYP2E1 inducibility is suggested. The authors speculate on a link between induction of CYP2E1 and the development of alcoholic liver disease.

19. In a further study by the same group, CYP2E1 by CHZ metabolism was assessed in 40 chronic alcoholics and 18 controls (<20g alcohol/day) (Dupont et al 2000). Parameters of oxidative stress, plasma lipid peroxides (LPO) and plasma vitamin E were determined and antibodies to HER and MDA adducts provided a measure of oxidative damage. CYP2E1 activity was significantly correlated with alcohol consumption and plasma levels, though the association was weak. However, the markers of oxidative stress in alcoholics, although discussed by the authors in terms of being increased in alcoholics, (by up to 20%) were not statistically different from controls (with exception of HER adducts)

20. CYP2E1 and CYP3A activities were assessed in 20 subjects with moderate alcohol consumption (140-210g/week) and compared to 20 age, gender BMI matched, non-drinker controls (Liangpunsakul et al 2005). mRNA of each CYP was measured in peripheral lymphocytes and enzyme activity was measured by clearance of midazolam (MDZ) and CHZ for CYP3A and 2E1 respectively. CYP2E1 mRNA and clearance of CHZ were significantly increased by moderate alcohol consumption, and whilst CYP3A4 mRNA was increased, MDZ clearance and PK parameters were unaffected.

21. In a study using liver biopsy samples from patients with ALD, it was shown that there was a strong correlation between CYP2E1 expression, protein bound 4-HNE and the formation of etheno-DNA adducts (Wang et al 2009). The association was corroborated with results from a rat model of ALD as described above. In HepG2 cells transfected with human CYP2E1, immunohistochemical detection of εdA was noted after EtOH administration but not in vector mock cHepG2 cells. Formation of εdA was prevented by the CYP2E1 inhibitor chlormethiazole (CMZ).

22. An evaluation of oxidative damage endpoints was undertaken in patients with alcohol dependency (Chinese population sample - 79 alcoholics – mean consumption 196.5 g/day, 63 non-drinker controls). Levels of 8-OHdG and MDA were measured in blood taken at baseline and 1 week after alcohol detoxification to investigate changes in these markers during alcohol withdrawal syndrome (Chen et al 2011). At baseline, MDA and 8-OH-dG were higher in alcoholics compared to controls. Following alcohol withdrawal, MDA was reported to be reduced but 8-OH-dG was increased. However the authors state that the 8-OH-dG levels may be confounded by factors such as age, smoking and vitamin status.

23. Bianchini et al (2001) examined the relationship between alcohol consumption and 8-OH-dG adducts in lymphocytes from groups of non-smoking women from different parts of Europe (n= 24, 27, 28 and 36). There was an inverse relationship between adducts and consumption in all four study groups which persisted after re-adjusting for possible confounders such as fruit and vegetable consumption, plasma carotinoids.

24. An investigation of human oesophageal tissue biopsies aimed to examine the hypothesis that CYP2E1 induction may also contribute to alcohol's MOA in oesophageal cancers (Millionig et al 2011). The study aimed to determine if chronic alcohol consumption induces CYP2E1 in oesophagus similar to the liver and if there is a correlation between induction, exocyclic etheno adducts and a cell proliferation marker, Ki67 (all were measured using immunohistochemical techniques). Tissues were taken from 37 patients with oesophageal cancer (2 teetotallers, 10 1-60g/day, 25 >60g/day) and compared with tissues from 16 from non-cancer patients (12 teetotallers, 4 0-25g/day). Smoking status was also taken into consideration. CYP2E1 expression and etheno-adduct levels were strongly correlated in the individual samples examined. Smokers who drank had a significantly higher proliferation rate, CYP2E1 expression and nuclei containing etheno adducts than those that didn't – similarly patients with cancer had higher levels of all three markers compared to those without cancer. Alcohol consumption correlated with CYP2E1 expression and etheno adducts but not proliferation. Whilst this study looks robust there are also reports of a number of other CYP forms (e.g. CYP2C8, CYP3A4, CYP3A5), elevated in oesophageal cancer, and therefore the role of CYP2E1 is not clear cut (Bergheim et al 2007)

The role of CYP2E1 Polymorphisms in alcohol's mode of action

25. The human *CYP2E1* gene exhibits several polymorphisms (see the Human Cytochrome P450 Allele Nomenclature Database http://www.cypalleles.ki.se/cyp2e1.htm), some of which affect gene expression at the

transcriptional level, whereas others affect enzyme activity. (Pst I/Rsa polymorphism has three genotypes wild type homozygous c1/c1; heterozygous c1/c2 and a rare homozygous form c2/c2). Some published reviews suggest that certain polymorphic forms are associated with cancer susceptibility (Danko et al 2005; Trafalis et al 2010) and it is known that CYP2E1 polymorphisms impact on genotoxicity biomarkers induced by vinyl chloride in exposed workers (Dhillon et al 2011). However it is also reported that there is no association between CYP2E1 polymorphisms and cancer of the oesophagus (Yang et al 2005).

