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Review

Evaluation of the genotoxic potential of acrylamide: Arguments for the derivation of a tolerable daily intake (TDI value)

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ABSTRACT

This opinion of the Senate Commission on Food Safety (SKLM) of the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) presents arguments for an updated risk assessment of diet-related exposure to acrylamide (AA), based on a critical review of scientific evidence relevant to low dose exposure. The SKLM arrives at the conclusion that as long as an appropriate exposure limit for AA is not exceeded, genotoxic effects resulting in carcinogenicity are unlikely to occur. Based on the totality of the evidence, the SKLM considers it scientifically justified to derive a tolerable daily intake (TDI) as a health-based guidance value.

Abbreviations

AA	acrylamide
AAMA	N-acetyl-S-(2-carbamoylethyl)-L-cysteine
AC	acrolein
Ade	adenine
AUC	area under the curve
$BMDL_{10}$	benchmark dose lower confidence limit of 10%
CI	confidence interval
CYP	cytochrome P450
DFG	Deutsche Forschungsgemeinschaft
EFSA	European Food Safety Authority
FFQ	food frequency questionnaires
FPG	formamidopyrimidine-DNA glycosylase
GA	glycidamide
GAMA	N-acetyl-S-(2-hydroxy-2-carbamoylethyl)-L-cysteine
Gua	guanine
gpt	glutamate pyruvate transaminase
GSH	glutathione
Gua	guanine
Hb	haemoglobin
3-HPA	3-hydroxypropanal
hprt	hypoxanthine-guanine-phosphoribosyl-transferase
HR	Hazard Ratio
LOD	limit of detection
MA	mercapturic acid
MAK	Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area
3-MCPD	3-monochloropropane diol
MF	mutation frequencies
MN	micronuclei
MNNG	N-methyl-N′-nitro-N-nitrosoguanidine
MoA	mode of action
MOE	margin of exposure
PBBK	physiologically based biokinetic
Pig-a	phosphatidylinositol glycan class A
PBMC	peripheral blood mononuclear cells
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
SKLM	Senate Commission on Food Safety
TDI	tolerable daily intake
TK	thymidine kinase

Preamble

This manuscript presents arguments in support of the derivation of a tolerable daily intake (TDI) value for acrylamide (AA), based on evidence for a non-linear dose-response for AA genotoxicity in the low dose range. Moreover, the authors consider that endogenous AA formation in animals and humans has not been adequately taken into account in previous evaluations of AA. The authors therefore primarily focus on scientific evidence generated at low doses/concentrations better reflecting realistic human dietary exposure scenarios. In contrast, high dose toxicology is operationally defined as effects observed at dose levels by far exceeding consumers' exposure, such as those chronically applied in carcinogenicity bioassays. In this manuscript, AA doses $\geq$1 mg/kg bw/day, which are several orders of magnitude above the estimated human exposure (mean: 0.4–1.9 μg/kg bw/day; 95th percentile: 0.6–3.4 μg/kg bw/day) are considered to be high doses from a toxicological point of view. Of note, such *in vivo* doses translate into rather low plasma levels. For instance, administration of a single dose of 100 μg/kg bw AA to rats resulted in peak plasma concentrations close to 2 μM of AA and 60 nM of the metabolite glycidamide (GA) (Berger et al., 2011), whereas repeated application of approximately 1 mg/kg bw to rats were reported to result in steady state levels of about 0.5–0.65 μM AA and GA (Doerge et al., 2005).

The approach presented here is driven by evidence arguing for the existence of nonlinear, thresholded dose-response relationships inherent in the toxicological characteristics of AA in the low dose range. This is at variance with risk assessments driven by preferential consideration of dose-related effects observed at experimentally accessible high concentrations/dosages, with subsequent extrapolation to human exposure levels following a best fit modelling function. The most relevant toxicological effect of AA observed in long-term studies in experimental animals is tumorigenicity. Genotoxicity of AA is considered to contribute to these effects, as comprehensively summarized by the European Food Safety Authority (EFSA, 2015; EFSA, 2022). Based on positive findings in *in vitro* and *in vivo* genotoxicity studies (DNA and/or chromosomal damage and gene mutations), AA was concluded to be a genotoxic carcinogen and, therefore, the margin of exposure approach was considered to be appropriate for the safety assessment (EFSA, 2015; EFSA, 2022). However, evidence from studies on AA conducted at low doses, which more closely reflect dietary exposure, suggests a sublinear dose response for AA-mediated genotoxicity, which is in line with the concept of threshold levels for genotoxicity/mutagenicity/carcinogenicity.

Therefore, the aim of the present paper is to complement earlier opinions and statements by specifically considering low dose AA toxicology and to provide arguments in favor of the derivation of a health based guidance value for AA.

1. Introduction

α,β-Unsaturated aliphatic carbonyl compounds are naturally widespread in food, but are also formed during thermal treatment of food.

The Senate Commission on Food Safety (SKLM) of the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) repeatedly evaluated various α,β-unsaturated carbonyl compounds. In 2002, health effects of certain 2-alkenals naturally occurring in food and/or added as flavourings to food, such as 2-hexenal or 2,4-nonadienal, were evaluated (SKLM, 2005). These compounds can readily react with proteins and DNA to cause cytotoxic and genotoxic effects similar to other α,β-unsaturated carbonyl compounds. Metabolically, 2-alkenals are rapidly detoxified by oxidation or reduction as well as by glutathione conjugation (SKLM, 2005). Although the available data were considered inadequate for a comprehensive risk assessment at that time, there was evidence to suggest that toxicity and genotoxicity become significant only at high doses that exceed the metabolic detoxification capacity (SKLM, 2005).

In 2012, the SKLM adopted an opinion focusing on the potentially genotoxic process-related contaminants AA and acrolein (AC), which may occur naturally, but appear to be primarily formed during thermal treatment of food (SKLM, 2013; Guth et al., 2013). Data on the formation, occurrence, exposure, metabolism, biological effects, toxicity and carcinogenicity of AC and AA available in the scientific literature at that time were evaluated and knowledge gaps as well as research needs were defined. The SKLM also considered the available evidence for the endogenous formation of AC and AA as a consequence of physiological energy metabolism. However, to what extent such endogenous formation might contribute to the total exposure and the probable sources were not known at that time (Guth et al., 2013).

In 2020, a joint working group of the DFG Senate Commissions SKLM and MAK (Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area) proposed an updated risk evaluation for genotoxic carcinogens, also addressing AA as one of several selected examples (Hartwig et al., 2020). Although effective mitigation measures have been implemented, certain levels of carcinogenic substances are still present in food, and consumers can often not avoid intake (Hartwig et al., 2020). Up to now, the distinction between genotoxic and non-genotoxic carcinogens has been considered critical for risk assessment. In the case of non-genotoxic carcinogens, the existence of no-effect levels (threshold levels) is assumed, whereas genotoxic carcinogens are considered to represent a risk even at extremely low exposure levels, since even few DNA lesions may in principle result in mutations and increased tumour risk (Hartwig et al., 2020). Based on updated mechanistic knowledge, an alternative approach to the risk assessment of genotoxic carcinogens has been considered by the MAK/SKLM joint working group within a comprehensive risk evaluation of diet-borne putative genotoxic carcinogens (Hartwig et al., 2020). Essentially, in this publication three categories of mechanisms have been identified encompassing effects of compounds that "at the doses at which an increased cancer incidence is observed may act primarily by DNA damage induction (1), may additionally exert promoting activity (2) or may (possibly additionally) affect the processing of DNA modifications (3)" (Hartwig et al., 2020).

