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Assessment of the genotoxicity of acrylamide

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Abstract

EFSA was requested to deliver a statement on a recent publication revisiting the evidence for genotoxicity of acrylamide (AA). The statement was prepared by a Working Group and was endorsed by the CONTAM Panel before its final approval. In interpreting the Terms of Reference, the statement considered the modes of action underlying the carcinogenicity of AA including genotoxic and non-genotoxic effects. Relevant publications since the 2015 CONTAM Panel Opinion on AA in food were reviewed. Several new studies reported positive results on the clastogenic and mutagenic properties of AA and its active metabolite glycidamide (GA). DNA adducts of GA were induced by AA exposure in experimental animals and have also been observed in humans. In addition to the genotoxicity of AA, there is evidence for both secondary DNA oxidation via generation of reactive oxygen species and for non-genotoxic effects which may contribute to carcinogenesis by AA. These studies extend the information assessed by the CONTAM Panel in its 2015 Opinion, and support its conclusions. That Opinion applied the margin of exposure (MOE) approach, as recommended in the EFSA Guidance for substances that are both genotoxic and carcinogenic, for risk characterisation of the neoplastic effects of AA. Based on the new data evaluated, the MOE approach is still considered appropriate, and an update of the 2015 Opinion is not required at the present time.

Keywords: Acrylamide, glycidamide, genotoxicity, DNA adducts, modes of action, food

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

Background

The European Food Safety Authority Panel on Contaminants in the Food Chain (CONTAM Panel) has adopted a scientific opinion on acrylamide in food.[1]

The CONTAM Panel concluded that acrylamide is a genotoxic carcinogen. Acrylamide is extensively metabolised, mostly by conjugation with glutathione but also by epoxidation to glycidamide. Formation of glycidamide is considered to represent the route underlying the genotoxicity and carcinogenicity of acrylamide.

In a recent review article,[2] it is concluded that the totality of available scientific evidence clearly argues against a genotoxic mode of action underlying the neoplastic effects of acrylamide.

It is appropriate to provide a scientific statement on this review article and to assess if based on the evidence provided in the article, if an update to the scientific opinion on acrylamide in food would be appropriate.

Terms of reference

In accordance with Article 31 (1) of Regulation (EC) No 178/2002 the Commission asks EFSA for a statement on a recent publication revisiting the evidence for genotoxicity of acrylamide.

1.2. Interpretation of the Terms of Reference (if appropriate)

In interpreting the Terms of Reference, the statement considered the modes of action underlying the carcinogenicity of AA including genotoxic and non-genotoxic effects. It was noted that the Eisenbrand (2020a) review and its erratum (Eisenbrand, 2020b) was not a comprehensive review and therefore a new literature search on recent data on the genotoxicity and modes of action of AA was carried out.

2. Data and methodologies

2.1. Collection and appraisal of literature

The methodology used to inform the current statement is detailed in **Appendix A**. Details on the literature searches carried out to identify relevant studies available in the public domain since the publication of the Scientific Opinion on Acrylamide in Food (EFSA CONTAM Panel, 2015) on the genotoxicity of acrylamide (AA), studies on adducts of AA and glycidamide (GA) and on epidemiological studies on the association of AA exposure and risk of cancer can be found in **Appendix B**.

The studies retrieved were reviewed by the Working Group on AA Genotoxicity using expert judgement. Any limitations of the information used are documented in this statement.

3. Assessment

3.1. Genotoxicity of AA

Main conclusions on genotoxicity from the 2015 Opinion on AA in food

In 2015, the CONTAM Panel published its Opinion on the risk for human health related to the presence of AA in food (EFSA CONTAM Panel, 2015). The Panel evaluated the data on the genotoxicity of AA available at that time, and concluded that:

- *'In vitro genotoxicity studies indicate that AA is a weak mutagen in mammalian cells but an effective clastogen.*
- *GA is a strong mutagen and a clastogen. It induces mutations via a DNA adduct mechanism.*
- *In vivo, AA is clearly genotoxic in somatic and germ cells.*

[1] https://www.efsa.europa.eu/en/efsajournal/pub/4104

[2] Eisenbrand G, 2020. Revisiting the evidence for genotoxicity of acrylamide (AA), key to risk assessment of dietary AA exposure. Archives of Toxicology, 94, 2939–2950. https://doi.org/10.1007/s00204-020-02794-3

- *AA exerts its mutagenicity via metabolism by CYP2E1 to GA. AA can also induce gene mutations by a pathway involving the generation of reactive oxygen species (ROS) and oxidative DNA damage'.*

Since AA was of concern with respect to genotoxicity, the CONTAM Panel did not consider it appropriate to establish a health-based guidance value, e.g. a tolerable daily intake (TDI). Thus, the risk characterisation for neoplastic effects was performed using the margin of exposure (MOE) approach for compounds that are both genotoxic and carcinogenic. The Panel used as the reference point the $BMDL_{10}$ of 0.17 mg/kg body weight (bw) per day, i.e. the lowest $BMDL_{10}$ from data on incidences of Harderian gland adenomas and adenocarcinomas in male $B6C3F_1$ mice exposed to AA for 2 years (NTP, 2012; EFSA CONTAM Panel, 2015). The CONTAM Panel at that time noted that the Harderian gland is absent in humans, but that in rodents it is a sensitive target tissue for compounds that are both genotoxic and carcinogenic. Taking into account that target tissues for tumour formation by a given genotoxic carcinogen may differ between species, the CONTAM Panel considered the Harderian gland to be a conservative endpoint for assessment of the risk for neoplastic effects of AA in humans.

New studies since the 2015 Opinion on AA in food

Since the publication of the Opinion on AA in food in 2015, a number of studies on the genotoxicity of AA have become available. These are listed in **Table 1** (*in vitro* genotoxicity studies) and **Table 2** (*in vivo* genotoxicity studies) and a summary of the main observations is described below.

In some of these studies there is evidence of a potential role of reactive oxygen species (ROS) and this is discussed in **Section 3.2**.

Chromosomal damage

In vitro

Several new studies since the 2015 Opinion (see **Table 1**) reported on the ability of AA to induce chromosomal damage both *in vitro* and *in vivo*. Micronucleus tests following *in vitro* exposure to AA of human (Zamani et al., 2018) or rat lymphocytes (Ankaiah et al., 2018) were positive. In these publications the major emphasis was on the ability of the antioxidant L-carnitine and resveratrol in preventing AA-induced DNA damage (see **Section 3.2**).

In a specific *in vitro* assay to investigate potential hazards to female fertility, AA increased in a dose-dependent manner the percentage of chromosome misalignment in Metaphase II-stage mouse oocytes and induced significant alterations of spindle morphology. These were found to be associated with a decreased maturation of mouse oocytes (Liu et al., 2015). The positive results in these *in vitro* studies were independent of added metabolic activating systems.

In vivo

In three strains of mice (B6C3F1, Swiss albino, C57BL/6J), *in vivo* oral exposure to AA was consistently positive in micronucleus tests in the bone marrow or peripheral blood (Hobbs et al., 2016; Algarni, 2018; Hagio et al., 2021) (see **Table 2**). In more limited studies, a single i.p. injection of AA in Kunming mice also resulted in positive effects (Zhao et al., 2015a,b). In Zhao et al. (2015a,b), both AA-induced alteration of liver antioxidant enzymes and genotoxic effects (micronuclei and DNA breaks, see below) were alleviated by feeding the mice with a diet containing various fruits (or extracts) containing antioxidants (see **Section 3.2**). Analysis of AA-induced chromosome aberrations in mouse bone marrow cells revealed increased levels of polyploidy as well as chromosomal fragments, deleted chromosomes and Robertsonian centric fusions (Algarni, 2018). Maturation of mouse oocytes were significantly impaired by AA treatment, with metaphase II oocytes being characterised by a reduction in meiotic spindle mass and chromosome misalignments (Aras et al., 2017).

Increased micronuclei were observed in orally treated Wistar and Sprague–Dawley rats (Jangir et al., 2016; Shimamura et al., 2017; Şekeroğlu et al., 2017), but not in Fisher 344 (F344) rats (Dobrovolsky et al., 2016; Hobbs et al., 2016; Chepelev et al., 2017). It appears that mice are more sensitive to micronuclei formation by AA than rats and this correlates with the level of DNA adducts produced by AA dosing in rats being generally lower than in mice (Doerge et al., 2005).

Comet assays

In vitro

AA induced DNA strand breaks in several human cell lines (THP-1 monocyte, liver HepaRG, CaCo-2) (Xiao et al., 2016; Mandon et al., 2019; Nowak et al., 2020), human lymphocytes (Wang et al., 2021) and mouse and human spermatozoa (Katen et al., 2017). In the case of mouse spermatozoa, increased DNA strand breakage was observed only following exposure to GA, the active AA metabolite. Positive results were, however, obtained following exposure of spermatozoa to AA in the presence of conditioned media from epididymal mECap18 cells that express high levels of the CYP2E1 enzyme required for the metabolic activation of AA (Katen et al., 2017). Comet assays in the presence of Fpg (a DNA glycosylase that removes 8-oxoguanine and other lesions from DNA) revealed large increases in DNA strand breaks in AA-treated CaCo-2 cells, spermatozoa and human lymphocytes (Katen et al., 2017; Hansen et al., 2018; Nowak et al., 2020). In contrast, modest changes in the levels of DNA breaks were observed in assays supplemented with the human OGG1 enzyme (8-oxoguanine DNA glycosylase involved in maintaining genomic integrity), an enzyme that is specific for excision of DNA 8-oxoguanine (Katen et al., 2017). Since under the alkaline conditions of the comet assay, the N7-GA-Gua adduct can be converted to a ring-open form that is a substrate for Fpg but not OGG1, this finding indicates that the majority of DNA breaks induced by AA or GA are not due to the presence of DNA 8-oxoguanine (Hansen et al., 2018). Some observations indicate however that at least a fraction of AA-induced DNA strand breaks might be due to DNA oxidation. These include elevation of ROS levels together with mitochondria depolarisation occurring in parallel with DNA strand breaks in CaCo-2 cells (Nowak et al., 2020) and modulation of these by antioxidants such as curcumin and epigallocatechin in human lymphocytes (Wang et al., 2021) (see **Section 3.2**).

Finally, increased levels of DNA strand breaks and sister chromatid exchanges were observed in GA-treated lymphocytes from individuals carrying polymorphisms in the *CASP10* and *CASP8* genes, respectively. The authors suggested that these caspase polymorphisms might decrease the apoptotic rate, increasing cell survival and consequently cellular yields of genotoxic effects caused by GA exposure (de Lima et al., 2016).

In vivo

Lymphocytes and liver of i.p. AA-treated mice (Zhao et al., 2015a,b), liver of i.p. treated Wistar rats (Ansar et al., 2016), liver of orally treated F344 rat (Dobrowolsky et al., 2016) and kidney and brain of orally treated Wistar rats (Shimamura et al., 2017) were positive in the *in vivo* comet assay. Increased levels of DNA damage were induced in spermatocytes and spermatozoa in Swiss CD-1 mice following a short AA exposure *via* i.p. or a 6-month treatment via the oral route (Katen et al., 2016a,b, 2017). In addition, the male offspring of AA treated male mice had significant increased levels of DNA breaks in their spermatozoa (Katen et al., 2016a).

In conclusion, these data indicate that, in addition to liver, AA can induce DNA damage in several rodent organs and chronic paternal AA exposure in mice has consequences for their male offspring.

Gene mutation

In vitro

AA and GA treatment increased the *TK* gene mutation frequency in the human MCL-5 lymphoblastoid cell line engineered to express several CYPs including CYP2E1 (David and Gooderham, 2018). AA induced a modest increase (2-fold at the highest tested dose) in mutation frequency at the knock-in *lacZ* gene present in Hupki mouse embryo fibroblasts (Hölzl-Armstrong et al., 2020a) or a marginal increase (1.5-fold) in the metabolically competent FE lung cell line (Hölzl-Armstrong et al., 2020b). In the presence of S9, *gpt* gene mutations were induced in lung cells in pulmonary organoid structures from *gpt* delta C57BL/6J mice (Komiya et al., 2021). The mutational spectrum (increases in GC > AT transitions, AT > TA and AT > CG transversions and deletions/insertions) was similar to that previously reported *in vivo* in the same transgenic mice (Ishi et al., 2015).

The relative mutagenicity of AA and GA in several cell lines (Hupki mouse embryo fibroblast, MCL-5 cells and FE cells) indicates that GA is the more potent. In particular, a comparison of AA and GA mutagenicity at concentrations that caused similar levels of cytotoxicity showed that GA induced three times more *lacZ* mutants than AA (Hölzl-Armstrong et al., 2020a).

