Committee on the Toxicity of Chemicals in Food, Consumer Products and the Environment.

Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs

Genotoxicity

Background

Previous 2015 EFSA conclusion

1. In the 2015 EFSA opinion on Bisphenol A (BPA) (EFSA CEF Panel, 2015), the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) Panel concluded that BPA is not mutagenic (in bacteria or mammalian cells), or clastogenic (micronuclei and chromosomal aberrations). The potential of BPA to produce aneuploidy *in vitro* was not expressed *in vivo*. The positive findings in the post labelling assays *in vitro* and *in vivo* were judged unlikely to be of concern, given the lack of mutagenicity and clastogenicity of BPA *in vitro* and *in vivo*.

Current new data examined, literature search timeline and screening methodology

2. For the health outcome category (HOC) genotoxicity, the time span of the literature search was extended until 21 July 2021 and the studies assessed in the 2015 EFSA opinion were also re-considered by the EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP).

3. The methods that were used for data collection through literature searches were conducted in the following bibliographic databases: PubMed, Web of Science and Core Collection.

4. For the additional time span considered in the literature search, the screening question was: ‘Is the paper reporting information about exposure to BPA and genotoxicity?’

5. For screening the additional genotoxicity studies, the categorisation was made into different subgroups of genotoxicity endpoints (genotoxicity, epigenetics, oxidative stress). An additional screening of the relevance of the studies was done by experts in this field following the full-text screening.

6. A specific internal validity approach was applied and a specific Weight of Evidence (WoE) approach was applied, as described in detail in Annex A. to this paper. The CEP Panel examined whether new data from the published literature could provide new evidence on the potential genotoxicity of BPA. The references from the previous CEF Panel opinion (EFSA CEF Panel, 2015) were also included in the current assessment using the same appraisal criteria applied to the newly published data and considering the EFSA Scientific Committee guidance documents on genotoxicity published after 2015 (EFSA Scientific Committee, 2017, 2021).

Methods for assessing genotoxicity

7. The evaluation of data quality for hazard/risk assessment includes the evaluation of reliability and relevance (Klimisch *et al*., 1997; OECD, 2005; ECHA, 2011; EFSA Scientific Committee, 2017c; EFSA Scientific Committee, 2021).

8. In the assessment of genotoxicity studies, the data quality has been evaluated based on reliability and relevance. Reliability has been assessed using a scoring system based on criteria published by Klimisch *et al*. (1997).

9. In a second step, the relevance (high, limited, low) of the study results was assessed based on reliability of the study and other aspects, *e.g.* genetic endpoint, purity of test substance, route of administration and status of validation of the assay.

10. Genotoxicity studies evaluated as of high or limited relevance have been considered in a WoE approach as described in Annex A. Genotoxicity studies evaluated as of low relevance have not been further considered in the assessment. The different steps of the evaluation of reliability and relevance are described in Annex A to this paper.

Method for uncertainty analysis for genotoxicity

11. Details on how the uncertainty analysis was carried out as well as the results discussion can be found in Annex A to this paper.

Genotoxicity studies considered for the EFSA CEP assessment

12. A total of 88 *in vitro* and *in vivo* studies were retrieved from the literature search along with 15 *in vitro* and *in vivo* studies considered in the Scientific opinion on the risks to public health related to the presence of BPA in foodstuffs (EFSA CEF Panel, 2015) (see Annex A to this paper).

13. *In vitro and in vivo* studies were grouped based on the genotoxicity endpoint investigated:

- gene mutations (*e.g.* bacterial reverse mutation assay);
- chromosomal damage (CA and micronucleus assays);
- DNA damage (comet assay).

14. These studies were summarized in synoptic tables (see Annex A to this paper), evaluated for reliability and relevance and grouped into lines of evidence in a WoE approach (see Annex A to this paper).

15. Studies not investigating classical genotoxicity endpoints (*e.g.* γH2AX, oxidative DNA damage, DNA binding, ROS generation) and studies in humans are considered in the Mode of Action (MoA) analysis and as supportive evidence.

Weight of Evidence

Gene mutations *in vitro* and *in vivo*

In vitro gene mutation

16. Of the six available studies of the mutagenicity of BPA in bacteria, only one describes the application of the Ames test in a comprehensive battery of Salmonella Typhimurium strains (TA1535, TA97, TA98, 11407 TA100 and TA102) at a range of

concentrations up to 5000 µg/plate. It reports negative results both in the presence and absence of metabolic activation (Xin *et al*., 2015).

17. Three studies reported negative results in TA98 and TA100 (Masuda *et al*., 2005; Fic *et al*., 2013; Zemheri and Uguz, 2016). A further study shows negative results in TA98, TA100 and TA102 strains (Tiwari *et al*., 2012). The sixth used the bacterial SOS/umuC assay with a range of concentrations from 1 to 1000 µg/L in presence and absence of S9 mix. It also reported negative results (Balabanič *et al*., 2021). The CEP Panel concluded that BPA does not induce gene mutations in bacteria.

Summaries of studies

18. Summary of Xin *et al* study 2015: The study evaluated the cytotoxic, genotoxic and clastogenic activity of BPA (purity 99%) in Chinese hamster ovary cells (CHO) cells and its mutagenicity in the Ames test. The battery of assays applied in CHO cells included the MTT assay for the evaluation of cytotoxicity, and the comet, micronucleus and chromosome aberration tests. In the Ames test, BPA (10-5000 µg/plate) was uniformly negative in all Salmonella Typhimurium strains (TA1535, TA97, TA98, TA100 and TA102), with and without metabolic activation. Exposure of CHO cells to four BPA doses (40, 80, 100 and 120 µM) for 12 and 24 h resulted in a significant decrease in cell viability at 80 µM and above which, however, remained above 50% in all cases; a concentration-related increase of DNA damage was observed in a comet assay [increased Olive tail moment (OTM), tail length and % tail DNA, statistically significant at all doses] after 12 and 24 h exposure to BPA; after 24 h treatment, an increase in micronuclei (MN) (statistically significant at 100 and 120 µM) and structural chromosomal aberrations (chromatid breaks and chromosome fragments, statistically significant at 80 µM and above) was also observed.

19. Summary of Masuda *et al*., 2005: The study evaluated the mutagenicity of BPA in Ames test in the presence or absence of S9-mix. BPA (Tokyo Kasei Kogyo Co., Ltd) was tested on S. Typhimurium strains TA98 and TA100 at the single dose of 0.1 µmole/plate (100 µL of 1 mM solution). No mutagenic effect was observed.

20. Summary of Fic *et al*., 2013: In this study the mutagenic and genotoxic potential of eight BPA (purity >99%) structural analogues [BPF, BPAF, bisphenol Z (BPZ), BPS, bis(4-hydroxy-3-methylphenyl)propane (DMBPA), 4,4'-sulfonylbis(2-methylphenol) (DMBPS), [sulphonylbis(benzene-4,1-diyloxy)]diethanol (BP-1), and 4,4'-sulphanediyldiphenol (BP-2)] were investigated using the Ames and comet assay. None of these bisphenols were mutagenic in Salmonella Typhimurium strains TA98 and TA100 either in the presence or absence of external S9-mediated metabolic activation (Aroclor 1254-induced male rat liver). Potential genotoxicity of bisphenols was determined in the HepG2 human hepatoma cell line following 4-h and 24-h exposure to non-cytotoxic concentrations 0.1 µmol/L to 10 µmol/L. In the comet assay, BPA and its analogue BPS induced significant DNA damage only after the 24-h exposure, while analogues DMBPS, BP-1, and BP-2 induced a transient increase in DNA strand breaks observed only after the 4-h exposure. BPF, BPAF, BPZ, and DMBPA did not induce DNA damage.

21. Summary of Zemheri and Uguz, 2016: The study evaluated the mutagenicity of BPA (Merck) in a limited Ames test, using two tester strains (TA98 and TA100) and four dose levels (0.1, 1, 10 and 100 µg/plate). The results were negative, with and without metabolic activation.

22. Summary of Tiwari *et al*., 2012: The study evaluated the mutagenicity of BPA in Ames test. BPA (purity 99%) was tested at concentrations from 6.25 to 200 µg/plate on different strains of S. Typhimurium (TA 98, TA 100 and TA 102). The mutagenic response was not observed in any of the tester strains at the various concentration of BPA in absence of S9 fractions. A slight increase in the numbers of revertants was observed in the presence of S9 fractions from the 6.25 - 25 µg/plate of BPA in each strain, but the increase was statistically significant only in strain TA 102 at 25 µg/plate.

23. Summary of Balabanič *et al*., 2021: The study evaluated cytotoxic and genotoxic effects of some endocrine disrupting chemicals (EDCs), including BPA, which have been previously identified in effluents from two paper mills. BPA (Sigma-Aldrich) tested at concentrations of 1, 10, 100, 1000 µg/L with the bacterial SOS/umuC assay in S. Typhimurium TA1535/pSK1002 strain did not induce toxic

nor genotoxic effects in the presence or absence of S9 metabolic activation. The compound was also assessed in HepG2 cells with MTT assay for cell viability and with comet assay at 1, 10, 100 and 1000 µg/L for 4 and 24 h. No significant reduction of the viability. A statistically significant concentration-dependent increase of DNA damage, expressed as percent of DNA in tail, was reported starting from 10 µg/L.

In vivo gene mutation

24. No studies on gene mutation assays in mammalian cells following the OECD guidelines were available.

Induction of chromosomal aberrations/micronuclei *in vitro and in vivo*

In vitro chromosomal aberrations/micronuclei

25. Fifteen *in vitro* studies of micronuclei (MN) and structural chromosomal aberrations (CA) induction in different cell lines were available for evaluation. Of these, nine were further considered in the assessment, classified as having high (1 study) or limited relevance (8 studies).

26. All showed positive results in both blood cells and established cell lines. In the single study classified as of high relevance, a concentration-dependent increase of MN frequency over a wide range of concentrations (1.5 to 37 µg/ml corresponding to 6.6 µM and 162 µM) was observed in the AHH-1 human lymphoblastoid cell line (Johnson and Parry, 2008). Positive CA results were also reported from cultures of human peripheral lymphocytes in two studies with limited relevance (Santovito *et al*., 2018; Di Pietro *et al*., 2020). In one of these (Santovito *et al*., 2018), MN frequency was also measured. A study of MN in bovine peripheral blood lymphocytes also reported positive findings (Šutiaková *et al*., 2014).

27. In murine macrophage RAW264.7 cells, positive MN results were associated with an increase in reactive oxygen species (ROS), and a decreased level of antioxidant enzymes (GPx, SOD and CAT. Concomitant phosphorylation of P53 and release of cytochrome C from mitochondria were detected along with increased apoptosis. Pretreatment with N-acetylcysteine (NAC) reduced BPA-induced

cytotoxicity, apoptosis and genotoxicity (MN frequency was reduced by 30%). These results indicate that the toxic effect of BPA in macrophages was mainly through the oxidative stress-associated mitochondrial apoptotic pathway (Huang FM *et al*., 2018).

28. Finally, two studies in the Chinese hamster ovary (CHO) and V79 cell lines reported positive results (Xin *et al*., 2015; Yu *et al*., 2020). Xin and co-workers reported a concentration dependent increase of both MN and CAs in CHO cells in the absence of metabolic activation. In contrast, the BPA-induced increase in MN frequency in V79, reported by Yu and colleagues, apparently required CYP1A1 and CYP1B1 expression.

29. Overall, the significant increases of chromatid and chromosome breaks observed in several studies *in vitro* indicated that BPA has clastogenic activity also at non-cytotoxic concentrations. Two reports indicated that oxidative stress is implicated in the observed induction of chromosomal damage. In addition, Johnson and Parry (2008) reported the formation of aberrant mitotic spindles, with multiple poles, in cells treated with BPA.

30. In conclusion, the *in vitro* studies on CA and MN induced by BPA indicated that both clastogenic and aneugenic mechanisms may operate.

Summary of studies

31. John and Parry 2008: In this mechanistic study the aneugenicity of two known spindle poisons model compounds, namely rotenone and BPA, has been investigated following low dose-exposure to mammalian cells, using the cytokinesis blocked micronucleus assay (CBMA) and immunofluorescence methods to visualize modifications of the microtubule organizing centres (MTOCs) of the mitotic spindles. For induction of MN BPA (Sigma-Aldrich) was added over a range of narrowed low concentrations (1.5, 3.1, 6.2, 7.7, 9.2, 10.8, 12.3, 18.5, 24.6, and 37.0 µg/ml) to cultures of human (AHH-1) lymphoblastoid cell line for a complete cell cycle (22-26 h dependent upon any cell cycle delay) in the presence of cytochalasin-B. A minimum of five separate experiments were performed. A concentration-related and statistically significant increase of binucleate-micronucleated cells from 12.3 µg/mL

was reported with a clear threshold for induction of MN (NOEL at 10.80 µg/mL and LOEL at 12.3 µg/mL). A NOEL and LOEL for percentage of binucleate cells was also observed at 9.2 µg/mL and 10.8 µg/mL BPA respectively. For mechanistic evaluation of the aneugenic effects of BPA, fluorescently labelled antibodies were used to visualize microtubules (α-tubulin) and MTOCs (γ-tubulin) in V79 culture. BPA in this case was added to V79 cells growing on sterile glass microscope slides placed in Petri dishes at concentrations 4.2, 4.9, 5.6, 7.0, 8.4, 9.8, 11.2 and 14 µg/mL for 20 h (*i.e*. one cell cycle for V79). Similarly for induction of aberrations in the mitotic machinery a NOEL was observed at 7.0 µg/mL and a LOEL at 8.4 µg/mL BPA in V79 cells. Aberrant mitotic divisions, in the form of multiple spindle poles were detected and it was suggested by the study authors to be the mechanism for the production of chromosome loss into MN.