2. Risk assessment of AA by the margin-of-exposure concept

Based on evidence for genotoxicity, recommendations by risk management bodies have been developed to confine consumer exposure to AA to levels as low as reasonably achievable (ALARA principle). Consequently, various mitigation measures, in combination with adequate consumer information, have contributed to a reduction of mean dietary consumer exposure (Hartwig et al., 2020). However, reduction of dietary exposure to a level that would bring about a margin of exposure (MOE) close to 10,000 or higher (see below) does not appear within reach. This may be inferred not only from the limited perspectives to further implement effective mitigation measures "from farm to fork", but also from the fact that the formation of process-related contaminants such as AA during domestic meal preparation is difficult to control. Moreover, endogenous formation of AA is considered to add to a substantial degree to exogenous exposure and means to reduce endogenous exposure are unknown at present. Considering the limited effects expected for further mitigation measures, this seems to be a difficult situation for all stakeholders to cope with (Hartwig et al., 2020).

In its 2015 assessment, the EFSA CONTAM Panel applied the MOE approach for compounds that are both genotoxic and carcinogenic for the risk characterisation of neoplastic effects supposedly associated with a dietary exposure to AA (EFSA, 2015). This assessment was reconfirmed in 2022 (EFSA, 2022). The point of departure for risk characterisation was the lowest $BMDL_{10}$ (benchmark dose, lower confidence limit of 10%) of 0.17 mg/kg bw/day derived from data on incidences of Harderian gland adenomas and adenocarcinomas in male B6C3F1 mice exposed to AA for two years (NTP, 2012; EFSA, 2015). Mean and 95th percentile dietary AA exposures in Europe have been estimated to range between 0.4 and 1.9 μg/kg bw/day and between 0.6 and 3.4 μg/kg bw/day, respectively, across surveys and age groups (EFSA, 2015). By dividing the $BMDL_{10}$ by these mean (95th percentile) dietary AA exposures, MOE values below 500 were derived (EFSA, 2015). However, for substances that are both genotoxic and carcinogenic, a MOE of 10,000 or higher would be considered to be of low concern from a public health point of view (EFSA, 2005).

3. Considerations for an updated risk assessment

In the opinion of the SKLM, new data have become available that justify the classification of AA as a chemical that does not significantly contribute to cancer risk in humans, provided an appropriate exposure limit is not exceeded. The SKLM substantiates the recommendation to reconsider the risk assessment of AA by the following science-based arguments:

- Glycidamide is a weak mutagen/genotoxic agent (3.1)
- At low dose level, AA only induces minimal DNA damage *in vivo*, which does not exceed the background range of similar human DNA damage (3.2)
- At low dose level, metabolically formed GA is almost entirely scavenged by coupling with glutathione (3.3)
- AA is formed endogenously in the body at concentrations in the lower range of human dietary exposure (3.4)

These main arguments are outlined in detail in the following sections.

3.1. Glycidamide is a weak mutagen/genotoxic agent

The formation of the reactive AA metabolite glycidamide (GA, oxirane-2-carboxamide) and its interaction with nucleic acids causing DNA damage have long been considered to represent a key event governing genotoxicity of AA. However, GA is not considered to be a potent genotoxic agent or a potent mutagen (Baum et al., 2005, 2008; Glatt et al., 2005; Johansson et al., 2005; Puppel et al., 2005; Thielen et al., 2006), as discussed in the following section.

3.1.1. Comparison to potent genotoxic mutagens/carcinogens

To exemplify the low genotoxic/mutagenic potency of GA and the observed nonlinear dose response characteristics, *in vitro* evidence from concentration/response studies in comparison to potent genotoxic standard mutagens/clastogens may be considered. The induction of DNA damage, mutations of the hypoxanthine-guanine-phosphoribosyltransferase (hprt) gene and of micronuclei (MN) were analysed in V79 cells and human peripheral lymphocytes incubated with AA and GA, using bleomycin and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) as positive controls (Baum et al., 2005). With AA (100–10,000 μM), no significant induction of hprt mutations was observed in V79 cells, whereas GA (400–2,000 μM) induced significantly increased mutation frequencies (MF) from 800 μM upwards. However, MNNG

induced mutations in a concentration-dependent manner already at concentrations of 0.5 μM and higher, thus showing an orders of magnitude higher potency in comparison to GA. Similar results were obtained by monitoring DNA damage in human blood lymphocytes with the comet assay: AA was found to be inactive at all concentrations tested (1,000–6,000 μM), whereas GA induced DNA damage in a concentration-dependent manner, with significant effects at 300 μM and higher (Baum et al., 2005). Furthermore, MN induction in human blood lymphocytes (blood samples from 15 donors) was tested with AA (500–5,000 μM) and GA (50–1,000 μM), using bleomycin (4 μM) as positive control. The highest AA concentration tested (5,000 μM) induced an about two-fold increase in the mean MN frequency in 7 out of 15 donors, whereas the rest showed no reaction, resulting in a non-significant overall effect. GA at the dose range tested (50–1,000 μM) did not significantly induce enhanced MN frequencies, whereas bleomycin (4 μM) was strongly active at a more than 200-fold lower concentration when compared to the highest, still negative GA concentration (Baum et al., 2005). Since such a high concentration was deemed to exceed by various orders of magnitude any blood level to be expected under realistic dietary exposure conditions, no higher GA concentrations were tested by the authors (Baum et al., 2005). Not unexpectedly, the data from this study also demonstrated that DNA damage, as measured in the comet assay, was detected at a lower concentration than the concentration that led to the manifestation of mutagenicity. In later studies, highly potent food borne carcinogens were investigated in comparison to GA using similar genotoxicity and mutagenicity testing methodology. In all tests, GA proved to exert orders of magnitude lower potency, as discussed in detail in Hartwig et al. (2020). In the comet assay in V79 cells and in human lymphocytes, GA induced DNA damage down to a concentration of 300 μM (4 h) (Baum et al., 2008). By comparison, the preactivated N-nitroso compound 3-N-nitroso-oxazolidine-2-one (NOZ-2) and (±)-anti-benzo[a]pyrene-7, 8-dihydrodiol-9,10-epoxide ((±)-BPDE), showed much stronger genotoxic activity, significantly inducing DNA damage already at 3 μM (15 min) (Baum et al., 2008). In the hprt mutagenicity test in V79 cells, GA induced mutations at concentrations of 800 μM and above, whereas NOZ-2 as well as (±)-BPDE significantly induced hprt mutations at > 200-fold lower concentration (Baum et al., 2005, 2008; Thielen et al., 2006). The results confirm the strong difference in potency between GA and highly potent food borne genotoxic mutagens/carcinogens, especially pre-activated forms of nitrosamines and polycyclic aromatic hydrocarbons.

In summary, these results strongly argue for a rather low genotoxic/mutagenic activity of GA in comparison to standard laboratory or food borne genotoxic mutagens. Moreover, the existence of thresholds for both effects can be deduced. For genotoxicity in human blood lymphocytes, as measured in the comet assay, a threshold between 100 (negative outcome) and 300 μM (first significant positive outcome) is apparent. For mutagenicity, as detected in the hprt test with V79 cells, the apparent threshold level is higher and is localized at a concentration range of 400–800 μM. In contrast, even a 1 mM concentration of GA did not induce a significant rise in MN in human white blood cells. It may be assumed that the observed sublinear/thresholded *in vitro* dose-response relationships may to some extent depend on the tested cell types, chosen incubation conditions and the sensitivity of the test systems.