In Hupki mouse embryo fibroblasts, however, neither analysis of a human knock-in *TP53* gene (Hölzl-Armstrong et al., 2020a) nor exome or whole genome sequencing provided evidence of

AA-induced mutations (Zhivagui et al., 2019; Hölzl-Armstrong et al., 2020a). These negative results were attributed to the limited ability of Hupki cells to activate AA (Zhivagui et al., 2019).

GA exposure (in the absence of metabolic activation) did, however, increase mutation frequency at the *TP53* gene in Hupki mouse embryo fibroblasts (Hölzl-Armstrong et al., 2020a). The majority of GA-induced mutations occurred at A:T base pairs (59%) with AT > TA and AT > GC mutations being the most common types. These base substitutions occurred at specific *TP53* codons that have also been found to be mutated in human tumours. The most common cancers with the *TP53* mutations characteristic for the GA-induced mutations were ovarian (10% of tumours analysed) and breast (10%) cancer followed by ~ 8% each colorectal and lung cancer.

Two studies in Hupki mouse embryo fibroblasts analysed mutational spectra induced by GA exposure. In the first performed by whole exome sequencing, 3 mM GA increased the number of single base substitutions (SBS) by 2.5-fold (from an average of 190 mutations in untreated clones to 485 in GA-treated ones), with AT > GC transitions and AT > TA and GC > TA transversions being the main mutational classes affected. Parallel measurements of N7-GA-Gua and N3-GA-Ade in GA-treated cells were also performed (see **Table 3** for details). The authors stressed that these DNA adducts provided a possible mechanistic basis for the mutation types and the mutational signature arising upon treatment with GA, the reactive metabolite of AA. A specific GA mutational signature was identified and compared to the Pan-Cancer Analysis of Whole Genomes (PCAWG) database SBS mutational signatures. The highest enrichment of the GA signature was observed in the cancers of the lung (88% of the interrogated tumours), liver (73%), kidney (> 70%), bile duct (57%), cervix (50%), and, to a lesser extent, additional cancer types (Zhivagui et al., 2019).

In a parallel study in the same cells performed by whole genome sequencing, GA (1.1 mM) induced an increase in mutational load but this did not reach statistical significance (Hölzl-Armstrong et al., 2020a). The mutational spectrum was, however, different from the control, with the main classes affected being AT > GC, AT > TA and AT > CG. A comparison with the Catalogue Of Somatic Mutations In Cancer (COSMIC) database identified similarities between this GA specific signature and mutational signatures previously found in human tumours (e.g. breast, ovary, pancreas), with mutations at adenine being similar to those found mostly in smokers' lung cancer.

The authors concluded that these studies suggest a contribution of AA and/or GA-associated mutagenesis to human cancers (Zhivagui et al., 2019; Hölzl-Armstrong et al., 2020a).

In vivo

The *in vivo* mutagenic potential of AA has been largely investigated at the endogenous *Pig-a* gene in reticulocytes and red blood cells. These studies were either equivocal (no dose response; positive at a single dose or single cell type) or negative for AA in orally treated rats and mice (Dobrovolsky et al., 2016; Hobbs et al., 2016; Horibata et al., 2016; Chepelev et al., 2017).

Both AA and GA increased *cII* gene mutation frequency in the brain of BigBlue mice. Mutational spectra were similar for the two compounds that mainly affected GC > TA, AT > TA and AT > CG mutations (Li et al., 2016). Increased mutation frequencies were also reported at the *gpt* gene in the testis, lung (two- to threefold increase) and sperm (sixfold increase) of C57BL/6J mice treated with AA (Hagio et al., 2021). The mutational classes involved varied between organs: GC > TA, GC > AT and 1-bp deletions predominated in sperm, whereas AT > TA mutations were the major class involved in the lung. Analysis of AA mutagenicity in male germ cells indicates that cells in the late stages of spermatogenesis are more sensitive than spermatogonial stem cells.

In conclusion, analysis of mutational classes from both *in vitro* and *in vivo* studies highlights the relationship between DNA adduct profiles originating from the metabolic conversion of AA and the mutational signature of AA/GA. Both N3-GA-Ade and N7-GA-Gua can undergo depurination leading to the formation of apurinic/ apyrimidinic sites. During replication, these may lead to misincorporation of deoxyadenine, resulting into the respective AT > TA and GC > TA transversions observed in the GA signature. The AT > GC transitions enriched in the GA signature correspond to the miscoding of another commonly identified adenine adduct *in vitro*, i.e. N1-GA-Ade.

DNA adducts

A summary of the main observations on DNA adducts formation following *in vitro* or *in vivo* exposure to AA is made below with individual studies being listed in **Table 3**.

In vitro

DNA adducts induced by AA (N7-GA-Gua and N3-GA-Ade) were identified by LC-MS in calf thymus DNA (Hansen et al., 2018). Following exposure of Hupki mouse embryo fibroblast to 5,000 μM AA + S9, low levels of N7-GA-Gua (11 adducts per 10^8 nucleotides) were reported, while no increase was observed without metabolic activation (limit of detection (LOD): 5.5 adducts per 10^8 nucleotides). In contrast, very high levels of N7-GA-Gua and N3-GA-Ade were observed in the same cells following 3,000 μM GA treatment (49,000 adducts per 10^8 and 350 adducts per 10^8 nucleotides, respectively) (Zhivagui et al., 2019).

In a second study in Hupki cells, measurements of N7-GA-Gua by UPLC–ESE-MS/MS did not identify any increase over background levels following exposure to AA (up to 1,500 μM), while 20–30 DNA adducts per 10^8 nucleotides were produced by GA (750–1,500 μM) (Hölzl-Armstrong et al., 2020).

A non-linear concentration-response for N7-GA-Gua (by UHPLC–ESI+-MS/MS) was observed in primary cultures of rat hepatocytes treated *in vitro* with increasing concentrations of AA (range 2–2,000 μM). The increase in DNA adduction over background levels was observed only at 1,000 and 2,000 μM (20–30 N7-GA-Gua per 10^8 nucleotides) (Hemgesberg et al., 2021a).

In vivo

Median urinary levels of N7-GA-Gua (by measurements of the nucleoside) and the *N*-acetyl-*S*-(propionamide)-cysteine (AAMA) mercapturic acid derivative, a metabolite of AA considered a biomarker for current exposure (investigated by SPE LC–MS/MS) were found to be 0.93 and 1.41 μg/g creatinine in 33 non-smokers and 30 smokers, respectively (a not-statistically significant difference). A significant correlation was observed between the urinary AAMA and N7-GA-Gua levels in non-smokers, smokers, and all study subjects combined. Multiple linear regression analysis of data to adjust confounding factors revealed that N7-GA-Gua levels were significantly associated with the levels of urinary AAMA, but not urinary cotinine and other factors. The authors concluded that urinary N7-GA-Gua of non-smokers and smokers is significantly associated with dietary AA intake (Huang et al., 2015).

Urinary N7-GA-Gua, AAMA and the mercapturic acids of AA and GA, namely *N*-acetyl-*S*-(2-carbamoylethyl)-L-cysteine (AAMA) and *N*-(*R*,*S*)-acetyl-*S*-(1-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA) were also analysed in 8 male AA-exposed workers and 36 controls (administrative workers) from the same AA production plants (collection of the urines at pre- and post-shift). Significantly higher levels were found in AA-exposed workers than in controls at both time points, with median urinary N7-GA-Gua level in exposed workers being 2.46 and 1.57 μg/g creatinine (at pre- and post-shift) and 0.36 and 0.39 μg/g creatinine in the control group, respectively (Huang et al., 2018).

In B6C3F1 mice orally treated (drinking water) for 28 days with AA concentrations in the 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 per day for males, and to 1.10, 2.23, 4.65 and 9.96 mg/kg bw per day for females), a dose-dependent increase in the levels of N7-GA-Gua and N3-GA-Ade (900 and 4 adducts per 10^8 nucleotides at 700 μM, respectively) were found both in the liver and in the lung (De Conti et al., 2019). On analysis of these data, the WG found evidence of dose linearity in the adduct formation in the dose range applied.

DNA adducts (N7-GA-Gua) were also investigated in urine and tissues of rats treated with AA (35 mg/kg bw) for 7 and 14 days. N7-GA-Gua adducts per 10^8 nucleotides were approximately 900 and 1,300 in liver, 1,300 and 2,100 in kidney and 1,000 and 1,900 in lung at the two time points, respectively (Wang et al., 2019).

Low levels of N7-GA-Gua were identified in peripheral blood mononuclear cells (PBMCs) of 56 healthy human volunteers with N7-GA-Gua being detected in 80% of participants (Hemgesberg et al., 2021b). The mean levels ranged from the LOD value (0.2 adducts per 10^8 nucleosides) to 26.6 adducts per 10^8 nucleosides. The study aimed to identify possible correlations between dietary habits, biometric and some blood parameters and N7-GA-Gua levels. Measurements included age, gender, body weight, height, smoking, vegetarian, vegan, frequent consumption of coffee, French fries and cookies. Although no correlation was found between DNA adducts and dietary habits, blood glucose levels or glycated haemoglobin (an indicator of blood glucose levels averaged over 2–3 months) and DNA adducts significantly correlated with the body mass index. No statistically significant difference was found between non-smokers and smokers (1.01 and 2.05 per 10^8 nucleosides, respectively).

Identification and quantification of N7-GA-Gua adducts by LC–ESI-MS/MS was also performed in the blood DNA of 17 healthy volunteers (aged between 18 and 65 years) following a 24-h dietary

exposure to AA present in carbohydrate rich foods as part of the normal human diet (Jones et al., 2021). N7-GA-Gua adducts, quantified in 13 out of 17 samples, were in the range 0.3–6.3 adducts per 10^8 nucleotides, with a mean value of 1.9 per 10^8 nucleotides. The AA intake calculated from the food frequency questionnaires was in the range 20.0–78.6 μg per day (0.29–1.14 μg/kg bw per day[3]). There was no direct correlation between the estimated 24-h AA intake, based on the food frequency questionnaire and N7-GA-Gua levels in this volunteer study. The authors stressed that this might be due to the relatively small sample size and possible inaccuracies of the questionnaire with large variation in AA levels in the same food/drink category. It was noted that the levels of DNA adducts observed in these two studies in human volunteers are in the same range.

[3] Converted using the mean body weight of 69 kg according to Jones et al. (2021).

Table 1: ***In vitro*** genotoxicity studies with acrylamide (AA) and/or glycidamide (GA)

Test	Cellular system	Experimental design	Results	Comments	Reference
Micronucleus test	Human lymphocytes from healthy, young (20–24 years) male	**AA**: 250, 50,000, 100,000 μM Exposure: 20 h Markers of oxidative stress: ROS, MDA, GSH	**Positive AA:** at 50,000 and 100,000 μM Inhibition of AA genotoxicity by the antioxidant L-Carnitine. Modulation of markers of oxidative stress by AA.	See **Table 4** for further information.	Zamani et al. (2018)
Micronucleus test	Rat lymphocytes Exposure AA: 24 h	**AA**: 100, 200, 300 mg/L (corresponding to 1,407, 2,814, 4,221 μM) ± resveratrol pretreatment: 24 h at 100 μM	**Positive AA**: all AA concentrations Reduction of micronuclei by resveratrol pretreatment.	See **Table 4** for further information.	Ankaiah et al. (2018)
Chromosome alignment and spindle morphology during oocytes maturation	Cumulus–oocyte complexes isolated from ICR mice (F) (5–7 weeks of age)	**AA**: 0, 5, 10, 20 μM Exposure: 14 h Oocyte maturation *in vitro* following fertilisation	Increased percentage of misaligned chromosomes in AA-treated oocytes at MII-stage. Abnormal spindle morphology in the AA-treated oocytes. Decreased fertilisation and capacity to form two-cell from the zygote.		Liu et al. (2015)
Comet assay	Human THP-1 monocyte cell line	**AA**: 100, 200, 400, 600, 800, 1,000 μM Exposure: 1.5 h Or in combination with chrysoidine G, Sudan I, acid orange II, malachite green and potassium bromate.	**Positive AA**: increase in % tail DNA from 600 μM No significant differences in the % tail DNA values between the single and combined tests at most cases.		Xiao et al. (2016)
Comet assay	Liver HepaRG cell line in 3D	**AA**: 250, 500, 1,000, 2,000 μM Exposure: 24 and 48 h	**Positive AA**: from 500 μM (% DNA tail) No cytotoxicity at any concentrations.		Mandon et al. (2019)