32. Santovito *et al*., 2018: In this study the possible induction of chromosomal damage by BPA (Sigma-Aldrich) was tested in human peripheral blood lymphocytes cultures applying the CA assay and the micronucleus test (MN). Cell cultures were exposed to a range of concentrations from 0.01 to 0.20 µg/mL, (including the reference dose established by United States Environmental Protection Agency (US EPA) (0.05 µg/mL), the tolerable daily intake established by European Union (0.01 µg/mL) and the highest concentration of unconjugated BPA found in human serum (0.02 µg/mL)) for 24 h for the chromosomal aberration test and for 48 for the micronucleus test. A statistically significant increase of cells with structural chromosomal aberrations, with a prevalence of chromatid breaks, was reported starting from 0.05 µg/mL; no numerical aberration was observed. A concentration related increase in MN frequency was detected starting from 0.02 µg/mL in which a four-fold increase with respect to the control level was observed.

33. Di Pietro *et al*., 2020: The study investigated the effects of BPA exposure on cell proliferation, cell cycle progression and DNA damage in human peripheral blood mononuclear cells (PBMC) and the BPA-induced neurotoxicity in rats exposed to environmental relevant doses of BPA during development. Human PBMC from five unrelated healthy donors (adult males and females) were cultured and treated with BPA (Merck) from 5 nM to 200 µM. The treatment with BPA of unstimulated resting PBMC did not affect cell proliferation (determined by the colorimetric MTT) at all the

concentrations tested except for 200 μM for which a marked inhibition of cell proliferation was observed at 24 and 48 h after the treatment. By contrast, in PHA-stimulated cells, BPA caused a pronounced increase of cell growth starting from 10 nM to 100 nM and a concentration-dependent decrease of cell proliferation from 25 to 200 μM. The cell cycle was analyzed by flow cytometry. BPA at 50 nM increased the percentage of cells in S phase of the cell cycle at 24 h and this effect was higher at 48 h with an increase of about 17% of cells in the S phase compared with the control. At 100 μM, BPA induced a significant increase of the percentage of cells in the G0/G1 phase, suggesting that BPA affected cell growth in a non-monotonic way. BPA-treatment at 25, 50 and 100 nM for 48 h induced a significant increase ($p < 0.001$) of both the percentage of aberrant cells (about 20% at 100 nM) and structural aberrations (about 27% at 100 nM) including chromatid and chromosome breaks, rings and fragments. BPA also increased significantly the percentage of highly fragmented metaphases (shattered cells). In PHA-stimulated PBMC treated with BPA (50 nM) for 24 h, γH2AX was significantly increased in CD3+ T lymphocytes and was also detected in a higher proportion of CD8+ T lymphocytes than the CD4+ T lymphocytes and a slight percentage of γH2AX was reported among the B cells. The treatment of PHA-stimulated PBMC with BPA (50 nM) induced p21/Waf1 and PARP1 protein expressions approximately within the same time interval. These findings suggest that BPA could affect the p53-p21/ Waf1 checkpoint and PARP1 levels resulting in DNA damage repair defects. BPA (50 nM) for 24 h modulated the expression of ER-α and ER-β in both sexes inducing or inhibiting its expression in males and in females with effects similar to the variations induced by pharmacological concentrations of E2 (100 nM). The study investigated also the BPA-induced neurotoxicity in terms of DNA damage. After the coupling period, three females/group received BPA (0.1 mg/L), or vehicle (ethanol 0.1 mL/L) in the drinking water during gestation, lactation and weaning of their offspring. Five female and three male pups from BPA-exposed mothers and five female and three male newborns from vehicle-treated dams were then sacrificed at PND 17. BPA was shown to induce ɣH2AX phosphorylation in cells possessing immune function in the CNS, such as microglia and astrocytes of rat hippocampus. In BPA-exposed rats a marked decreasing trend of ERα expression was found therefore proposing a role for this receptor in the effects induced by BPA.

34. Šutiaková *et al*., 2014: The study evaluated the genotoxic and cytotoxic effects of BPA (Sigma-Aldrich) on bovine peripheral lymphocytes *in vitro*. Lymphocyte cultures from two animals were exposed to four different concentrations of BPA (1×10−4, 1×10−5, 1×10−6 and 1×10−7 mol. L−1) 24 h after stimulation by L-phytohemagglutinin, and incubated for total 72 h. Micronucleus frequency was determined using the cytokinesis block method, adding 6 µg/mL cytochalasin B at 44 h. A significant increase in the number of MN (p= 0.018) was observed at the highest concentration of BPA; at lower concentrations micronucleus frequency was not significantly different from vehicle (DMSO) control. The nuclear division index (NDI) was not affected by BPA treatment at any concentration level.

35. Huang FM *et al*., 2018: The study reported positive results for induction of DNA strand breaks (evaluated by comet assay) and MN frequency in murine macrophage RAW264.7 cells. Cell cultures were treated at 0, 3, 10, 30, and 50 µM of BPA (Sigma-Aldrich) dissolved in DMSO for 24 h. Concentration-dependent increase of tail length, based on the analysis of 50 cells/slide, and of MN frequency by the evaluation of 1000 binucleated cells per concentration were observed. No positive controls were used. The genotoxic effects were observed starting from 10 µM and were associated with an increase of reactive oxygen species (ROS), measured by Dichlorofluorescein Diacetate Assay (DCFH-DA) and a decrease of antioxidant enzymes, including GPx, SOD and CAT. Concomitant phosphorylation of P53 and release of cyto C from mitochondria into cytosol were reported. A reduced expression of antiapoptotic proteins BCL2 and BCL-XL significant from 10 and 3 µM respectively and an increase of the expression of proapoptotic proteins BAX, BID, and BAD beginning at 10, 10 and 30 µM respectively were observed in a concentration-dependent manner. Increased level of the apoptosis-inducing factor (AIF) in the nucleus and a decrease in the mitochondria was detected. Expression of pro-caspase-3 and pro-caspase-9 is reduced by BPA in a concentration-dependent manner and PARP-1 cleavage was induced by BPA. Pre-treatment of the cell cultures with N-acetylcysteine (NAC), a cysteine precursor of the antioxidant glutathione, at the concentration of 10 µM for 30 min reduced BPA-induced cytotoxicity, apoptosis, and genotoxicity. The results of this study indicates that the toxic effects induced by BPA in macrophages was mainly through oxidative stress-associated mitochondrial apoptotic pathway.

36. Xin *et al* 2015: See summary in the *in vitro* gene mutation section.

37. Yu *et al* 2020: In this study, induction of MN and double-strand DNA breaks by BPA, BPF, and BPS were investigated in Chinese hamster V79-derived cell lines expressing various human CYP enzymes and a human hepatoma (C3A) (metabolism-proficient) cell line. In a first step a prediction of BPA, BPF, and BPS as potential substrates for several human CYP enzymes, which are commonly involved in the metabolic activation of compounds, was conducted by molecular docking. The results of the analysis showed a similar affinity of the compound with all the enzymes tested: CYP1A1, 1A2, 1B1, 2B6, 2E1, and 3A4. BPA (99.6% analytical purity) tested at 40, 80 and 160 µM for 9 h, followed by 15 h of recovery induced a concentration related increase of MN frequency in V79-hCYP1A1. In V79-hCYP1B1 cells MN were observed only at the two highest concentrations. No induction of MN was reported in V79-Mz, V79-hCYP1A2, V79-hCYP2E1, or V79-hCYP3A4-hOR cells. A consistency with the results of the molecular.

In vivo chromosomal aberrations/micronuclei

38. Eleven *in vivo* studies addressing BPA-induced MN and structural CA after oral exposure were evaluated. After a screening for the reliability and relevance of the results, six studies from four publications, all ranked as of limited relevance, were selected for further consideration (Table 1). Of these, three studies were considered positive for the induction of MN and CA in the same publication (Tiwari *et al*., 2012) or of MN (Panpatil *et al*., 2020) in rats following daily oral BPA administrations for 6 and 28 days, respectively. Tiwari *et al*. (2012) applied a range of doses from 2.4 µg up to 50 mg/kg bw per day. In a separate publication, the same authors (Tiwari and Vanage, 2017) reported that these experimental conditions were associated with the induction of lipid peroxidation (malonaldehyde, MDA) and oxidative stress (decreased SOD, CAT, GSH) in rat bone marrow and peripheral blood lymphocytes. In Panpatil *et al*. (2020) the dose range was much lower (50 and 100 µg/kg bw per day). A fourth study tested positive in the mouse bone marrow MN test after the administration of a daily dose of 50 mg/kg bw for 28 days in presence of high level of cytotoxicity (Fawzy *et al*., 2018). A study by Naik and Vijayalaxmi (2009) reported

negative findings in the mouse bone marrow MN test and CAs following a single dose in the range 10 to 100 mg/kg bw.

39. Overall, the available data provided evidence of chromosomal damage after multiple oral administrations but not after single oral administration of BPA.

Table 1. Summary table of test results of MN and CAs *in vivo* studies.

Test System	Dose	Results	Reference
MN and CA in bone marrow Swiss albino mice 6 animals /group	10, 50 and 100 mg/kg bw, single dose by gavage; 10 mg/kg for 5 days (50mg by gavage	**Negative** No significant decrease of PCE/NCE ratio but significant increase of gaps and C mitoses.	Naik and Vijayalaxmi, 2009
MN in bone marrow Holtzman rats 10 animals /group	2.4µg, 10 µg, 5 mg snf 50 mg/kg bw per day orally for 6 days	**Positive** Dose related increase of CA and MN PCE starting from 10 µg	Tiwari *et al*., 2012
MN in bone marrow Male Swiss albino mice 10 animals /group	50 mg/kg bw per day orally for 28 days	**Positive** Significant reduction in the ratio of PCE/NCE	Fawzy *et al*., 2018
MN in bone marrow Male Wistar rats 6 animals / group	50 and 100 µg/kg/bw per day orally for 28 days	**Positive** Dose related increase of MDA in blood and of urinary 8OHdG	Panpatil *et al*., 2020.

Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021)

Summary of studies

40. Tiwari *et al*., 2012: This study was aimed to assess potential genotoxic effects of BPA (Sigma-Aldrich) in rats (five males and five females per group) following oral administration of test compound once a day for 6 consecutive days at dose-levels of 2.4 µg, 10 µg, 5 mg and 50 mg/kg bw by measuring induction of MN and structural chromosome aberrations in bone marrow cells and primary DNA damage in blood lymphocytes using single cell gel electrophoresis (comet assay). Furthermore, plasma concentrations of 8-hydroxydeoxyguanosine (8-OHdG), lipid peroxidation and glutathione activity were evaluated to assess potential induction of oxidative DNA damage. Results obtained for genotoxicity endpoints show marked dose-

related increases of both MN and structural chromosome aberrations in bone marrow cells of male and female rats exposed to BPA. The observed increases achieved statistical significance at dose-levels as low as 10 µg/kg bw per day. Similarly, primary DNA damage evaluated by comet assay, in isolated peripheral blood lymphocytes showed marked and dose-related increases that were statistically significant at dose-levels as low as 10 µg/kg bw per day. Significant increase in plasma concentration of 8-OHdG was detected only at 50 mg/kg bw. A dose-related increase of malonaldehyde and decrease of glutathione were observed in liver.

41. Panpatil *et al*., 2020: The study evaluated the protective action of turmeric acid on the genotoxic effects of BPA in Wistar rats. Six groups of six animals were administered with BPA (Sigma-Aldrich) at 0, 50 and 100 µg/kg by oral gavage for a period of 4 weeks: three groups were fed with a normal diet, the others with a diet containing 3% turmeric. At the end of the experiment the animals were sacrificed. Urine was collected 24 h before the sacrifice. 8-OHdG was measured in urine using an ELISA kit. DNA damage by comet assay was evaluated in blood, liver and kidney: 50 cells per slide were counted twice. Micronucleus assay was applied in bone marrow: 2000 PCE were evaluated. A weak but statistically significant and dose related increase of tail length was observed in liver. In kidney an increase of DNA damage was observed only at the dose of 50 µg/kg. A dose related increase of 8-OHdG in urine and of the concentration of MDA in blood serum was observed. A dose related increase of MNPCE was reported associated with a low decrease of the PCE/NCE ratio. A significant decrease of the genotoxic effects was observed in animal fed with diet with turmeric.

42. Tiwari and Vanage, 2013: This study investigated the induction by BPA of dominant lethal mutations in the different stages of spermatogenesis in the rat. Furthermore, the induction of DNA damage by BPA in epididymal sperm was investigated. Holtzman male rats (7 per group) were treated by oral gavage with BPA (Sigma Chemical Co.) dissolved in ethyl alcohol and diluted in sesame oil, at dose-levels of 10 µg/kg bw and 5 mg/kg bw once a day for 6 consecutive days. Negative controls were treated with vehicle. Each treated male was mated with two females per week over a period of eight weeks. The mated females were then sacrificed on the day 15th of their gestation and uterine content examined. DNA damage in

epididymal sperm was evaluated by alkaline comet assay in sperm samples from treated males (4 animals per group) sacrificed after completion of the mating phase. In the dominant lethal study, a significant decrease in total implants/female and live implants/female, with a concurrent significant increase in the number of resorbed embryos per female, was observed during the fourth week and sixth week in females mated with males treated with 5 mg BPA/kg bw, suggesting the induction of post-implantation loss due to dominant lethal mutations in mid-spermatids and spermatocytes. No significant change was observed in the pre-implantation and post-implantation losses in pregnant female mated with males exposed to 10 µg/kg bw of BPA. In the comet assay with epididymal sperm, a significant increase in comet parameters (tail length, tail moment and % tail DNA) was observed in rats treated with 5 mg/kg bw compared with control.