3.1.2. Evidence from DNA adduct formation and toxicokinetic studies

At variance to results obtained in toxicological studies with high AA doses, in the here defined low dose range, exclusively *N*7-GA-guanine (Gua) lesions were detected in rat tissues at levels close to background (Watzek et al., 2012). In the high dose range, evidence for the formation of *N*3-GA-adenine (Ade) adducts, albeit at various orders of magnitude lower yields when compared to *N*7-GA-Gua-adducts, was also provided. For illustration, in mice given single doses of 10–50 mg AA/kg bw, up to about 100–1000-fold differences in adduct levels of *N*7-GA-Gua vs *N*3-GA-Ade were reported (Gamboa da Costa et al., 2003). Similar differences in adduct levels were reported in lung and liver of B6C3F1 mice following administration of about 1–9 mg AA/kg bw in the drinking water for 28 days (de Conti et al., 2019), encompassing the concentration range used in the 2-year cancer bioassay (87.5–700 μM, equivalent to 1.04, 2.20, 4.11 and 8.93 mg/kg bw/day for males, and to 1.10, 2.23, 4.65 and 9.96 mg/kg bw/day for females). Dose-dependent increases in the *N*7-GA-Gua and *N*3-GA-Ade levels (900 and 4 adducts per 10^8 nucleotides at 700 μM AA, respectively) were likewise detected in the liver, providing evidence of dose linearity for adduct formation in this high dose range (de Conti et al., 2019). Both DNA base adducts are depurinating and considered to exert similarly weak biological effects with respect to mutagenicity. Especially *N*7-GA-Gua adducts are easily and frequently formed, but although they may to some extent cause apurinic sites and/or generate 5-N-alkyl-2,6-diamino-4-hydroxyformamidopyrimidine (N-alkyl-FAPy-G lesions), they are considered to have low relevance as cause of mutations in cells and tissues (Boysen et al., 2009). Other adducts may form under exaggerated *in vitro* conditions, e.g., *N*1-GA-Ade that has been reported to be formed when exposing salmon sperm DNA to high concentrations of GA (Gamboa da Costa et al., 2003). However, to the best of our knowledge this adduct has not been identified *in vivo*. Likewise, adducts at positions of supposedly higher mutagenic potential like, for instance, *O*6 of Gua have not been found *in vivo*. A comparison to a closely related epoxide, ethylene oxide, may be useful for read-across purposes. Ethylene oxide was assessed to exert rather weak mutagenic potential concerning the ethylene oxide-induced DNA adducts in mammalian/human cells (Hartwig et al., 2020; Tompkins et al., 2009), which is in line with the observed low *in vivo* mutagenic potency in rodents (Recio et al., 2004; Tates et al., 1999). Ethylene oxide was reported to generate under forced *in vitro* conditions predominantly *N*7-Gua adducts, together with much smaller yields of adducts at *N*3 of adenosine, and traces of *O*6-guanosine (Segerbäck, 1990).

GA concentrations that begin to show significant genotoxic/mutagenic effects *in vitro* are orders of magnitude higher than those expected from *in vivo* exposure. Toxicokinetic studies in SD rats orally exposed to 100 μg AA/kg bw via drinking water or with food (French fries) showed rapid kinetics after ingestion in water, with a peak plasma concentration (c_{max}, 30 min) of 1.84 μM and an elimination half-life of 3 h (Berger et al., 2011). In contrast, after intake with food, plasma AA levels reached a plateau at about 0.44 μM, reflecting delayed release from food during gastro-intestinal passage. In contrast to AA, GA was barely detectable (i.e., plasma concentration: 60 nM) at just one single point in time (4 h) in both treatment groups (Berger et al., 2011). For comparison, a ten-fold higher dosage of about 1 mg AA/kg bw/day administered in drinking water to Fischer F344/N rats for up to 50 days resulted in a steady state in serum with an accumulation half-life of about 3–4 days and GA serum levels of about 0.5–0.65 μM (Doerge et al., 2005). Mice given a single gavage dose of 50 mg/kg AA showed a maximal AA serum concentration of 450 μM after 0.5 h and a maximal GA concentration of 190 μM after 2 h (Doerge et al., 2005). As mentioned above, these data show that blood levels reached after extended application of an AA dose selected within the range of tumour induction of AA (1 mg/kg/day) are far lower than concentrations beginning to exert genotoxic and/or mutagenic effects *in vitro*. Additional toxicokinetic evidence comes from studies in pigs, considered physiologically to be more close to humans than rodents (Aureli et al., 2007). AA intakes of about 0.8 and 8 μg kg bw/day did not result in measurable GA-haemoglobin (Hb) adduct levels whereas AA-Hb adducts were found directly proportional to the intake. Although the dosages were quite different (8 μg/kg bw in pigs vs. 100 μg/kg bw in rats) the results support the observation of Berger et al. (2011) who also found in rats dose dependently enhanced AA-Hb adduct levels but no response of GA-Hb adduct levels. At variance, in a further study by Vikström et al. (2008), AA was administered to mice through diet at five dose levels between 3 and 50 μg/kg bw/day. AA- and GA-Hb adduct levels showed a linear increase with AA intake. It is well established that oxidative metabolism to GA is more effective in the mouse

than in rats and humans, especially in the low dosage range. In a human study (Vikström et al., 2011), AA-rich foods were given to nonsmokers: a "high" intake of 11 μg AA/kg bw/day for 4 days or an extra "medium" intake of 2.5 μg AA/kg bw/day for a month. Hb-adduct levels from AA and GA, measured in blood before and after exposures, were used for calculation of the "area under the concentration-time curve" (AUC) of AA and GA using reaction rate constants for the adduct formation measured *in vitro*. Mean AA- and GA-adduct levels increased about twofold after the periods with enhanced intake. The AUC-AA and AUC-GA were determined to be approximately 200 and 50 nMh per μg AA/kg bw, respectively. Further insight into human toxicokinetics can be obtained from Fuhr et al. (2006). AA toxicokinetic was evaluated in healthy volunteers after the consumption of a meal containing 0.94 mg of AA per person (corresponding to approx. 13 μg/kg bw for a person of 70 kg). Conjugation of AA with glutathione was found to exceed the formation of the reactive metabolite GA. This finding is supported by results of a mechanistic study in primary human hepatocytes showing glutathione coupling of AA to proceed at up to 3 times higher rate than its epoxidation to GA (Watzek et al., 2013) (see also section 3.3). In addition, the Fuhr data also indicate an at least 2–4 fold lower internal GA exposure from dietary AA in humans as compared to rats and mice, respectively.

Altogether, the data from rats and pigs appear quite consistent whereas other data are at variance. Toxicokinetic data obtained in humans may be useful for physiologically based biokinetic (PBBK) based dosimetry and risk assessment (see section 6. "Previous approaches for the derivation of a TDI in the literature").

3.1.3. Combined evidence from in vitro and oral in vivo genotoxicity and mutagenicity studies

An updated comprehensive review of *in vitro* and *in vivo* genotoxicity and mutagenicity studies on AA and/or GA since 2015 is available from EFSA (2022). Most of the evaluated studies reported positive genotoxicity findings (induction of micronuclei, DNA strand breaks, gene mutations) at *in vitro* and *in vivo* exposure conditions encompassing concentrations/dosages much higher than those expected under realistic exposure scenarios (positive *in vitro* concentrations: 0.5–4 mM AA and 0.1–1.1 mM GA; positive *in vivo* doses: 1–6 mg/kg bw/day and higher). Of note, these *in vivo* studies in general did not consider any potential additional AA exposure by AA containing feed and/or endogenous AA generation. Fig. 1 and 2 give an overview of the findings of some relevant *in vitro* and oral *in vivo* studies. In more detail, mutations of the *TK* gene in human MCL-5 cells expressing various CYPs were observed with 4 mM AA and 0.1 mM GA (David and Gooderham, 2018). The same applies to human *TP 53* knock-in mutations in mouse embryo fibroblasts (1.1 mM GA), to *lacZ* gene mutations in the same cells (3 mM AA; 0.75 mM GA) (Hölzl-Armstrong et al., 2020a) as well as in lung FE cells (AA + S9, GA - S9, positive at concentrations ≥2 mM) (Hölzl-Armstrong et al., 2020b) and to *gpt* gene mutations in murine pulmonary organoids from *gpt* delta M C57BL/6 J mice (1.4 mM AA) (Komiya et al., 2021). The micronucleus and *Pig-a* mutation assays were negative and equivocal, respectively, in F344 rats in a dose range of up to 12 mg/kg bw given in drinking water, whereas in mice (up to 24 mg/kg bw) a dose-dependent increase in micronucleus formation (statistically significant at 6–24 mg/kg bw) and an equivocal response in the *Pig-a* mutation assay (increased mutation frequency only at an intermediate dose) were reported (Hobbs et al., 2016). At these high dosages, even exceeding those applied in long-term carcinogenicity studies, "induction of structural DNA damage, as opposed to point mutations is most relevant to the genotoxic mode of action of acrylamide", prompting the notion that "non-genotoxic mechanisms contribute to acrylamide-induced carcinogenicity in rodents" (Hobbs et al., 2016). Clearly, genotoxicity was not detected in this study at dosages <6.0 mg/kg bw/day. Similar results were observed in a study in mice with single intraperitoneal application of seven AA doses ranging from 0 to 30 mg/kg bw (0, 1, 3, 6, 12, 24, and 30 mg/kg bw) (Abramsson-Zetterberg, 2003). A significant increase in micronuclei formation at ≥ 6 mg/kg bw was documented. Although the authors noted the absence of a threshold dose, the data suggest a thresholded response which might be located between 3 and 6 mg/kg bw. This would be in agreement with the data presented by Hobbs et al. (2016).