Test	Cellular system	Experimental design	Results	Comments	Reference
Comet assay +/− Fpg and EndoIII digestion	CaCo-2 cells Measurements ROS and mitochondrial membrane potential and apoptosis	**AA**: 200, 800, 3,200, 6,400, 12,500, 50,000 μM Comet assay: 1 h exposure ROS levels: 6 h exposure Mitochondria depolarisation and apoptosis: 24 h exposure	**Positive AA:** increase in % tail DNA from 800 μM (non-cytotoxic concentrations). + Fpg: increase from 3,200 μM. + EndoIII: increase from 6,400 μM. Role of oxidative damage in AA: ROS: increased from 3,200 μM. Depolarisation of mitochondria: from 3,200 μM. Apoptosis: from 3,200 μM.	See **Table 4** for further information.	Nowak et al. (2020)
Comet assay +/− Fpg and Endo III digestion	Human lymphocytes and whole blood + EGCG and/or curcumin	**AA**: 0.001, 0.005, 0.01 μM Exposure: 1 h	**Positive AA:** concentration related increase in DNA strand breaks (tail DNA %). Fpg and EndoIII increase AA-induced DNA strand breaks (0.005 μM) (modest change). ECGC and curcumin decrease AA-induced DNA strand breaks (0.01 μM).	See **Table 4** for further information.	Wang et al. (2021)
Comet assay +/− Fpg and OGG1 digestion	Mouse and human spermatozoa and mouse epididymal mECap18 cells	Spermatozoa treatment: **AA**: 10, 100, 1,000, 10,000, 100,000 μM **GA**: 0.05, 0.5, 5, 50, 500 μM Exposure 1 or 2 h mECap18 cells treatment: **AA**: 10, 1,000, 100,000 μM **GA**: 5 μM	Mouse spermatozoa treatment: **Negative AA:** at all concentrations **Positive GA:** at all concentrations Epididymal mECap18 cells treatment: **Positive AA** + **Fpg:** large increase in DNA breaks. + **OGG1:** modest changes in DNA breaks. **Positive**: mouse and human sperm in the presence of	The large difference in the number of DNA breaks following Fpg and OGG1 incubation indicates that Fpg recognises and incises AA-induced DNA adducts while OGG1 recognises only 8-oxoguanine in DNA. See **Table 4** for further information.	Katen et al. (2017)

Test	Cellular system	Experimental design	Results	Comments	Reference
			conditioned media from AA-or GA-treated mECap18 cells.		
Comet assay +Fpg	Human lymphocytes Comet analysis + Fpg in various lysis and pH conditions	**GA**: 10,000, 25,000, 50,000 μM	**Positive GA**: all concentrations (linear response, % tail DNA)	GA-induced lesions are predominantly N7-GA-dG adducts slowly undergoing imidazole ring opening at pH 10; such structures are incised by Fpg leading to DNA strand breaks.	Hansen et al. (2018)
DNA adducts (N7-GA-Gua and N3-GA-Ade)	Calf thymus DNA Analysis by LC–MS		At neutral conditions DNA adducts are released from DNA (spontaneous depurination)	See **Table 3** and **4** for further information.	
Comet assay	Human lymphocytes from several donors with polymorphisms in *CASP7*, *CASP8*, *CASP9*, *CASP10*, *LTA* and *TNFRSF1B* genes	**GA**: 250 μM in non-stimulated (for Comet assays) and stimulated (for SCEs) lymphocytes	**Positive GA**: increase in % tail DNA in homozygous individuals for the CASP10 I522L T allele in comparison to heterozygous individuals or individuals with at least one variant allele.		de Lima et al. (2016)
SCEs			**Positive GA**: increase in GA-induced SCE for heterozygous individuals with CASP8 rs1035142 G > T and for individuals with at least one variant allele in comparison to G homozygous individuals.		
Comet assay	Human HCE-T corneal cells in 3D	**AA**: 420–422,100 μM Exposure: 1 min	**Negative AA** Positive for other genotoxic agents (including MMS)	Limited information from this study because of unusual animal model for genotoxicity testing and AA delivery.	Tahara et al. (2019)
Mutations (*TK* gene)	Human MCL-5 cell line expressing CYP1A1, CYP1A2, CYP2E1,	**AA**: 0.1, 1, 10, 100, 1,000, 2,000, 3,000, 4,000 μM	**Positive** **AA:** only at 4,000 μM **GA:** only at 100 μM		David and Gooderham (2018)

Test	Cellular system	Experimental design	Results	Comments	Reference
	CYP2A6, CYP3A4 and microsomal epoxide hydrolase	**GA**: 0.001, 0.01, 0.1, 1, 10, 100, 15, 25, 50, 75, 100 μM Exposure: 24 h Positive control: EMS			
Mutations (human *TP53* knock-in)	(Hupki) mouse embryo fibroblasts Immortalisation of untreated and AA/GA treated cell cultures Control: 30 cultures AA: 198 cultures GA: 24 cultures Cell viability by crystal violet staining	**AA**: 1,500 μM (48 h) **GA**: 1,100 μM (24 h) 40–60% survival	**Negative AA** **Positive GA:** *TP53* mutations in 21/198 cultures (11%), 0% mutations in controls. Enrichment in AT > TA (35%); AT > GC and GC > CG (18%). 59% mutations at AT and 36% at GC base pairs.	Comparison with *TP53* mutation IARC data base of human tumours: similarities with ovarian (10%), breast (10%) and lung and colorectal cancer (8%).	Hölzl-Armstrong et al. (2020a)
Mutations by whole genome sequencing	(Hupki) mouse embryo fibroblasts Number of immortalised clones analysed: Control: 4 clones AA: 3 clones/ concentration. GA: 4 clones.	**AA**: 1,500, 3,000 μM (48 h) **GA**: 1,100 μM (24 h)	**Negative AA**: no difference in the number of mutations/ genome and mutational profile similar to control. **Positive GA**: increased number of mutations (but not significant); mutation pattern different from control (main classes: AT > GC, AT > TA, AT > CG).	Comparison with 49 COSMIC mutational signatures: similarities with ovary, breast, pancreatic and lung cancer signatures.	
Mutations (*lacZ* gene)	(Hupki) mouse embryo fibroblasts	**AA**: 0, 1,000, 1,500, 3,000 μM (48 h) **GA**: 0, 750, 1,100, 1,500 μM (24 h + 24 h recovery)	**Positive AA:** only at 3,000 μM; Mutant frequency: 22 *vs* 9×10^{-5} in controls. **Positive GA:** dose-dependent increase (Mutant frequency: up to 45×10^{-5}).		
DNA adducts (N7-GA-Gua)	UPLC–ESI-MS/MS		**Negative AA** **Positive GA**	See **Table 3** for further information.	

Test	Cellular system	Experimental design	Results	Comments	Reference
Elevation of DNA damage response proteins (p-p53, p-Chk, p21, γ-H2AX) Induction of Cyp2E1	Western blotting QRT-PCR	**AA**: 0, 1,000, 1,500, 3,000 μM **GA**: 0, 750, 1,100, 1,500 μM Exposure time: 24 and 48 h	**Positive AA**: elevation of p-p53, p-Chk1 and g-H2AX (not p21). **Positive GA**: elevation of all DNA damage response proteins. **Positive Cyp2E1 induction AA**: sixfold increase only at 3,000 μM.	See **Table 4** for further information.	
Mutations (*lacZ* gene)	Lung FE cells (metabolically competent cells from MutaMouse)	**AA** -S9: 2,000, 4,000, 6,000, 8,000, 10,000 μM **AA** +S9: 500, 1,000, 2,000, 4,000 μM **GA** -S9: 500, 1,500, 2,000, 3,000, 3,500 μM Exposure: 6 h	**Positive AA**: -S9 (8,000 μM) **Positive AA**: +S9 (2,000–4,000 μM) **Positive GA**: -S9 (2,000–3,500 μM) No induction of *Cyp2e1* by AA treatment.		Hölzl-Armstrong et al. (2020a)
Mutations (*gpt* gene)	Murine lung cells (pulmonary organoids) from *gpt* delta M C57BL/6J mice in the presence of metabolic activation	**AA**: 280, 1,400 μM Treatment time: 24 h (repeated 3 times after passaging the organoids)	**Positive AA:** increased mutation frequency at 1,400 μM (fourfold). Increases in GC > AT transition (16%), AT > TA and AT > CG transversions and deletions (54%). Increase expression of Cyp2e1 in AA-treated lung organoids (only at 1,400 μM).	Mutational spectrum similar to that reported *in vivo* in the same transgenic mice (Ishi et al., 2015)	Komiya et al. (2021)
Mutations by whole exome sequencing	(Hupki) mouse embryo fibroblasts Immortalisation of untreated and AA/GA treated cell cultures Control: 3 clones AA +S9: 2 clones AA -S9: 5 clones GA: 5 clones	**AA**: 5,000 μM + 2% human S9 **AA**: 10,000 μM -S9 **GA**: 3,000 μM Treatment time: 24 h	**Negative AA:** 208 and 190 mutations in AA and control, respectively. **Positive GA:** 485 mutations in GA *vs* 190 in control; enrichment in AT > TA, AT > GC, GC > TA base substitutions. 3 specific mutational signatures (mutations mostly in the transcribed strand).	Enrichment of GA signatures in cancer of the lung (88%), liver (73%), kidney (> 70%), bile duct (57%), cervix (50%).	Zhivagui et al. (2019)

Test	Cellular system	Experimental design	Results	Comments	Reference
DNA adducts (N7-GA-Gua and N3-GA-Ade)	DNA adducts by LC–MS/MS		**Negative AA** -S9 **Positive AA** +S9 **Positive GA**	See **Table 3** for further details.	

AA: acrylamide. COSMIC: Catalogue of Somatic Mutations in Cancer. EGCG: epigallocatechin gallate; EMS: ethyl methanesulfonate; Fpg: DNA-formamidopyrimidine glycosylase; GA: glycidamide; GSH: glutathione; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; LC–MS/MS: liquid chromatography with tandem mass spectrometry; LO limit of detection; LOQ: limit of quantification; M: male; MDA: malonaldehyde; MMS: methyl methanesulfonate; N3-GA-ade: N3-(2-carbamoyl-2-hydroxyethyladenine; N7-GA-Gua: N7-(2-carbamoyl-2-hydroxyethyl)guanine; QRT-PCR: quantitative real-time polymerase chain reaction; ROS: reactive oxygen species; SCE: sister chromatid exchange; UHPLC–ESI-MS/MS: ultra-high-performance liquid chromatography-electron spray ionisation-tandem mass spectrometry.

Table 2: ***In vivo*** genotoxicity studies with acrylamide (AA) and/or glycidamide (GA)

Test	Species	Experimental design and doses	Results	Comments	Reference
Micronucleus test in RET	F344 rats and B6C3F1 mice (M) (8 weeks old; 7 animal/ dose group)	Oral (drinking water) **AA** in rat: 0, 0.5, 1.5, 3, 6, 12 mg/kg bw per day **AA** in mice: 0, 0.5, 1.5, 3, 6, 12, 24 mg/kg bw per day For 30 days. Positive control: ENU	**Negative AA: in rats** **Positive AA: in mice** (6–24 mg/kg per day)		Hobbs et al. (2016), Chepelev et al. (2017)
Mutation in RET and RBC (*Pig-a* gene)			**Equivocal AA in rats:** increased mutation frequency only at highest dose; only in RET (but not in RBC); only in 3 out of 7 animals. **Equivocal AA in mice:** increased mutation frequency only at an intermediate dose; only in RET (but not in RBC).		
Micronucleus test in bone marrow	Swiss albino mice (M, F) (10–12 weeks of age, 4 animal/dose group)	Oral (drinking water) **AA**: 0, 2, 4, 8, 16, 32 mg/kg bw per day Exposure: 30 days Analysis: 24 h after the last exposure Positive control: doxorubicin	**Positive AA:** dose-related increase from the lowest dose.		Algarni (2018)
Chromosome aberrations in bone marrow			**Positive AA**: dose-dependent increase in polyploidy, chromatid gaps, Robertsonian centric fusions and stickiness. Decreased mitotic index (slow progression of cells from S- to M-phase of the cell-cycle).		
Micronucleus test in RET	*gpt* delta mice (M) (8 weeks of age, 5–6 animals/dose group)	Gavage **AA**: 7.5, 15, 30 mg/kg bw per day for 28 days Analysis: 3 days (for micronuclei) and 3 and 49 days (expression time for mutation) at the end of the treatment. Positive control: ENU	**Positive AA:** dose- dependent increase in micronuclei (significant increases at 15 and 30 mg/kg bw per day).	Changes in mutational classes: sperm (GC > TA, GC > AT, 1 bp deletions); AT > TA (lung). Important discussion on sensitivity of germ cells to AA genotoxicity (spermatogonial cells less sensitive).	Hagio et al. (2021)
Mutations at the *gpt* gene in testes, sperm and lung			**Positive AA**: increased mutation frequency **Testis** and **lung:** two- to threefold increases at 30 mg/kg bw per day. No difference between 3 and 49 days of analyses. **Sperm**: sixfold increase at 30 mg/kg per bw (only after 3 days)		