43. Fawzy *et al*., 2018: The study was conducted to evaluate the protective action of pumpkin seed oil (PSO) against adverse effects induced by BPA. BPA (Sigma-Aldrich) was administered orally to male Swiss albino mice at 50 mg/kg bw once a day for 28 days. PSO was administered at 1 mL/kg bw either before, with or after treatment of BPA, for 28 days. Seven groups of animals (n = 10) were treated: group 1 (control); group 2 (vehicle); group 3 (PSO); group 4 (BPA); group 5 (PSO before BPA); group 6 (PSO with BPA) and group 7 (PSO after BPA). DNA damage was evaluated by comet assay in liver and testes. Fifty randomly selected nuclei per experimental group were analysed. MN frequencies were evaluated in bone marrow. Two thousand polychromatic erythrocytes (PCE) were scored per animal. A significant ($p<0.05$) increase of tail DNA % in liver and testes of BPA-treated group with respect to controls (19.93 ± 0.68 vs 13.15 ± 0.22 and 23.56 ± 0.45 vs 15.00 ± 0.50) was observed. A significant increase of MNPCEs (66.40 ± 9.94 vs 10.40 ± 2.96) and a decrease in the ratio of PCE/NCE were also detected. The histopathological examination revealed hepatocyte vacuolar degeneration with many necrotic cells. A defective spermatogenesis was also observed characterized by severe necrosis and loss of the spermatogonial layers with multiple spermatid giant cells formation in most of the seminiferous tubules and a congestion of the interstitial blood vessels. The treatment with PSO reduced the genotoxic effects induced by BPA. PSO before BPA treatment was the best regimen in the alleviation of the adverse effects.

44. Naik and Vijayalaxmi, 2009: This study evaluated potential genotoxic effects of BPA by induction of chromosomal aberrations and MN in bone marrow cells of Swiss albino mice. To assess for potential interference of BPA with mitotic spindle apparatus, induction of c-mitoses was also performed. BPA (Loba Chemie, Mumbai, India) was administered orally in a 2% acacia gum suspension at dose-levels of 10, 50 and 100 mg/kg bw to groups of three male and three female mice, as single acute dose. Cumulative dose-level experiments were also performed at the lowest (10 mg/kg bw) dose-level for five consecutive days. In single treatment schedule, sampling of bone marrow was performed at 6, 24, 48 and 72 h from beginning of treatment for both micronucleus and chromosome aberration assays. Ina cumulative treatment schedule, bone marrow was sampled in both assays 24 h after the last administration of BPA. For induction of c-mitoses, the same dose levels used for micronucleus and chromosome aberration assays were applied as single dose and sampling of bone marrow was performed at 2, 6, 12, 24, 48 and 72 h. Results showed that no significant increases of chromosomal aberrations or MN were induced at any dose-level and sampling time used. Conversely, significant increases in the frequencies of gaps were observed in all dose-levels assayed at the 48 and 72 h sampling time and at the two higher dose-levels (50 and 100 mg/kg bw) at the 24 h sampling time. The significant increases of achromatic lesions (gaps) are not considered relevant for clastogenicity. In addition, BPA also induced c-mitotic effects through increases of mitotic indices and decrease in anaphase for both higher dose-levels at 24, 48 and 72 h sampling times.

Comet Assay

In vitro comet assay

45. Twenty-two in vitro studies using a comet assay in different cell lines were available for evaluation. Twelve were classified as of limited relevance and further considered in the assessment. Most cell lines used in these studies were of human origin from blood, mammary gland and prostate. Rodent cell lines from rat, mouse and hamster and one cell line from monkey were also considered.

46. Eleven of the 12 studies reported positive results. Three studies on HepG2 cell line yielded both positive (Li XH *et al*., 2017); Balabanič *et al*., 2021) and negative (Fic *et al*., 2013) results. In a non-tumorigenic human prostatic cell line, BPA induced a significant increase in DNA strand breaks paralleled by a decrease in total GSH, antioxidant capacity, glutathione peroxidase 1 (GPx1) and SOD activity and an increase in glutathione reductase (Kose *et al*., 2020). Positive results were also reported in CHO cells (Xin *et al*., 2015). Positive results were reported from two studies in which human PBMC were analysed by both alkaline and neutral comet assays (Mokra *et al*., 2017). Evidence of oxidative damage to DNA bases was provided by the addition of endonuclease III (Nth) and 8-oxoguanine DNA glycosylase (hOGG1) DNA repair enzymes (Mokra *et al*., 2018). DNA strand breaks induction by BPA was associated with increased ROS, MDA and reduced SOD activity in HepG2 (Li XH *et al*., 2017). In murine macrophage RAW264.7 cells, positive DNA strand breaks were associated with an increase in ROS and decreased level of antioxidant enzymes (Huang FM *et al*., 2018). In Marc-145 rhesus monkey embryo renal epithelial cells, DNA strand breaks induction was associated with increased ROS and Thiobarbituric Acid Reactive Substances (TBARS) and decrease in glutathione (GSH) and Superoxide Dismutase (SOD) activity (Yuan *et al*., 2019).

47. DNA strand breaks induction in mouse embryonic fibroblast cell line (NIH3T3) is associated with elevated ROS and a modest increase in DNA 8-hydroxy-2′-deoxyguanosine (8-OHdG) at the highest concentration tested (Chen *et al*., 2016). In rat INS-1 insulinoma cells, DNA strand breaks and ROS level increased in parallel along with the induction of DNA damage-associated proteins (p53 and p-Chk2). At the highest concentration of 100 µM, pre-treatment with NAC reduced the number of induced DNA strand breaks by two-fold (Xin *et al*., 2014). Finally, ER-positive MCF-7 cells were more sensitive than Oestrogen receptor (ER)-negative MDA-MB-231 cells to BPA-induced DNA damage, as measured by comet assay (Iso *et al*., 2006).

48. The available *in vitro* studies provided evidence that BPA induces DNA strand breaks most likely related to the induction of oxidative stress.

Summary of studies

49. Li XH *et al*., 2017: The study investigated the cytotoxic effects and oxidative stress induced by BPA (Sigma-Aldrich) alone and in combination with dibutyl phthalate (DBP) or cadmium (Cd) in vitro in HepG2 cells. The cell cultures were exposed for a period of 6 h to a range of concentrations of the single substances ensuring a cell viability above 50%. BPA tested from 10-8 to 10-4 mol/L for 6 hours induced a concentration dependent increase of reactive oxygen species (ROS), measured by DCFH-DA, and malondialdehyde (MDA) level and a decreased activity of SOD. An increase of DNA strand breaks (up to eight- fold with respect to the control value) applying the comet assay, was detected after BPA treatment at 10-8, 10-7, 10-6 mol/L for 24 h without a clear concentration response. The co-exposure treatments (BPA and DBP or BPA and Cd) showed higher ROS and MDA levels and lower SOD activity than the mono-exposure treatments. The combined treatments with BPA and Cd had stronger DNA damage effect.

50. Balabanič *et al*., 2021: See summary in the *in vitro* gene mutation section.

51. Fic *et al*., 2013: See summary in the *in vitro* gene mutation section.

52. Kose *et al*., 2020: This study investigated the relative toxicity, potential oxidative stress and genotoxicity induced by BPA (>99% purity), BPS and BPF on the RWPE-1 non-tumorigenic prostatic cell line. RWPE-1 cells were incubated with BPA at concentrations of 50–600 μM for 24 h exposure. The IC_{20} and IC_{50} values, concentrations that causes 20 and 50% of cell viability loss, after a 24 exposure to BPA were 45 and 113.7 μM. BPA induced significant decreases in the activities of glutathione peroxidase (GPx1) and SOD, an increase in glutathione reductase and total GSH and a decrease in total antioxidant capacity. At a single concentration (IC20), BPA produced significantly higher levels of DNA damage vs the control both in the standard (2.5-fold increase) and Fpg-modified comet assays. No changes in the mRNA levels of p53 and the OGG1, Ape-1, DNA polymerase β base excision repair (BER) proteins were induced by BPA. The single exception was a small decrease in the expression levels of MYH expression.

53. Xin *et al* study 2015: See summary in the *in vitro* gene mutation section.

54. Mokra *et al*., 2017: The study reported concentration-related induction of DNA single and double strand breaks (detected with alkaline and neutral comet assay) by

BPA (Sigma-Aldrich) and its analogues, BPS, BPF and BPAF in human peripheral blood mononuclear cells (PBMC) treated in the concentrations ranging from 0.01 to 10 µg/mL after 1 and 4 h treatment. No significant decrease of cell viability, evaluated using calcein-AM/PI stains, was observed at the concentrations tested for DNA damage. After 1 h incubation, BPA caused statistically significant increase in DNA strand breaks at 0.1 mg/mL. The highest effects were induced by BPA and BPAF, which produced single strand breaks starting from 0.01 µg/mL, while BPS caused the lowest effect at 10 µg/mL after 4 h of exposure. Statistically significant increases of DNA double strand breaks were induced by BPA at concentrations of 1 µg/mL and 10 µg/mL after 1 h incubation and at 0.1 µg/mL and 1 µg/mL after 4 h incubation. The strongest effect was observed with BPAF. DNA repair was also evaluated at different times (30, 60 and 120 min) after the treatment with BPA at 10 µg/mL. A significant decrease of the DNA damage was observed at 60 min, but the repair was not complete after 120 min.

55. Mokra *et al*., 2018: The study reported that BPA (Sigma-Aldrich) and its analogues, BPS, BPF and BPAF caused oxidative DNA damage to purine and pyrimidines in human peripheral blood mononuclear cells (PBMC) treated at concentrations of 0.01, 0.1 and 1 µg/mL for 4 h and 0.001, 0.01 and 0.1 µg/mL for 48 h. BPA was dissolved in ethanol. No significant decrease of cell viability, evaluated using calcein-AM/PI stains, was observed at the concentrations tested. DNA damage was detected with alkaline comet assay coupled with repair enzyme endonuclease III (Nth) and 8-oxoguanine DNA glycosylase (hOGG1). Statistically significant and concentration related oxidative damage to purines (from 0.01 µg/mL) and to pyrimidines (from 0.1 µg/mL) was reported after 4 h treatment. After 48 h treatment significant damage to purine was observed from 0.001 µg/mL and to pyrimidines from 0.01 µg/mL. Statistically significant differences for DNA damage between 4 h and 48 h exposure at the highest concentrations tested (0.01 and 0.1 µg/mL).

56. Huang FM *et al*., 2018: See summary in the *in vitro* chromosomal aberrations/micronuclei section.

57. Yuan *et al*., 2019: In this study, markers of oxidative stress and DNA damage were evaluated in Marc-145 rhesus monkey embryo renal epithelial cells exposed to

BPA (Sigma-Aldrich, purity > 99%) in the range 10-1, 10-2, 10-3, 10-4, 10-5 and 10-6 M (24 hr exposure). The results showed that BPA induced a concentration-dependent decrease in cell viability (from 20% at the lowest concentration up to almost 80% at the highest concentration), in SOD activity and GSH level. Concomitant concentration-dependent increases in apoptosis, lactate dehydrogenase (LDH) activity, ROS and thiobarbituric acid reactive substances content were observed. BPA also induced a concentration-dependent increase in DNA strand breaks by comet assay in the range of concentrations measured (10-3 - to 10-6 M).

58. Chen *et al*., 2016: The study investigated the cytotoxic and genotoxic effects induced by BPA alone and in combination with cadmium (Cd) *in vitro* in mouse embryonic fibroblast cell line (NIH3T3). The treatment of the cell cultures with BPA (Sigma-Aldrich) at 2, 10 and 50 µM was shown to induce, only at the highest concentration tested, a decrease in the cell viability and an increase of the oxidative damage as reactive oxygen species (ROS), measured by DCFH-DA and as 8-OHdG. Significant increase of DNA strand breaks was also detected as tail DNA% and tail moment by comet assay. Higher number of γH2AX foci detected through the use of immunofluorescence and increased γH2AX expression evaluated by western blot in BPA treated cells are indicative of DNA double strand breaks. In addition, 50 µM BPA treatment did significantly decrease the percentage of cells in G1 phase and increased the percentage of cells in G2 phase but not in S phase. Pre-treatment of cells with Cd was observed to aggravate BPA- induced cytotoxicity, and increase ROS production, DNA damage, G2 phase arrest, total TUNEL positive cells and cleaved-PARP expression levels.

59. Xin *et al*., 2014: The aim of this study was to assess how BPA can influence the function of pancreatic islets. To measure DNA damage, rat INS-1 insulinoma cells were exposed to different concentrations of BPA (Sigma-Aldrich, 99% purity) (0, 25, 50, 100 µM for 24 h) and analysed by the single-cell gel electrophoresis (comet assay). To investigate the possible mechanism of DNA damage induced by BPA, p53 and p-Chk2 levels were also analysed by western blotting together with measurements of intracellular ROS and glutathione (GSH). The results show that BPA caused an increase in DNA strand-breaks at 50 and 100 µM (as measured by

tail moment, tail length and tail DNA %). The authors state that these experimental conditions did not cause any significant toxicity (90% survival; no data provided). Pre-treatment with NAC decreased to half the number of DNA strand breaks induced at the highest dose. A significant increase in intracellular ROS, which was decreased by NAC pre-treatment, was also observed. A significant reduction in the level of GSH levels was observed at all BPA concentrations. Finally, expression of DNA damage-associated proteins (p53 and p-Chk2) was significantly increased by BPA exposure at all concentrations.

60. Iso *et al*., 2006: In this study the effects of BPA and 17β-oestradiol (E2) on DNA damage was analysed in ER-positive MCF-7 cells by comet assay. One thousand higher concentrations of BPA (Wako Pure Chemicals Industries, Ltd.) were needed to induce the same levels of effects of E2. Levels of γH2AX foci measured by immunofluorescence microscopy were increased after treatment with E2 or BPA. Foci of γH2AX co-localized with the Bloom helicase, an enzyme involved in the repair of DSBs. In comparison with MCF-7 cells, DNA damage was not as severe in the ER-negative MDA-MB-231 cells. In addition, the ER antagonist ICI182780 blocked E2 and BPA genotoxic effects on MCF-7 cells. These results together suggest that BPA causes genotoxicity ER dependently in the same way as E2.

In vivo comet assay

61. In the current assessment only 5 of 21 *in vivo* comet assay studies of DNA strand breaks induction by BPA were classified as of high (one study) or limited relevance and have been considered for evaluation. Among the five oral studies selected, three were positive and two were negative. A single study of high relevance reported negative results in multiple mouse organs (liver, kidney, testes, urinary bladder, colon and lungs) after single treatment at three doses up to the Maximum Tolerated Dose (MTD) of 500 mg/kg bw (Sharma *et al*., 2018). Negative results were also reported in rats exposed to 200 mg/kg bw per day orally for 10 days (De Flora *et al*., 2011). In contrast, dose-related increases in DNA strand breaks were reported at doses greater than 10 µg/kg bw in rats treated for 6 days with a range of doses between 2.4 µg and 50 mg/kg bw per day (Tiwari *et al*., 2012). A weak and dose-dependent increase in liver DNA strand breaks was observed at

50 and 100 mg/kg bw per day, whereas the increase in kidney was limited to 50 µg/kg bw (Panpatil *et al*., 2020). Finally, in a study on BPA neurotoxicity, a significant increase of strand breaks in brain cells was observed after treatment in a range of doses from 0.5 to 5000 µg/kg bw per day for 8 weeks (Zhou YX *et al*., 2017).

