ACETALDEHYDE WORKING GROUP

Comments on the CLH Report on Acetaldehyde; Proposal for Harmonised Classification and Labeling (June 2015) (Submitted 11 September 2015)

I. Introduction and Summary

The Acetaldehyde Working Group (AWG) submits the following comments in response to the CLH Report on acetaldehyde; Proposal for Harmonised Classification and Labeling (June 2015) prepared by RIVM, The Netherlands (hereinafter referred to as the RIVM Proposal). AWG is a not-for-profit association, located in Washington DC, whose mission is to address human health and relevant risk assessment/regulatory issues of interest to the membership.

The RIVM proposal seeks to alter the classification of acetaldehyde as it pertains to carcinogenicity and germ cell mutagenicity. Acetaldehyde is currently classified for carcinogenicity in Category 2 (suspected human carcinogen) and is not classified for mutagenic activity. RIVM is proposing to upgrade the carcinogenicity classification to Cat. 1B and to establish a category 1B classification for germ cell mutagenicity.

As noted in the United Nations Guide to the Globally System of Classification and labeling (GHS):

> *Classification is the starting point for hazard communication. It involves the identification of the hazard(s) of a chemical or mixture by assigning a category of hazard/danger using defined criteria.*

By definition, hazard communication is intended to illicit a behavioral response (usually avoidance or caution) by those people that may be exposed to the substance. In that context, it is important to consider application of the CLP guidance to compounds that are ubiquitous and endogenously produced, particularly given the multitude of sources of exposure.

As the RIVM proposal notes, acetaldehyde occurs naturally in coffee, bread, and ripe fruit, and is produced by plants as part of their normal metabolism. Section II of these comments briefly summarize the numerous food and beverages sources contain acetaldehyde - be it orange juice consumed in the morning or beer in the afternoon/evening. One of the most comprehensive analyses of food sources not discussed in the RIVM proposal, was published in 2011 by researchers from the German Laboratory - *Chemisches und Veterinäruntersuchungsamt (CVUA) Karlsruhe, Weissenburger Straße 3, 76187 Karlsruhe, German (*Uebelacker and Lachenmeier, 2011). The results from 140 food samples reveals that a wide range of food products contain natural levels of acetaldehyde that contribute to background exposures. For example, the acetaldehyde content in apples was 0.97 +/- 0.80 mg/kg, while orange juice was found to

contain 3.86 +/- 2.88 mg/kg. The maximum content of foods for direct consumption was found in a yoghurt (17.42 mg/kg).

With this backdrop, it is important to consider the key scientific elements of the proposed classification change. The Netherlands Technical Support document on which the RIVM proposal is based: Health Council of the Netherlands Acetaldehyde: Re-evaluation of Carcinogenicity and Genotoxicity (Pub. No. 2014/28, December 2014) attempts to support the classification as follows (see Executive Summary (p. 11)):

> *"The committee concludes that acetaldehyde is presumed to be carcinogenic to man, and recommends classifying the substance in category 1B. Based on the available data, the committee furthermore recommends classifying acetaldehyde as a germ cell mutagen in category 1B (substance to be regarded as if it induces heritable mutations in the germ cells of humans). <u>The substance acts by a stochastic genotoxic mechanism."</u>*

A critical underlying premise to this hypothesis is that this type of mutagen produces a molecular interaction (i.e., DNA adduct), irrespective of dose, and this leads to subsequent mutations over background. As further explained in the attached comments, available studies, some recent and not considered in the RIVM proposal, demonstrate that this stochastic mechanism is not the mode of action for acetaldehyde. Rather, homeostatic mechanisms have evolved to keep intra-cellular concentrations within physiological ranges given the variations in metabolic processes.

AWG believes that a rational classification approach to an endogenous, ubiquitous compound like acetaldehyde can be supported by employing the recent June 2015 Guidance on the Application of the CLP Criteria; Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures (Version 4.1). Of specific note is the discussion on carcinogenicity,

> *Tumours occurring only at excessive doses associated with severe toxicity generally have a more doubtful potential for carcinogenicity in humans. In addition, tumours occurring only at sites of contact and/or only at excessive doses need to be carefully evaluated for human relevance for carcinogenic hazard.*
>
> *If a test compound is only found to be carcinogenic at the highest dose(s) used in a lifetime bioassay, and the characteristics associated with doses exceeding the MTD as outlined above are present, this could be an indication of a confounding effect of excessive toxicity. This may support a classification of the test compound in Category 2 or no classification.*

This guidance combined with the data on acetaldehyde supports the conclusion that <u>no change in the classification of acetaldehyde is justified.</u>

II. Acetaldehyde's Ubiquitous Presence Should Be Considered as Part of the Classification Process

Extensive information has recently become available on levels of acetaldehyde in foods and beverages. The following provides an overview summary, and a more comprehensive consideration of acetaldehyde levels in food and beverages is encouraged before arriving at a classification decisions.

Beer and Other Alcoholic Beverages - While acetaldehyde is found in many natural food substances, a major source of human exposure to acetaldehyde comes from alcoholic beverages. Relatively small amounts are found in the beverage itself and considerably more is produced as part of ethanol metabolism. It is significant to note that there are generally eight compounds that are related to the distinctive flavor of beer, including acetaldehyde[1] (e.g., typical concentration of acetaldehyde in Heineken is 1.6 mg/L).