Test	Species	Experimental design and doses	Results	Comments	Reference
Micronucleus test in bone marrow Comet assay in lymphocytes and liver	Kunming mice (M) (6–7 weeks old; 10 animals/dose group)	i.p. injection **AA**: 50 mg/kg bw per day, for 5 days. Oxidative stress markers: SOD, GSH-Px, MDA.	**Positive AA** **Positive AA:** as measured by tail length, olive tail moment and tail % DNA.	Main focus on inhibitory role of several antioxidants on AA genotoxicity. See **Table 4** for further information.	Zhao et al. (2015a)
Micronucleus test in bone marrow Comet assay in lymphocyte and liver cells	Kunming mice (M) (6–7 weeks old; 10 animals/dose group)	i.p. injection **AA**: 50 mg/kg bw per day, for 7 days. Oxidative stress markers: ROS, SOD, GSH-Px, GST, GSH, γ-GCS, P450 2E1.	**Positive AA** **Positive AA:** as measured by tail length, olive tail moment and tail % DNA	Main focus on inhibitory role of blueberry anthocyanin extract antioxidant properties on AA genotoxicity. See **Table 4** for further information.	Zhao et al. (2015b)
Meiotic maturation in mouse oocytes	Germinal vesicle-stage mouse oocytes from BALB/c mice (F)	i.p. injection F BALB/c mice (n = 12) **AA:** 25 mg/kg per day, for 7 days.	**Positive AA**: significant reduction in % of metaphase II oocytes compared to controls, meiotic spindle mass reduced, chromosome misalignments.	Reduced maturation in *in vivo* experiments but negative results in *in vitro* experiments.	Aras et al. (2017)
Micronucleus test in bone marrow RET	Wistar rat (M) (6 animals/dose group)	Gavage **AA**: 0, 10, 15, 20 mg/kg bw, for 28 days. Markers of oxidative stress: MDA, GSH, SOD. Positive control: Cyclophosphamide	**Positive AA**: dose-dependent increase from 10 mg/kg per bw; altered PCE/NCE.	Alterations: in several liver haematological parameters; in brain, spinal cord, liver, kidney, myocardium by histopathology; in neurobehavioural parameters. No significant changes in oxidative stress markers in liver and kidney.	Jangir et al. (2016)
Micronucleus test in bone marrow Comet assay in liver, kidney, brain	Wistar rats (M) (5-week-old)	Oral **AA**: 100 mg/kg bw per day Unclear exposure time	**Positive AA:** significant increase in micronuclei. **Positive AA:** significant induction of DNA measured by tail intensity in all organs. No difference in bw and relative weights of liver and kidney.	Main focus on horseradish allyl isothiocyanate antioxidant properties in inhibiting AA-induced DNA damage (micronuclei and comet)	Shimamura et al. (2017)

Test	Species	Experimental design and doses	Results	Comments	Reference
Micronucleus test in bone marrow	Sprague-Dawley rats (F) (12–14 week of age< 5 animals/dose group)	Oral **AA**: 50 mg/kg bw per day, for 30 days.	**Positive AA**: increased micronuclei at decreased PCE/NCE ratio. Measurements of MPO activity, urinary 8-OHdG, levels of GSH, MDA, PCO, TBARS.	Main focus on argan oil antioxidant properties on AA-induced clastogenicity. See **Table 4** for further information.	Şekeroğlu et al. (2017)
Micronucleus test in peripheral blood	F344 rats (M) (8 weeks old; 6 animals/ dose group)	Gavage **AA**: 0, 0.33, 0.66, 1.32, 2.7, 5, 10, 20 mg/kg per day, for up to 29 days Analysis micronuclei: day 4 and 29. Positive control: scoring controls.	**Negative AA**.		Dobrovolsky et al. (2016)
Comet assay in liver, kidney, bone marrow		Analysis Comet: day 29 (analysis 3 h after final dose) For short-term comet assay: 250 mg/kg per day **GA** for 3 days starting at ~12 weeks of age. Positive control: MMS	**Positive AA:** in liver (weak), but not kidney and bone marrow.		
Mutation in RET and RBC (*Pig -a* gene)		Analysis Pig-a: day 15, 29, 56. Positive control: mutant-mimic samples.	**Positive AA:** only at 20 mg/kg per day at day 56, only in RBC.		
Micronucleus test in RET	F344/DuCrl rats (M)	Drinking water **AA**: 0, 0.5, 1.5, 3, 6, 12 mg/kg bw per day for 30 days.	**Negative AA**	No changes in final bw or bw gain in exposed versus control animals. Data on transcriptional profiling. See **Table 4** for further information.	Chepelev et al. (2017), Hobbs et al. (2016)
Mutation in RET and RBC (*Pig-a* gene)			**Equivocal AA**		

Test	Species	Experimental design and doses	Results	Comments	Reference
Comet assay in liver cells	Wistar rats (M) (6 animals/ dose group)	i.p. injection **AA**: 50 mg/kg bw Analysis: 48 h after injection Oxidative stress markers, GST, 8-OHdG; Histopathological findings	**Positive AA:** as measured by tail length, tail moment and tail % DNA	Main focus on inhibitory role of quercetin antioxidant properties on AA genotoxicity. See **Table 4** for further information.	Ansar et al. (2016)
Comet assay in mouse spermatozoa	Swiss CD1 mice (M) (3 animals/dose group)	Oral (drinking water) **AA**: 1 ug/mL, for 6 months	**Positive AA:** increased DNA breaks in spermatozoa without a concomitant reduction in overall fertility. Increased DNA breaks also in the spermatozoa of offspring (F1). Small increase in DNA 8-OHdG.	See **Table 4** for further information.	Katen et al. (2016a)
Comet assay in spermatozoa 8-OHdG by immunostaining γ-H2AX by immuno-fluorescence	Swiss CD-1 mice (M) (5–6 week of age; 3 and 6 mice for 3 and 6 month time points, respectively)	Oral (drinking water) **AA**: 0.18 mg/kg bw per day Exposure: 3 and 6 months +/– resveratrol: once a week.	**Positive AA** **Positive AA**: both at 3 and 6 months AA exposure. **Positive AA:** increase in post-meiotic germ cells (late spermatids and spermatozoa) both at 3 and 6 months AA exposure.	See **Table 4** for further information (small increases in 8-OHdG and γ-H2AX foci)	Katen et al. (2016b)
Comet assay in spermatocytes and spermatozoa of mice	Swiss CD-1 mice (M) (6 animals/dose group)	i.p. injection **AA**: 25 mg/kg bw per day, for 5 consecutive days Analysis: 3–5 days (spermatozoa) or 24–26 days (spermatocytes) following the last injection.	**Positive AA:** at both stages of spermatogenesis.	Epididymal CYP2E1 plays a critical role in AA-induced DNA damage in spermatozoa and paternally mediated embryonic resorptions. See **Table 4** for further information.	Katen et al. (2017)

Test	Species	Experimental design and doses	Results	Comments	Reference
Comet assay in corneal epithelial cells	Japanese white rabbits (M) (11–12 weeks of age, 2-4 animals/dose group)	Eye instillation **AA**: 0.6% and 3% AA, 50 μL per eye. Analysis: 2 h after instillation Also tested: ethidium bromide, paraquat, MMS, 4-NQO	**Negative AA**: comet assay as measured by % tail DNA.	Limited information from this study because of unusual animal model for genotoxicity testing and AA delivery.	Tahara et al. (2019)
γ-H2AX foci in the urinary bladder	B6C3F1 mice (M) (5 weeks of age; 10 animals/dose group)	Oral (drinking water) **AA**: 0.005% (corresponding to 9.85 mg/kg bw daily intake) Exposure time: 4 weeks	**Negative AA**	Limited information from this study because of the use of AA as a negative control (a genotoxic non bladder carcinogen).	Sone et al. (2019)
UDS in corneal epithelial cells	Japanese white rabbits (M) (11–12 weeks of age, 2-4 animals/dose group)	Eye instillation **AA**: 3% AA, 50 μL per eye. Analysis: 2 h after instillation Also tested: paraquat, acridine orange, ethidium bromide, 4-NQO	**Negative AA**	Limited information from this study because of unusual animal model for genotoxicity testing and AA delivery.	Tahara et al. (2021)
Mutation in RET and RBC (*Pig-a* gene)	F344 rats (M)	Oral (drinking water) 0, 25, 50, 100, 137.5, 175 mg/kg per day, for 28 days Analysis: 2, 7, 14, 28 days Positive control: ENU	**Negative AA**	Body weight gain reduced in two highest dose groups; evidence of reticulocytosis at high doses.	Horibata et al. (2016)
Mutation in brain (*cII* gene)	Big Blue mice (M, F)	Oral (drinking water) **AA** and **GA**: 0, 1.4, 7.0 mM, for 4 weeks	**Positive AA and GA**: increased mutation frequency only in males at 7.0 mM. twofold increase in GC > TA; AT > TA and AT > CG.	Similar spectra for AA and GA. Data also for lung, liver and testis from Wang et al. (2010); Manjanatha et al. (2015).	Li et al. (2016)

AA: acrylamide; bw: body weight; ENU: *N*-ethyl-*N*-nitrosourea; F: female; GA: glycidamide; γ-GCS: γ-glutamylcysteine synthetase; GST: gluthathione *S*-transferase; GSH: glutathione; GSH-Px: glutathione peroxidase; 8-OHdG: 8-hydroxy-2′-deoxyguanosine; M: male; MDA: malonaldehyde; MMS: methyl methanesulfonate; MPO: myeloperoxidase; 4-NQO: 4-nitroquinoline 1-oxide; PCE/NCE: polychromatic erythrocytes/normochromatic erythrocytes; PCO: protein carbonyl; ROS: reactive oxygen species; RBC: red blood cells; RET: reticulocytes; SOD: Superoxide dismutase; TBARS: thiobarbituric acid reactive substances; UDS: unscheduled DNA synthesis.

Table 3: Studies on *in vitro* and *in vivo* **DNA adducts** induced by AA and/or GA

Test	Analytical method	Experimental design and doses	Results	Comments	Reference
In vitro					
DNA adducts (N7-GA-Gua and N3-GA-Ade) in calf thymus DNA	LC–MS	**GA**: 10,000, 25,000, 50,000 μM	**Positive GA** At neutral conditions DNA adducts are released from DNA (spontaneous depurination)	GA-induced lesions are predominantly N7-GA-dG adducts slowly undergoing imidazole ring opening at pH 10; such structures are incised by Fpg leading to DNA strand breaks. See **Table 4** for further information.	Hansen et al. (2018)
DNA adducts (N7-GA-Gua and N3-GA-Ade) in (Hupki) mouse embryo fibroblasts	LC–MS/MS LOD: 5.5 adducts per 10^8 nucleotides	**AA**: 5,000 μM + 2% human S9 **AA**: 10,000 μM –S9 **GA**: 3,000 μM Treatment time: 24 h	AA –S9: no N7-GA-Gua adducts AA +S9: 11 adducts per 10^8 nucleotides GA: 49,000 and 350 adducts per 10^8 nucleotides for N7-GA-Gua and N3-GA-Ade, respectively. Control: 5.5 adducts per 10^8 nucleotides	Mutations by whole exome sequencing in (Hupki) mouse embryo fibroblasts (Negative AA; Positive GA).	Zhivagui et al. (2019)
DNA adducts (N7-GA-Gua) in (Hupki) mouse embryo fibroblasts	UPLC–ESI-MS/MS LOQ: 1.7 adducts per 10^8 nucleosides	**AA**: 0, 1,000, 1,500, 3,000 μM (48 h) **GA**: 0, 750, 1,100, 1,500 μM (24 h)	**Negative AA:** < LOQ **Positive GA:** 20 (at 750 and 1,100 μM) and 30 (at 1500 μM) N7-GA-Gua per 10^8 nucleosides; below LOQ at 48 h; no adducts in control.	Mutations by whole genome sequencing in (Hupki) mouse embryo fibroblasts (Negative AA; Positive GA). See **Table 4** for further information.	Hölzl-Armstrong et al. (2020a)
DNA adducts (N7-GA-Gua) in rat hepatocytes		**AA**: 2, 20, 200, 500, 1,000, 2,000 μM Treatment time: 16 and 24 h	**Positive AA:** non-linear concentration response; increases in N7-GA-Gua at 1,000 and 2,000 μM (20–30 adducts per 10^8 nucleotides); background levels: 5–10 adducts per 10^8 nucleotides		Hemgesberg et al. (2021a)