Likewise, induction of chromosomal aberrations/micronuclei *in vivo* was observed at high dosage (≥2 mg/kg bw/day for 30 days) in the bone marrow of Swiss albino mice (Algarni, 2018) and in *gpt* delta mice (≥15 mg/kg bw/day for 28 days), also showing mutations in testes, lung and sperm at 30 mg/kg bw (Hagio et al., 2021). A whole series of similar *in vivo* micronucleus tests with AA given by different routes has been listed in the EFSA review, showing positive responses at high dosages (>1 mg/kg bw/day; EFSA, 2022). Similarly, *in vitro* positive micronucleus responses were observed in human lymphocytes (0.25–100 mM AA, becoming positive at 50 mM after a 20 h exposure) (Zamani et al., 2018) and in rat lymphocytes (1.4–4.2 mM, after 24 h) (Ankaiah et al., 2018).

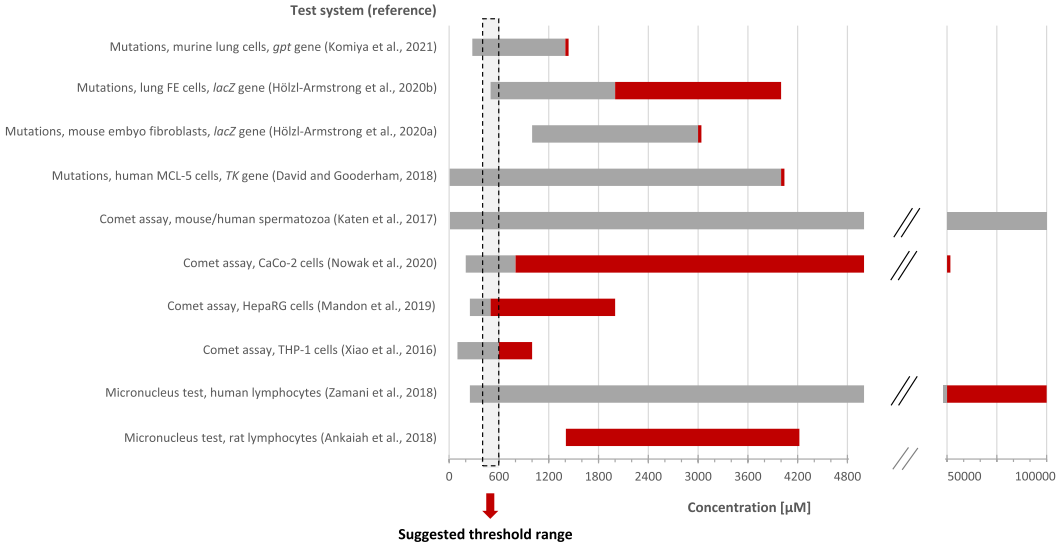

Fig. 1. Evidence from relevant *in vitro* genotoxicity studies on AA since 2015. Significant positive responses were detected in the range of 400–600 μM (suggested threshold range), which agrees with the range described by Baum et al. (2005) and Hemgesberg et al. (2021b). Grey: negative response; red: positive response. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

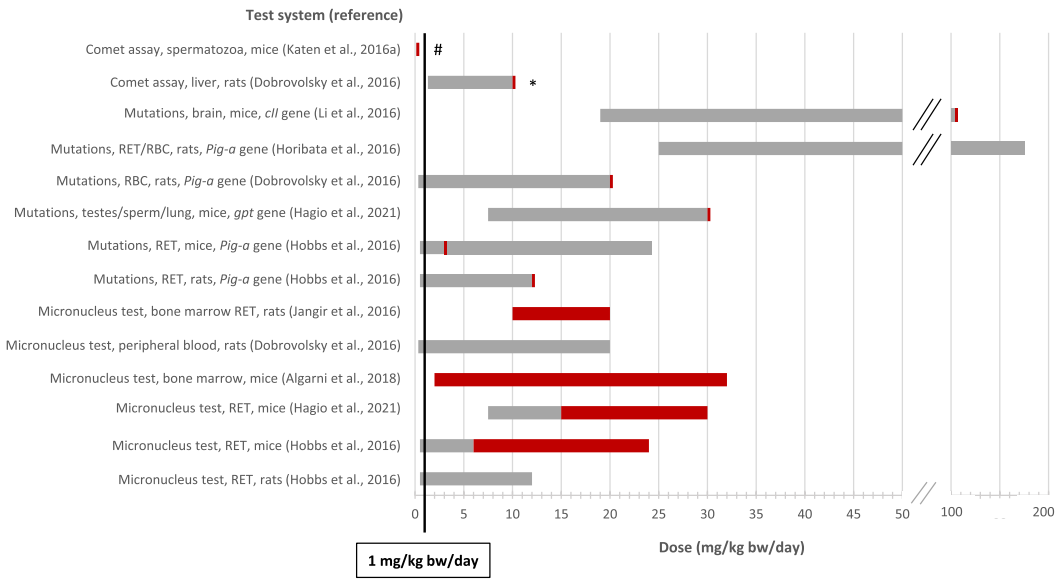

Fig. 2. Evidence from relevant *in vivo* genotoxicity studies on oral AA administration since 2015. AA doses ≥1 mg/kg bw/day, which are several orders of magnitude above the estimated human exposure (approx. 0.4–3.4 μg/kg bw/day), are considered to reflect high doses in toxicological studies. Grey: negative response; red: positive response; *indicates a weak response, #according to the SKLM, the validity of these findings needs substantiation (see text). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.) (Dobrovolsky et al., 2016; Horibata et al., 2016; Jangir et al., 2016; Li et al., 2016).

Positive responses in the comet assay were observed in a variety of cell systems, including human THP monocytic cells (at 0.6 mM AA and higher; Xiao et al., 2016), 3D liver HepaRG cells (at 0.5 mM AA and above; Mandon et al., 2019) and human lymphocytes (≥10 mM or 0.25 mM GA, respectively; Hansen et al., 2018; de Lima et al., 2016). In Caco-2 cells, the comet assay became positive at 0.8 mM AA and above after 1 h, with minor enhancing effects following treatment with EndoIII and formamidopyrimidine-DNA glycosylase (FPG) (Nowak et al., 2020).