Lachenmeier et al. (2009a) estimated exposure to acetaldehyde due to alcoholic beverage consumption, based on products from the EU. According to these estimates:

> *"….a 60-kg person with mean alcoholic beverage consumption in Europe and a mean content of acetaldehyde would be exposed to 0.112 mg/kg body weight/day of acetaldehyde. A heavy drinker (99th percentile) exposed to a mean content of acetaldehyde would be exposed to 0.305 mg/kg/day. An average drinker consuming beverages with high content of acetaldehyde (99th percentile) would be exposed to 0.56 mg/kg/day. Lastly, a heavy drinker of beverages with high acetaldehyde content would be exposed to 1.639 mg/kg/day of acetaldehyde. "*

Furthermore, Lachenmeier et al. (2009b) estimated that twice-daily use of alcohol containing mouthwashes leads to a systemic acetaldehyde exposure of 0.26 µg/kg/day on average.

Acetaldehyde is Found in Non-Alcoholic Food Sources Including Fruit - Acetaldehyde occurs naturally in coffee, bread, and ripe fruit, and is produced by plants as part of their normal metabolism. In foods, acetaldehyde may occur either naturally or because of intentional addition as a flavor compound as acetaldehyde is recognized by most food agencies as safe. In the United States, acetaldehyde is deemed to be GRAS (Generally Recognized as Safe; citation). Some food substances derive their distinct flavor based on the concentration of acetaldehyde.

One of the most comprehensive analyses of food sources was published in 2011 by researchers from the German Laboratory - *Chemisches und Veterinäruntersuchungsamt (CVUA) Karlsruhe, Weissenburger Straße 3, 76187 Karlsruhe, German (*Uebelacker and Lachenmeier, 2011). Their

[1] Acetaldehyde, acetone, ethyl formate, ethyl acetate, 1-propanol, isobutanol, isoamyl acetate, isopentanol, and ethyl hexanoate.

analysis relied on headspace gas chromatography with flame ionization detector (limit of detection was 0.01 mg/L and the limit of quantification was 0.04 mg/L.)

The results from 140 food samples are presented. The acetaldehyde content in apples was 0.97 +/- 0.80 mg/kg, orange juice contained 3.86 +/- 2.88 mg/kg. The maximum content of foods for direct consumption was found in a yoghurt (17.42 mg/kg). In food ingredients, 26.3 mg/kg were found in a baking flavor, while an industrial orange flavor contained 1416 mg/kg of acetaldehyde, which was the maximum of all analyzed samples.

In fruits, the highest acetaldehyde contents were found in bananas and in citrus fruits. While all fruits were generally analyzed in fresh state, the researchers investigated changes in acetaldehyde content that occur during the ripening process, using bananas. The bananas for this experiment were stored in a refrigerator for up to 22 days. Similar to the blackening of the color, the acetaldehyde content rose up to an increase of 80% compared to the initial content.

The Joint FAO/WHO Expert Committee on Food Additives has estimated that 9.7–11 mg per person per day of acetaldehyde is ingested due to its use as food flavor additive. The Food Safety Commission of Japan has estimated a similar range between 9.618 mg (Europe) and 19.211 mg (USA), which was assumed to be 20% of the acetaldehyde that is contained in foods while the other 80% can be traced as natural occurrence. The US Flavor and Extract Manufacturers Association (FEMA) has estimated the possible average daily intake as 35 mg (0.47 mg/kg bw/day) while Morris et al. [estimated a range of 40–80 mg (0.53–1.07 mg/kg bw/day), with worst case levels up to 200 mg (2.67 mg/kg bw/day).

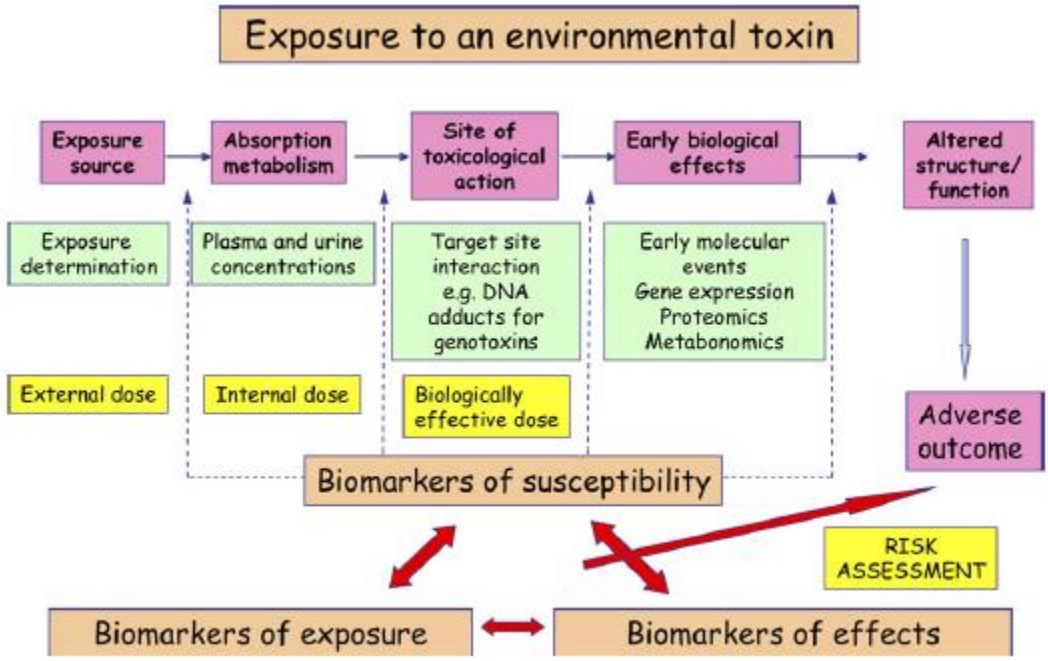
III. Overview – Considerations Regarding the Evaluation of Carcinogenicity and Genotoxicity Classification for Acetaldehyde