Test	Analytical method	Experimental design and doses	Results	Comments	Reference
In vivo					
DNA adducts (N7-GA-Gua and N3-GA-Ade) in lung and liver of B6C3F1 mice (F) (5-6 weeks of age)	Analysis by HPLC–ESI-MS/MS LOD: 0.5 adducts per 10^8 nucleotides for both N7-GA-Gua and N3-GA-Ade LOQ: 1 and 1.5 adducts per 10^8 nucleotides for N7-GA-Gua and N3-GA-Ade, respectively	Oral (drinking water) **AA**: 0, 87.5, 175, 350, 700 μM (2-year cancer bioassay concentrations) (equivalent to 1.04, 2.20, 4.11 and 8.93 mg/kg bw per day for M, and to 1.10, 2.23, 4.65 and 9.96 mg/kg bw per day for F) Treatment time: 28 days	**Positive AA:** dose-dependent increase in both DNA adducts in lung and liver. Similar levels of adduction in the two organs. N7-GA-Gua: range 100–1,000 N3-GA-Ade: range 0.5–4 per 10^8 nucleotides	Important role of epigenetic alterations in determining the target organ for AA tumorigenesis. See **Table 4** for further information.	de Conti et al. (2019)
DNA adducts (N7-GA-Gua) and GAMA3 in urine and tissues (liver, kidney and lung) of Sprague-Dawley rats (F) (7-8 weeks of age, 12 animals/dose group)	UPLC–MS/MS LOD and LOQ for N7-GA-Gua: 1 and 3 adducts per 10^8 nucleotides (0.02–0.06 pmol, respectively).	Gavage **AA:** 35 mg/kg bw per day Treatment time: 7 and 14 days Urine collection: 0 (0.5 h), 0.5 (1 h), 1 (2 h), 2 (4 h), 4 (8 h) following 1st AA gavage, sacrifice at 14th days.	**Positive** N7-GA-Gua adducts per 10^8 nucleotides at 7 and 14 days, respectively: – Liver: approx. 900 and 1,300 – Kidney: approx. 1,300 and 2,100 – Lung: approx. 1,000 and 1,900 Pre-treatment with blueberry anthocyanin extract: significant block of AA epoxidation to GA.		Wang et al. (2019)
DNA adducts (DNA N7-GA-Gua) and AAMA in urine of smokers (n = 30) and non-smokers (n = 33)	Isotope dilution SPE LC–MS/MS LOD for N7-GA-Gua: 0.25 ng/mL in urine	–	**Positive:** median urinary N7-GA-Gua level: 0.93 and 1.41 μg/g creatinine (1.50 and 2.01 ng/mL urine) in non-smokers and smokers, respectively (this difference is not statistically significant). Urinary N7-GA-Gua levels significantly associated with AAMA levels.	The authors conclude that urinary N7-GA-Gua of non-smokers and smokers is significantly associated with a very low level of dietary AA intake.	Huang et al. (2015)

Test	Analytical method	Experimental design and doses	Results	Comments	Reference
DNA Adducts (DNA N7-GA-Gua) and mercapturic acid derivatives AAMA and GAMA in the urine of 8 male AA-exposed workers and 36 controls (administrative workers) from the same AA production plants (four plants). Collection of urines pre- and post-shifts at work	Analysis of DNA N7-GA-Gua by isotope-dilution two-step SPE LC–MS/MS Analysis of AAMA and GAMA: LC–ESI-MS/MS LOD of N7-GA-Gua: 0.25 ng/mL in urine (50 fmoles)	-	**Positive:** Significantly higher levels in AA-exposed workers than in controls at both time points; Median urinary N7-GA-Gua level in exposed workers: 1.85 ng/mL and 2.26 ng/mL (at pre- and post-shift, respectively). Median urinary N7-GA-Gua level the control group: 0.33 ng/mL and 0.29 ng/mL (at pre- and post-shift, respectively). The elimination half-life of urinary N7-GA-Gua was 346.5 h (140.3–737.2 h). N7-GA-Gua level correlated positively with AAMA and GAMA levels.	The two groups had similar BMIs, periods of employment, smoking status and preferences for fried food.	Huang et al. (2018)
DNA adducts (N7-GA-Gua) in PBMC from 56 healthy volunteers (age 18–65 years)	UHPLC–ESI^+-MS/MS LOD: 0.1–0.2 adducts per 10^8 nucleosides LOQ: 0.3–0.5 per 10^8 nucleosides	Measurements of biometric, dietary and biochemical parameters: (age, gender, body weight, height, smoking, vegetarian, vegan, frequent consumption of coffee, French fries, cookies)	N7-GA-Gua DNA adducts: mean: 1.1 per 10^8 nucleosides; range: 0.2–26.6 per 10^8 nucleosides Correlation with BMI. No correlation with dietary habits, blood glucose levels and haemoglobin.		Hemgesberg et al. (2021b)
DNA adducts (N7-GA-Gua) in blood samples of 17 healthy volunteers	LC–ESI-MS/MS LOD: 25 fmoles LOQ: 0.50 fmoles		**Positive:** DNA adducts quantified in 13 out of 17 samples N7-GA-Gua range: 0.3–6.3 adducts per 10^8 nucleotides N7-GA-Gua mean: 1.9 adduct per 10^8 nucleotides	Range of AA intake from the food frequency questionnaires: 20.0–78.6 μg per day (corresponding to 0.29–1.14 μg/kg bw per day, using the mean body weight of 69 kg provided by the authors)	Jones et al. (2021)

AA: Acrylamide; AAMA: *N*-acetyl-S-(2- carbamoylethyl)-L-cysteine; BMI: body mass index; F: females; Fpg: DNA-formamidopyrimidine glycosylase; GA: glycidamide; GAMA3: *N*-acetyl-S-(3-amino-2-hydroxy-3-oxopropyl)-cysteine; GAMA: *N*-(*R*,*S*)-acetyl-*S*-(1-carbamoyl-2-hydroxyethyl)-L-cysteine; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; LOD: limit of detection; LOQ: limit of quantification; M: male; N3-GA-Ade: N3-(2-carbamoyl-2-hydroxyethyl)adenine; N7-GA-Gua: N7-(2-carbamoyl-2-hydroxyethyl) guanine; LC–MS/MS: liquid chromatography tandem mass spectrometry; PBMC: peripheral blood mononuclear cell; SPE: solid-phase extraction; UHPLC–ESI-MS/MS: ultra-high-performance liquid chromatography-electron spray ionisation-tandem mass spectrometry.

3.2. Non-genotoxic effects and genotoxicity secondary to oxidative stress that may contribute to AA carcinogenicity

Main conclusions in the 2015 EFSA Opinion on AA in food

In addition to observations relating to genotoxicity produced by AA or its metabolites, the CONTAM Panel noted that in situations of limited CYP2E1 activity, genotoxicity may involve the generation of ROS and induction of oxidative DNA damage (EFSA CONTAM Panel, 2015). This alternative pathway appears to take place only with very high toxic doses of AA. It was also noted that it was postulated by several investigators that the clastogenic effects of AA on germ cells may be mediated through interference with the kinesin motor proteins that are involved in spindle fibre formation and chromosomal segregation during cell division or alkylation of protamines in sperm. Alternatively, AA may alkylate proteins associated with chromatin via its affinity for sulfhydryl groups, resulting in clastogenic effects.

Since tumours originating from tissues involved in the endocrine system, such as mammary gland, testis and thyroid, were significantly increased in rat bioassays, several publications had raised the possibility that AA may act as a carcinogen via adverse effects on endocrine regulation. One such proposal was that AA acts as an agonist at dopamine D1-receptors in rat ovaries thus increasing prolactin release which may stimulate the mammary gland resulting in increased rates of mammary gland fibroadenoma in female F344 rats. Another report concluded that both genotoxic and growth stimulation may be relevant in thyroid tumour formation. One publication reported that thyroxine (T3) and triiodothyronine (T4) and corticosterone levels were lower in rats treated with AA than that in control rats, leading the authors to conclude that endocrine disturbance may contribute to tumours in thyroid and adrenal glands. However, the Panel noted that inconsistent changes in thyroid hormones were reported and concluded that mechanistic hypotheses on local endocrine effects of AA which may explain tumour formation in certain hormone- or paracrine-regulated target tissues lack experimental proof (EFSA CONTAM Panel, 2015).

New studies since the 2015 EFSA Opinion on AA in food

The studies identified are summarised in **Table 4.**

Several studies provide further support for oxidative stress in various cellular systems and tissues *in vivo* and for an associated oxidative damage to DNA. Such evidence comes from an association of AA-induced DNA strand breaks with oxidative stress, both *in vitro* and *in vivo*. Both oxidative stress and DNA strand breaks were reduced by antioxidants (Zhao et al., 2015a,b; Ansar et al., 2016; Nowak et al., 2020; Wang et al., 2021). The demonstration of enhanced DNA breaks induced by enzymes involved in the repair of oxidative DNA damage (Fpg, EndoIII or OGG1) in the comet assay was shown to implicate DNA oxidation in various studies, however, the interpretation of the studies using Fpg to imply DNA oxidation should be taken with caution since Hansen et al. (2018) provided evidence that DNA damage revealed through the use of Fpg may also be produced by GA directly. Furthermore, since Fpg (less specific) was more effective in inducing DNA breaks during repair than OGG1 (which is more specific for repair of 8-oxoguanine), this may imply that oxidative DNA damage is a relatively small component of the damage detected in the comet assay (Katen et al., 2017). Nevertheless, studies show the formation of 8-oxoguanine via the production of ROS following treatment of biological systems with AA (Wang et al., 2015, 2020; Katen et al., 2016a,b; Şekeroğlu et al., 2017; Zamani et al., 2018; Salimi et al., 2021a,b). Several studies also demonstrated an association between micronucleus formation and oxidative stress both *in vitro* and *in vivo* (Zhao et al., 2015a,b; Şekeroğlu et al., 2017; Ankaiah et al., 2018; Zamani et al., 2018). Although oxidative DNA damage was reduced by various antioxidants, it should be noted, however, that it cannot be assumed that the various antioxidants studied necessarily inhibit DNA damage via the antioxidant activity. Thus, one study showed the inhibition of CYP 2E1 and hence implicates inhibition of the metabolic oxidation of AA to GA by blueberry anthocyanins (Zhao et al., 2015b).

Some gene expression studies showed evidence (albeit limited) for a DNA-damage response to AA. Collí-Dulá et al. (2016) reported AA-induced transcriptomic changes in the Wistar rat thyroid which included genes that are involved in DNA damage and repair as well as various other changes relating to, e.g. oxidative stress and motor proteins and kinases which may also relate to carcinogenicity. Chepelev et al. (2017), in contrast, did not find evidence of a DNA damage response in F344 rat thyroid (including no response in *Gadd*45 (commonly associated with a genotoxic insult). However, in the rat liver there was evidence of a DNA damage response including the induction of p53. p53 gene

expression was not elevated in F344 rat testis *in vivo* by AA under conditions where increased transcription of genes involved in calcium signalling and the cytoskeleton was observed (Recio et al., 2017). The WG noted that the strain of rat used by Collí-Dulá et al. (2016) (Wistar) and Chepelev et al. (2017) and Recio et al. (2017) (F344) differed. Chepelev et al. (2018) found that in mice treated with AA, genes involved in the p53 pathway were not significantly enriched in those genes with altered expression in the Harderian gland or the lung. However, there was an elevation of phospho-p53, phospho-Chk1 and γ-H2ax proteins implicating a DNA damage response in mouse embryo fibroblasts treated with either AA or GA (Hölzl-Armstrong et al., 2020a). In this study, the cell cycle regulator (p21) was elevated by GA but not by AA.

Several toxicogenomic studies also implicate changes in the expression of genes involved in calcium signalling and the cytoskeleton as a major transcriptome response (Chepelev et al., 2017, 2018; Recio et al., 2017). The authors note that changes in calcium signalling and actin filaments may lead to impaired microtubule and microfilament integrity and interfere with chromosome segregation during cell division. The alteration of calcium signalling and the cytoskeleton might contribute to the observed increase in the percentage of misaligned chromosomes and abnormal spindle morphology produced in AA treated oocytes (Liu et al., 2015). Despite the potential ways in which calcium signalling might contribute to carcinogenesis, a specific role is unproven.

The ability of AA to bring about epigenetic modifications (specifically changes in histone acetylation and methylation, and DNA methylation in liver and lung of mice) is noted as a potential epigenetic influence on carcinogenesis (de Conti et al., 2019). In this study, AA caused an increase in histone acetylation and hypomethylation of DNA in liver in contrast to a decrease in histone methylation and hypermethylation of DNA associated with transcriptional silencing in lung.

The potential for enhanced cell proliferation was evidenced by an increased expression of cell cycle regulators and an increase in the migratory ability of prostate cancer cells by GA (Ekanem et al., 2019).