26. Genetic polymorphisms have been investigated with a view to understanding the variation in susceptibility to alcohol related liver diseases. Lee et al (1997) concluded that there is a lack of association of the c1/c2, c2/c2 polymorphisms of CYP2E1 with the risk of HCC in humans. A meta-analysis of the association of 2E1 gene polymorphism and alcohol drinking on the risk of HCC showed that CYP2E1 Pst I/Rsa polymorphism was not associated with HCC risk, while the interaction between Pst I/Rsa polymorphism and alcohol consumption increased the risk of HCC (Liu et al 2014).

27. Ishikawa et al (2006) examined the relationship between alcohol-drinking, ALDH2 and CYP2E1 polymorphisms and MN frequency in 248 Japanese men. The CYP2E1 classification genotypes were CYP2E1 *1/*1 (wild-type), *1/*3 or *3/*3. CYP2E1 *1/*1 genotype was associated with higher MN frequency in habitual drinkers and CYP2E1*2 was associated with lower MN. [These are a different polymorphic variation to c1/c1, c1/c2 classification above].

Conclusions:

28. It is hypothesized that alcohol-induced oxidative stress is of importance in the pathogenesis of liver-induced injury, including carcinogenesis.

29. Reactive oxygen species generated from oxidative metabolism or inflammatory processes gives rise to lipid peroxidation products which in turn may yield mutagenic exocyclic adducts.

30. It is known that ethanol consumption 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 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 CYP2E1 polymorphisms and ALD/alcohol-induced carcinogenesis appears to be weak.

Questions for Members:

- What are Members views on the hypothesis that CYP2E1 induction, ROS generation and etheno adduct formation are part of alcohol's mode of mutagenic and carcinogenic action?
- Do Members know of any other studies or information which they feel should be included in the alcohol mode of action discussions?
- Do Members feel that the topic has been covered in sufficient detail to write a section suitable for the statement on alcohol and mutagenicity?
- If sufficient, Members are invited to provide their comments and suggestions for the content of the section in the statement.

References

Albano E, Clot P, Morimoto M, Tomasi A, Ingelman-Sundberg M, French SW. (1996) Role of cytochrome P4502E1-dependent formation of hydroxyethyl free radical in the development of liver damage in rats intragastrically fed with ethanol. Hepatology. **23(1):**155-63.

Albano (2006) Alcohol, oxidative stress and free radical damage Proc. Nutr. Soc. **65** 278-290

Barbin A (2000) Etheno-adduct-forming chemicals: from mutagenicity testing to tumor mutation spectra. Mutat Res. **462(2-3)**:55-69.

Bartsch H, Nair J. (2006) Chronic inflammation and oxidative stress in the genesis and perpetuation of cancer: role of lipid peroxidation, DNA damage, and repair. Arch Surg. **391(5):** 499-510.

Bergheim I, Wolfgarten E, Bollschweiler E, Hölscher AH, Bode C, Parlesak A. (2007) Cytochrome P450 levels are altered in patients with esophageal squamous-cell carcinoma. World J Gastroenterol. **21;13(7)**:997-1002.

Bianchini F, Jaeckel A, Vineis P, Martinez-Garciá C, Elmstahl S, van Kappel AL, Boeing H, Ohshima H, Riboli E, Kaaks R. (2001) Inverse correlation between alcohol consumption and lymphocyte levels of 8-hydroxydeoxyguanosine in humans. Carcinogenesis.;**22(6)**:885-90.

Blair IA. (2008) DNA adducts with lipid peroxidation products. J Biol Chem. **283(23):**15545-9.

Bradford BU, Kono H, Isayama F, Kosyk O, Wheeler MD, Akiyama TE, Bleye L, Krausz KW, Gonzalez FJ, Koop DR, Rusyn I. (2005) Cytochrome P450 CYP2E1, but not nicotinamide adenine dinucleotide phosphate oxidase, is required for ethanol-induced oxidative DNA damage in rodent liver. Hepatology. 41(2):336-44.

Chen CH, Pan CH, Chen CC, Huang MC. (2011) Increased oxidative DNA damage in patients with alcohol dependence and its correlation with alcohol withdrawal severity. Alcohol Clin Exp Res. 35(2):338-44.

Danko IM, Chaschin NA. (2005) Association of CYP2E1 gene polymorphism with predisposition to cancer development. Exp Oncol**. 27(4)**:248-56.

Dhillon VS, Thomas P, Iarmarcovai G, Kirsch-Volders M, Bonassi S, Fenech M. (2011) Genetic polymorphisms of genes involved in DNA repair and metabolism influence micronucleus frequencies in human peripheral blood lymphocytes. Mutagenesis. **26(1):**33-42.