62. Overall, the comet assays provided only limited evidence of DNA damage following multiple administrations of BPA, but not following single dose administrations.

Table 3. Summary table of test results of Comet *in vivo* studies.

Test system	Dose	Results	Reference
Comet assay in liver, kidney testes, urinary bladder, colon and lungs			
CD-1 male mice 5 animals/group	125, 250 and 500 (MTD) mg/kg bw Single dose by gavage	**Negative**	Sharma *et al*., 2018
Comet assay in liver, kidney, testes, urinary bladder, colon and lungs			
Sprague Dawley rats 8 animals/group	200 mg/kg bw per day orally for 10 days	**Negative**	De Flora *et al*., 2011
Holtzman rats 10 animals/group	2.4 µg, 10 µg, 5mg and 50 mg/kg per day orally for 6 days	**Positive** Dose-related increase starting from 10 µg/kg	Tiwari *et al*., 2012
Comet assay in liver and kidney			
Male Wistar rats (WNIN) 6 animals/ group	50 100 µg/kg orally for 4 weeks	**Positive** Weak dose-related in liver, only at 50 µg/kg in kidney	Panpatil *et al*., 2020
Comet assay in brain cells			
KM male mice 11 animals/group	0.5, 50 and 5000 µg/kg bw per day Orally for 8 weeks	**Positive**	Zhou YX *et al*., 2017

Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021).

Summary of studies

63. Sharma *et al*., 2018: The *in vivo* genotoxic potential of BPA in mouse organs was investigated using the alkaline comet assay. Male CD-1 mice (5 per group) were administered BPA (Sigma-Aldrich) by gavage in corn oil suspensions prepared by ultrasonication at three dose levels (125, 250 and 500 mg/kg bw), given twice, 24 h apart. Ethyl methane sulphonate, given once by gavage at 300 mg/kg bw, served as positive control. Animals were sacrificed 3 h after the last treatment and DNA damage investigated by a commercial kit for comet assay in liver, kidney, testes, urinary bladder, colon and lungs cells. For each mouse, 200 cells were analysed (100 per gel) using an automatic comet assay scoring imaging system. Median values for each tissue from each animal were used, and the mean of the median values was evaluated in a statistical analysis. The results of comet assay did not show BPA related effects in any tissue, except for the testes, in which an increased level of DNA strand breaks ($p < 0.01$ compared with control group) was observed at the lowest dose; however, no dose response relationship was observed as the effects at the medium and highest doses were at the same level as the control group. A modified alkaline comet assay was conducted on human sperm cells treated with BPA 0, 1, 1.5, 2 and 3 µmol/L for 1h. BPA 3 µmol/L reduced cell viability to 60%, therefore it was the highest concentration tested. Ethyl methanesulfonate (EMS) was used as positive control. In total, 600 cells were scored for each concentration. No increase in % tail DNA was observed compared with the negative control.

64. De Flora *et al*., 2011: The ability of BPA to form DNA adducts was investigated in two human prostatic cell lines: PNT1a non tumorigenic epithelial cells and PC3 cells androgen-independent prostate cancer cells originated from bone metastasis of prostatic carcinoma. PNT1a and PC3 cells were treated with BPA (Sigma-Aldrich), dissolved in ethanol at a concentration corresponding to the IC50 (200 µM for PNT1a and 250 µM for PC3) for 24 h. PNT1a cells were also treated at a concentration of 1 nM, for 2 months. Significant levels of DNA adducts were detected by 32P-postlabeling technique in prostate cell lines treated with high-concentration of BPA for 24 h (4.2-fold increase over controls) in PNT1a cells and a

2.7-fold increase over controls in PC3 cells) and in a lower extent in PNT1a cells treated at low-concentration for 2 months.

65. Tiwari *et al*., 2012: See summary in the *in vitro* gene mutation section.

66. Panpatil *et al*., 2020: See summary in the *in vivo* chromosomal aberrations/micronuclei section.

67. Zhou YX *et al*., 2017: The study investigated the neurotoxicity of low-dose exposure to BPA in a mouse model, examining brain cell damage and the effects of learning and memory ability after 8 weeks exposure to BPA at 0.5, 50 and 5000 µg/kg bw (daily dose, by gavage). The comet assay was used to detect brain cell damage. At the end of treatment 11 mice per group were sacrificed and brain processed for comet assay. Forty cells from each brain were analysed. Based on tail DNA percentage, the damage level was divided into five grades, from 0 (undamaged) to 4 (maximum damage). The results obtained indicated that with increasing exposure concentrations, the fraction of damaged cells (all types) increased significantly from 23.0% in the control group to 47.3%, 66.6% and 72.5% in the low-, medium and high exposed groups, respectively. Also, the severity of DNA damage, expressed as arbitrary units (AUs), increased with AUs of 0.28 in the control to AUs of 0.59, 0.96 and 1.28 in the low-, medium and high-exposed groups, respectively.

Other studies

Induction of γH2AX foci

68. Several studies have investigated the induction of γH2AX foci (generally regarded as a marker of DNA DSBs) following BPA treatment (Iso *et al*., 2006; Pfeifer *et al*., 2015; George and Rupasinghe, 2018; Kim *et al*., 2018b; Mahemuti *et al*., 2018; Hercog *et al*., 2019; Hercog *et al*., 2020; Nair *et al*., 2020; Yin *et al*., 2020; Escarda-Castro *et al*., 2021; Yuan *et al*., 2021).

69. Iso *et al*. (2006) reported increased levels of γH2AX foci after treatment with 17β-E2 or BPA in ER- positive MCF-7 cells (1000x higher concentrations of BPA

were needed to induce the same levels of effects as E2). Induction was less severe in ER-negative MDA-MB-231 cells and the ER antagonist ICI182780 blocked BPA-induced γH2AX focus formation in MCF-7 cells. Taken together, these findings indicate that BPA-induced genotoxicity is ER-dependent

70. The effects of low-dose BPA were studied in the ERα-negative MCF10A and in 184A1 normal breast epithelial cell lines and the ERα-positive MCF7 and MDA-MB-231 human breast epithelial adenocarcinomas. Low doses (10 and 100 nM) induced DSBs as measured by γH2AX foci in all cell lines and increased the level of c-Myc and of the cell-cycle regulatory proteins cyclins D1 and E and E2F1. Silencing c-Myc reduced BPA-induced γ-H2AX foci and abolished BPA-mediated mitochondrial ROS production. BPA also induced proliferation in ERα-negative mammary cells. The authors conclude that low-dose BPA exerts a c-Myc–dependent genotoxicity and mitogenicity in ERα-negative mammary cells (Pfeifer *et al*., 2015).

Summary of studies (in order of mention)

71. Iso *et al*., 2006: See summary in the *in vitro* comet assay section.

72. Pfeifer *et al*., 2015: The objective of this study was to investigate the effects of low-dose BPA (Sigma-Aldrich) in mammary gland cells. The human cell lines used in the study are the ERα-negative immortalized benign and normal breast epithelial cell lines (MCF10A and 184A1, respectively) and the ERα-positive MCF7 and MDA-MB-231 cell lines originate from human breast epithelial adenocarcinomas. Low concentrations BPA (10 and 100 nM) induced double strand breaks (DSBs) as measured by γH2AX foci in all cell lines. Both MCF10A and MCF7 cells had also a greater number of ATM-pS1981–positive nuclei after 24 h treatment compared with the control. Low-concentration BPA significantly increased the level of c-Myc protein and other cell-cycle regulatory proteins (cyclin D1, cyclin E and E2F1) and induced proliferation in parallel in ERα-negative 184A1 mammary cells. Silencing c-Myc reduced BPA-mediated increase of γH2AX suggesting that c-Myc plays an essential role in BPA-induced DNA damage. The increased level of DNA double strand breaks induced by BPA exposure in 184A1 cells was also confirmed in a neutral comet assay and was found to be reduced by c-Myc silencing. Similarly, silencing c-Myc

abolished BPA-mediated ROS production, which was localized to mitochondria. The authors concluded that low-concentration BPA exerted a c-Myc–dependent genotoxic and mitogenic effects on ERα-negative mammary cells (results reported as tail moment only and a single BPA concentration was analysed).

73. George and Rupasinghe, 2018: This study investigated the relative toxicity of BPA (Sigma-Aldrich) and BPS on human bronchial epithelial cells (BEAS-2B). The tested endpoints included cytotoxicity, induction of ROS, DNA fragmentation, γH2AX foci and DNA tail damage. To evaluate the mechanism of cell death, the DNA Damage response (DDR) and activation of caspase-3 were also investigated. In all the assays, only a single concentration and single exposure time were used (200 μM BPA for 24 h). According to the authors this concentration caused 50% loss of cell viability (IC50). However, the data reported indicate high levels of toxicity (90%), with all the results being unreliable at this level of toxicity.

74. Kim *et al*., 2018: BPA (> 99% purity, Sigma-Aldrich) promoted cell proliferation in undifferentiated and differentiated human hepatocyte cell lines (HepG2 and NKNT-3, respectively) at sub-micromolar concentrations (0.3-5 μM for 24 h). The proliferative effects of BPA disappeared at concentrations higher than 5 μM and cell viability decreased at concentrations higher than 10 μM. Exposure to BPA in the sub-micromolar range induced DNA damage in both cell lines as shown by a dose-dependent increase in phosphorylation of histone H2AX (γH2AX), p53 activation and induction of cyclin B1. Increased levels of γH2AX were also observed in liver tissue of juvenile rats (PND 9) orally exposed to a relatively low dose of BPA (0.5 mg/kg for 90 days). At a higher BPA dose (250 mg/kg) no increase in hepatocyte proliferation or cyclin B1 was observed. BPA promoted ROS generation as measured by DCF-DA-enhanced fluorescence in HepG2 cells. Increased levels of ROS were suggested to play a role in BPA-induced proliferation and DNA damage as shown by the partial reversion of both processes upon pre-treatment with NAC.

75. Mahemuti *et al*., 2018: The aim of this study was to investigate the key molecular pathways involved in the developmental effects of BPA on human fetal lung fibroblasts and their potential implications in the link between pre-natal exposure to BPA and increased sensitivity to childhood respiratory diseases. Global

gene expression profiles and pathway analysis was performed in cultured HFLF exposed to non-cytotoxic concentrations of BPA (0.01, 1 and 100 μM BPA for 24 h, 99% purity, Sigma-Aldrich). Molecular pathways and gene networks were affected by 100, but not 0.01 and 1 μM BPA. These changes were confirmed at both gene and protein levels. The pathways affected by BPA included the cell cycle control of chromosome replication and a decreased DDR. BPA increased DNA DSBs as shown by phosphorylation of H2AX and activated ATM signalling (increased phosphorylation of p53). This resulted in increased cell cycle arrest at G1 phase, senescence and autophagy, and decreased cell proliferation in HFLF. Finally, BPA increased cellular ROS level and activated Nrf2-regulated stress response and xenobiotic detoxification pathways. The authors suggest that pre-natal exposure to BPA may affect fetal lung development and maturation, thereby affecting susceptibility to childhood respiratory diseases.

76. Hercog *et al*., 2019: With the aim of comparing the toxicological profiles of possibly safer analogues of BPA, the authors investigated the cytotoxic/genotoxic effects of BPS, BPF and BPAF and their mixtures in human hepatocellular carcinoma HepG2 cells. Single exposure to BPA (99% analytical purity, Sigma-Aldrich) did not induce any significant changes in cell viability at the tested concentrations (2.5, 5, 10, 20 μg/mL for 24 or 72 h). Induction of a significant increase in DNA double strand breaks, as determined by γH2AX assay, was observed only at the highest dose (20 μg/mL for 72 h). BPA (tested at the 10 μg/mL concentration) induced changes in the expression of some genes involved in the xenobiotic metabolism (CYP1A1, UGT1A1, but not GST1), response to oxidative stress (GCLC but not GPX1, GSR, SOD1, CAT), while no changes were observed in any of the genes involved in the DDR (TP53, MDM2, CDKN1A, GADD45A, CHK1, ERCC4). Similar results were obtained when cells were exposed to BPA as a single compound or in mixtures with its analogues at concentrations relevant for human exposure (10 ng/mL). The relevance of these changes is of uncertain biological significance.

77. Hercog *et al*., 2020: In a follow-up study by Hercog *et al*. (2020) the genotoxic effects induced by co-exposure of the cyanotoxin cylindrospermopsin (CYN)(0.5 μg/mL) and BPA (Sigma-Aldrich), BPS and BPF(10 μg/mL, 24 and 72 h exposure)

were investigated on HepG2 cells using the same techniques and experimental conditions of Hercog *et al*. (2019). The results obtained with BPA confirm the previously published observations, but the relevance of these changes remains of uncertain biological significance.

78. Nair *et al*., 2020: The effects of BPA (Sigma-Aldrich) as a single agent, or in combination with 4-tert-octylphenol (OP) and hexabromocyclododecane (HBCD), were studied in the HME1 mammary epithelial cells and in the MCF7 breast cancer cell line. Following a 2-month exposure to a low non-toxic BPA concentration (0.0043 nM), increased levels of DNA damage were evidenced by upregulation in both cell lines of phosphorylated DNA damage markers (γ-H2AX, pCHK1, pCHK2, p-P53). Disruption of the cell cycle was observed both after short exposures (24 h and 48 h, G2/M arrest) as well as after the 2-month exposure treatment (G1 and S phase increases). BPA increased cellular invasiveness through collagen. Methylation changes were investigated by Methylation Specific Multiplex-Ligation Dependent Probe Amplification (MS-MLPA) using a panel of 24 tumour suppressor genes (all hypomethylated) and identified hypermethylation of TIMP3, CHFR, ESR1, IGSF4 in MCF7 cells and CDH13 and GSTP1 genes in HME1 cells. Finally, BPA induced phosphorylation of six protein kinases in HME1 cells (EGFR, CREB, STAT6, c-Jun, STAT3, HSP60) and increased levels of several other proteins involved in potential oncogenic pathways (HSP27, AMPKα1, FAK, p53, GSK-3α/β, and P70S6).