In rats exposed *in utero* to AA (Collí-Dulá et al., 2016), transcriptomic changes involved genes related to cell cycle. In these animals, plasma T3 and T4 were increased in AA-treated rats, lending further evidence to support the disturbance of thyroid hormones by AA which was noted in the 2015 EFSA Opinion (EFSA CONTAM Panel, 2015) as being inconsistent. It is recognised that modulation of thyroid hormone levels has been associated with thyroid carcinogenesis in rodents, particularly via raised thyroid-stimulating hormone (TSH). However, the mechanism of thyroid hormone dysregulation by AA is not known and there is no clear evidence that AA leads to raised TSH. Furthermore, the WG noted that the relevance to human of rodent thyroid tumours induced by alteration of the level of thyroid hormones is questionable (Bartsch et al., 2018).

Table 4: Non-genotoxic effects and genotoxicity secondary to oxidative stress that may contribute to carcinogenicity of AA

Test	Cellular system	Experimental design	Results	Comments	Reference
Comet assay and 8-OHdG	Wistar rats (M) (6 animals/dose group) Liver analysed	i.p. injection of **AA**; 50 mg/kg bw Analysis: 48 h after injection ± Quercetin (10 mg/kg bw) Oxidative stress markers, GST, 8-OHdG	**Positive AA:** DNA breaks (as measured by tail length, tail moment and tail % DNA) and 8-OHdG were increased by AA. DNA breaks and 8-OHdG were inhibited by quercetin.	Association of positive comet response with oxidative stress.	Ansar et al. (2016)
Comet assay ± Fpg and OGG1 in mice and human spermatozoa	Mouse and human spermatozoa and mouse epididymal spermatozoa mECap18 cells	Spermatozoa treatment: **AA**: 10, 100, 1,000, 10,000, 100,000 μM **GA**: 0.05, 0.5, 5, 50, 500 μM Exposure 1 or 2 h mECap18 cells treatment: **AA**: 10, 1,000, 100,000 μM **GA**: 5 μM	Mouse spermatozoa treatment: **Negative AA:** at all concentrations **Positive GA:** at all concentrations Epididymal mECap18 cells treatment: **Positive AA** + **Fpg:** large increase in DNA breaks. + **OGG1:** modest changes in DNA breaks. **Positive**: mouse and human sperm in the presence of conditioned media from AA- or GA-treated mECap18 cells.	The large difference in the number of DNA breaks following Fpg and OGG1 incubation indicates that Fpg can incise AA-induced DNA adducts as well as 8-oxoguanine while OGG1 recognises only 8-oxoguanine in DNA.	Katen et al. (2017)
Comet assay using Fpg	Human lymphocytes	**GA**: 0–60 μM tested with Fpg enzyme under different lysis conditions	DNA breaks detected using Fpg (linear response) irrespective of alkaline lysis conditions.	Results suggest that the positive response in the Comet assay with Fpg is not due to DNA oxidation since alkaline lysis conditions did not influence the detection of lesions induced by the phototoxic compound Ro 12–9786 inducing mostly 8-oxoguanine lesions.	Hansen et al. (2018)

Test	Cellular system	Experimental design	Results	Comments	Reference
Comet assay with Fpg and EndoIII	CaCo-2 cells	**AA**: 200, 800, 3,200, 6,400, 12,500, 25,000, 50,000 μM Comet assay: 1 h ROS levels: 6 h Mitochondria depolarisation and apoptosis 24 h	**Positive:** increase in % tail DNA from 800 μM (non-cytotoxic concentrations) with elevation of ROS. +Fpg: increase from 3,200 μM +EndoIII: increase from 6,400 μM Depolarisation of mitochondria and apoptosis from 3,200 μM.	Suggests DNA oxidation by AA. Apoptosis at later time point.	Nowak et al. (2020)
Comet assay using Fpg and EndoIII	Human lymphocytes	**AA**: 1, 5, 10 mM; 1 h exposure Comet assay +/− Fpg and Endo III digestion +/− epigallocatechin gallate and curcumin (10 mM)	**Positive:** concentration related increase in DNA strand breaks. Fpg and EndoIII increase AA (5 mM)-induced DNA strand breaks (modest). Epigallocatechin gallate and curcumin decrease AA-induced DNA strand breaks.	Suggests DNA oxidation by AA, inhibited by the antioxidants epigallocatechin gallate and curcumin.	Wang et al. (2021)
Comet assay and Micronucleus assay	Kunming mice (M) (6–7 week old; 10 animals/dose group) Comet assay: Lymphocytes and liver Micronucleus: bone marrow	i.p. injection **AA**: 50 mg/kg bw per day, for 5 days. Control diet or diet containing freeze-dried strawberry, grape and blueberry powder. Oxidative stress markers: SOD, GSH-Px, MDA.	Comet assay positive as measured by tail length, olive tail moment and tail % DNA. Micronucleus assay positive	Protective effect of freeze-dried strawberry, grape and blueberry powder on AA genotoxicity may imply an oxidative mechanism	Zhao et al. (2015a)

Test	Cellular system	Experimental design	Results	Comments	Reference
Comet assay and Micronucleus assay	Kunming mice (M) (6–7 weeks old; 10 animals/dose group) Comet assay: Lymphocytes and liver Micronucleus: bone marrow	i.p. injection **AA**: 50 mg/kg bw per day, for 7 days +/– Blueberry anthocyanins extract (0, 50, 150, 250 mg/kg bw per day) Oxidative stress markers: ROS, SOD, GSH-Px, GST, GSH, γ-GCS, P450 2E1.	Comet assay positive as measured by tail length, olive tail moment and tail % DNA. Micronucleus assay positive.	AA-induced genotoxicity, oxidative stress and cytochrome P450 2E1 activation were all inhibited by blueberry anthocyanins extract (antioxidant).	Zhao et al. (2015b)
Micronucleus assay in bone marrow and urinary 8-OHdG	Sprague-Dawley rats (F) (12–14 week of age); 5 animals/dose group	Oral **AA**: 50 mg/kg bw per day, for 30 days. +/– Argan oil (6 mL/kg bw per day) Measurements of MPO activity, urinary 8-OHdG, levels of GSH, MDA, PCO, TBARS	**Positive AA**: increased micronuclei at decreased PCE/NCE ratio. Evidence of oxidative stress and increased 8-OHdG, inhibited by Argan oil.	Protective effect of antioxidant Argan oil.	Sekeroglu et al. (2017)
Measurement of micronuclei and markers of ROS	Rat lymphocytes	**AA**: 100, 200, 300 mg/L (corresponding to 1,407, 2,814, 4,221 μM) 24 h +/– resveratrol pretreatment: 100 μM	**Positive micronuclei**: all AA concentrations. Reduction by resveratrol pre-treatment. ROS induction by AA: 5, 50, 100, 200 mg/L: decreased by resveratrol at 100, 200 mg/L AA	Suggests ROS production (inhibited by the antioxidant resveratrol) contributes to micronucleus formation.	Ankaiah et al. (2018)

Test	Cellular system	Experimental design	Results	Comments	Reference
Measurement of micronuclei and markers of ROS and oxidative stress	Human lymphocytes	**AA**: 250, 50,000, 100,000 μM, for 20 h +/− L-carnitine (100 and 200 μM) Markers of oxidative stress: ROS, MDA, GSH	Significant elevation of micronuclei at 50 and 100,000 μM in association with oxidative stress. These effects were inhibited by L-carnitine.	Suggests ROS production (inhibited by the antioxidant L-carnitine) contributes to micronucleus formation.	Zamani et al. (2018)
Analysis of 8-OHdG and measurements of ROS and oxidative stress	Kidney, liver, brain and lung of M and F mice	i.p. injection **GA**: 50 mg/kg bw per day, for 7 days +/− allicin: 5, 10 or 20 mg/kg, intragastric for 15 days	8-OHdG was elevated in tissues by GA as was ROS along with an inhibition of various antioxidant systems. Allicin at all doses tested reduced all of the above oxidant effects produced by GA.	Demonstrates DNA oxidation by AA, inhibited by the antioxidant allicin.	Wang et al. (2015)
Analysis of 8-OHdG	Swiss CD1 mice (M) (3 animals/dose group) Analysis of spermatozoa	Oral (drinking water) **AA**: 1 μg/mL (equivalent to 0.13 mg/kg bw per day) for 6 months	Small increase in DNA 8-OHdG	Evidence of DNA oxidation	Katen et al. (2016b)
8-OHdG by immunostaining	Swiss CD-1 mouse spermatozoa (M) (5–6 week of age; 3 and 6 mice for 3 and 6 month time points, respectively)	Oral (drinking water) **AA**: 0.18 mg/kg bw per day Exposure: 3 and 6 months. +/− resveratrol (4 mg/mL), once a week.	**Positive**: both at 3 and 6 months AA exposure. Inhibited by resveratrol.	Evidence of DNA oxidation inhibited by the antioxidant resveratrol.	Katen et al. (2016a)
Elevation of 8-OHdG in cellular DNA	Human lymphocytes	**AA**: 50 μM; 4 h AA exposure: 4 h 8-OHdG measured ROS, MDA, GSH and GSSG measured Ellagic acid (10, 25, 50 μM)	AA induced DNA 8-OHdG (2.5-fold) which was inhibited by ellagic acid and associated with ROS production.	Demonstrates DNA oxidation by AA, inhibited by the antioxidant ellagic acid.	Salimi et al. (2021a)

Test	Cellular system	Experimental design	Results	Comments	Reference
Elevation of 8- OHdG in cellular DNA	Human lymphocytes	**AA**: 50 μM +/− Chrysin (10, 25, 50 μM) ROS, MDA, GSH and GSSG measured.	AA induced DNA 8-OHdG and oxidative stress which were inhibited by chrysin.	Demonstrates DNA oxidation by AA, inhibited by the flavonoid chrysin.	Salimi et al. (2021b).
Urinary measurements of 8-oxoguanine and AA metabolites	Human subjects. Analysis of urine	Statistical analysis of the association between AA metabolites and 8-oxoguanine in urine.	A significant linear positive dose-response relationship was found between urinary AA metabolites and 8-oxoguanine.	Provides evidence of an association between AA and oxidative DNA damage in humans.	Wang et al. (2020)
Transcriptomic analysis of thyroid glands and measurement of plasma thyroid hormones	RccHan Wistar rats exposed *in utero* to AA	Carcinogenic dose of AA (3 mg/kg bw per day) from GD6 to delivery and then through their drinking water to PND35. RatV 18×60K oligonucleotide microarray. Measurement of plasma TSH, T3 and T4.	Plasma T3 and T4 were increased in AA-treated rats sampled during the night. DNA repair, transcriptomic changes involved genes related to cell cycle cell death with notable changes in kinesins and gene expression related to oxidative stress.	In addition to some transcriptomic responses indicative of DNA damage and repair, the findings implicate potential contributions of oxidative stress and thyroid hormone changes in thyroid carcinogenicity.	Collí-Dulá et al. (2016)
Transcriptomic analysis (Qiagen miRNeasy Mini Kits) and measurement of serum thyroid hormone levels	Thyroids and livers of Fischer 344/DuCrl rats (M)	Oral (drinking water) **AA**: 0.0, 0.5, 1.5, 3.0, 6.0, 12.0 mg/kg bw per day For 5, 15, 31 days	Marginal gene expression changes associated with DNA damage response (more evident in liver than thyroid) or thyroid hormone regulation. Altered gene expression gave evidence for perturbation of calcium signalling in the thyroid. Decreased serum T3 at 1.5 mg/kg bw per day on day 5, and increased T4 on day 5 (high dose) and day 31 (1.5 mg/kg bw per day dose).	The most marked gene expression response related to calcium signalling and the cytoskeleton.	Chepelev et al. (2017)

Test	Cellular system	Experimental design	Results	Comments	Reference
Transcriptomic analysis (Illumina NextSeq500)	Testes from F344 rats	Oral (drinking water) **AA**: 0.0, 0.5, 1.5, 3.0, 6.0, 12.0 mg/kg bw per day, for 5, 15, 31 days	The most prominent functional clusters affected by AA exposure were actin filament organisation, response to calcium ion and regulation of cell proliferation. Lack of p53 damage response pathway.	The authors considered that the gene expression changes associated with calcium signalling and cytoskeleton may contribute to dominant lethal mutations in the rat.	Recio et al. (2017)
Transcriptomic analysis RNA-seq analysis in the lungs (ion proton sequencer) or Harderian gland (IlluminaNextSe)	CD-1 mouse (M) lungs and Harderian gland	Oral (drinking water) **AA**: 0.0, 1.5, 3.0, 6.0, 12.0, 24.0 mg/kg bw per day, for 5, 15, 31 days	Pronounced effect on gene expression relating to calcium signalling and cytoskeleton in Harderian gland. Changes in Integrin-linked kinase signalling was most prominent in lungs.	The authors report limited support for genotoxicity as a key event and highlight the marked response in calcium signalling.	Chepelev et al. (2018)
Measurement of DNA damage response proteins (p-p53, p-Chk, p21, γ-H2AX)	(Hupki) mouse embryo fibroblasts Western blotting 24 and 48 h	**AA**: 0, 1,000, 1,500, 3,000 μM (48 h) **GA**: 0, 750, 1,100, 1,500 μM (24 h)	**Positive AA**: elevation of p-p53, p-Chk1 and γ-H2ax (not p21). **Positive GA**: elevation of all DNA damage response proteins.	Results give support for a genotoxic response but the cell cycle regulator p21 was affected by GA and not AA.	Hölzl-Armstrong et al. (2020a)
Modulation of expression of genes related to cell cycle and epithelial-to-mesenchymal transition	Prostate cancer cells (PC3 and LNCaP cell lines)	**GA**	Increased expression of the cell cycle regulators and increased the migratory ability of prostate cancer cells.	GA promoted the growth ability of prostate cancer cells.	Ekanem et al. (2019)