Further studies addressed the induction of DNA damage in the germline of mice (Katen et al., 2016a, 2016b, 2017). Male Swiss CD mice (n = 3) received AA in the drinking water for 6 months (1 μg/mL, corresponding to about 130 μg/kg bw/day). This exposure regime was reported to increase DNA damage in the spermatozoa, as measured by an alkaline comet assay. No effect on overall fertility was found. After mating with unexposed females, increased DNA damage was also observed in the male offspring of AA-treated males. In addition, the F0 as well as F1 mice showed increased levels of CYP2E1 protein in their germ cells. The authors interpreted these findings as potentially pointing to epigenetic alterations (Katen et al., 2016a). In another study with a similar experimental design, essentially similar findings were reported by the same group, in addition pointing to some modifying effects of resveratrol (Katen et al., 2016b). Based on the fact that the test system is quite unique, the number of AA-treated F0 animals was very low (n = 3) and only one dose group was included (obviating a dose response analysis for the reported effects), the SKLM considers that these data cannot be used for risk assessment.

This also applies to a subsequent high dose study (Katen et al., 2017), in which AA was administered intraperitoneally to mice (12.5 and 25 mg/kg bw/day i.p. for 5 days). Induction of DNA damage in epididymal sperm cells and in spermatocytes was reported. In the same paper, it was also shown that *in vitro* exposure to AA (up to 100 mM) did not lead to DNA damage in mouse spermatozoa, whereas DNA damage was observed with GA already at 50 nM. However, within the wide concentration range of 50 nM–500 μM GA (after a 1 h incubation), the results did not show a clear dose response (Katen et al., 2017).

3.2. At low dose level, AA induces only minimal DNA damage in vivo, which does not exceed the background range of similar human DNA damage

In rats, AA at low exposure levels (encompassing diet-related intake levels) induced only minimal DNA damage, as monitored by adduct formation. DNA adducts were monitored at single oral doses of 0.1–100 μg/kg bw, the highest dose by far exceeding average consumer exposure ranging from 0.4 to 3.4 μg/kg bw/day (EFSA, 2015). However, DNA adduct formation reported in response to AA in the above-mentioned dose range was far from being linear. Moreover, it did not exceed the lower bound of human background DNA damage of comparable DNA *N*7-Gua lesions (Watzek et al., 2012). Significant dose-dependent increases in DNA adduct formation were only observed at a higher dose range (see below).

In more detail, formation of *N*7-GA-Gua DNA adducts in liver, kidney, and lung was measured in rats following a single oral dose of 0.1–10,000 μg/kg bw administered by gavage (Watzek et al., 2012). The animals received an experimental diet prepared in-house, reflecting a theoretical maximum daily background AA intake of 0.1 μg/kg bw/day, based on the analytical detection limit of AA in this diet. An increased formation of *N*7-GA-Gua adducts was not detected in the liver, kidney, and lung at 0.1 μg/kg bw/day. In the dose range of 1–100 μg/kg bw/day, no linear dose-response relationship was evident in any organ, with adduct levels not exceeding a level of about 1–2 adducts/10^8 nucleotides. However, a clearly dose-related increase was observed at 500 μg/kg bw and above. In control animals receiving the experimental diet low in AA, adduct levels were below the LOD (0.2 adducts/10^8 nucleotides). This dose-response DNA adduct study by Watzek et al. (2012) is of substantial value, since it included dose levels encompassing mean consumer exposure, enabling DNA adduct dosimetry at ultrahigh analytical sensitivity and accuracy levels (limit of quantification: 0.25 in 100 million nucleotides; inter and intraday accuracy: 3%). In addition, it allowed parallel dosimetry of urinary mercapturic acids, which reflect the detoxifying coupling of AA and GA with glutathione in the liver. This parallel dosimetry of toxifying and detoxifying dose-response revealed a clear dose dependence of the detoxifying mercapturic acid pathway, down to 1 μg/kg bw AA. In contrast, for DNA *N*7-GA-Gua adduct

formation, no dose response was observed in the low dose range encompassing 100 μg/kg bw down to 0.1 μg/kg bw (Watzek et al., 2012). This lack of dose dependence supports the existence of a threshold level for AA, which has to be exceeded in order to induce dose-dependent genotoxic DNA damage. In the study by Watzek et al. (2012), this threshold level may be localized in a dose range between 100 and 500 μg/kg bw.

To put this into perspective, background *N*7-GA-Gua adduct levels in the vast majority of samples of human blood DNA or human peripheral blood mononuclear cells (PBMC) taken from healthy volunteers are within a range of 1–2 adducts/10^8 nucleotides (Hemgesberg et al., 2021a; Jones et al., 2021). For comparison, in human tissues, background levels of DNA adducts related to electrophilic genotoxic agents of various origins cover a range of up to 500 specific adducts/10^8 nucleotides (Hartwig et al., 2020). As an example, for *N*7-(2-carboxyethyl)-Gua, considered closely related to *N*7-GA-Gua, a background level of about 8 adducts/10^8 nucleotides has been reported in liver DNA (Cheng et al., 2010). Thus, at the low dose range, *N*7-GA-Gua adduct levels in rats are not expected to exceed the range of human physiological background DNA damage (Watzek et al., 2012; Hartwig et al., 2020). This conclusion is supported by results of an *in vitro* dose response study in primary rat hepatocytes, in which a background level of 5–10 *N*7-GA-Gua adducts/10^8 nucleosides was measured (Hemgesberg et al., 2021b). Incubation with AA up to a concentration of 500 μM for 24 h did not induce a significant increase in DNA adduct formation, only becoming measurable in the mM concentration range. The authors calculated a composite lower bound of the 95% confidence interval of the benchmark concentration associated with a 10% increase in *N*7-GA-Gua levels over background ($BMCL_{10}$) of 6.35 μM AA. According to the model, up to this benchmark value, an increase in *N*7-GA-Gua of more than 10% over the background observed in untreated hepatocytes may not be expected (Hemgesberg et al., 2021b). According to the authors, this value may be considered as a practical threshold for genotoxicity of AA in the cell culture model used.

3.3. At low dose level, metabolically formed GA is almost entirely scavenged by coupling with glutathione

In rats, repeated intake of AA in foods/water at doses of 50–100 μg/kg bw resulted in linear time- and exposure-related cumulative build-up of AA-Hb adducts in blood erythrocytes (Berger et al., 2011). In contrast, no corresponding increase in Hb-GA adducts was observed, although urinary excretion of N-acetyl-S-(2-hydroxy-2-carbamoylethyl)-L-cysteine (glycidamide mercapturic acid, GAMA) indicated significant GA formation (Berger et al., 2011). It was concluded that in this low exposure range most of the GA formed metabolically in the liver is effectively scavenged by conjugation with glutathione (GSH) (Berger et al., 2011). This conclusion is further supported by *in vitro* data in primary rat hepatocytes demonstrating that the detoxification of GA via GSH binding is clearly faster than GA formation (Watzek et al., 2013). In this context, it should be mentioned that the primary rat hepatocytes used in the above-mentioned study displayed GSH levels and glutathione transferase activities similar to those in liver tissue (Langley-Evans et al., 1996; Watzek et al., 2013). In addition to coupling with GSH, direct reaction of AA with other nucleophilic biomolecules might additionally play some role at low dose levels. For instance, a direct reaction of radiolabelled AA/GA with plasma proteins and erythrocytes was shown to contribute to consume AA and GA (Eisenbrand, 2020, and references therein). Furthermore, minor metabolic detoxification pathways, such as the enzymatic hydrolysis of GA to 2,3-dihydroxy-propanamide by epoxide hydrolase described in rodents and humans, may additionally contribute (summarized by Hartwig et al., 2020, and references therein).

The findings mentioned above are also supported by results of the toxicokinetic study in healthy volunteers (Fuhr et al., 2006). After the consumption of a meal containing 0.94 mg of AA the conjugation of AA with GSH was found to exceed the formation of GA. Furthermore, a 2–4 fold lower internal GA exposure from dietary AA was observed in humans as compared to rats and mice.