The reason for the proposed classification (re-classification) of acetaldehyde is clearly stated in the Executive Summary (p. 11) of this document as:

> *"The committee concludes that acetaldehyde is presumed to be carcinogenic to man, and recommends classifying the substance in category 1B. Based on the available data, the committee furthermore recommends classifying acetaldehyde as a germ cell mutagen in category 1B (substance to be regarded as if it induces heritable mutations in the germ cells of humans). <u>The substance acts by a stochastic genotoxic mechanism."</u>*

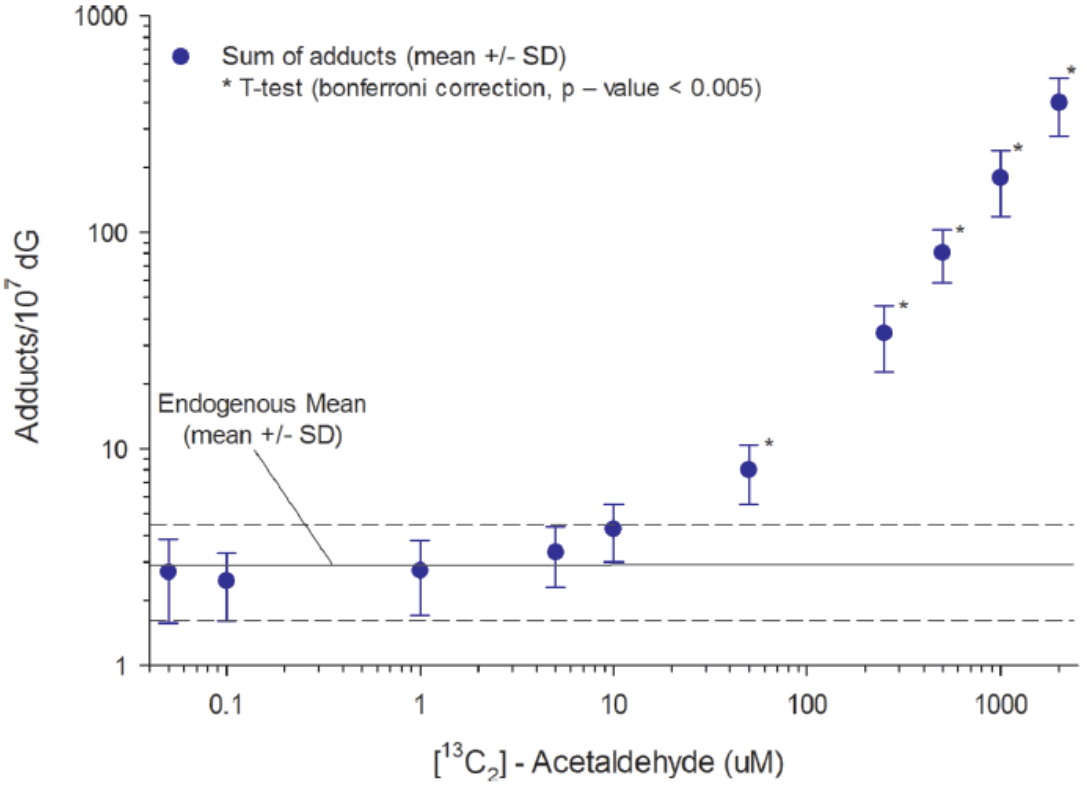
A critical underlying premise to the hypothesis that acetaldehyde <u>*acts by a stochastic genotoxic mechanism*</u> is that this type of mutagen produces a molecular interaction (i.e., DNA adduct) over background, irrespective of dose, and that this molecular interaction leads to a finite probability of inducing subsequent mutations over background. As further explained in this and the next section, available studies, some recent and not considered in the RIVM proposal, demonstrate that this stochastic mechanism is not the mode of action for acetaldehyde.

Generally, DNA adduct formation is considered the initial event (molecular interaction) in the mutagenic process as illustrated by the below figure from Farmer and Singh (2000).



Acetaldehyde is certainly capable of interacting with the DNA to form a variety of DNA adducts, at least as evaluated in cell free systems (Albertini 2013 and references therein). In cells, the N^2-ethylidine-dG adducts (measured as N^2-ethyl-dG) and the N^2-propano-dG mono-adducts as well DNA-protein cross-links have been the most frequently studied, with primary emphasis on the first of these. Although the mutagenic potential of N^2-ethylidine-dG is uncertain, it is formed at lower acetaldehyde exposure concentrations than N^2-propano-dG or the cross-links, which are intrinsically more mutagenic, and can serve as a biomarker of exposure.