79. Yin *et al*., 2020: The scope of the study was developing a novel *in vitro* three-dimensional testicular cell co-culture mouse model that enables the classification of reproductive toxic substances. BPA (99%, Sigma-Aldrich) as well as BPS, TBBPA, and BPAF were used as model compounds. A concentration-dependent increase in BPA toxicity was found in the range 2.5 - 400 µM following 24, 48 and 72 h exposures. The large variations in the number of gH2AX foci observed at 72 h make the relevance of these results questionable. No increase in gH2AX used as marker of DNA damage was found up to a dose of 100 mM (70% cell viability).

80. Escarda-Castro *et al*., 2021: The ability of BPA to induce genotoxic and epigenetic changes was investigated before and during cardiomyocyte differentiation in H9c2 rat myoblasts exposed to 10 and 30 µM BPA (92% and 73% of cell viability,

respectively). Exposure to BPA (no information on purity or the supplier company) before differentiation repressed the expression of the Hand2 and Gata4 heart transcription factors and three genes belonging to the myosin heavy chain family (Myh1, Myh3, and Myh8), whereas exposure after the 5 days of differentiation reduced the expression of cardiac-specific Tnnt2, Myom2, Sln, and Atp2a1 genes. BPA did not induce ROS and did not increase DNA 8-oxodG levels (as measured by immunostaining) in either myoblasts or cardiomyocytes. After BPA exposure the percentage of DNA repair foci formed by co-localization of the γH2AX and 53BP1 proteins increased in a concentration-dependent manner in myoblasts (from 44% in the control group to 61% and 86% at 10 and 30 μM BPA, respectively), with no increase in MN. Repair foci also increased in cardiomyocytes (from 45% in the control group to 59% and 72% at 10 and 30 μM BPA, respectively). A small increase (up to 13%) in MN was also reported only in cardiomyocytes treated with 10 μM BPA. A decrease in the epigenetic markers H3K9ac and H3K27ac was also reported. The authors concluded from these *in vitro* data that BPA interferes with the process of cardiomyocyte differentiation. However, the reliability and significance of the data on BPA-induced DNA damage is questioned by several negative factors (high background levels of DNA repair foci, lack of information on methods for micronucleus assays and the small increase of MN over high background).

81. Yuan *et al*., 2021: This study investigated the combinatorial toxicity of BPA (≥ 99.8% purity), decabrominated diphenyl ether and acrylamide to HepG2 cells. Increased number of γH2AX foci were induced in HepG2 by a 24h exposure to a single BPA dose that induced 25% toxicity. The majority of the data (ROS measurements, Ca2+ flux, DNA damage, Caspase-3 and decreased mitochondrial membrane potential) refers to additive/synergistic effects induced by varying combinations of contaminants. The authors conclude that BPA induced an increase in γH2AX fluorescence and in the number of γH2AX foci/nucleus. However, this conclusion is not fully supported by the data presented.

Changes in gene expression and DNA methylation

82. Changes in DNA methylation have been investigated in several studies (De Felice *et al*, 2015; Porreca *et al*., 2016; Karmakar *et al*., 2017; Karaman *et al*., 2019).

83. No specific discussion on DNA repair or DDR genes is reported in these publications.

84. None of the information present in these studies is relevant for the clarification of the genotoxic potential of BPA.

Studies in humans

85. Overall, human studies are not considered to provide additional relevant information for the evaluation of BPA genotoxicity

Mode of action

86. BPA did not induce gene mutations in bacteria. All the available *in vitro* studies on chromosomal damage, classified as of high or limited relevance, reported positive results such as increase of CA or MN frequency, in different cellular systems. The increases in BPA-induced chromatid and chromosome breaks observed in some studies (Xin *et al*., 2015; Santovito *et al*., 2018; Di Pietro *et al*., 2020) in association with the induction of DNA strand breaks, detected by a comet assay (Xin *et al*., 2015) are consistent with a clastogenic activity. Moreover, the potential of BPA to affect the spindle integrity and interfere with the chromosome segregation machinery was demonstrated in some reliable studies. Johnson and Parry (2008) reported the formation of aberrant mitotic spindles, with multiple poles, in V79 cells treated with BPA. Altered cytoskeleton organization, with multipolar spindles, failure of microtubule attachment to the kinetochore with the concomitant activation of spindle assembly checkpoint (SAC) and chromosome misalignment, were also observed in HeLa cells (Kim *et al*., 2019). Studies on spindle morphology of mouse (Yang *et al*., 2020) and bovine (Campen *et al*., 2018) oocytes during in vitro maturation reported a pattern of alterations similar to that observed in permanent cell lines, namely shorter and multipolar spindles, with altered kinetochore-microtubule attachment and chromosome misalignment at M II.

87. The conclusion, based on these *in vitro* studies, is that BPA may act by both clastogenic and aneugenic mechanisms.

88. The large majority (11 out of 12) of the *in vitro* studies on the comet assay, classified as of limited relevance, reported BPA-induced increases of DNA strand breaks. In some studies, the increase of DNA damage was associated with a parallel increase of ROS and MDA and decrease in antioxidant capacity and in total GSH (Xin *et al*., 2014; Li XH *et al*., 2017; Huang FM *et al*., 2018; Yuan *et al*., 2019; Kose *et al*., 2020). A study in macrophages reported also a release of cytochrome c from mitochondria along with increased apoptosis with the indication that the DNA strand breaks could be mainly through the oxidative stress-associated mitochondrial apoptotic pathway (Huang FM *et al*., 2018). In a study on human PBMC, the application of comet assay with the addition of endonuclease III (Nth) and 8-oxoguanine DNA glycosylase (hOGG1) DNA repair enzymes allowed the detection of oxidative damage to DNA bases (Mokra *et al*., 2018). Further indication of the role of oxidative damage in induction of DNA strand breaks was provided by the protective effects on DNA damage induced by the pre-treatment with NAC (Xin *et al*., 2014; Huang FM *et al*., 2018).

89. In conclusion, the evidence of DNA strand breaks *in vitro* is in agreement with the ability of BPA to induce clastogenic damage. In addition, the studies using comet assays provide consistent evidence that BPA induces DNA strand breaks most probably related to the induction of oxidative stress.

90. The available *in vivo* studies for BPA-induced chromosomal damage in somatic cells reported mixed results. No increase of CA and MN frequency was reported after a single administration of BPA to mice in a range of doses inducing toxicity at the bone marrow level (Naik and Vijayalaxmi, 2009). In contrast, in another study in mice, increased MN frequency was detected in the presence of high bone marrow toxicity (Fawzy *et al*., 2018). Positive results were observed in two rat studies (Tiwari *et al*., 2012; Panpatil *et al*., 2020) after repeated dose administration, possibly associated with lipid peroxidation and oxidative stress in the first study. No induction of hyperploidy or polyploidy was observed in these studies.

91. These results indicate that the *in vivo* induction of chromosomal damage requires specific conditions such as repeated exposure to BPA.

92. Induction of DNA strand breaks, detected by comet assay *in vivo*, was observed only after repeated exposure for extensive periods of time up to 8 weeks (Tiwari *et al*., 2012; Zhou YX *et al*., 2017; Panpatil *et al*., 2020). Only one study of high relevance was available on single administration of BPA reporting negative results in multiple mouse organs in a range of doses up to the MTD of 500 mg/kg bw (Sharma *et al*., 2018). An indication of a possible role of oxidative stress in inducing DNA strand breaks by BPA was provided by the results of several studies (Abdel-Rahman *et al*., 2018; Fawzy *et al*., 2018; Kazmi *et al*., 2018; Majid *et al*., 2019; Mohammed *et al*., 2020) showing the protective effects of natural extracts with antioxidant properties. However, these studies were evaluated as low relevance.

93. Finally, studies on germ cells, carried out by four laboratories in the framework of a collaborative project on aneugenic chemicals, did not provide any evidence of increased frequency of aneuploidy in mouse oocytes and zygotes and in sperm cells following exposure to low BPA doses (Pacchierotti *et al*., 2008).

94. BPA is genotoxic *in vitro* inducing chromosomal damage and DNA breaks. However, *in vivo* the evidence of genotoxic properties of BPA is contradictory. This might depend on multiple mechanisms of action described or proposed for BPA. A major difficulty in the interpretation of these contradictory results is the lack of knowledge on the role of BPA metabolism that could be operational in genotoxic activity. Indeed, the role of the proposed DNA adducts has not been clarified. Other uncertainties include the role of ER receptors in the oxidative stress induced by BPA.

Summary of studies

95. Xin *et al*., 2015: summary in the *in vitro* gene mutation section.

96. Santovito *et al*., 2018: summary in the *in vitro* chromosomal aberrations/micronuclei section.

97. Di Pietro *et al*., 2020: summary in the *in vitro* chromosomal aberrations/micronuclei section.

98. Johnson and Parry (2008): summary in the *in vitro* chromosomal aberrations/micronuclei section.

99. Kim *et al*., 2019: *In vitro* effects of BPA (Sigma-Aldrich) on mitotic progression were examined in HeLa cells exposed to 100 nM BPA for 5 h. Proteins involved in mitotic processes were detected by western blot, live cell imaging, and immunofluorescence staining. Under the applied treatment conditions, BPA was shown to disturb spindle microtubule attachment to the kinetochore, with the concomitant activation of SAC. Spindle attachment failure was attributed to BPA interference with proper localization of microtubule associated proteins, such as Hepatoma Upregulated protein (HURP) to the proximal ends of spindle microtubules, Kif2a to the minus ends of spindle microtubules, and TPX2 on the mitotic spindle. BPA also caused centriole overduplication, with the formation of multipolar spindle.

100. Yang *et al*., 2020: The effect(s) of exposure to BPA (Sigma-Aldrich) on assembled spindle stability in ovulated oocytes were studied. Mature M II oocytes, recovered from the oviducts of superovulated B6D2F1 mice, were cultured for 4 h in the presence of increasing concentrations (5, 25, and 50 µg/mL) of BPA. After treatment oocytes were analysed by immunofluorescence and live cell imaging to investigate the effect of BPA on spindle dynamics. BPA disrupted spindle organization in a dose-dependent manner, resulting in significantly shorter spindles with unfocused poles and chromosomes congressed in an abnormally elongated metaphase-like configuration, with increased erroneous kinetochore-microtubule interactions.

101. Campen *et al*., 2018: The aim of the study was to compare the effects of *in vitro* exposure to either BPA (Sigma-Aldrich) or BPS on meiotic progression, spindle morphology and chromosome alignment in the bovine oocyte. Bovine ovaries were sourced from an abattoir. Groups of 5–20 cumulus–oocyte complexes (COCs) extracted from the bovine ovaries were treated with BPA or BPS at 10 concentrations between 1 fM and 50 µM and underwent to *in vitro* maturation for 24 h, then the oocytes were extracted. For BPA experiments, a total of 939 oocytes were analysed for meiotic stage (including 250 vehicle-only control oocytes), of which a total of 767 were at metaphase II (MII) (including 211 MII oocytes in the

control) and were included for analysis of spindle and chromosome configuration. Immunocytochemistry was used to label the chromatin, actin and microtubules in the fixed oocytes. The meiotic stage was assessed using immunofluorescence, and the MII oocytes were further assessed for spindle morphology and chromosome alignment (in all MII oocytes regardless of spindle morphology). No difference in the proportion of bovine oocytes that reached MII was observed for BPA treatment. Significant effect on spindle morphology ($p < 0.0001$) was induced by BPA treatment at very low concentration (1 fM). Fewer oocytes with bipolar spindles were seen following exposure to BPA at concentrations of 1 fM, 10 fM, 100 fM, 10 pM, 1 nM, 10 nM, 100 nM and 50 µM, compared with the control. There was no effect of BPA on spindle morphology at concentrations of 1 or 100 pM. Increased chromosome misalignments were observed at BPA concentrations of 10 fM, 10 nM and 50 µM of BPA, no effect was detected at any other concentration. The study presents limitations: in the ovaries the effects were evaluated in a specific period of development (namely, the 24 h window of oocyte maturation), without considering potential prior historical exposures *in vivo*.

102. Xin *et al*., 2014 : summary in the *in vitro* comet assay section.

103. Li XH *et al*., 2017: summary in the *in vitro* comet assay section.

104. Huang FM *et al*., 2018: summary in the *in vitro* chromosomal aberrations/micronuclei section.

105. Yuan *et al*., 2019: summary in the *in vitro* comet assay section.

106. Kose *et al*., 2020: summary in the *in vitro* comet assay section.

107. Mokra *et al*., 2018: summary in the *in vitro* comet assay section.

108. Naik and Vijayalaxmi, 2009: summary in the *in vitro* chromosomal aberrations/micronuclei section.

109. Fawzy *et al*., 2018: summary in the *in vitro* chromosomal aberrations/micronuclei section.

110. Tiwari *et al*., 2012: Summary in the *in vitro* gene mutation section.

111. Panpatil *et al*., 2020: summary in the *in vivo* chromosomal aberrations/micronuclei section.

112. Zhou YX *et al*., 2017: summary in the *in vitro* comet assay section.

113. Sharma *et al*., 2018: summary in the *in vivo* comet assay section.

114. Abdel-Rahman *et al*., 2018: The study evaluated the protective action of lycopene (LYC), an antioxidant agent, on the toxic effects of BPA (Sigma-Aldrich). Four groups of seven Wistar rats were treated daily for 30 days via gavage: the first group (controls) received corn oil, the second group was given lycopene at a dose of 10 mg/kg bw, the third group was given BPA at 10 mg/kg bw, the fourth group was given both BPA and LYC at the 10 mg/kg. Rats were sacrificed immediately after the last administration. Liver was frozen at -80 °C. Single-cell suspensions for use in a comet assay were prepared from frozen livers. No positive controls were used. The comet method applied was not reported. A significant ($p < 0.05$) increase of tail DNA % in liver of BPA-treated group with respect to controls (25.05 vs 6.68) was observed. Higher activities ($p < 0.05$) of liver enzymes (serum ALT, alkaline phosphatase (ALP) and GGT and lower levels of total protein and albumin than control rats were detected in serum. Antioxidant enzymes (GPx, SOD and CYPR450 activities) significantly ($p < 0.05$) decreased while MDA level significantly increased in liver of BPA treated animals. Caspase-3 protein in liver of BPA-treated rats is overexpressed. Histopathological analyses showed deleterious hepatic changes ranging from hepatocytes' vacuolization and eccentric nuclei to focal necrosis and fibrosis. LYC administration reduced the cytotoxic effects of BPA on hepatic tissue, through improving the liver function biomarkers and oxidant-antioxidant state as well as DNA damage around the control values.