Dupont I, Lucas D, Clot P, Menez C, Albano (1998) Cytochrome P4502E1 inducibility and hdroxyehyl radical formation among alcoholics. J. Hepatol. **28** 564-571

Dupont 1, Bodenez P, Berthou F, Simon b, Bardou LG, Lucas D. (2000) Cytochrome P450 2E1 activity and oxidative stress in alcoholic pateients Alcohol and Alcoholism **35(1)** 98-103

Filaire E, Dupuis C, Galvaing G, Aubreton S, Laurent H, Richard R, Filaire M. (2013) Lung cancer: what are the links with oxidative stress, physical activity and nutrition. Lung Cancer. **82(3)**:383-9.

Hirano T. (2011) Alcohol consumption and oxidative DNA damage. Int J Environ Res Public Health. **8(7):**2895-906

IARC (2010) IARC Monographs on the Evaluation of Carcinogenic Risks to Humans VOLUME 96 Alcohol Consumption and Ethyl Carbamate , Lyon France.

Ingelman-Sundberg M, Johansson I, Yin H, Terelius Y, Eliasson E, Clot P, Albano E. (1993) Ethanol-inducible cytochrome P4502E1: genetic olymorphism, regulation, and possible role in the etiology of alcohol-induced liver disease. Alcohol. 10(6):447-52.

Khan AJ, Ruwali M, Choudhuri G, Mathur N, Husain Q, Parmar D. (2009) Polymorphism in cytochrome P450 2E1 and interaction with other genetic risk factors and susceptibility to alcoholic liver cirrhosis. Mutat Res. **664(1-2)**:55-63.

Lee HS, Yoon JH, Kamimura S, Iwata K, Watanabe H, Kim CY. (1997) Lack of association of cytochrome P450 2E1 genetic polymorphisms with the risk of human hepatocellular carcinoma. Int J Cancer. **71(5):**737-40

Liangpunsakul S, Kolwankar D, Pinto A, Gorski JC, Hall SD, Chalasani N. (2005) Activity of CYP2E1 and CYP3A enzymes in adults with moderate alcohol consumption: a comparison with nonalcoholics. Hepatology. **41(5)**:1144-50.

Ling S, Feng T, Jia K, Tian Y, Li Y. (2014) Inflammation to cancer: The molecular biology in the pancreas (Review). Oncol Lett. **7(6)**:1747-1754.

Linhart K, Bartsch H, Seitz HK (2014) The roel of reactive oxygen species (ROS) and cytochrome P450 2E1 in the generation of carcinogenic etheno-DNa adducts Redox Biol **3** 56-62

Liu C, Wang H, Pan C, Shen J, Liang Y. (2012) CYP2E1 PstI/RsaI polymorphism and interaction with alcohol consumption in hepatocellular carcinoma susceptibility: evidence from 1,661 cases and 2,317 controls. Tumour Biol. 33(4):979-84.

Lonkar,P. Dedon, P.C. (2010) Reactive species and DNA damage in chronic inflammation: reconciling chemical mechanisms and biological fates Int.J.Cancer **128** 1999-2009

Millonig G, Wang Y, Homann N, Bernhardt F, Qin H, Mueller S, Bartsch H, Seitz HK. (2011) Ethanol-mediated carcinogenesis in the human esophagus implicates CYP2E1 induction and the generation of carcinogenic DNA-lesions . Int J Cancer. 1;128(3):533-40

Morgan K, French SW, Morgan TR. (2002) Production of a cytochrome P450 2E1 transgenic mouse and initial evaluation of alcoholic liver damage. Hepatology. **36(1)**:122-34.

Morimoto M, Hagbjörk AL, Wan YJ, Fu PC, Clot P, Albano E, Ingelman-Sundberg M, (1995) Modulation of experimental alcohol-induced liver disease by cytochrome P450 2E1 inhibitors. Hepatology. **21(6):**1610-7.

Nair U, Bartsch H, Nair J (2007) Lipid peroxidation-induced DNA damage in cancer-prone inflammatory diseases: A review of published adduct types and levels in humans. Free Rad Biol Med **43** 1109-1120

Nanji AA, Zhao S, Sadrzadeh SM, Dannenberg AJ, Tahan SR, Waxman DJ. Alcohol (1994) Markedly enhanced cytochrome P450 2E1 induction and lipid peroxidation is associated with severe liver injury in fish oil-ethanol-fed rats. Clin Exp Res. **18(5):**1280-5.

Navasumrit, P., Ward, T. H., O'Connor, P. J., Nair, J., Frank, N., and Bartsch, H. (2001) Ethanol enhances the formation of endogenously and exogenously derived adducts in rat hepatic DNA. *Mutat. Res*. **479**, 81-94.

Novak RF, Woodcroft KJ. (2000) The alcohol-inducible form of cytochrome P450 (CYP2E1): roel in toxicology and regulation of expression. Arch. Pharm. Res **23(4):** 267-282

Oneta CM, Lieber CS, Li J, Rüttimann S, Schmid B, Lattmann J, Rosman AS, Seitz HK. (2002) Dynamics of cytochrome P4502E1 activity in man: induction by ethanol and disappearance during withdrawal phase. J Hepatol.**36(1)**:47-52.