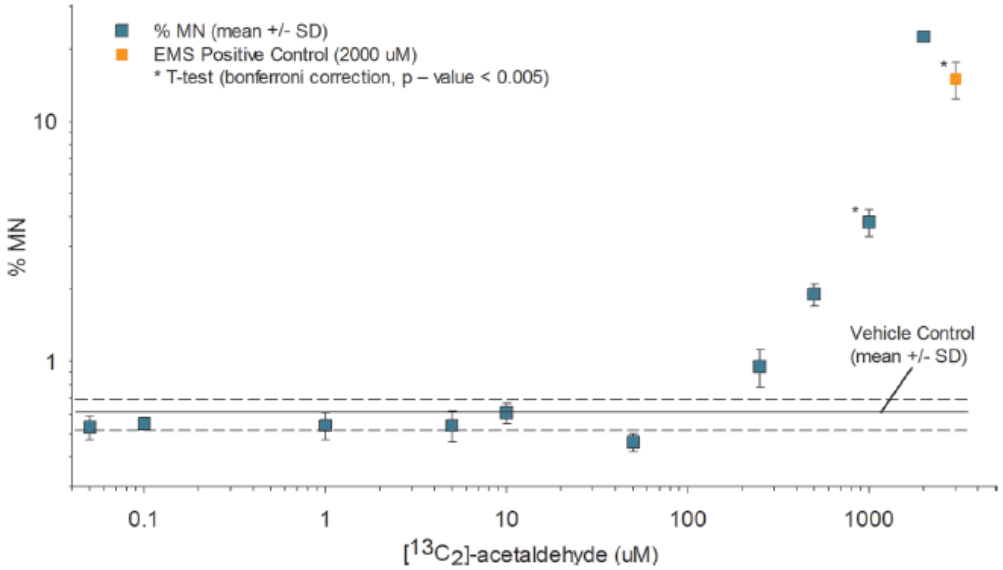
The hypothesis that any acetaldehyde exposure results in a single DNA interaction that triggers subsequent adverse effects is not only testable; it has been tested. The recent study by Moeller et al. (2013) has demonstrated that exposure of human TK6 lymphoblastoid cells to acetaldehyde does not produce N^2-ethylidine-dG adducts above background level until an exposure concentration of 50 µM is exceeded (see figure). (This study had limits of detection for the adducts in the amol range.)



Therefore, a "single molecular interaction" is demonstrably not triggered by a low acetaldehyde exposure concentration. This same study (Moeller et al., 2013) also explored the dose-response for acetaldehyde induced micronuclei in the rapidly proliferating TK6 cells with result as shown in the figure.

Clearly, these chromosome level mutations <u>are not stochastic events</u> induced by acetaldehyde; rather, there are a wide range of low-dose concentrations that do not induce mutations above background.

It is to be expected that an endogenously produced substance, such as acetaldehyde, could not be a stochastic genotoxic agent. Acetaldehyde is a product of normal metabolism in all living organisms, including humans. To avoid adverse effects, homeostatic mechanisms have had to evolve to keep intra-cellular concentrations within physiological ranges given the variations in metabolic processes. Although these homeostatic mechanisms can be overwhelmed by massive exposures, <u>such a mechanism of mutagenic action is not consistent with the definition of a stochastic genotoxic agent.</u>



It is apparent that the hypothetical assertion that acetaldehyde "acts by a stochastic genotoxic mechanism" is demonstrably not true. It is also biologically unreasonable. As this assertion forms the basis for the re-evaluation of acetaldehyde as a category 1B carcinogen and germ cell mutagen, this classification is clearly not warranted for either of the adverse effect.

IV. Acetaldehyde Should Not Be Classified As Cat. 1B Germ Cell Mutagen

The Netherlands propose that acetaldehyde be classified as a Category *1B* germ cell mutagen. The justification for the proposed classification is "new information" that was not heretofore considered, specifically, a 2002 study of sister-chromatid exchanges (SCEs) in spermatogonial cells of mice exposed to exogenous acetaldehyde by i.p. injections (Madrigal-Dejaidar et al. 2002).

The ECHA guidance document describes the criteria for designating a substance as a *Category 1B* germ cell mutagen (p.360, Annex 1.3.5.2.2; *Hazard Categories for Germ Cell Mutagens*):

> *1. Positive result(s) from in vivo heritable germ cell mutagenicity tests in mammals, or*
>
> *2. Positive result(s) from in vivo somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has the potential to cause mutations in germ cells. It is possible to derive this supporting evidence from mutagenicity/genotoxicity tests in germ cells in vivo, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells, or*

3. Positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny; for example, an increase in aneuploidy in sperm cells of exposed people.

Criterion # 1 is not fulfilled by results of experimental studies; in fact, there are negative studies of germ cell mutagenesis in mammals as detailed below.

There is no evidence in the genetic toxicology profile of acetaldehyde that would support *criterion #3*.

Criteria #2 requires a more comprehensive review of the available studies.