115. Kazmi *et al*., 2018: The study evaluated the protective role of Quercus dilatate (green or holly oak) extracts against BPA (no information on purity) induced hepatotoxicity. Ten groups of Sprague Dawley (SD) rats (7 animals/group) were considered, including untreated control group and a group receiving the vehicle. The

distilled water-acetone (QDDAE) and methanol-ethyl acetate (QDMEtE) extracts were administered in high (300 mg/kg bw) or low (150 mg/kg bw) doses to rats, intraperitoneally injected with BPA (25 mg/kg bw). A group of rats was treated only with BPA. Rats were sacrificed after 4 weeks of treatment and blood and liver were collected. The comet method applied was not sufficiently detailed. An increase of DNA strand breaks in hepatocytes was reported for animals treated with BPA alone. However, the results reported using the different parameters (tail length, % of DNA in tail, tail moment) are not consistent. The % of DNA in tail is 28.35 ± 1.2 in BPA treated animals vs 0.01 ± 0.005 in controls. The value of % of DNA in tail in controls is extremely low with respect to the data reported in the scientific literature. Significant reduction in haemoglobin level, red blood cells and platelet count, whereas elevated levels of white blood cells and erythrocyte sedimentation rate (ESR) were observed in the BPA treated group. Administration of BPA significantly ($p < 0.05$) decreased the endogenous antioxidant enzyme (CAT,GPx, superoxide dismutase (SOD) and GSH) levels compared with control group. In addition, in the BPA treated group, H2O2, nitrite and TBARS levels in the hepatic tissue were found to be higher when compared with controls. Histopathological examination of BPA treated animals revealed intense hepatic cytoplasm inflammation, centrilobular necrosis, cellular hypertrophy, fatty degeneration, vacuolization, steatosis and distortion of portal vein. A dose dependent hepatoprotective activity was exhibited by both the extracts of Quercus dilatate in different extent for the parameters analysed.

116. Majid *et al*., 2019: The study evaluated the protective role of sweet potato (*Ipomoea batatas L. Lam*.) against BPA-induced testicular toxicity. Sixteen groups of seven Male SD rats were established, including controls, animals treated with the vehicle, with ethyl acetate and methanol extracts from tuber and aerial part of Ipomoea batatas, with BPA (Merck KGaA) and with BPA and different extracts of Ipomea batatas. The BPA group received 50 mg/kg bw dissolved in 10% DMSO, injected intraperitoneal on alternate days for 21 days. The rats were sacrificed 24 h after the last treatment. Comet assay was applied to evaluate the DNA damage. An average 50–100 cells were analysed in each sample for comet parameters (head length, comet length, tail moment, tail length, and amount of DNA in head) of gonadal cell's nuclei. A statistically significant increase of % DNA in tail (3 folds with respect to the control value) was reported in the group of rats treated with BPA.

Endogenous antioxidant enzymes were measured in supernatant from the testicular homogenates: BPA decreased the levels of peroxidases (POD), CAT, SOD. BPA induced also gonadotoxicity measured as size and weight of testes and epididymis, concentration and quality of sperms. The treatment with extracts of Ipomea batatas significantly reduced the gonadotoxicity induced by BPA, the DNA damage and restored the levels of antioxidant enzymes.

117. Mohammed *et al*., 2020: The study evaluated the protective role of ginger extract (GE) against BPA-induced toxic effects on thyroid. Four groups of 20 male albino rats were treated orally with BPA (Sigma–Aldrich), GE or both once a day for 35 days as follow: Control group: 0.1 ml/rat of corn oil; BPA group: 200 mg/kg bw per day (1/20 of the oral LD50); GE group: ginger extract 250 mg/kg bw; BPA + GE group: ginger extract followed by BPA after 1 h with the same doses as the other groups. The animals were sacrificed 24 h after the last administration. DNA damage was evaluated by comet assay. A statistically significant increase of DNA damage expressed as tail % DNA, tail length and tail moment were shown in thyroid follicular cells of animals treated with BPA. A concurrent increase of MDA and a decrease of GSH, and SOD were also observed. Adverse effects on the thyroid gland were reported with a significant decrease in serum levels of T3 and T4 accompanied by a significantly increase in serum Thyroid Stimulating Hormone (TSH) level. A decrease of Nrf-2 mRNA relative expression and protein concentration and of HO-1 mRNA expression in the BPA-induced thyroid injured rats were also described. The histopathological analysis revealed an alteration of the thyroid gland follicles most of which containing scanty colloid secretion and some others atrophied. The treatment with GE significantly reduced the genotoxic damage and the alteration of thyroid hormones regulating genes.

118. Pacchierotti *et al*., 2008: The study evaluated the potential aneugenic effects of BPA on mouse male and female germ cells and bone marrow cells following acute, subacute or subchronic oral exposure. For experiments with acute and subacute exposure, female C57BL/6 mice were treated by gavage with BPA (from Sigma-Aldrich) dissolved in corn oil once with 0.2 and 20 mg/kg bw, or with seven daily administrations of 0.04 mg/kg bw. In subchronic experiments, mice received BPA in drinking water at 0.5 mg/L for 7 weeks. The dose levels tested for subacute

effects in bone marrow and male germ cells were 0.002, 0.02 and 0.2 mg/kg bw for 6 days. For the assessment of aneugenicity in female germ cells, M II oocytes were harvested 17 h after induced superovulation, and cytogenetically analysed after C-banding. The percentages of metaphase I-arrested oocytes, polyploid oocytes and oocytes that had undergone Premature Centromere Separation (PCS) or Premature Anaphase II (PA) were calculated. To evaluate the aneugenic effects of BPA upon the second meiotic division, zygote metaphases were prepared from superovulated females mated with untreated C57Bl/6 males. Zygote metaphases were prepared, C-banded and cytogenetically analysed for the occurrence of polyploidy and hyperploidy. Experiments on male germ cells were performed with 102/ElxC3H/El)F1 males. Epididymal sperms were collected and hybridized with fluocrochrome-labelled DNA probes for chromosomes 8, X and Y and 10,000 sperm per animal were analysed to evaluate the incidence of hyperhaploid (X88, Y88, XY8) and diploid (XY88, XX88, YY88) sperm cells. Micronucleus test was performed with four groups of five (102/ElxC3H/El) F1 male mice treated with 0, 0.002, 0.02 or 0.2 mg/kg BPA by gavage on 2 consecutive days and sacrificed 24 h after the second administration. In total, 2000 PCE from two slides were scored per animal for the presence of MN. No significant induction of hyperploidy or polyploidy was observed in oocytes and zygotes at any treatment condition. The only detectable effect was a significant increase of M II oocytes with prematurely separated chromatids after chronic exposure; this effect, however, had no consequence upon the fidelity of chromosome segregation, as demonstrated by the normal chromosome constitution of zygotes under the same exposure condition. Similarly, with male mice no induction of hyperploidy and polyploidy was shown in epididymal sperm after six daily oral BPA doses, and no induction of MN in PCE.

Conclusion on hazard identification for genotoxicity effects of BPA

119. In 2015, the CEF Panel concluded that: The available data support that BPA is not mutagenic (in bacteria or mammalian cells), or clastogenic (MN and CAs). The potential of BPA to produce aneuploidy *in vitro* was not expressed *in vivo*. The positive finding in the post labelling assays *in vitro* and *in vivo* is unlikely to be of concern, given the lack of mutagenicity and clastogenicity of BPA *in vitro* and *in vivo*.

120. Based on the scientific literature considered in the previous EFSA opinions and published thereafter until 21 July 2021, the CEP Panel concluded that:

•BPA does not induce gene mutations in bacteria;

• BPA induces DNA strand breaks, clastogenic and aneugenic effects in mammalian cells *in vitro*;

• oxidative stress related mechanism(s) are likely to be involved in the DNA damaging and clastogenic activity elicited by BPA *in vitro*;

• there is some evidence for DNA and chromosomal damaging activities of BPA *in vivo* following repeated administrations, but not following single administrations;

• the available studies do not provide evidence of aneugenicity of BPA in germ cells *in vivo*.

121. In contrast with consistent positive *in vitro* findings, the *in vivo* findings in several studies with high/limited reliability were inconsistent. The CEP Panel concluded that the evidence does not support an *in vivo* genotoxic hazard posed by BPA through direct interaction with DNA.

Uncertainty analysis for the genotoxicity assessment

122. It was concluded that it is Unlikely to Very Unlikely (5 – 30% probability) that BPA presents a genotoxic hazard, the causes of which include a direct mechanism (combining subquestion 1 and 2 (see Annex A to this paper)). Accordingly, it was concluded that it is Likely to Very Likely (70 - 95% probability) that BPA either presents a genotoxic hazard only through indirect mechanism(s) or is not genotoxic. The likelihood terms used in these conclusions are taken from the approximate probability scale, which is recommended by EFSA (EFSA Scientific Committee, 2018) for harmonised use in EFSA assessments.

123. EFSA Scientific Committee (2017) has advised that, where the overall evaluation of genotoxicity for a substance leaves no concerns for genotoxicity, Health Based Guidance Value (HBGV)s may be established. However, if concerns

for genotoxicity remain, establishing a HBGV is not considered appropriate and a Margin of Exposure (MoE) approach should be followed.

124. Considering the WoE for probabilities closer to either 70% or 95% that BPA does not present a genotoxic hazard by a direct mechanism, the CEP Panel concluded that probabilities close to 95% are more strongly supported by the evidence than probabilities close to 70% and, therefore, the balance of evidence allows a HBGV to be established.

Overall conclusions on genotoxicity

125. The analysis of the available literature data indicate that BPA does not induce gene mutations in bacteria. BPA induces DNA strand breaks, clastogenic and aneugenic effects in mammalian cells *in vitro*. Oxidative stress-related mechanism(s) are likely to be involved in this DNA damaging and clastogenic activity.

126. In contrast with consistent positive *in vitro* findings, the *in vivo* findings in several studies with high/limited reliability were inconsistent. The CEP Panel concluded that the evidence does not support an *in vivo* genotoxic hazard posed by BPA through direct interaction with DNA.

127. The CEP Panel concluded that it is unlikely to very unlikely that BPA presents a genotoxic hazard, the causes of which include a direct mechanism, and that the balance of evidence allows a HBGV to be established.

Questions to the Committee

i) Do Members have any comments on the approach taken by the EFSA panel to assess genotoxicity? Including the weight of evidence and uncertainty analyses?
ii) Do Members have any comments on the expert elicitation process used in the genotoxicity assessment?
iii) Do Members have any comments on the overall conclusions reached by EFSA?

Secretariat

February 2022

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Abbreviations

BP-1	Sulphonylbis(benzene-4,1-diyloxy)]diethanol
BP-2	4,4'-Sulphanediyldiphenol
BPA	Bisphenol A
BPAF	Bisphenol AF
BW	birth weight
CA	chromosomal aberrations
Cd	cadmium
CBMA	cytokinesis blocked micronucleus assay
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEP -	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
CHO cells	Chinese hamster ovary cells
DCFH-DA	Dichlorofluorescein Diacetate Assay
DBP	dibutyl phthalate
DDR	DNA damage response
E2	Oestradiol
ER	Oestrogen receptor
ECHA	European Chemicals Agency
GE	Ginger Extract
GSH	Glutathione
HBCD	hexabromocyclododecane

HBGV	Health Based Guidance Value
HFLF	Human fetal lung fibroblasts
HOC	health outcome category
HURP	Hepatoma Upregulated protein
LYC	lycopene
MDA	Malondialdehyde
MN	micronuclei
MTOCs	microtubule organizing centres
MoA	mode of action
MTD	Maximum Tolerated Dose
NAC	N-acetylcysteine
NDI	nuclear division index
OTM	olive tail moment
OP	4-tert-octylphenol
8-OHdG	8-hydroxydeoxyguanosine
SAC	spindle assembly checkpoint
SD	Sprague Dawley
PBMC	peripheral blood mononuclear cells
PCE	Polychromatic erythrocytes
PSO	pumpkin seed oil
ROS	reactive oxygen species
SOD	Superoxide Dismutase
TBARS	Thiobarbituric Acid Reactive Substances
TSH	Thyroid Stimulating Hormone

WG	working group
WoE	weight of evidence

Annex A

Evaluation of reliability of results of genotoxicity studies – general considerations

1. Reliability is defined as “evaluating the inherent quality of a test report or publication relating to preferably standardized methodology and the way that the experimental procedure and results are described to give evidence of the clarity and plausibility of the findings” (Klimisch *et al*., 1997).

2. In assigning the reliability score, the compliance with the Organization for European Economic Cooperation and Development (OECD) Test Guidelines (TGs) or standardized methodology and the completeness of the reporting as detailed below were considered.

3. The reliability scores were:

1) reliable without restriction : This includes studies or data from the literature or reports which were carried out or generated according to generally valid and/or internationally accepted testing guidelines (preferably performed according to Good Laboratory Practice (GLP)) or in which the test parameters documented are based on a specific (national) testing guideline (preferably performed according to GLP) or in which all parameters described are closely related/comparable to a guideline method.