Polavarapu R, Spitz DR, Sim JE, Follansbee MH, Oberley LW, Rahemtulla A, Nanji AA. (1998) Increased lipid peroxidation and impaired antioxidant enzyme function is associated with pathological liver injury in experimental alcoholic liver disease in rats fed diets high in corn oil and fish oil. Hepatology. **27(5)**:1317-23.

Ingelman-Sundberg M, Johansson I, Yin H, Terelius Y, Eliasson E, Clot P, (1993) Ethanol-inducible cytochrome P4502E1: genetic polymorphism, regulation, and possible role in the etiology of alcohol-induced liver disease. Alcohol. **10(6):**447-52.

Seitz HK & Stickel F (2006). Risk factors and mechanisms of hepatocarcinogenesis with special emphasis on alcohol and oxidative stress. *Biol Chem*, 387: 349–360.

Seitz HK & Stickel F (2007). Molecular mechanisms of alcohol-mediated carcinogenesis. Nature Reviews (Cancer) 7: 599-612

Seitz HK, Wang XD. (2013) The role of cytochrome P450 2E1 in ethanol-mediated carcinogenesis Subcell Biochem. 67:131-43.

Singh R, Gromadzinska J, Mistry Y, Cordell R et al (2012) Detection of acetaldehyde derived N2-ethyl-2'-deoxyguanosine in human leukocyte DNA following alcohol consumption. Mut. Res **737** 8-11

Swenberg JA, Fedtke N, Ciroussel F, Barbin A, Bartsch H. (1992) Etheno adducts formed in DNA of vinyl chloride-exposed rats are highly persistent in liver. Carcinogenesis. **13(4):**727-9.

Tatematsu K, Koide A, Morimura K, Fukushima S, Mori Y. (2013) The enhancing effect of ethanol on the mutagenic activation of N-nitrosomethylbenzylamine by cytochrome P450 2A in the rat oesophagus. Mutagenesis. 28(2):161-9.

Trafalis DT, Panteli ES, Grivas A, Tsigris C, Karamanakos PN. (2010) CYP2E1 and risk of chemically mediated cancers. Expert Opin Drug Metab Toxicol. 6(3):307-19.

Valavanidis A, Vlachogianni T, Fiotakis K, Loridas S. (2013) Pulmonary oxidative stress, inflammation and cancer: respirable particulate matter, fibrous dusts and ozone as major causes of lung carcinogenesis through reactive oxygen species mechanisms. Int J Environ Res Public Health. **10(9):**3886-907.

Wang Y, Millonig G, Nair J, Patsenker E, Stickel F, Mueller S, Bartsch H, Seitz HK. (2009) Ethanol-induced cytochrome P4502E1 causes carcinogenic etheno-DNA lesions in alcoholic liver disease. Hepatology. 50(2):453-61.

Wong NA, Rae F, Simpson KJ, Murray GD, Harrison DJ. (2000) Genetic Polymorphisms of cytochrome p4502E1 and susceptibility to alcoholic liver disease and hepatocellular carcinoma in a white population: a study and literature review, including meta-analysis. Mol Pathol. **53(2)**:88-93.

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

ANNEX 1:

Linhart K, Bartsch H, Seitz HK (2014) The roel of reactive oxygen species (ROS) and cytochrome P450 2E1 in the generation of carcinogenic etheno-DNa adducts Redox Biol **3** 56-62

CC/08/10: Mechanisms contributing to the synergism of alcohol and tobacco in human cancers

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Annex 2

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

Mechanisms contributing to the synergism of alcohol and tobacco in human cancers

Following the initial discussion on mixtures at the last meeting, it was decided to consider a well-known carcinogenic interaction of significance to public health and to review the literature for information on potential mechanisms which explain the interaction. The synergistic interaction between alcohol and smoking on certain cancer endpoints was selected. The Department of Health Toxicology Unit has produced the attached paper which presents some of the studies which describe the combined effect of alcohol and smoking, and considers some of the mechanisms which have been proposed in the literature to explain the synergism.

The committee is asked to consider the attached paper and to answer the following questions:

1. What is members' opinion of the mechanisms which have been proposed to explain the synergistic effect of alcohol and tobacco?

2. Do members consider that any other mechanisms may be of importance?

3. Are there any other known interactions which it would be useful to consider in this way?

Secretariat

July 2008

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Mechanisms contributing to the synergism of alcohol and tobacco in human cancers

1. Following on from the initial discussion paper on the carcinogenicity of chemical mixtures presented at the COC meeting in April 2008, it was decided to examine interactions which may be of importance to public health. One such example is the known potentiation of some tobacco-induced cancers by the consumption of alcohol. This paper attempts to examine the extent of this interaction and the mechanisms which underpin this synergism.

2. Tobacco and alcohol are well established risk factors for cancers of the head and neck, larynx, and oesophagus and these are the cancers which have most commonly been investigated with respect to the combined impact of these life-style factors. A few studies have been designed to look specifically for the effects of combined use and in other larger studies, it is possible to extract the relevant data to address this end-point.