<u>Rationale on the assessment of the validity of criterion #2 for acetaldehyde:</u>

Criterion #2 requires

- *Positive result(s) from in vivo somatic cell mutagenicity tests in mammals*
- *in combination with some evidence that the substance has the potential to cause mutations in germ cells*

It is possible to derive this supporting evidence

- *from mutagenicity/genotoxicity tests in germ cells in vivo, or*
- *by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells.*

It appears that the thrust of the proposed 1B classification is intended to be the results of a murine germ cell study (Madrigal-Dejaidar et al. 2002; new information) in addition to results from older studies of somatic mutations *in vivo* in mammals. The CLH report seems to regard these results as "*some evidence that the substance has the potential to cause mutations in germ cells".* This evidence cannot be established by positive sister chromatic exchange (SCE) results in an animal study requiring administration of acetaldehyde via intraperitoneal administration.

Mutagenicity versus Genotoxicity

<u>Annex 1: 3.5.1.2.</u> The more general terms "genotoxic" and "genotoxicity" apply to agents or processes which alter the structure, information content or segregation of DNA, including those which cause DNA damage by interfering with normal replication processes, or which in a non-physiological manner (temporarily) alter its replication. Genotoxicity test results are usually taken as ***indicators*** *mutagenic effects.*

"Positive result(s) from in vivo somatic cell mutagenicity tests in mammals"

In vivo germ cell effects

A report of the germ cell genotoxic effects of acetaldehyde**,** i.e. SCE frequencies in the male germ cells of mice apparently seems to constitute the "new information" that underlies the proposal for reclassification. Madrigal-Dejaidar et al. (2002) reported that SCEs frequencies were elevated over control frequencies in spermatogonial cells of adult NIH mice following single acetaldehyde i.p. injections at doses as low as 0.40 mg/kg (400 µg/kg). Effects were observed approximately 55 hours after exposure with greater induction at higher doses. At the same time, a review of the data reveals no clear dose-response effect. When the animals were given disulfiram to inhibit Aldh enzyme activity shortly after the acetaldehyde, SCE frequency elevations over control were seen in these cells at doses of 0.04 mg/kg and 0.004 mg/kg – doses that were ineffective in the absence of disulfiram.

For the following reasons, these SCE results cannot be considered as a relevant end-point for determining mutagenicity and cannot be used as basis for the reclassification of acetaldehyde**:**

- SCE are not mutational endpoints. SCEs most likely represent error free repair of single strand breaks in the DNA (ubiquitous events) by homologous recombination (Wilson and Thompson, 2001). This is in agreement with the most recent ECHA *"Guidance on the Application of the CLP Criteria"* (version 4.1, 2015), which defines *"A mutation means a **permanent** change in the amount or structure of the genetic material in a cell. The term "mutation" applies both the heritable genetic changes that may be manifested at the phenotypic level and to the underlying DNA modifications when known (including specific base pair changes and chromosomal translocations). The term "mutagenic" and "mutagen" will be used for agents giving rise to an increased occurrence of mutations in populations of cells and/or organisms."* (p.358)"3.5. *Germ cell mutagenicity, Annex 1: 3.5.11.)*

- There is a general lack of understanding over the mechanisms of action associated with this type of test. In fact, the *in vit*ro SCE test was recently removed from the list of OECD recommended tests for genotoxicity testing (OECD Guidelines for Testing of Chemicals, 2014). According to the OECD, "TG 479 was also deleted because of a lack of understanding of the mechanisms of action detected by the test". In vivo SCE tests were never on the list of OECD recommended tests.

- The OECD Testing Guidelines discuss the approaches to evaluate mutagenic potential: "For the evaluation of the mutagenic potential of substances, only tests which measure a mutation endpoint (gene or chromosome mutations) that cannot be repaired anymore and could be transmitted to daughter cells should be preferred". The SCE test clearly does not fit these criteria. In fact, the lack of biological significance of in vivo SCEs (in somatic cells of humans) has been clearly demonstrated by epidemiological studies. While true chromosome mutations (chromosome aberrations and micronuclei) predict the subsequent occurrence of cancer in humans at the population level, SCEs are not predictive (Bonassi et al, 2004; Norppa et al. 2006).

- Yauk et al. (2015) evaluated the various methodological and interpretative considerations for assessing Germ Cell Mutagens. Their recommendations and guidance are presented as a summary of a 2013 International Workshop on Genotoxicity Testing, which identified several experimental protocols for evaluating germ cell mutagenicity. None of these methods recognize SCE as a legitimate endpoint for establishing germ cell mutagenicity. Notably, the IWGT workshop summary does not mention the use of sister chromatid exchange results as a basis for establishing germ cell mutation classification.

- Madrigal-Dejaidar et al. (2002) , the authors of the publication regarded as relevant new information to revise the classification for mutagenicity, state: "The present results raise the question of whether the observed damage could be maintained during the development of spermatozoa and even passed on to the zygote"… "Thus, at present, there is no clear evidence that the observed damage by Ace [acetaldehyde] could produce abnormal zygotes".