There are numerous publications reporting on AA/GA exposure biomarkers in animals and humans, primarily Hb adducts in red blood cells or mercapturic acids (MA) in urine. Some relevant studies have already been described in section 3.1.2 ('Evidence from DNA adduct formation and toxicokinetic studies'). A European human study monitored trends of exposure to AA in several European countries by measuring urinary mercapturic acid levels (2000–2021). Multiple linear regression analysis for time trends on data from 2000 to 2021 indicated an overall increase in AA exposure between the years 2001 and 2017 in adults, with declining values after 2018 (Poteser et al., 2022). Of note, to the best of our knowledge, all as yet available animal or human biomarker studies have not taken into consideration the consistent endogenous exposure to AA and, therefore, do not appear to represent a convincing metric concerning dietary AA exposure (see 3.4). Although at present the contribution of endogenous AA exposure to biomarker levels in humans is far from being quantitatively fully explored, it is certainly not negligible, reaching levels similar to dietary exposure, as demonstrated in human mercapturic acid biomonitoring studies (Ruenz et al., 2016; Goempel et al., 2017). Given this is the case, it cannot be excluded that extrahepatic formation and metabolism of AA/GA may contribute to exposure biomarker levels reported in humans and, therefore, may reflect endogenous exposure to an as yet unknown extent.

3.4. AA is formed endogenously in the body at concentrations in the lower range of human dietary exposure

Findings in animal experiments and in human intervention studies under tightly controlled conditions indicate that AA is also formed endogenously in the organism, resulting in substantial background exposure (summarized by Rietjens et al., 2018). At present, the origin of the endogenous AA background is not fully understood. AA may arise from the manifold biochemical reactions occurring during physiological energy metabolism of dietary substrates in the human organism. Another pathway of endogenous formation may occur via AC generated by the gut microbiome. It appears that glycerol acts as the major molecular precursor for AC in the human gut: the microbial glycerol metabolite 3-hydroxypropanal (3-HPA) equilibrates with AC in the intestine and may easily react with ammonia or amines to generate AA and/or corresponding reaction products. This pathway was discovered when identifying an acrolein reaction product of the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), termed PHIP-M1, in urine and faeces of volunteers after consuming well done chicken. Formation of this PhIP-metabolite was ascribed to a bacterial transformation process mediated by strains isolated from human faecal samples (*Enterococcus species*, *Lactobacillus Reuteri*), which were found to convert faecal glycerol into 3-HPA (Vanhaecke et al., 2008; Rietjens et al., 2022, and further references therein).

In a study in rats, environmental and dietary conditions were controlled to achieve minimal exogenous AA exposure (≤0.4 nmol AA per day, corresponding to ≤0.1 μg/kg bw/day). In untreated controls, a background urinary excretion of approximately 0.8 nmol (cumulative excretion 16 h post-application) of GA/AA mercapturic acids was consistently observed, estimated to be equivalent to an internal exposure of approximately 1.6–2 nmol (0.6–0.7 μg/kg bw) of ingested AA (Watzek et al., 2012).

Two human intervention studies confirmed these results observed in rats (Ruenz et al., 2016; Goempel et al., 2017). In the first study, 14 healthy male volunteers received a low vs. a high AA diet over a period of 9 days under controlled conditions, minimizing any other dietary sources of AA exposure (Ruenz et al., 2016). In the washout phases, the volunteers consumed an AA-minimized diet, resulting in dietary AA exposures not exceeding 41 ng/kg bw/day. At the end of the initial

three-day washout period, the AA mercapturic acid (N-acetyl-S-(2-carbamoylethyl)-L-cysteine, AAMA) baseline level approached 93 ± 31 nmol/day. Assuming that 30% of AA is excreted within 24 h as AAMA, a baseline endogenous exposure to AA, equivalent to an intake of 0.2–0.3 μg AA/kg bw/day was estimated (Ruenz et al., 2016). A subsequent extended duplicate diet follow-up study was conducted, encompassing washout periods of up to 13 days (n = 12), to confirm these findings using stable isotope-labelled AA as a toxicokinetic tracer (Goempel et al., 2017). Six volunteers ingested $^{13}C_3D_3$-AA (1 μg/kg bw), while the other six consumed freshly prepared meals with dosimetry of AA content by duplicate meal analysis. At the end of the 13-day washout period the $^{13}C_3D_3$-AA group excreted an unlabelled AAMA baseline level of 0.14 ± 0.10 μmol/day, whereas AA intake was only about 0.06 μmol/day. This sustained disproportionally high AAMA background indicated an endogenous AA background exposure of 0.3–0.4 μg/kg bw/day (Goempel et al., 2017). The $^{13}C_3D_3$-AA tracer was almost completely excreted within 72–96 h. This rules out delayed release of AA (or any other GAMA/AAMA precursor) from deep body compartments as an explanation for the observed long-term background and provides compelling support for the hypothesis of sustained endogenous AA formation in the human body (Goempel et al., 2017; Rietjens et al., 2018).

Additional evidence for endogenous AA formation in the organism comes from a recent cohort study (n = 56 healthy volunteers), which found a background of AA-derived *N*7-GA-Gua adducts in peripheral blood mononuclear cell DNA. The *N*7-GA-Gua adduct levels were not associated with dietary habits in this study, but were significantly correlated with body mass index (BMI) (Hemgesberg et al., 2021a). Results from another study with 17 healthy volunteers support these findings (Jones et al., 2021). *N*7-GA-Gua adducts were detected in human blood DNA in 13 out of 17 samples, but no direct correlation with the estimated 24-h AA intake of 0.29–1.14 μg/kg bw/day (based on food frequency questionnaires [FFQ]) was reported (Jones et al., 2021). Inherent inaccuracies of FFQ and large, natural variation in AA levels in the same food/drink category were discussed as potential confounders but endogenous background exposure may as well be considered. Similarly, in primary rat hepatocytes from AA-unexposed rats, background levels of 5–10 *N*7-GA-Gua adducts/10^8 nucleosides were reported (Hemgesberg et al., 2021b). To further consolidate data on endogenous background exposure, extended strictly controlled animal or human intervention studies will be required to exactly quantify respective biomarker responses to endogenous and dietary AA exposure on a population level and to learn more about the ratio of external vs endogenous exposure.

4. Contribution of non-genotoxic effects to major tumour responses in rodents

In long-term studies in rodents, AA induced neoplastic effects in multiple tissues at doses >0.5 mg/kg bw/day. Increased formation of mammary gland adenomas and fibroadenomas, thyroid follicular cell neoplasms, and testicular mesotheliomas were observed in F344 rats (Johnson et al., 1986; Friedman et al., 1995; NTP, 2012), while tumours in the Harderian gland, mammary gland, lung, ovary, skin, and stomach/forestomach were observed in mice (NTP, 2012).

EFSA based its assessment of health risks related to AA intake on increased incidences of Harderian gland adenomas and adenocarcinomas in male B6C3F1 mice (EFSA, 2015). Although the Harderian gland is not present in humans, EFSA considered that this rodent organ represents a sensitive endpoint for the detection of compounds that are both genotoxic and carcinogenic (EFSA, 2015). Therefore, EFSA concluded that Harderian gland neoplasms found in mice cannot be disregarded in the risk assessment of AA (EFSA, 2015). While the mode of action (MoA) of tumour formation by AA in this target organ has not been established, a recent toxicogenomic study in CD-1 mice exposed to doses of 1.5–24.0 mg AA/kg bw in drinking water for up to 31 days provided no support for a genotoxic MoA in the Harderian gland. The most prominent transcriptional response to AA in the Harderian gland involved altered expression of genes associated with calcium signalling and cytoskeletal function (Chepelev et al., 2018). Similar transcriptional changes were identified in CD-1 mice and F344 rats in other target tissues of AA carcinogenicity, such as thyroid, testes and lung (Chepelev et al., 2017, 2018; Recio et al., 2017), while no changes in the expression of genes involved in pathways typically associated with a genotoxic MoA (i.e., p53-regulated pathways as well as pathways involved in DNA repair and cell cycle regulation) were observed. Interference with calcium signalling and cytoskeletal processes may affect microtubule dynamics and thus perturb chromosome segregation during mitosis. However, to what extent these changes contribute to AA-mediated carcinogenicity has not been firmly established.