3. A study which specifically aimed to investigate the combined effects of alcohol and tobacco smoking on the incidence of cancer of the larynx collected data on tobacco smoking (g/day) and alcohol intake (g/week) from 326 cases and 1134 control subjects (Olsen et al 1985a). Age and sex adjusted risk ratios indicated, in general, dose related increases in OR for laryngeal cancer for both intakes as follows

Alcohol g/week	Tobacco g/day				Adjusted for tobacco
	0-10	11-20	21+	unknown	
0-100	1.0	1.5	1.7	0.7	1.0
101-200	1.2	1.6	3.2	-	1.5
201-300	1.5	6.0	15.5	-	3.2
301+	5.4	4.4	7.5	-	4.1
Unknown	0.5	2.8	-	0.5	0.8
Adjusted for alcohol	1.0	1.7	2.3	0.5	

4. These analyses showed a synergistic effect for all intakes with the exception of moderate alcohol and tobacco use. The authors conclude that even if the collection of data on residual confounding factors is flawed, this would be unlikely to account for the entirety of the synergistic response seen.

5. The same group also examined the impact of alcohol and tobacco exposure on the risk of cancer of the hypopharynx, although the numbers of patients evaluated was much smaller (Olsen et al 1985b). Assessment of a total of 32 case of cancer of the hypopharynx and 321 with laryngeal cancer and 1141 controls similarly demonstrated a more than additive effect of combined intakes of tobacco and alcohol (maximum OR=5.1).

6. Talamini et al (2002) report a case-control study in which 527 cases of squamous –cell carcinoma of the larynx were age, sex and area of residency matched with 1297 controls. ORs for current smokers

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compared to never smokers was 19.8 (95% CI 11.9-32.9) and for drinkers 1.8 (95% CI 1.0-3.3). Their assessment was detailed, including stratification across those with low, moderate and high intakes of both alcohol and tobacco. The data clearly demonstrate that both alcohol and tobacco are independent risk factors for laryngeal cancer and that heavy alcohol consumption and tobacco use produced a remarkable multiplicative effect.

Smoking habit no cigs/day	Alcohol intake drinks/week				
	Never and <14	14-27	28-55	>56	OR (95% CI)
Never	1[a]	0.8 (0.19-2.95)	1.7 (0.42-7.01)	8.5 (2.39-30.17)	1[a]
1-14	9.7 (3.56-26.13)	8.0 (2.82-22.80)	18.7 (6.99-50.22)	63.7 (20.97-193-42)	9.2 (5.15-16.38)
15-24	27.6 (10.19-74.73)	31.5 (11.96-82.94)	66.5 (26.83-165.03)	139.9 (54.73-357.86)	28.0 (16.01-48.96)
>25	18.9 (5.69-62.70)	52.5 (18.28-150.62)	83.9 (30.92-227.52)	177.2 (64.99-483.28)	34.3 (18.57-63.47)
OR (95% CI)	1[a]	1.2 (0.73-2.03)	2.5 (1.54-4.08)	6.1 (3.58-10.43)	

a: reference category

7. Laryngeal cancer is substantially less common in women than in men (sex ratio of 10-30 fold) and thus there is a paucity of data assessing risk factors in women. This was addressed in two pooled case-control studies, although only 78 cases were collected (compared to 340 controls) (Gallus et al 2003). The fact that alcohol and tobacco usage could not explain the marked sex difference was also explored. The ORs for <3 drinks/day increased with the number of cigarettes smoked to 19.1 (95% CI 6.0-60.3) for 0-15 cigarettes and 88.4 (95% CI 22.5-347.2) for >15 cigarettes/day. In those that consumed >3 drinks/day the ORs were 1.3 for never or ex-smokers and 20.4 (95% CI4.7-88.8) and 317.9 (95% CI 70.5-1434.0) for those smoking 0-15 and >15 cigaretees/day respectively. Cigarette smoking was shown conclusively to be the most prominent risk factor and again, the data clearly demonstrate the multiplicative interaction of alcohol and tobacco on risk for this kind of cancer.

8. A large study in an Indian population investigated tobacco smoking and chewing and alcohol drinking on the risk of oral (lip, tongue and mouth), pharyngeal and oesophageal cancer incidence (Znaor et al 2003). Data were collected from 1563 oral, 636 pharyngeal and 566 oesophageal male cancer patients and compared to 1711 controls. A large number of analyses were undertaken including the combinations of smoking, chewing and drinking. The OR data for smoking and alcohol drinking only are presented:

Smoke	Drink	Oral		Pharynx		Oesophagus	
		OR	95% CI	OR	95% CI	OR	95% CI
No	No	1		1		1	
Yes	No	2.45	1.94-3.10	3.54	2.54-4.94	3.57	2.51-5.06
No	Yes	2.56	1.42-4.64	No cases		3.41	1.46-7.99
Yes	Yes	4.81	3.74-6.19	8.41	5.94-11.90	7.33	5.06-10.62

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9. Chewing tobacco further increased the odd ratios particularly for the oral cancers.