It is important to recognize that the Lähdetie, 1988 study evaluated mutagenicity of acetaldehyde in mice, also by i.p injection at an even higher dose. Lähdetie (1988)evaluated meiotic micronuclei induction of germ cells in stage I pre-leptotene spermatids in mice and found no significant increases at any acetaldehyde dose following single i.p. injections of , 125 mg/kg, 250 mg/kg, 375 mg/kg or 500 mg/kg (Lähdetie, 1988). Although all animals died at the highest dose, all animals survived at the other doses thus demonstrating that acetaldehyde should not be considered to pose a mutagenic potential to male germ cells.

"some evidence that the substance has the potential to cause mutations in germ cells"

Criterion #2 in the ECHA Guidance document states that "some evidence that the substance has the potential to cause mutations in germ cells" is required for a *Category 1B* germ cell mutagen classification. While SCEs may be supporting data as to the mutagenic potential, such data should not over-ride definitive data from well conducted mutagenicity studies.
Moreover, the ECHA Guidance document indicates that the likely route of human exposure should be taken into consider in arriving at an appropriate classification. There should be no doubt that i.p. injection is an irrelevant route of exposure. Annex 1.3.5.2.3.9. (p. 361) states:

"The classification of individual substances shall be based on the total weight of evidence available, using expert judgement. In those instances where a single well-conducted test is used for classification, it shall provide clear and unambiguously positive results. If new, well validated tests arise, these may also be used in the total weight of evidence to be considered. ***The relevance of the route of exposure used in the study of the substance compared to the most likely route of human exposure shall also be taken into account"[2] .***

[2] A recent study determined blood concentrations of acetaldehyde following i.p. single dose administration of 400 mg/Kg to mice (Isse et al. 2005). Blood concentrations of > 5000 µM were achieved at approximately 3 minutes post exposure in both Aldh $^{+/+}$ and $^{-/-}$ animals, with decreases towards baseline by 2 hours post exposure. These

While acknowledging the fact that a SCE is not a mutagenic endpoint, the current CLH proposal justifies the use of this endpoint as an indicator that exogenously administered acetaldehyde reaches the testes and can "---- interact with the genetic material of germ cells" (*Criterion #2*). However, this has to be a quantitative and not a qualitative assertion because acetaldehyde, as a product of normal cellular metabolism, is present in cells continuously. Cellular sensitivity or resistance to acetaldehyde is critically determined by intracellular ALDH activity, which varies among cell types (EU Risk Assessment, 2008 and references therein). As acetaldehyde is produced endogenously as well as being formed from many external agents, intra-cellular AA concentrations are kept at physiological concentrations by this enzyme's activity. However, when exposures to acetaldehyde are high, the physiological concentrations may be exceeded and adverse effects produced. Therefore, an additional mutational load resulting from exogenous AA would only be manifested when **physiological concentrations are exceeded.**

A recent review of the mutagenic potential of acetaldehyde was recently published (Albertini 2013), which documents a clear threshold in studies of mutagenicity. Specifically, this threshold effect has been unambiguously demonstrated in the in vitro study of MN induction in human TK6 cells (Budinsky et al., 2013) Additionally, Moeller et al. (2013) have shown that the mechanistic basis of this threshold is that the combined total of exogenous AND endogenous N^2-ethylidine dG adducts in the exposed cells does not exceed the background level of these adducts until an exposure acetaldehyde concentration of 50 µM is reached. Observed mutations are produced at much higher concentrations. Furthermore, N2-ethylidene dG adducts are of uncertain mutagenicity but are more significant as biomarkers of exposure. For comparison, blood concentrations of acetaldehyde in $Aldh^{+/+}$ mice exposed to 125µM or 500 ppm acetaldehyde by inhalation for 24 hrs/day for 14 days are only 1.65 µM or 1.72 µM, respectively (Oyama et al. 2007) Even for $Aldh^{-/-}$ animals, the blood acetaldehyde concentration achieved by the higher exposure level is only 8.9 µM. All of these blood concentration values are well below the threshold for even DNA adduct formation and certainly for mutation induction, e.g. a threshold between 50 and 250 µM (Budinsky et al., 2013).

A clear demonstration that the endogenous acetaldehyde is capable of inducing SCEs when the homeostatic mechanism is paralyzed has been provided by the results of an earlier study by Madrigal-Dejaidar et al. (1999) in which they show that disulfiram alone (without exogenously administered acetaldehyde) is capable of producing increases in SCEs in spermatogonial cells. The authors of this paper offer an explanation for this effect: "Another probable explanation for the detected genotoxic damage may be related to the acetaldehyde fraction of endogenous origin that has been detected in subjects not drinking alcohol".

<u>*Criterion #2 for a cat 1B germ cell mutagen classification calls for p*</u><u>ositive result(s) from *in vivo* somatic cell mutagenicity tests in mammals.</u> There have been several studies of *in vivo* chromosome level mutations induced in mammals by exogenous acetaldehyde (reviewed in

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acetaldehyde blood concentrations compare with concentrations of 1.72 µM or 8.9 µM produced in these two groups of mice following inhalation exposure of acetaldehyde 500 ppm for 14 days (Isse et al., 2005).