Arguments in support of the view that other major tumour responses in the thyroid, mammary gland and testes observed in long-term studies in rodents may be considered species- and strain-specific and not necessarily predictive for humans have been reported in a number of previous publications (Maronpot et al., 2009, 2015, 2016; Shipp et al., 2006; Alison et al., 1994; Capen, 1997; Neumann, 1991; Ben-Jonathan et al., 2008; Laube et al., 2019). It has been suggested that AA-mediated tumorigenesis in these hormone-sensitive tissues may be driven primarily via interference with species and strain specific endocrine regulation. Whether other (mostly minor) tumour types found in rodents chronically exposed to AA at dosages >0.5 mg/kg bw/day (e.g. in skin, lung, ovary, and stomach/forestomach) may reflect responses to AA of potentially higher human relevance, remains to be investigated.

The MAK Commission in 2007 discussed various mechanisms underlying tumour formation by AA, e.g., genotoxicity, cell proliferation, oxidative stress and changes in hormonal regulation (MAK, 2009). MAK at that time regarded AA as a genotoxic substance, but also considered its potential to affect hormone-sensitive tissues such as the mammary gland, testes, and thyroid. It was considered that, in principle, tumours observed in response to AA exposure may result from the combined effect of genotoxicity and stimulation of proliferation, but that mechanistic aspects of tumour induction and their human relevance still remained to be clarified (MAK, 2009). AA was classified into carcinogen category 2,[1] but it was also noted that, should new studies become available to elucidate the mechanisms involved in AA-mediated tumour formation, a possible reclassification of AA into carcinogen categories 4[2] or 5[3] should be discussed (MAK, 2009). The MAK

[1] 'Substances that are considered to be carcinogenic for man because sufficient data from long-term animal studies or evidence from animal studies substantiated by evidence from epidemiological studies indicate that they can contribute to cancer risk. Limited data from animal studies can be supported by evidence that the substance causes cancer by a mode of action that is relevant to man and by results of *in vitro* tests and short-term animal studies.' (Extracted from MAK, 2021).

[2] 'Substances that cause cancer in humans or animals or that are considered to be carcinogenic for humans and for which a MAK value can be derived. A nongenotoxic mode of action is of prime importance and genotoxic effects play no or at most a minor part, provided the MAK and BAT values are observed. Under these conditions no contribution to human cancer risk is expected. The classification is supported especially by evidence that, for example, increases in cellular proliferation, inhibition of apoptosis or disturbances in cellular differentiation are important in the mode of action. The classification and the MAK and BAT values take into consideration the manifold mechanisms contributing to carcinogenesis and their characteristic dose-time-response relationships.' (Extracted from MAK, 2021).

[3] 'Substances that cause cancer in humans or animals or that are considered to be carcinogenic for humans and for which a MAK value can be derived. A genotoxic mode of action is of prime importance but is considered to contribute only very slightly to human cancer risk, provided the MAK and BAT values are observed. The classification and the MAK and BAT values are supported by information on the mode of action, dose-dependence and toxicokinetic data.' (Extracted from MAK, 2021).

Commission proposed criteria, which can be applied to justify the classification of a compound into categories 4 and 5. By a case-to-case approach, the classification decision should be based on a founded and comprehensible combination of criteria (MAK, 1998; Neumann et al., 1998).

The SKLM noted that new data have become available since 2007 that justify the classification of AA as a chemical that does not significantly contribute to cancer risk in humans, provided an appropriate exposure limit is not exceeded. The SKLM therefore recommends reconsidering the risk assessment of AA, as further outlined in the conclusions section.

5. Evidence from epidemiological studies

It has to be pointed out that epidemiological studies on specific process-related contaminants, if not supported by appropriate biomarker dosimetry, merely inform about potential associations with exposure to a whole series of co-occurring process-related contaminants, such as, for instance, other alkenals including AC, furan and alkylfurans, fatty acid esters of 3-monochloropropane diol (3-MCPD) and glycidol/glycidyl esters. Such process-related contaminants may be present at widely varying concentrations in foods, making it particularly difficult to detect associations between cancer risk and a specific agent within the frame of dietary exposure. In addition, there is evidence for human endogenous exposure to AA at a level rather close to dietary exposure. This may be subject to physiological interindividual variability and can thus be expected to make it even more difficult to identify possible associations of cancer risk with dietary exposure to AA. Finally, endogenous exposure to a closely related α,β-unsaturated aldehyde, namely AC, far exceeds exposure to AA (Ruenz et al., 2019). Given these major confounders, epidemiological observations and conclusions regarding potential causality need careful analysis, especially when trying to uncover potential associations with a single process-related contaminant like AA.

Of note, the available epidemiological studies do not indicate an increase in cancer risk from exposure to AA. EFSA concluded in 2015 that AA intake was not associated with an increased risk of most common cancers, including those of the gastrointestinal or respiratory tract, breast, prostate and bladder (EFSA, 2015). Some studies have suggested an increased risk of renal, endometrial and ovarian cancer, and one study suggested lower survival in non-smoking women with breast cancer. However, the available evidence was considered limited and inconsistent (EFSA, 2015).

Likewise, the results of prospective epidemiological studies published after 2015 appear to add to the body of evidence that dietary AA is not a relevant cancer risk factor in humans. In the Japan Public Health Center-based Prospective Study on diet and cancer (85,303 participants), the intake of dietary AA was not associated with the risk of haematological malignancies (Zha et al., 2021), pancreatic cancer (Kito et al., 2020), liver cancer (Zha et al., 2020), lung cancer (Liu et al., 2020), oesophageal, gastric, or colorectal cancer (Liu et al., 2019), endometrial or ovarian cancer (Kotemori et al., 2018a) and breast cancer (Kotemori et al., 2018b) in the Japanese population. Moreover, dietary AA was not associated with renal cell cancer risk in the US American CPS-II Nutrition Cohort (McCullough et al., 2019) and in two long-term US American prospective cohorts (Health Professionals Follow-up Study and Nurses' Health Study) with dosimetry of dietary AA intake (Graff et al., 2018). In a meta-analysis, dietary AA was not related to the risk of renal cell carcinoma (Jiang et al., 2020).

A prospective cohort study (median follow up: 11.1 years) in men and women (n = 4000, age 65+ years), originally designed to investigate risk factors of osteoporosis, reported dietary AA to be associated with increased overall cancer mortality (Hazard Ratio (HR) for the highest quartile: 1.9; 95% confidence interval (CI) 1.3–2.8; $P_{trend}<0.01$) as well as with cancer mortality of the digestive (HR 1.9; 95% CI 1.0–3.6; $P_{trend} = 0.05$) and respiratory tract (HR 2.0; 95% CI 1.0–4.0; $P_{trend} = 0.06$). In men, associations were attenuated to null after further adjustment for circulating free estradiol (Liu et al., 2017). Limitations of the study, as in part also discussed by the authors, relate to: intake estimated at baseline only, the outcome cancer mortality not reflecting morbidity/cancer cases, and no consideration of potential confounding by anticancer therapy. Likewise, there was no consideration of potential co-exposure to other food borne carcinogens or to those of endogenous origin, neither were previous occupational exposures to potential carcinogens (including AA) taken into account.