10. Oesophageal cancer is broadly classified into two histological types; squamous cell carcinoma (SCC) which arises principally in the upper and middle thirds of the oesophagus, and oesophageal adenocarcinoma (EAC) which occurs in the lower third of the oesophagus. There are strong associations with SCC and alcohol and tobacco consumption but these are considered to be of less importance in the etiology of EAC. Whereas the SCC cancer is more closely related to the head and neck cancers such as pharyngeal, EAC is associated with (and included within?) the gastric cancers. This was also discussed in the COC paper reviewing oesophageal cancer (CC/04/14)

11. The studies described previously did not specify from which part of the oesophagus the cancer had arisen. However, Lagergren (2000) compared the incidences of the two oesophageal cancer types. For alcohol alone, high consumption of hard liquor was associated with an increased risk of SCC (OR 5.0, 95% CI 2.8-9.0) for >30g/week ethanol, although there was not a clear association trend across dose. For tobacco the incidence of SCC was strongly associated with smoking; the adjusted OR was 9.3 (95% CI 5.1-17.) among current smokers vs those never smoked. Assessment of combined alcohol and tobacco use (>35y + >70g week) was 23.1 (95%CI, 9.6-56.0) compared with never users for SCC, but was not significant for risk of EAC (2.3 95% 0.9-5.7) (Lagergren *et al.*, 2000).

12. Lee et al (2007) specifically assessed the impact of tobacco, alcohol and combined exposure on the incidence of SCC. Statistical models were applied to analyse the multiplicative nature of the interaction. Tobacco was shown to interact with light to moderate alcohol (0.1-30g/day) in a supra-multiplicative manner (OR 5.5-5.7) whereas it interacted with heavy alcohol consumption in a simple multiplicative model (OR 1.7-2.3). Regular alcohol intake was most strongly associated with the increased risk of tumour development,

13. A Scandinavian cohort study collected data from 69,962 individuals and a total of 1,117,648 person-years between 1984 and 2002 (Sjodahl et al 2006). Cardia and non-cardia gastric cancers were analysed separately. The following relevant data were retrieved:

Smoking	Alcohol[a]	Gastric cancer	Noncardia gastric cancer
		OR(95% CI)	OR(95% CI)
Never	Never	Reference	Reference
<20	Never	1.34 (0.40-4.51)	1.40 (0.41-4.75)
>20	Never	-	-
Never	<4	1.08 (0.66-1.78)	1.06(0.64-1.78)
Never	>5	1.57 (0.47-5.28)	1.69 (0.50-5.73)
<20	<4	1.79 (1.07-2.99)	1.57 (0.91-2.68)
>20	<4	1.78 (0.85-3.73)	1.66 (0.75-3.66)
<20	>5	2.15 (0.93-4.94)	2.33 (1.00-5.40)
>20	>5	4.38 (1.72-11.17)	4.90 (1.90-12.62)

a: data presented as number of occasions per 14 days

14. Although the risk associated with the use of both alcohol and tobacco is greater than the risk associated with the individual factors, for these cancers the interaction between alcohol and tobacco was not statistically significant

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15. The studies presented indicate that the combined use of alcohol and tobacco interact to induce cancers of the mouth, neck and squamous cell carcinoma of the oesophagus in a greater than additive manner. However the synergism is not apparent for oesophageal adenocarcinoma and cancers of the gastric cardia.

Induction of CYP enzymes by ethanol

16. Alcohol itself is not considered to be a carcinogen in experimental animals (IARC 1988). The mechanism by which alcohol produces enhanced carcinogenicity is not well understood although there are several hypotheses which have received attention. Although alcohol is metabolized principally by alcohol dehydrogenase, CYP2E1 is thought to be responsible for metabolizing 20% of ethanol at low blood concentrations (Schoedel and Tyndale 2003). Furthermore, ethanol is also known to induce CYP2E1, via protein stabilization and prevention of the degradation of the 26S proteosome (Anderson et al 1995). The significance of this induction to ethanol's apparent synergy with tobacco in the carcinogenic process is apparent when it is considered that most carcinogenic nitrosamines present in tobacco smoke are metabolically activated by the CYP family of enzymes.

17. There are a number of studies in which the relevance of the various CYP enzymes on the metabolism of different nitrosamines is considered which are relevant to understanding the association of alcohol and tobacco intake on cancer incidences. A discussion of the organ distribution of the CYP isoforms or which of these is capable of metabolically activating which specific nitrosamines is out side the scope of the current assessment. However a number of papers are included to provide an indication of how ethanol induced enzyme induction may impact on the carcinogenicity of nitrosamines in tobacco. Of particular note is the induction of CYP2E1 by ethanol.

18. Mori et al (2002) assessed CYP1A1/2, 2B1/2, 2E1 and 3A2 levels using western blotting in rats given N-nitrosomethylbenzylamine (NMBA; 0.5mg/kg) with or without ethanol in the drinking water (10%) and also used S9 prepared from these livers in an Ames test with a variety of N-nitrosamines and heterocyclic amines. NMBA did not increase any of the CYPs measured and ethanol induced 2E1 only. This in turn was shown to increase the mutagenic activity of dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) in the Ames test, but not NMBA mutagenicity.