Albertini, 2013 and in the current RIVM: The Netherlands CLH report). The reviews reveal the following:

- The only formal mutagenicity study that investigated the ability of acetaldehyde to induce mutations at either the gene or chromosome levels in mice, following administration by physiological routes, found that neither were induced in normal Aldh2 animals (Kunugita et al., 2008). Acetaldehyde was administered to groups of Aldh2 knockout (Aldh2-/- = deficient) and Aldh2 normal (ALDH2 +/+ = proficient) mice at 125 ppm or 500 ppm by continuous inhalation for 14 days or at 100 mg/kg orally for 14 days. Micronuclei frequencies in reticulocytes and gene mutations in the T-cell receptor genes (Tcr) of splenic lymphocytes were both assessed by cytometry. No significant inductions of either mutational end-point were observed in the Aldh2+/+ proficient (normal) mice at any administered acetaldehyde dose by either route. The study did observe significant increases in both endpoint in the Aldh2-/- enzyme deficient mice, again clearly demonstrating the critical role of Aldh2 in modulating the mutagenicity of this endogenous chemical.

- All positive studies of mutations (chromosome level; micronuclei) induced *in vivo* in mammals have employed i.p. injections as the route of administration (Morita et al. 1997; Wakata et al., 1998; Hynes et al., 2002).

- In addition, acetaldehyde (delivered as a metabolite of vinyl acetate) was positive for inducing micronuclei in mice when administered under non-physiological conditions, i.e., i.p. injections at doses of 250 mg/kg, 500 mg/kg, 1000 mg/kg or 2000 mg/kg with the last two doses inducing 43% and 45% lethality, respectively (Mäki-Paakkanen and Norppa, 1987). While approximately two-fold increases in micronuclei frequencies were observed at the lethal doses, significant increases were not observed at the non-lethal doses demonstrating a clear threshold**.**

In summary, although mutations have been induced in mammals following acetaldehyde exposure, all positive studies have employed the non-physiological i.p. route of administration. When normal laboratory animals are exposed via relevant physiological routes, there are no meaningful positive responses. The i.p. route, bypassing site-of-contact detoxification mechanisms, is not a realistic route of exposure for humans[3].

The non-physiological i.p. route of exposure allows the normal homeostatic mechanisms that protect against mutations from this endogenous agent to be overwhelmed. Specifically, the capacity of ALDH to detoxify acetaldehyde is presumably saturated when exogenous

[3] Collateral evidence as to the importance of route of administration may be obtained from studies in Drosophila. While a SLRL study was reported as positive when acetaldehyde was administered to larvae by injection, it was negative when the route of administration was by feeding (Woodruff, 1985). Similarly, an X-chromosome segregation test was also negative when acetaldehyde was administered by feeding (Rey et al., 1994).

acetaldehyde is delivered directly in this manner. The known exposure pathways for acetaldehyde in humans, provided high levels are achieved via the oral or inhalation route, would result in site-of-contact effects (e.g., irritation) but do not lead to systemic effects that could impact the testicular compartment. Hence, the irrelevance of any hazard characterization based on intraperitoneal administration. For these reasons, it is highly unlikely that acetaldehyde via the oral or inhalation route and susceptible to detoxification at sites-of-contact would be capable of reaching the testis in sufficient concentrations to produce mutagenic changes in spermatogonia.

V. Acetaldehyde Should Retain the Cat 2 Classification for Carcinogenicity

The AWG maintains that no change in classification is warranted because:

- There is no new hazard data in either laboratory animals or humans (as noted in the Health Council of the Netherlands, Acetaldehyde: Reevaluation of the Carcinogenicity and Genotoxicity[4], Nov 2014, pg 48) that would warrant a reevaluation and a more severe cancer classification for acetaldehyde.

- Based on prior EU assessments (EU Summary Risk Assessment Report, 2008[5] and ECHA RAC harmonized opinion, ECHA/RAC/ CLH-O-0000001742-77-01/A1, 2011), reviewers concluded that "acetaldehyde is a physiological intermediate with low background concentrations. Its adverse effects (genotoxicity and mutagenicity) are limited to non-physiologically high concentrations."

As acknowledged in the Netherlands proposal, there are no reliable human studies or oral or drinking water chronic rodent bioassays of acetaldehyde. Moreover, tumors observed following oral administration (Soffritti et al., 2002) have low reliability due to possible intercurrent respiratory infection in the rat colony[6] and by methodological shortcomings.

AWG believes that particularly for an endogenous, ubiquitous compound like acetaldehyde, it is critically important to consider the recent June 2015 guidance that ECHA has provided (https://echa.europa.eu/documents/10162/13562/clp_en.pdf, pg 380). Of particular relevance is the guidance provided for the classification of substances that induce cancer through excessive toxicity leading to cell death with associated regenerative hyperplasia. Acetaldehyde fits the description of such a substance.

Acutely, acetaldehyde is a skin, eye and respiratory tract irritant. The nature of acetaldehyde's nasal injury following chronic inhalation exposure at high concentrations (sufficient to cause

[4] http://www.gr.nl/sites/default/files/201428_acetaldehyde.pdf
[5] EU 2008: EU Risk Assessment Report of Vinyl Acetate Monomer, document R059_0805_env_hh http://echa.europa.eu/documents/10162/6434698/orats_final_rar_vinylacetate_en.pdf
[6] Health Council of the Netherlands, Acetaldehyde: Reevaluation of the Carcinogenicity and Genotoxicity, Nov 2014, pg 43.

marked reductions in body weights and survival[7]) suggests degenerative changes initially followed by hyperplastic and metaplastic transformation, along with cell proliferation at higher exposure concentrations; these changes precede tumor development. Indeed, all concentrations of acetaldehyde in the rat inhalation studies induced chronic tissue damage in the respiratory tract (Feron et al. 1982; Woutersen et al., 1986, Woutersen and Feron 1987).