Test	Cellular system	Experimental design	Results	Comments	Reference
Analysis of epigenetic changes	Lung and Liver of B6C3F1 mice (F)	Oral (drinking water) **AA**: 0, 0.0875, 0.175, 0.35, 0.70 mM (equivalent to 1.04, 2.20, 4.11 and 8.93 mg/kg bw per day for M, and to 1.10, 2.23, 4.65 and 9.96 mg/kg bw per day for F) For 28 days	Lung: AA induced decrease of histone H4 lysine 20 trimethylation along with DNA hypermethylation and transcription silencing. Liver: increased acetylation of histone H3 lysine 27 and DNA hypomethylation.	The results demonstrate a potential contribution of epigenetic changes and gene expression in AA carcinogenesis; liver and lung showing different responses.	de Conti et al. (2019)

AA: acrylamide; F: female; Fpg: DNA-formamidopyrimidine glycosylase; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; GA: glycidamide; GD: gestational day; GSH: glutathione; GSH-Px: glutathione peroxidase; GSSG: oxidised GSH; GST: gluthathione S-transferase; γ-GCS: γ-glutamylcysteine synthetase; M: male; MDA: malonaldehyde; PCE/NCE: polychromatic erythrocytes/normochromatic erythrocytes; PCO: protein carbonyl; PND: postnatal day; TBARS: thiobarbituric acid reactive substances; T3: thyroxine, T4: triiodothyronine; TSH: thyroid-stimulating hormone; ROS: reactive oxygen species; SOD: superoxide dismutase.

3.3. Other relevant data

Endogenous formation of AA

It has been suggested that endogenous formation of AA occurs at a rate relatively close to average dietary exposure (Eisenbrand, 2020a). The CONTAM Panel previously noted some evidence indicating possible endogenous formation of AA. However, no conclusions could be drawn and it was recommended to consider the possibility that cysteine adducts of AA in dietary proteins could be present in food and feed, which could be absorbed from the GI tract after proteolytic degradation and subsequently excreted in urine (EFSA CONTAM Panel, 2015). Since then, further evidence for endogenous formation of AA has been provided by two studies in healthy, non-smoker, human volunteers performed under strictly controlled conditions.

In the first study (Ruenz et al., 2016), an initial 3-day washout period involved an AA-minimised diet, with dietary AA exposure not exceeding 0.004 μg/kg bw per day (analysed in duplicate diets). Subsequent dietary exposure to both low (0.6–0.8 μg/kg bw per day) or high (1.3–1.8 μg/kg bw per day) levels of AA resulted in 58% of the AA being excreted in urine as AA mercapturic acid (AAMA) within 3 days. Based on this conversion, and the urinary AAMA concentration at the end of the initial washout period, the authors estimated an endogenous AA formation of 0.2–0.3 μg/kg bw per day. They noted however, that there may have been substantial dietary AA exposure before the start of the 3-day washout, resulting in residual AAMA excretion. Therefore, the authors performed a follow-up study with extended washout periods and stable isotope labelled AA.

The follow-up study (Goempel et al., 2017) included one group of volunteers who received a minimized AA diet (0.05–0.06 μg/kg bw per day) for the whole of the 13-day study period and were given $^{13}C_3D_3$-AA (1 μg/kg bw) on day 6. A second group received the minimized diet with the addition of a low exposure to AA in coffee (0.15–0.17 μg/kg bw) on day 6, and a high exposure AA meal (14.1–15.9 μg/kg bw) on day 10. The coffee consumption on day 6 increased the urinary excretion of AAMA compared to the preceding day, but within the range of the control group. The high exposure on day 10 resulted in a steep increase (up to about 10-fold) in the urinary excretion of AAMA, and also of the mercapturic acid of GA (GAMA). The elimination curve of the $^{13}C_3D_3$-AA mercapturic acid intersected with the unlabelled background signal after 30 h, and approximately 41% was excreted as the mercapturic acid after 96 h. The high AA-containing meal, but not the coffee consumption, resulted in a statistically significant increase in N-terminal valine haemoglobin adducts of both AA and GA. The authors estimated endogenous AA formation of 0.3–0.4 μg/kg bw per day and suggested this could result from Maillard type chemistry.

Another candidate precursor for endogenous AA formation is acrolein (Tareke et al., 2008; Ruenz et al., 2019) formed endogenously (Cleusix et al., 2008; Zhang et al., 2018). Oxidative stress has been discussed as an endogenous source of acrolein (Uchida, 1999). The association between GA-N7-Gua in peripheral blood mononuclear cells from human volunteers with the BMI was suggested to be due to lipid peroxidation and acrolein formation (Hemgesberg et al., 2021b).

Overall, the WG noted that estimates of endogenous formation of AA are in the range 0.2–0.4 μg/kg bw per day. Since estimates of dietary exposure are 0.4–1.9 μg/kg bw per day at the mean and 0.6–3.4 μg/kg bw per day at the P95 (EFSA CONTAM Panel, 2015), the WG concluded that endogenous formation adds to the dietary exposure.

Measurement of haemoglobin adducts

It has been suggested that GA is entirely detoxified at dietary exposure levels by conjugation with glutathione (Eisenbrand, 2020a). However, a large number of studies have reported measurement of Hb adducts of both AA and GA in humans exposed to background levels of AA in their diet, thereby demonstrating that GA is produced and is not entirely detoxified. It is beyond the scope of the current statement to review all of these in detail, but the following provides an indication of the data.

Results from the 2003–2004 US National Health and Nutrition Examination Survey (NHANES) were discussed in the section on biomarkers in the CONTAM Panel Opinion (EFSA CONTAM Panel, 2015). More recently, results of subsequent surveys have continued to demonstrate that Hb adducts of GA are measurable in the US population. For example, Liu et al. (2021) reported Hb-GA/Hb-AA ratios in the region of 0.7–1.1 in 3,234 participants of the 2003–2006 and 2013–2016 surveys. Similarly, in Europe, Hb adducts of both AA and GA have been measured in the European Prospective Investigation into Cancer and Nutrition (EPIC). For example, median Hb-GA/Hb-AA ratios of 0.7–1.0 were reported in a subgroup of 801 non-smoking postmenopausal women from 8 European countries (Obon-Santacana

et al., 2017). Therefore, it is clear that GA formed as a result of dietary exposure to AA is not entirely detoxified and is systemically available.

Epidemiological studies on cancer risk

In its 2015 Opinion, the CONTAM Panel noted that associations between AA exposure through diet and risk of several types of cancer had been investigated in 16 epidemiological studies. The CONTAM Panel concluded that there was no consistent indication for an association between AA exposure and increased risk of cancer in various organs (EFSA CONTAM Panel, 2015). There were severe limitations to these studies as outlined in the 2015 Opinion, such as uncertainties in the exposure assessment of AA and lack of statistical power (EFSA CONTAM Panel, 2015).

3.4. Summary and discussion

Genotoxicity

Several new studies on the clastogenic properties of AA/GA have been identified since 2015. These included 2 *in vitro* and 10 *in vivo* studies on micronuclei formation in the bone marrow or peripheral blood of mice or rats orally exposed to AA. The majority of these studies provided positive results. Exceptions include studies performed in F344 rats, possibly because of their relatively low metabolic activating capability. Analysis of AA-induced chromosomal aberrations in mouse bone marrow showed increases in both polyploidy and chromosome breakage. These new data confirm and extend previous conclusions on the clastogenic potential of AA reported in the 2015 EFSA Opinion.

AA induced DNA strand breaks, as identified by alkaline Comet assays, both in mammalian cells *in vitro* (8 studies) and *in vivo* (9 studies). DNA damage was observed in several organs (liver, kidney, brain) of mice or rats as well as in lymphocytes, spermatocytes and spermatozoa. These data are in agreement with the previous conclusions on increased levels of DNA damage associated with AA exposure including effects on male reproductive cells (i.e. germ cell mutagen).

Recently in mammalian cells *in vitro* a large data set has been published on the capacity of AA/GA to induce gene mutations (see **Table 1**). The molecular analysis of mutations induced by AA/GA at different target genes (*TK*, l*acZ*, *gpt*) indicate that: (a) GA is more mutagenic than AA; (b) the mutational spectra of AA and GA are similar (and different from controls); (c) *in vitro* and *in vivo* treatments result in similar mutational spectra; (d) AA/GA exposure is associated with a particular enrichment of mutations at A:T base pairs (AT > TA, AT > GC, AT > CG).

Studies of mutations by whole genome and exome sequencing confirmed these conclusions. They provided additional information on mutation localization and defined a specific AA/GA mutational signature. A comparison of this GA-specific fingerprint with those present in the Catalogue of Somatic Mutations in Cancer (COSMIC) data base identifies similarities between the GA signature and signatures found in some human tumours (e.g. breast, ovary, pancreas, lung). These studies might indicate a contribution of AA and/or GA-associated mutagenesis to human cancers.

Six additional studies on gene mutations were performed *in vivo*. Although studies on the endogenous *Pig-a* gene provided equivocal or negative results, those in Big Blue transgenic mice were positive with increased *cII* mutations being reported in the brain and testis, and *gpt* mutations in lung and sperm following AA/GA treatment. Similar mutational spectra were again observed for the two compounds. These results confirm and extend previous studies on gene mutations in transgenic mice exposed *via* drinking water to AA and GA reported in the 2015 EFSA Opinion (Manjanatha et al., 2006, 2015; Wang et al., 2010; Ishi et al., 2015). In conclusion, these studies show that: (a) AA and GA induce similar mutational spectra which differ significantly from that of spontaneous mutations; (b) mutational spectra may vary between tissues/organs (lung *vs* liver, testis and sperm) suggesting a different contribution of specific DNA adducts to mutations occurring in those tissues.

In conclusion, both *in vitro* and *in vivo* data highlight the relationship between DNA adduct profiles originating from the metabolic conversion of AA to GA and the mutational signature of AA/GA. The increased levels of AT > TA and GC > TA transversions observed in the GA signature might be explained by replication errors at apurinic sites derived from N3-GA-Ade and N7-GA-Gua depurination events. In addition, the enrichment of AT > GC transitions in the GA signature corresponds to the miscoding of another commonly identified adenine adduct (i.e. N1-GA-Ade). It should be noted that the specific mutation fingerprint of GC > TA transversions due to 8-oxoguanine accumulation in DNA (Viel et al., 2017; Alexandrov et al., 2020) was not among the mutational signatures associated with GA exposure. The new studies are perfectly in line with several *in vitro* and *in vivo* mutational spectra

reported in the 2015 EFSA Opinion. The recent identification of an AA mutational fingerprint highlights the specificity of the mutational events associated with AA exposure.

Four *in vitro* and four *in vivo* new studies on DNA adducts induced by AA/GA exposure were identified since the publication of the 2015 Opinion.

Measurements of DNA adducts in GA-treated cells confirm the presence of the previously identified N3-GA-Ade and N7-GA-Gua adducts, with the expected high ratio of N7-GA-Gua/N3-GA-Ade adduct level (EFSA CONTAM Panel, 2015). There were, however, large variations among these *in vitro* studies in the number of DNA adducts induced by exposure to GA (0.75–3 mM GA in Hupki mouse cells, range: 49,000–30 N7-GA-Gua per 10^8 nucleotides) and AA (1–2 mM AA in rat hepatocytes: 20–30 N7-GA-Gua per 10^8 nucleotides).

In vivo exposure of mice to drinking water with AA concentrations as used in a cancer bioassay resulted in a dose-dependent increase in N7-GA-Gua and N3-GA-Ade in liver and lung (900 and 4 adducts per 10^8 nucleotides following a 28-day exposure to around 9 mg/kg bw per day, respectively), while increased N7-GA-Gua levels were reported in the urine as well as in liver, kidney and lung of AA-treated rats (range: 900–2,100 adducts per 10^8 nucleotides). These new data provide evidence of the formation of DNA adducts following both *in vitro* and *in vivo* exposure of mice and rats to AA.