2) reliable with restrictions: This includes studies or data from the literature or reports (mostly not performed according to GLP), in which the test parameters documented do not totally comply with the specific testing guideline, but are sufficient to accept the data or in which investigations are described which cannot be subsumed under a testing guideline, but which are nevertheless well documented and scientifically acceptable.

3) insufficient reliability: testing guideline, but are sufficient to accept the data or in which investigations are described which cannot be subsumed under a testing guideline, but which are nevertheless well documented and scientifically acceptable.

4) reliability cannot be evaluated: This includes studies or data from the literature, that do not give sufficient experimental details and that are only listed in short abstracts or secondary literature (books, reviews, etc.).

5) reliability not evaluated, since the study is not relevant and/or not required for the risk assessment (in case the study is reported for reasons of transparency only): The study is not relevant and/or not useful for the risk assessment.

Evaluation of relevance of results of genotoxicity studies -general considerations

4. The relevance of the study (high, limited or low) is based both on its reliability and on the relevance of the test results.

5. The relevance of the test results was mainly, but not exclusively, based on:

• Genetic endpoint (high relevance for gene mutations, structural and numerical chromosomal alterations as well as results obtained in an in vivo comet assay, which belongs to the assays recommended by the EFSA Scientific Committee (2011) for the follow-up of a positive in vitro result; lower relevance for other genotoxic effects). Other test systems although potentially considered of limited or low relevance may provide useful supporting information.

• Route of administration (*e.g.* oral *vs*. intravenous, intraperitoneal injection, subcutaneous injection, inhalation exposure) in case of in vivo studies.

• Status of validation (*e.g.* for which an OECD TG exists or is in the course of development, internationally recommended protocol, validation at national level only, no validation).

• Reliability and relevance of the test system/test design irrespectively of whether a study has been conducted in compliance with GLP or not.

• Information on BPA purity grade and/or the supplier. If only the supplier was available, the company's website was consulted to retrieve the purity grade, or the authors were contacted to ask for it. If none of the two information were reported or obtained, the relevance was considered low and the study was excluded from the WoE assessment.

6. Studies for which the relevance of the result was judged to be low were not considered further.

WoE approach

7. The WoE approach applied to the evaluation of genotoxicity data is based on EFSA Scientific Committee recommendations (EFSA Scientific Committee, 2011, 2017). As recommended by the EFSA Scientific Committee (EFSA Scientific Committee, 2011, 2017), a documented WoE approach for the evaluation and interpretation of genotoxicity data' has been applied, taking into account not only the quality and availability of the data on genotoxicity itself, but also all other relevant data that may be available. The main steps of the WoE approach applied in the genotoxicity assessment of BPA are described below.

Assembling of the evidence into lines of evidence of similar type

8. In a first step, the CEP Panel evaluated all available *in vitro* and *in vivo* studies addressing the three main endpoints of genotoxicity: gene mutations, structural and numerical chromosomal aberrations (CA) in addition to DNA damage endpoint (evaluated by Comet assay). The study results addressing each of these endpoints were grouped into lines of evidence. Only the studies of high and limited relevance were included.

9. Studies investigating the BPA MoA were considered, *e.g*. DNA oxidation, ROS (when genotoxicity was also investigated in the same study), DNA binding,

interference with proteins involved in chromosome segregation during cell division, modulation of expression of genes involved in DNA repair or in chromosome segregation and markers of DNA double strand breaks (DSBs) (e.g. γH2AX). Evidence from the mechanistic studies may support the lines of evidence for the genotoxicity endpoints

Weighting of the evidence

10. A quantitative method to weight the evidence was not considered appropriate due to the quantity and heterogeneity of the evidence to be integrated. A qualitative method based on expert judgment was applied. All studies evaluated for reliability and relevance (as described above) were listed in tables below). The evaluation of the studies of high and limited relevance was described in the opinion, including the conclusion for each line of evidence. The consistency of the evidence was assessed and presented in the opinion.

Integrating all the evidence

11. Integrating evidence from the MoA with lines of evidence from genotoxicity endpoints allows a reduction in the uncertainty on the potential genotoxicity. In case genotoxic effects were observed, evidence from the MoA may allow clarification if the genotoxicity is due to a direct or indirect mechanism.

Uncertainty analysis for genotoxicity including results

12. The purpose of the uncertainty analysis for genotoxicity was to assess the degree of certainty for the conclusion on whether BPA presents a genotoxic hazard by a direct mechanism (direct interaction with DNA), taking into account the available evidence and also the associated uncertainties. This overall question was divided into two sub-questions, which were assessed by three WG members with specialist expertise in genotoxicity assessment:
Sub-question 1: What is your probability (%) that there is a genotoxic hazard in humans from BPA?

Sub-question 2: If there would be a genotoxic hazard in humans from BPA, what is your probability that its causes include a direct mechanism?

13. When assessing the two sub-questions, the experts considered all the data they had reviewed for the genotoxicity assessment, including results from *in vitro* studies and animal models, taking into account their relevance to humans; the available data from human studies were considered not relevant.

14. The experts' judgements were elicited by the structured procedure described below:

15. The word 'include' in sub-question 2 was introduced to accommodate the possibility that both direct and indirect mechanisms could operate together.

16. The experts were provided with guidance on how to assess and express their probability judgements for the two questions. They were asked to consider all the data they had reviewed for the genotoxicity assessment, including results from *in vitro* studies and animal models, taking into account their relevance to humans; the available human data were considered not relevant.

17. The three experts first worked on the questions independently, based on the evidence they had already reviewed and evaluated for the opinion, and recorded their probabilities and the reasoning for their judgements in an excel template similar to that which was used for Question 1 in the uncertainty analysis for non-genotoxic endpoints. This was followed by a facilitated meeting, where the three experts presented their judgements and reasoning and discussed them together with the WG Chair. After the meeting, the three experts were invited to review and, if they wished, revise their judgements and reasoning in the light of the discussion.

18. Each expert's revised probabilities for the two sub-questions were multiplied to provide a probability for the overall question. This is appropriate because the second question is conditional on the first. The first sub-question provides a probability for BPA presenting a genotoxic hazard; the second question provides a

conditional probability that, if BPA presents a genotoxic hazard, there is a direct mechanism. So the product of these is a probability that both are true: that BPA does present a genotoxic hazard and that there is a direct mechanism. As the experts' probabilities were approximate (ranges), the calculation is done by interval arithmetic and the resulting probabilities are also approximate.

19. The three experts presented and discussed their revised judgements and reasoning in a facilitated meeting with the full WG. The WG discussed the results of the calculations combining the experts' probabilities for the two questions and expressed the conclusion of the WG both as a probability range and using verbal likelihood terms from the approximate probability scale, which is recommended by EFSA (EFSA Scientific Committee, 2018) for harmonised use in EFSA assessments. Finally, the WG discussed the implications of their conclusion for whether a TDI could be set for BPA or whether a Margin of Exposure approach was required.

20. Table 1 shows the revised judgements provided by the three experts together after sharing and discussing their initial judgements and reasoning. The third row of Table 1 shows their probabilities for the overall question, which were obtained by multiplying each expert's probabilities for the two sub- questions. These are their probabilities that BPA does present a genotoxic hazard and that there is a direct mechanism. The bottom row of Table 1 shows the complement of the probabilities in the third row, obtained by subtracting each probability from 100%. These are the experts' probabilities for the opposite outcome: that BPA does not present a genotoxic hazard by a direct mechanism. The fifth column of Table 1 shows the 'envelope' of the probabilities for the three experts, obtained by taking the lowest and highest probabilities in each row. These express the range of opinion across the three experts.

Table 1 Results of the uncertainty analysis for the genotoxicity assessment

	Expert A	**Expert B**	**Expert C**	**Envelope of three experts**	**Assessment (rounded values)***

Experts' probabilities that BPA presents a genotoxic hazard in humans (sub-questions 1)	70-90%	66-90%	70-90%	66-90%	66-90%
Experts' probabilities that, if BPA is genotoxic, there is a direct mechanism (sub-question 2)	10-33%	10-33%	20-30%	10-33%	10-33%
Calculated probabilities that BPA is genotoxic by a direct mechanism ((sub-question 1) x (sub-question 2)	7-29.7%	6.6-29.7%	14.27%	6.6-29.7%	5-30%
Calculated probabilities that BPA is not genotoxic by a direct mechanism (100% minus row above)	70.3%-93%	70.3%-93.4%	73-86%	70.3-93.4	70-95%

*The calculated probabilities were rounded to the nearest 5%. The experts probabilities of 33% and 66% were not changed because they correspond approximately to a 1 in 3 chance and a 2 in 3 chance, respectively.

Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021)

21. The results in Table 1 and the reasoning of the three experts were presented and discussed in detail at a facilitated meeting with the full WG. It was agreed to take the envelope of the 3 experts' results as the consensus of the WG, taking account of the available evidence and associated uncertainties. The WG also agreed that their consensus probability that BPA is genotoxic by a direct mechanism should be rounded to 5 – 30%, as shown in the right-hand column of Table 1, to take account that it is based on expert judgement and avoid the implied precision of the calculated

values. Similarly, the WG rounded their consensus probability that BPA is not genotoxic by a direct mechanism to 70 – 95%.

22. The width of the consensus probability range for BPA not being genotoxic by a direct mechanism, reflects the uncertainty of the three experts and the other WG members about the judgements on sub- questions 1 and 2. The WG discussed in more detail which lines of evidence tended to support probabilities in the lower end of this range, and which tended to support the upper end of the range (Table 2).

Table 2. Summary of lines of evidence supporting either lower or higher probabilities that BPA does not present a genotoxic hazard by a direct mechanism, within the range assessed by the WG (70-95%).

Evidence supporting probabilities closer to 95 %	• Consistent negative Ames tests • Indications of carcinogenic effects of BPA do not indicate direct genotoxic mechanism because only at very low doses and not higher doses (non monotonic), only after development exposure (up to weaning) and only in one target tissue • Reactive non-conjugated metabolites of BPA are observed in animals but not in humans • Effects only from repeated exposure, so might be secondly • Evidence for several indirect mechanisms
Evidence supporting probabilities closer to 70%	• Presence of uncharacterised DNA adducts • Mutational spectrum from whole genome assessment •

Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021)

23. It was concluded that it is Unlikely to Very Unlikely (5 – 30% probability) that BPA presents a genotoxic hazard, the causes of which include a direct mechanism (combining subquestion 1 and 2, see third row of Table 1). Accordingly, it was concluded that it is Likely to Very Likely (70 - 95% probability) that BPA either

presents a genotoxic hazard only through indirect mechanism(s) or is not genotoxic. The likelihood terms used in these conclusions are taken from the approximate probability scale, which is recommended by EFSA (Table 2 in EFSA Scientific Committee, 2018) for harmonised use in EFSA assessments.

24. The EFSA Scientific Committee (2017) has advised that, where the overall evaluation of genotoxicity for a substance leaves no concerns for genotoxicity, HBGVs may be established. However, if concerns for genotoxicity remain, establishing a HBGV is not considered appropriate and a Margin of Exposure (MoE) approach should be followed.

25. Considering the WoE for probabilities closer to either 70% or 95% that BPA does not present a genotoxic hazard by a direct mechanism (Table 2), the CEP Panel concluded that probabilities close to 95% are more strongly supported by the evidence than probabilities close to 70% and, therefore, the balance of evidence allows a HBGV to be established.

Weight of Evidence Studies

26. The following are tables summarising new *in vitro* and *in vivo* genotoxicity studies on BPA identified in the literature (2013 –2021) and studies considered in the 'Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs' (EFSA CEF Panel, 2015). Key : *Indicates that more than one assay is reported/indicates when papers belong to more than one table. **Indicates that both *in vitro* and *in vivo* assays are reported in the same paper

27. The studies have been evaluated based on the criteria described above in Annex A.

Bacterial reverse mutation assay

Table 1. Bacterial reverse mutation assay (OECD TG 471 was considered for the evaluation of reliability

Test system/Test object	Exposure conditions (concentration/ duration/metabolic activation)	Information on the characteristics of the test substance	Results	Reliability/ Comments	Relevance of the result	Reference
Bacterial reverse mutation assay Salmonella Typhimurium strains TA 98 and TA 100 *In vivo* micronucleus assay (Table 7)**	BPA 1–10 µmoles/plate with or without S9; 3 replicates	BPA (Tokyo Kasei Kogyo Co., Ltd) Purity 99% not reported in the study but available in the website of the company	Negative	Reliability: 2 Only 2 strains Data on negative controls subtracted (but not shown) No positive control	Limited	Masuda *et al*., 2005[1]**
Bacterial reverse mutation assay Salmonella	BPA 0, 6.25, 12.5, 25, 50, 100, 150 and 200 µg/plate for 48 h; with	BPA, purity 99% (Sigma Chemical Company)	Negative	Reliability: 2 Only 3 strains used	Limited	Tiwari *et al*., 2012[1]**

Typhimurium strains TA98, TA100, TA102 *In vivo* chromosomal aberration (Table 6) micronucleus assay (Table 7) comet assay (Table 8)**	or without S9; preincubation method					
Bacterial reverse mutation assay Salmonella Typhimurium strains TA98 and TA 100 In vitro comet assay (Table 5)*	BPA 0, 4, 20, 100, 500 µg/plate for 48 h (TA100) and 72 h (TA98); 3 replicates; with or without S9	BPA, purity >99% (Sigma-Aldrich)	Negative	Reliability: 2 Only 2 strains	Limited	Fic *et al*., 2013[1]*
Bacterial reverse mutation assay Salmonella	BPA 10–5000 µg/plate; 48 h incubation; with or without S9; preincubation	BPA (purity 99%)[2], was purchased from Tianjin Guangfu Fine	Negative	Reliability: 1	High	Xin *et al*., 2015*