While acetaldehyde exhibits both cytotoxicity, regenerative cell proliferation and clastogenicity, these represent high exposure concentration effects, at least 750 ppm in chronic inhalation animal bioassays. Notably, as a consequence of its high irritancy, no human could tolerate these high levels of acetaldehyde exposure, even for brief periods. As such, it is not reasonable to anticipate that such a hazard potential could exist with acetaldehyde under any realistic and even realistic worst case situation (e.g.,. workplace, food, drink, etc.).

The relationship between acetaldehyde cytotoxicity as a non-stochastic, upper respiratory tract carcinogen was first discussed in the key (Woutersen et al., 1986) inhalation study in rats. In that publication, Woutersen et al. concluded:

> *These observations strongly support the hypothesis the nasal tumours arise from epithelium which is damaged by acetaldehyde, viz the olfactory epithelium in the low concentration group and both the olfactory and the respiratory epithelium in the mid- and top-concentration groups [13].*

Exposure levels of both formaldehyde and acetaldehyde resulting in nasal carcinomas are very irritating and cause much damage to the nasal mucosa[8] followed by regenerative hyperplasia and metaplasia. This process of recurrent tissue damage and repair is probably mainly responsible for the promoting activity of these compounds. Since formaldehyde is known to react preferentially with single-stranded DNA and the incidence of dividing cells in the normal intact nasal epithelium is low, it seems reasonable to suppose the initiating potential of formaldehyde (and probably also of acetaldehyde) in concentrations not leading to cell damage is very low.

On the other hand, concentrations of these aldehydes causing recurrent tissue damage may be very effective with respect to initiation. Moreover, the increased cell turnover may strongly enhance the fixation of relevant DNA damage, and subsequently strongly increase the chance of progression of preneoplastic cells to cancer. As a consequence, formaldehyde and acetaldehyde in subcytotoxic concentrations are likely to be very weak initiators (there are only a few dividing cells) with only minor, if any, promoting activity (there is no increased cell turnover). This means that in subcytotoxic concentrations these aldehydes cannot act as complete carcinogens, but only as weak initiators. These considerations suggest that the cytotoxic effects of these aldehydes may be very important for the induction of nasal

[7] Indeed, chronic acetaldehyde exposure in rats at all concentrations (750, 1500 or 3000/1000 ppm) was associated with growth retardation (>10% at 1500 ppm or greater) and reduced survival in all groups.

carcinomas in experimental animals. In this respect the question arises as to how far exposure of human beings to subcytotoxic (actual exposure) concentrations of formaldehyde or acetaldehyde constitutes a carcinogenic risk. If there is a risk, it seems to be extremely low. On the other hand, the risk may be more substantial if such exposure takes place under conditions of recurrent tissue damage due to other factors.

While a formal review of acetaldehyde's classification has not been undertaken for quite some time, it is relevant to recognize that acetaldehyde was reviewed in the course of recent assessment of vinyl acetate monomer (VAM), given that VAM metabolizes to acetaldehyde (see 2008 EU Risk Assessment Report of Vinyl Acetate Monomer and in the 2011 EU RAC Harmonized classification of Vinyl Acetate Monomer). Those reviews clearly support the more appropriate Cat 2 cancer classification for acetaldehyde rather than category 1B based on the recognition of a non-stochastic (i.e., threshold) mode of action. The 2011 EU report notes:

> *"Acetaldehyde is a physiological intermediate with low background concentrations. Its adverse effects (genotoxicity and mutagenicity) are limited to non-physiologically high concentrations. Therefore, a threshold mode of action is assumed for vinyl acetate.*
>
> *Above threshold concentrations, cytotoxicity (only at the olfactory mucosa), mitogenic actions and genotoxic actions occurred.*
>
> *In vivo genotoxicity tests showed that systemic genotoxicity appears to be limited to toxic doses. This is in line with the absence of systemic carcinogenic effects.*
>
> *Data on vinyl acetate are in line with the idea that vinyl acetate genotoxicity is mediated by acetaldehyde. Increasing concentrations of acetaldehyde produce genotoxic actions at the site of contact. It has to be taken into consideration that acetaldehyde occurs naturally in mammalians cells and is part of the physiological cellular metabolism."*

In summary, AWG contends that to the extent acetaldehyde may trigger a carcinogenic response, cancer is unlikely to be manifest unless an unrealistically high exposure level occurs that exceeds endogenous acetaldehyde levels and overwhelms acetaldehyde detoxification processes. Only under such conditions may the cytotoxicity and genotoxicity potential of acetaldehyde be expressed.

The CLH should acknowledge the multistep process involving acetaldehyde cytotoxicity with regenerative cell proliferation as a key step in tumorigenesis. The presence of an exposure threshold, supports a non-stochastic mode of action and the current Cat 2 EU classification for acetaldehyde.

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