19. Another approach indicating that induction of CYP2E1 may play a role in ethanol's synergistic effect with nitrosamines in oesophageal carcinogenesis utilized evaluation of the formation of oesophageal polyps, considered an early tumour marker (Tsutsumi et al 2006). In this study rats were given daily doses of N-nitrosomethylbenzylamine (NMBA) for 10 weeks together with ethanol in a liquid diet for 30 weeks (from 8 weeks before NMBA administration until 12 weeks after). An increase in polyps was recorded in treated animals when compared to control animals, and increased CYP2E1 expression was also apparent in the oesophageal mucosa. However there are some earlier studies which concluded that 2E1 was not expressed in rat oesophagus although microsomes from this tissue were shown to metabolise N-nitrosodiethylamine (Ribeiro Pinto et al 2001). However this study did not investigate the potential of ethanol to induce this isoform.

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20. There are a number of studies which investigate CYP2E1 induction by ethanol in man, the expression of oesophageal CYP enzymes and the role of various isoforms in the metabolic activation of nitrosamines associated with tobacco smoke.

21. Confirming the similarities of man and rat with regards to induction, Takahashi et al (1993) demonstrated that CYP2E1 expression is elevated in the centrilobular region of the livers of alcoholics. More specific to the current review, CYP expression in human oesophageal mucosa was investigated by Lechevrel et al (1999) using immunohistochemical techniques. CYP1A, 2E1, 3A and 4A isozymes were detected. CYP2E1 was consistently identified (17/19 samples) although there was up to 43-fold variation in expression. The effects of alcohol and tobacco exposure were monitored although no significant correlation was reported for any of the isoforms evaluated. Similarly, expression of CYP1A1, 1A2, 2A6/2A7, 2E1 and 3A4 mRNAs in human mucosal specimens taken from patients undergoing oesphagectomy was performed with a view to establishing a role in the etiology of oesophageal cancer (Godoy et al 2002 It was shown that 96% and 61% of patients expressed oesophageal CYP2E1 and 2A6/2A7 respectively although the 2A6/7 expression was somewhat variable between samples. It was tentatively suggested that this co-localised to areas susceptible to tumours. `In another report, human oesophageal microsomes were shown to metabolise tobacco nitrosamines and that these microsomes were immunoreactive for CYP2E1 (Smith et al 1998).

22. These observations provide a plausible hypothesis for the clear synergism of alcohol and tobacco in the induction of some cancers in that the induction of CYP enzymes by ethanol, particularly 2E1 increases the metabolic activation of some carcinogenic nitrosamines present in tobacco smoke. However, this is evidently not straightforward for a number of reasons. Induction of CYP in the liver would usually be associated with increased clearance of substrates and thus decreasing their availability to distant organs. However, Swann (1984) highlighted the fact that ethanol in fact significantly reduces nitrosamine metabolism in the liver thus blocking first pass clearance. This is likely a consequence of competitive inhibition, as ethanol itself will be concomitantly metabolised by the CYP enzymes it induces. Therefore it is more likely that the induction of the enzymes at the tumour site itself would be a more relevant mechanism of activation. Although CYP2E1 has not been shown to be consistently expressed in the oesophagus for example it is possible that induction by alcohol increases local concentrations of the metabolically activated nitrosamines present in tobacco. Anderson et al (1995) also supports the postulate that hepatic clearance of nitrosamines is reduced resulting in elevated levels at post-hepatic target tissues.

23. There are reports which have investigated polymorphisms of other CYP enzymes, such as CYP 2B1 and 1B1, which also have the capacity to metabolise nitrosamines, and polyaromatic hydrocarbons and their relevance to the induction of cancer (Schoedel and Tyndale 2003, Thier et al 2002). Alcohol and aldehyde dehydrogenase genes (ALD1B and ALDH2) and glutathione-s-transferase (GSTM1, GSTT1, GSTP1) polymorphisms have also been examined with regards to their impact on alcohol and tobacco carcinogenesis. Peters et al (2006) showed combined effects of alcohol and tobacco induced head and neck squamous cell carcinoma and deletion of GSTM1 enhanced this interaction. A Taiwanese study demonstrated an interaction between ADH1B and ALDH2 polymorphisms, smoking and alcohol consumption on the risk squamous cell carcinoma of the oesophagus (Lee et al 2008). Together it is clear that the presence and induction of enzymes responsible for metabolizing and detoxifying both

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carcinogenic nitrosamines contained in tobacco smoke and ethanol are likely to contribute to the synergism observed in the induction of tumours.