Case-control studies published after 2015 do not support a causal link between dietary AA and various cancer types. The International Pancreatic Cancer Case-Control Consortium (PanC4) found no association between dietary AA and pancreatic cancer (Pelucchi et al., 2017). A case-cohort analysis performed within the prospective Netherlands Cohort Study on diet and cancer did not observe associations for the risk of cutaneous malignant melanoma in women. In men, an increased risk was modeled per 10 μg increment of dietary AA after adjusting for age, educational level, body mass index and smoking (HR 1.13; 95% CI 1.01–1.26), but there was no clear linear trend over the quintiles ($P_{trend} = 0.12$) (Lipunova et al., 2017).

In an Italian case-control study, no association between dietary AA intake and endometrial cancer was reported (Pelucchi et al., 2016). In a nested case-control study in non-smoking postmenopausal women of the EPIC (European Prospective Investigation into Cancer and Nutrition) cohort, no clear association between biomarkers of exposure to AA (Hb adducts of AA and GA) and the risk of epithelial ovarian cancer risk (Obón-Santacana et al., 2016a) or endometrial cancer risk (Obón-Santacana et al., 2016b) was observed.

6. Previous approaches for the derivation of a TDI in the literature

In its 2015 opinion, EFSA briefly discussed an approach to derive a TDI for AA, but concluded that the derivation of a TDI is generally considered inappropriate for a chemical considered to be genotoxic and carcinogenic (EFSA, 2015). Tardiff et al. (2010) considered the results from chronic rodent studies as being primarily consistent with a mechanism involving hormonal dysregulation, based on PBBK model simulations of internal dosimetry for AA and GA reported by Sweeney et al. (2010). A nonlinear dose-response approach was applied for carcinogenicity (mixed: genotoxicity and non-genotoxic MoA) (Tardiff et al., 2010). Based on dose-response data for rats exposed to AA in drinking water (Johnson et al., 1986; Friedman et al., 1995), a geometric mean reference point was derived from the $BMDL_{10}$ values for thyroid tumours, tumours of the central nervous system, mammary gland tumours and peritesticular mesothelioma data (EFSA, 2015; Tardiff et al., 2010). Using the AUC for AA as the dose metric, the $BMDL_{10}$ values ranged from 0.006 to 0.10 mg/L*h (geometric mean = 0.027 mg/L*h), with the mammary gland tumours providing the lowest values (Tardiff et al., 2010). Using a human PBBK model, a human equivalent dose of AA of 0.2 mg/kg bw/day was calculated. Applying an uncertainty factor of 75,[4] an external TDI value for cancer of 2.6 μg/kg bw/day was derived

[4] An uncertainty factor of 10 for inter-species variation was considered to be comprised of separate components for toxicokinetics (4.0) and toxicodynamics (2.5). Because a PBPK model was used to account for species differences in kinetics between rats and humans, the kinetics component of inter-species variation was set equal to one, resulting in an overall value of 2.5 for inter-species variation (Tardiff et al., 2010). Intra-human variation was considered to be comprised of separate components for toxicokinetics (3.16) and toxicodynamics (3.16). Kinetic variation in humans was expected to have minimal impact on tissue dose and a value of 3 (3.16 rounded to one significant figure) was considered to be sufficient for potential toxicodynamic variation among humans (Tardiff et al., 2010). An additional uncertainty factor of 10 was used for the severity of the effect.

for AA. In a similar approach, a TDI for cancer of 16 μg/kg bw/day was derived for GA (Tardiff et al., 2010). Although this approach did not consider that the tumours selected as relevant key lesions appear to be rodent-specific and most probably not predictive for humans, these considerations may be useful as a first conservative approach to defining a TDI. Up to date PBBK modelling with a focus on low exposure conditions may provide improved information.

Apart from carcinogenicity, adverse effects on neurotoxicity, male reproduction, and development were identified as possible critical non-genotoxic endpoints for AA toxicity in experimental animal studies (EFSA, 2015). Neurotoxicity, which was also observed in humans, was considered as the most sensitive non-genotoxic endpoint and a nonlinear dose-response approach was applied, based on rat neuropathy results from 2-year exposures (Tardiff et al., 2010; EFSA, 2015; Johnson et al., 1986; Friedman et al., 1995). The TDI for neurotoxicity was estimated at 40 μg/kg bw/day for AA and at 70 μg/kg bw/day for GA (Tardiff et al., 2010).

An alternative approach could be to take the endogenous background exposure as a benchmark to inform the derivation of a TDI. However, to this end the database on endogenous background exposure needs to be extended, also addressing special population groups.

7. Conclusions

There is increasing evidence that agents acting through genotoxic and mutagenic mechanisms can show non-linear dose response relationships that may allow to identify a threshold. Such differential dose response, together with low dose non-linearity has been demonstrated for potent genotoxic mutagens, such as certain alkylating agents including alkyl methanesulfonates and N-nitrosoalkylureas. Dosimetry of DNA damage in connection with chromosome aberrations and gene mutations observed *in vitro* and *in vivo* (including transgenic animal tests) generated evidence for "practical thresholds in the low dose range, suggestive for cellular tolerance to low levels of many genotoxicants" (Guérard et al., 2015).

AA itself is not genotoxic, but can be converted metabolically to the epoxide GA, which may exert DNA damage by covalent binding. Such genetic damage may result in fixed mutations that eventually lead to neoplastic transformation. This has been considered in the past as the most probable key event involved in AA-induced neoplastic transformation. However, there is substantial evidence that genotoxic effects (DNA damage, mutations) follow a non-linear dose-response and that risks for genotoxic effects are negligible in the diet-related exposure range. Non-genotoxic mechanisms, which are only activated after exceeding particular threshold concentrations appear decisive for neoplastic transformation observed in animal experiments. This implies that as long as an appropriately low exposure limit is not exceeded, genotoxic effects and tumour induction may not be expected. Therefore, a TDI may be identified that ensures a level of dietary exposure not associated with a relevant contribution to human cancer risk (or to other toxicity risks).

Mean and 95th percentile dietary AA exposures in Europe have been estimated at 0.4–1.9 μg/kg bw/day and 0.6–3.4 μg/kg bw/day, respectively (EFSA, 2015). In animal experiments carried out at single doses that exceeded this dietary exposure by 1–2 orders of magnitude, DNA damage was reported to be very low and remained at the lower bound of human background DNA damage associated with comparable DNA *N*7-Gua lesions.

For human health risk assessment, sustained endogenous AA formation in the organism, estimated to result in a (physiological) background exposure of approximately 0.3–0.4 μg/kg bw/day, must be taken into account. Physiological background exposure may be proposed to serve as a future pivotal reference to evaluate potential health effects resulting from additional exogenous exposure (Rietjens et al., 2022). Endogenous as well as exogenous exposure are accessible to reliable analytical dosimetry and may be further supported by probabilistic exposure estimates (e.g., by using Monte Carlo methodology). This may allow a more reliable and improved risk assessment of nutritional AA exposure and the evaluation of the associated incremental health risk.

Overall, there is convincing evidence to support moving away from a risk assessment driven by the concept of classifying AA as a genotoxic carcinogen at present daily consumer exposure levels. The SKLM therefore considers it appropriate to derive a TDI as a health-based guidance value. Nevertheless, the SKLM considers the mitigation measures for AA developed and successfully applied in recent years as helpful to achieve and maintain compliance with such a health-based guidance value.

8. Gaps in knowledge and research needs

- Advanced biomarker monitoring with reverse dosimetry, also considering endogenous background exposure in humans, its source (s) and variables of influence
- PBBK modelling with a focus on defining thresholds of biomolecular key events with consequences for cancer induction in systems of high predictivity for humans
- Particular focus to be laid on non-genotoxic events
- Elucidation of the mechanisms and dose dependence underlying potential reproductive effects and other adverse outcomes
- Dose-related toxicogenomics *in vitro* and *in vivo* using experimental systems predictive for humans

Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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