The presence of N7-GA-Gua in humans has been reported in the urine of smokers/non-smokers with no significant association with the smoking habit, while increased levels were observed in AA-exposed workers when compared to administrative workers. Finally, in attempts to identify whether AA-induced DNA adducts were associated with specific dietary exposures, N7-GA-Gua levels were measured in the blood of human volunteers. Values for this adduct ranged between 0.2 and 26 adducts per 10^8 nucleotides, with mean values around 1–2 adducts per 10^8 nucleotides. In two studies, no significant correlation was found between DNA adducts and specific dietary habits. This lack of association may be due to the relatively small sample sizes (56 and 17) and limitations in the assessment of the exposure.

In order to understand the role of levels of DNA adducts associated with AA exposure, it is relevant to consider the endogenous formation of AA, and the balance of activation of AA vs detoxification of GA. Endogenous formation of AA has been demonstrated at levels below dietary exposure to AA. Thus, dietary exposure adds to endogenous formation and it is necessary to consider the impact of this additional exposure. Large scale studies in human populations have demonstrated the presence of Hb adducts of both AA and GA, at approximately equal concentrations. Furthermore, DNA adducts, both N7-GA-Gua and N3-GA-Ade, have been measured in humans.

These data show that GA is not entirely detoxified on formation, and that it is systemically available in humans with common levels of dietary exposure to AA. Furthermore, the profile of mutations in mice treated with AA differs from that seen in control mice.

Based on these lines of evidence, the WG concluded that dietary exposure to AA has the potential to result in formation of GA adducts and GA-related mutations.

Non-genotoxic effects and genotoxicity secondary to oxidative stress that may contribute to AA carcinogenicity

In addition to genotoxicity, the WG observed that there is the potential for both secondary DNA oxidation via generation of ROS and for non-genotoxic effects on the control of the cell cycle that may contribute to carcinogenesis by AA. Such effects were also considered in the 2015 EFSA Opinion on AA. It is not clear as to the origin of the ROS. However, one possibility is via the action of the enzyme CYP2E1 which is involved in the oxidative metabolism of AA. CYP2E1 has been demonstrated to generate ROS and lipid peroxidation leading to DNA oxidation (Linhart et al., 2014). This may involve reaction uncoupling which releases ROS during substrate metabolism (Denisov et al., 2005; Webster et al., 2021). The induction of CYP2E1 by AA, and its metabolism via this route, may elevate ROS.

Changes brought about by AA in histone acetylation and methylation and DNA methylation in liver and lung of mice and various studies showing increased expression of cell cycle regulators point to epigenetic influences that may contribute to enhanced cell proliferation and target organ carcinogenesis. Toxicogenomic studies and immunohistochemistry give some evidence for a DNA-damage response, albeit minor in some studies. However, genes involved in calcium signalling and the cytoskeleton were found to represent a major part of the transcriptome response. Although altered calcium signalling may involve modulation of microtubules and microfilaments and the action of kinesins during cell division, such effects have not been shown to contribute to the carcinogenicity of AA. The WG considered that these effects may be important in neurotoxicity.

Overall, the WG concluded that in addition to genotoxicity, non-genotoxic effects may contribute to the carcinogenicity of AA.

4. Conclusions

In conclusion, based on the evaluation of studies on genotoxicity and non-genotoxic effects of AA published since 2015 and consideration of the modes of action underlying the carcinogenicity of AA, the WG concludes that there is substantial evidence for the genotoxicity of AA mediated by the formation of GA in addition to a potential contribution of non-genotoxic effects towards AA carcinogenicity.

These studies extend the information assessed by the CONTAM Panel in its Opinion on the risks to human health related to the presence of AA in food (EFSA CONTAM Panel, 2015), and support its conclusions. The 2015 Opinion applied the MOE approach, as recommended in the Guidance for substances that are both genotoxic and carcinogenic (EFSA Scientific Committee, 2005), for risk characterisation of the neoplastic effects of AA. Based on the new data evaluated, the MOE approach is still considered appropriate, and an update of the 2015 Opinion is not required at the present time.

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Abbreviations

4-NQO	4-nitroquinoline 1-oxide
8-OHdG	8-hydroxy-2'-deoxyguanosine
AA	acrylamide
AAMA	*N*-acetyl-S-(2- carbamoylethyl)-L-cysteine
BMI	body mass index
CONTAM Panel	Panel on Contaminants in the Food Chain
COSMIC	Catalogue of Somatic Mutations in Cancer
EGCG	epigallocatechin gallate
EMS	ethyl methanesulfonate
ENU	*N*-ethyl-*N*-nitrosourea
ESI	electron spray ionisation
F	female
Fpg	DNA-formamidopyrimidine glycosylase
GA	glycidamide
GAMA	*N*-(*R*,*S*)-acetyl-*S*-(1-carbamoyl-2-hydroxyethyl)-L-cysteine
GAMA3	*N*-acetyl-S-(3-amino-2-hydroxy-3-oxopropyl)-cysteine
GD	gestational day
GSH	Glutathione
GSH-Px	glutathione peroxidase
GSSG	oxidised GSH
GST	gluthathione *S*-transferase
Hb	haemoglobin
HBGV	health-based guidance value
LC–MS/MS	liquid chromatography with tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
M	Male
MDA	malonaldehyde
MMS	methyl methanesulfonate
MPO	myeloperoxidase
MS/MS	tandem mass spectrometry
N3-GA-Ade	N3-(2-carbamoyl-2-hydroxyethyl)adenine
N7-GA-Gua	N7-(2-carbamoyl-2-hydroxyethyl)guanine
NHANES	US National Health and Nutrition Examination Survey
PBMC	peripheral blood mononuclear cells
PCE/NCE	polychromatic erythrocytes/normochromatic erythrocytes
PCO	protein carbonyl
PND	postnatal day
QRT-PCR	quantitative real-time polymerase chain reaction
RBC	red blood cells
RET	reticulocytes
ROS	reactive oxygen species
SCE	sister chromatid exchange
SOD	superoxide dismutase
SPE	solid-phase extraction
T3	thyroxine
T4	triiodothyronine

TBARS	thiobarbituric acid reactive substances
TSH	thyroid-stimulating hormone
UDS	unscheduled DNA synthesis
UHPLC	ultra-high-performance liquid chromatography
γ-GCS	γ-glutamylcysteine synthetase

Appendix A – Protocol for a statement on the genotoxicity of acrylamide

The current protocol reports on the problem formulation and approach selected by EFSA to address the request for a statement on a recent publication (Eisenbrand, 2020a,b) revisiting the evidence for genotoxicity of acrylamide (AA). The protocol is in accordance with the draft framework for protocol development for EFSA's scientific assessments (EFSA, 2020). This framework foresees that the extent of planning in the protocol (i.e. the degree of detail provided in the protocol for the methods that will be applied in the assessment) can be tailored to accommodate the characteristics of the mandate. Considering the short timeline and nature of the request, EFSA applied a low level of planning.

An EFSA WG will be formed to develop the draft statement. The draft statement will be then presented to the CONTAM Panel for endorsement before final approval.

A.1. Problem formulation

Objectives of the statement

The objective of the statement is to assess if, based on the scientific evidence provided in a recent review paper regarding the genotoxicity of AA (Eisenbrand, 2020a,b), an update of the 2015 Opinion is needed.

In 2015, the EFSA CONTAM Panel concluded that AA is genotoxic and carcinogenic, and thus did not find appropriate to establish a health-based guidance value, e.g. TDI (EFSA CONTAM Panel, 2015). In a recent review article (Eisenbrand, 2020a,b), the author concluded that the available scientific evidence argues against a genotoxic mode of action underlying the carcinogenicity of AA, and thus a TDI could be established.

Target populations

The target population of the statement is the European population.

Compound of concern

The statement will focus on AA.

Adverse effects and endpoints

The statement will focus on the *in vitro* and *in vivo* genotoxicity data, as well as on the mode of action of the carcinogenicity of AA.

Identification of the risk assessment sub-questions

The sub-questions identified that will be answered and combined to address the request are reported in Table A.1.

Table A.1: Sub-questions to be answered for the assessment

Risk assessment step	No	Sub-questions
Hazard identification	1	Are there new studies since the publication of the EFSA Opinion on AA in 2015 of relevance to the conclusions made in the 2015 Opinion on the genotoxicity of AA?
Hazard characterisation	2	What are the modes of action that can explain the carcinogenicity of AA?
Conclusions	3	Based on the above, is there a need to update the 2015 Opinion on AA in food?

A.2. Method for answering the sub-questions

The sub-questions formulated in Table A.1 will be answered by a narrative approach. The Eisenbrand (2020a) review and its erratum (Eisenbrand, 2020b) is not a comprehensive review of the publications available since the EFSA Opinion on AA in food published in 2015, thus a literature search will be performed to identify primary research studies as well as reviews and meta-analyses relevant to the sub-questions formulated and published since the 2015 Opinion on AA (EFSA CONTAM Panel, 2015). The studies cited in the review by Eisenbrand (2020a,b) will also be retrieved.

In addition, the bibliography of the key full text papers will be checked for further potential relevant studies. The expertise of the working group will be used in deciding whether to pursue these further to complement the evidence collection.

The details of the studies will be reported in tables and discussed in the statement.

The selection of the scientific studies for inclusion or exclusion will be done by the relevant domain experts from the EFSA WG on AA genotoxicity. It will be based on consideration of the extent to which the study is relevant to the assessment, and on general study quality considerations (e.g. sufficient details on the methodology, performance and outcome of the study, on dosing, substance studied and route of administration and on statistical description of the results), irrespective of the results. Major limitations in the information used will be documented in the statement.

The general principles of the risk assessment process for chemicals in food as described by WHO/IPCS (2009) will be applied. In addition, the following EFSA guidance documents pertaining to risk assessment will be followed:

- Guidance of the Scientific Committee on transparency in the scientific aspects of risk assessments carried out by EFSA. Part 2: General principles (EFSA Scientific Committee, 2009),
- Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA Scientific Committee, 2011),
- Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data (EFSA Scientific Committee, 2012a),
- Scientific Opinion on Risk Assessment terminology (EFSA Scientific Committee, 2012b),
- Scientific Opinion on the guidance on the use of the weight of evidence approach in scientific assessments (EFSA Scientific Committee, 2017b),
- Guidance on the assessment of the biological relevance of data in scientific assessments (EFSA Scientific Committee, 2017c).

Due to the time constraints and nature of the request, an uncertainty analysis following the EFSA Scientific Committee Guidance (2018) was not made.

Literature searches

The literature search to identify studies on the genotoxicity of AA since the publication of the 2015 EFSA Opinion will be performed searching the following bibliographic database:

1) Web of Science™, encompassing the following databases:
   - Web of Science™ Core Collection
   - BIOSIS Citation Index℠
   - CABI: CAB Abstracts®
   - Current Contents Connect®
   - Data Citation Index℠
   - FSTA® – the food science resource
   - MEDLINE®
   - SciELO Citation Index
   - Zoological Record®

The literature search will be performed by EFSA staff. The output from the searched databases, i.e. the bibliographic references including relevant information, e.g. title, authors, abstract, will be exported into Endnote files, allowing a count of the individual hits per database. The selection process will be performed using word files.

The data on the genotoxicity of AA will be evaluated, as well as on the mode of action underlying the neoplastics effects of AA.

The assessment and conclusions reached by the WG on AA genotoxicity will be presented to the CONTAM Panel for endorsement before final approval.

A.3. Plans for updating the literature searches and dealing with newly available evidence

Due to the short timeline of this request, the literature searches will be performed at the beginning of the work and further searches will be done as needed by the discussions and development of the

draft statement. The scientific papers retrieved during the development of the statement will be screened for relevance by the members of the WG on AA genotoxicity and EFSA staff and included in the draft statement as appropriate.

A.4. Public consultation

Due to the nature and short time frame of the request, no public consultation of the draft statement is foreseen.

A.5. History of the amendments to the protocol

Not applicable.

Appendix B – Literature search

B.1. Literature search terms

Genotoxicity of AA	
Search terms	TOPIC: (acrylamide) AND TOPIC: (genotoxicity) AND YEAR PUBLISHED: (2015–2021)
Date of search	23/3/2021, 24/9/2021
Adducts of AA or GA	
Search terms	TOPIC: (acrylamide) OR (glycidamide) AND TOPIC: (adducts) AND YEAR PUBLISHED: (2015–2021)
Date of search	9/12/2021
Epidemiological studies on the association of AA exposure and risk of cancer	
Search terms	TOPIC: (acrylamide) AND TOPIC: (cross-sectional) OR (cohort) OR (case-control) AND TOPIC: (cancer) OR (carcinogenicity) OR (tumours) AND YEAR PUBLISHED: (2015–2021)
Date of search	17/11/2021