Increased permeability of epithelial cells

24. Another postulate which remains plausible is that alcohol increases the permeability of the oral mucosa to carcinogens. It is known that ethanol potentiates the diffusion of co-administered chemicals across the skin (ref). With a view to investigating a potential mechanism for the synergistic effects of alcohol and tobacco on oral cancers, Du et al (2000) investigated the effects of ethanol on the penetration of nitrosonornicotine (NNN) in an *in vitro* model using porcine oral mucosal cells. A threshold concentration of 25% alcohol, nicotine at 0.2% and the combination of alcohol and nicotine enhanced the penetration of NNN across the oral mucosal cells in culture. The authors suggest that local permeabilising effects may be one mechanism which explains the synergy. However the concentrations used seem high and extrapolation is difficult with a lack of understanding of the relevance of the model used.

25. An in *vitro* system was also employed to assess the impact of ethanol on the rates of absorption of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (BaP) (Azzi et al 2005). The mucosal layer of whole porcine esophagus was established in Franz diffusion cells, concentrations of ethanol and menthol were selected in an attempt to mimic *in vivo* exposure (5% and 0.08% respectively) and the extent which chemicals permeated the reception chamber was measured. Alcohol and menthol did not effect the transit of the relatively water soluble NNK but did significantly enhance the penetration of the more lipophilic BaP. Confocal microscopy showed that BaP was more concentrated in the basement membrance adjacent to the smooth muscle tissue. Ethanol and menthol together showed synergism. It is concluded that the results support the supposition that the use of mentholated cigarettes and consumption of alcohol may have a marked effect on the absorption and fate of carcinogenic tobacco chemicals.

Conclusion

26. These data suggest that enhancing permeability of the oral mucosa by ethanol could increase local concentrations of carcinogenic nitrosamines present in tobacco. Two plausible mechanisms by which alcohol and tobacco act synergistically on the induction of a number of cancers, specifically those of head and neck, larynx, and oesophagus are presented. It is likely that there are other factors that contribute to this synergism, for example, other enzymes capable of metabolizing both ethanol and tobacco related nitrosamines. Furthermore the relative distribution, inducibility and substrate specificity of the different CYP family of enzymes may impact on other cancers at other sites. However, this provides an example of how chemicals may interact to enhance tumour formation.

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References:

Azzi C, Zhang J, Purdon C.H, et al (2005) Permeation and reservoir formation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (BaP)c across porcine esophageal tissue in the presence of ethanol andmethanol. Carcinogenesis **27** 137-145

Du X, Squier C.A, Kremer M.J, Wertz P.W. (2000) penetration of N-nitronornicotine (NNN) across oral mucosa in the presence of ethanol and nicotine. J.Oral.Pathol.Med **29** 80-85

Gallus S, Bosetti C, franceschi S et al (2003) Laryngeal cancer in women: tobacco, alcohol, nutritional and hormonal factors. Cancer Epidem.Biomarkers prev. **12** 514-517

Lagergren J, Bergstrom R, Lindgren A and Nyren O. (2000). The role of tobacco, snuff and alcohol use in theaetiology of cancer of the oesophagus and gastric cardia. Int. J. Cancer **85** 340-346

Lee C-H, Lee J-M, Wu D-C et al (2008) carcinogenetic impact of ADH1B and ALDH2 genes on squamous cell carcinoma risk of theesophagus wit hregard to the comsumption of alcohol, tobacco and betel quid. Int J. Cancer **122** 1347-1356

Mori, Y, Koide A, Kobayashi Y et al (2002) Effect of ethanol treatment on metabolic activation and detoxification of esophagus N-nitrosamiens in rat liver. Mutagenesis **17** 251-256

Olsen J, Sabero, S, Fasting U. (1985a) Interaction of alcohol and tobacco as risk factors in cancer of the laryngeal region. J. Epidemiol.Comm.Health **39** 165-168

Olsen J, Sabero, S, Ipsen J. (1985) Effect of combined alcohol and tobacco exposure on risk of cancer of the hypopharnx. J. Epidemiol.Comm.Health **39** 304-307

Talamini R, Bosetti C, La Vecchia, C et al (2002) Combined effect of tobacco and alcohol on laryngeal cancer risk: a case-comntrol study. Cancer Causes and Control **13** 957-964

Schoedel, K.A, Tyndale R.F (2003) Induction of nicotine-metabolizing CYP2B1 and ethanol-metabolizing CYP2E1 by nicotine: summary and implications. Biochim Biophys Acta **1619** 283-290

Smith T.J, Liao A, Wang L-D, et al (1998) Characterisation of xenobiotic-metabolizing enzymes anbd nitrosamine metabolism in the human esophagus. Carcinogenesis **19** 667-672

Sjodahl K, Lu Y, Nilsen T.I.L, et al (2006) Smoking and alcohol drinking in relation to risk of gastric cancer: a population-based prospective cohort study. Int.J.Cancer **120** 128-132

Swann P.F 91984) The possible roel of nitrosamines I nte link between alcohol consumption andesophageal cancer in man. Toxicol.Pathol. **12** 357-360

Znaor A, Brennan P, Gajalakshmi V et al (2003) Indepdent and combined effects of tobacco smoking, chewinf and alcohol drinking on the risk of oral pharyngeal and esophageal cancers in Indian men. Int.J.Cancer **105** 681-686