Typhimurium strains TA1535, TA97, TA98, TA100 and TA102 In vitro chromosomal aberration (Table 3) micronucleus assay (Table 4) comet assay (Table 5) in CHO cells*	method in triplicates; 3 independent experiments	Chemical Research Institute (Tianjin, China)				
Bacterial reverse mutation assay Salmonella Typhimurium strains TA98 and TA100	BPA 0.1, 1, 10 and 100 µg/plate with or without S9; plate incorporation assay in triplicates; 2 independent experiments	BPA (Merck) Purity >97% not reported in the study but available on the website of the company	Negative	Reliability: 2 Only 2 bacterial strains used	Limited	Zemheri and Uguz, 2016
SOS/umuC assay in Salmonella Typhimurium TA1535 pSK1002	BPA 0, 1, 10, 100, 1000 µg/L, without or with metabolic activation (S9)	BPA (Sigma-Aldrich) Purity >97% not reported in the study but available on the	Negative	Reliability: 2 Non-standard test applied as a preliminary analysis of toxicity and mutagenicity	Limited	Balabanič *et al.*, 2021*

In vitro comet assay (Table 5)*		website of the company				

[1]Studies considered in the Scientific Opinion on the Risks to Public Health Related to the Presence of Bisphenol A (BPA) in
[2]Foodstuffs (EFSA CEF Panel, 2015) Information on BPA purity provided by the study authors on 11 October 2021, upon EFSA request
Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021)

In vitro gene mutation in mammalian cells

Table 2: In vitro gene mutation in mammalian cells

Test system/Test object	Exposure conditions (concentration/ duration/metabolic activation)	Information on the characteristics of the test substance	Results	Reliability/ Comments	Relevance of the result	Reference
Analysis of mutational spectra in immortalised human embryonic kidney cells HEK 293T using whole genome sequencing (WGS) DNA double strand breaks as measured using γH2AX immunofluorescence staining	100 µM for 24 h exposure and WGS of clonally expanded cells populations No metabolic Activation Cell viability analysed in HEK 293T cells, treated for 24 h with 0.1, 1 and 100 µM BPA; cells were stained with crystal violet and results reported as colony area percentage	BPA from TCI (B04 94) purity ≥ 99% not reported in the study but available on the website of the company	Positive Increased levels of single base substitutions, doublestrand breaks and small insertions/deletions in BPA-treated HEK 293T cells in comparison with DMSO-treated controls Single base substitutions (C>A transversions) in BPA treated	Reliability: 2 Although there is no TG for this type of study, the research was adequately conducted and reported However, there is uncertainty in the level of toxicity of the	Limited	Hu *et al*., 2021

			cells preferentially occur at guanines Mutations at A:T bp were also reported Colony formation assay: concentration dependent decrease in % colony area Concentration dependent increase in DNA double strand breaks as increased number of nuclei with > 5 γH2AX foci	BPA treatment		

Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021)

In vitro chromosomal aberrations test

Table 3: *In vitro* chromosomal aberrations test (OECD TG 473 was considered for the evaluation of reliability)

Test system/Test object	Exposure conditions (concentration/ duration/metabolic activation)	Information on the characteristics of the test substance	Results	Reliability/ Comments	Relevance of the result	Reference
Chromosomal aberrations and SCE assays CHO-K1 cell line Cytotoxicity: cellcycle delay 'recognised by the metaphases without differently staining sister chromatids'	BPA 0, 0.1 to 0.6 mM for 3 h followed by 27 h recovery 100 metaphases SCE: 50 metaphases Without metabolic activation	BPA, purity > 99% (Tokyo Kasei Kogyo Co., Ltd)	Positive Only in presence of severe Cytotoxicity Increased CA (0.5, 0.55, 0.6 mM, % of differently staining sister chromatids 29%, 11%, and 0%, respectively) Increased endoreduplications (0.45 and 0.55	Reliability: 3 Only short-term treatment; high level of cytotoxicity The recovery time exceeded the recommended (18–21 h) Cells recovered in	Low	Tayama *et al.*, 2008[1]*

In vitro comet assay (Table 5)*			mM) Increased frequency of cmitosis-like figures (above 0.3 mM) Increased SCE (0.4 and 0.5 mM)	the presence of BrdU		
Chromosomal aberration assay CHO cells Cytotoxicity: MTT assay Bacterial reverse mutation assay (Table 1) In vitro micronucleus assay (Table 4), comet assay (Table 5)*	BPA 0, 80, 100 and 120 μM for 24 h 500 metaphases/group; without metabolic activation MTT assay: BPA 0, 40, 80, 100 and 120 μM for 12 and 24 h	BPA (purity 99%)2, was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China)	Positive Increase of structural chromosomal aberrations from 80 μM, with significant decrease in cell viability (but not lower than 50%) MTT assay: increase of cell proliferation at 40 μM; cytotoxicity from 80 μM	Reliability: 2 No short-term Treatment No positive control	Limited	Xin *et al*., 2015*

Chromosomal aberration assay in: - MCF-7 human breast cancer line; - human amniocytes from male [oestrogen receptors (ER) negative] and from female (ER positive) Cytotoxicity: MTT test	BPA 0, 0.4, 1, 4, 40 and 100 µg/mL for 48 h; 200 cells analysed for each treatment (less at highest concentrations in amniocytes for high toxicity) Without metabolic activation MTT test: BPA 0, 0.4, 1, 4, 40, 100 and 400 µg/mL for 48 h.	BPA, no information on purity or the supplier company	Positive Increase of cells with chromosome aberrations (from 1 µg/mL) in all cell types; the increase in cells with aberrations was not clearly concentration related and decreased at the highest concentrations, possible due to cytotoxicity that was not concurrently evaluated; no clear association with ER expression In a preliminary evaluation of cytotoxicity by the MTT test, the IC50 of BPA was 100, 40	Reliability: 2 Cells scored less than recommended in OECD TG 473 No short-term treatment No positive control No concurrent control of toxicity	Low No information on source and purity of BPA	Aghajanpour-Mir *et al*., 2016

			and 4 µg/mL in MCF-7 and ER-negative (male) and ERpositive (female) amniocytes, respectively			
Chromosomal aberration assay Human peripheral blood lymphocytes from 5 female subjects In vitro micronucleus assay (Table 4)*	BPA 0, 0.20, 0.10, 0.05, 0.02 and 0.01 µg/mL for 24 h 1000 metaphases (200/subject)/concentration Without metabolic activation	BPA (Sigma-Aldrich) purity ≥97% not reported in the study but available on the website of the company	Positive Increase from 0.05 µg/mL (prevalence of chromatid breaks) No numerical aberrations	Reliability: 2 No short-term treatment	Limited	Santovito *et al.*, 2018*
Chromosomal Aberrations Mouse embryonic fibroblasts (MEF) In vitro comet	BPA 150 µM for 24 h or co-exposure with camptothecin (CPT) 25 metaphases/treatment were analysed Without metabolic activation	BPA (Sigma-Aldrich) purity ≥97% not reported in the study but available on the website of the company	Negative No significant increase in CA frequency Cytotoxicity of BPA alone was not measured but the	Reliability: 3 Single concentration tested; low number of metaphases analysed No short-term treatment	Low	Sonavane *et al.*, 2018*

assay (Table 5)*			authors refer to 150 µM as concentration with minimal toxic effect from a previous publication			
Chromosomal aberrations Human peripheral blood mononuclear cells (PBMC) Cell proliferation: MTT test Cell-cycle analysis: FACS γH2AX: western blot and FACS analysis	BPA 0, 25, 50, 100 nM, cells stimulated with PHA for 16h and then treated with BPA for 48 h 30 metaphases/treatment/subject (5 donors) MTT test: BPA 0, 5, 10, 25, 50, 100, 200 nM and BPA 25, 50, 100, 200 µM, cells were treated with or without PHA for 16 h and then treated with BPA for 24 and 48 h γH2AX: cells treated with PHA and then with BPA 50 nM for 24 h or 48 h (western blot) or only for 24 h (FACS analysis analysing T and B lymphocytes) Without metabolic activation	BPA (Merck) Purity ≥97% not reported in the study but available on the website of the company	Positive Increased number of aberrant cells, structural chromosomal aberrations and highly fragmented metaphases MTT test: - unstimulated PBMCs: decreased cell proliferation only at 200 µM at both 24 and 48 h PHA stimulated PBMCs: - increased cell proliferation	Reliability: 2 No positive Control No short-term treatment	Limited	Di Pietro *et al.*, 2020

			from 10 nM to 100 nM; - concentration-dependent decreased cell proliferation from 25 to 200 µM Effect on cell proliferation confirmed using cell-cycle analysis γH2AX (western blot): - increase of protein phosphorylation only at 24 h (BPA 50 nM) γH2AX (FACS): increase in CD3+ and in CD4+ T cells			
Chromosomal aberrations assay in human peripheral blood lymphocytes	BPA 0, 5, 10, 20 and 50 µg/mL for 24 and 48 h Mitomycin C (MMC) at 0.10 µg/mL 'was added to the negative and a positive controls and to each concentration and chemical groups as well' Without metabolic activation	BPA, no information on purity or the supplier company	No data on chromosome aberrations were reported	Reliability: 3 MMC added to all Treatments No mitogenic Stimulation	Low No information on BPA purity	Özgür *et al*., 2021

				No short-term treatment		

Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021)

In vitro mammalian cell micronucleus test

Table 4: *In vitro* mammalian cell micronucleus test (OECD TG 487 was considered for the evaluation of reliability)

Test system/Test object	Exposure conditions (concentration/ duration/metabolic activation)	Information on the characteristics of the test substance	Results	Reliability/ Comments	Relevance of the result	Reference
Cytokinesis block micronucleus assay (CBMN) AHH-1 cell line (human lymphoblastoid cells) Effects on mitotic spindle using staining: brilliant blue and safranin O; α- and γ-tubulin immunofluorescence staining	BPA 0, 1.5, 3.1, 6.2, 7.7, 9.2, 10.8, 12.3, 18.5, 24.6, 37 µg/mL for a complete cell cycle (22–26 h), Five experiments: average of 8082 cells scored for each treatment Effects on mitotic spindle: BPA 0, 4.2–14 µg/mL for 20 h (one cell cycle); 100 cells undergoing mitosis scored in each experiment, 3 experiments	BPA (Sigma-Aldrich) purity ≥97% not reported in the study but available on the website of the company	Positive increased BNMN cells from 12.3 µg/ml Aberrant mitotic divisions (multiple spindle poles)	Reliability: 1 BN cells % as parameter of cytotoxicity High number of analysed binucleated cells	High	Johnson and Parry, 2008[1]

	Without metabolic activation					
Micronucleus test in: - human umbilical vascular endothelial cells (HUVEC); - human colon adenocarcinoma (HT29) cell line Immunofluorescence analysis of cytoskeleton organisation of HUVEC cells with anti-α-tubulin and anti-γ-tubulin Apoptosis using TUNEL assay and cell viability using CellTiter-Blue assay	BPA 0, 44 nM and 4.4 µM, (i.e. 10 ng/mL and 1 µg/mL) for 72 h BPA 10 ng/mL and 1 µg/ml for 24 or 72 h CellTiter-Blue assay: BPA 10 ng/mL and 1 µg/mL for 24, 48 or 72 h Without metabolic activation	BPA, no information on purity or the supplier company	Positive in HUVEC cells: slight increase of MN frequency Negative in HT29 cells Multipolar spindles and microtubule misalignment associated with BPA exposure No effects on cell viability, proliferation and apoptosis in both cell lines	Reliability: 2 No analysis of cell proliferation; no positive control; no short-term treatment	Low No information on source and purity of BPA	Ribeiro-Varandas et al., 2013
Cytokinesis block micronucleus assay; bovine peripheral blood lymphocytes;	BPA 1×10−4, 1×10−5, 1×10−6 and 1×10−7 mol/L for 48 h	BPA (Sigma-Aldrich) Purity ≥97% not reported in the study	Positive concentration-related increase in MN	Reliability: 2 No short-term	Limited	Šutiaková *et al*., 2014

cell proliferation: nuclear division index (NDI)	Without metabolic activation	but available on the website of the company	frequency, statistically significant at the highest concentration; no effect on NDI at any concentration	treatment; bovine lymphocytes are not commonly used in the micronucleus test, and their use has not been validate. However the study appears to be adequately performed and reported		
Micronucleus assay CHO cells Cytotoxicity: MTT test Bacterial reverse mutation assay (Table 1) In vitro chromosomal aberration (Table 3) comet assay (Table 5)*	BPA 0, 80, 100 and 120 µM for 24 h, without cytochalasin B; 1000 cells were scored for each sample; 3 independent experiments Without metabolic activation MTT test: - BPA 0, 40, 80, 100 and	BPA (purity 99%)2, was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China)	Positive increase in MN frequency at 100 and 120 µM MTT assay: concentration-related decrease in cell viability from 100 µM	Reliability: 2 No short-term Treatment No positive control	Limited	Xin *et al*., 2015*

	120 µM for 12 and 24 h					
Cytokinesis-blocked micronucleus assay in murine macrophage RAW264.7 cells 1000 binucleated cells/concentration Cell viability: MTT test In vitro comet assay (Table 5)*	BPA 0, 3, 10, 30, or 50 µM for 24 h BPA 10 µM tested for MN assay and cell viability, in the presence or absence of pretreatment with N-acetyl-L-cysteine (NAC) at the concentration of 10 µM for 30 min Without metabolic activation MTT test: BPA 0, 3, 10, 30, or 50 µM for 12 or 24 h	BPA (Sigma-Aldrich) Purity ≥97% not reported in the study but available on the website of the company	Positive Concentration dependent increase in MN frequency from 10 µM In the presence of NAC, MN frequencyand cytotoxicity were statistically significantly reduced (see also data on ROS in Table 5) MTT test: concentration- and time-dependent decrease of cell viability	Reliability: 2 No short-term treatments; no positive controls; no data on cell proliferation	Limited	Huang *et al*., 2018*

Cytokinesis block micronucleus assay Human peripheral blood lymphocytes from 5 female subjects 1000 binucleated lymphocytes/subject (5000 binucleated cells per concentration) In vitro chromosomal aberrations assay (Table 3)*	BPA 0, 0.20, 0.10, 0.05, 0.02 and 0.01 µg/mL for 48 h Without metabolic activation	BPA (Sigma-Aldrich) Purity ≥97% not reported in the study but available on the website of the company	Positive Increase in MN frequency from 0.02 µg/mL. At 0.2 µg/mL 4-fold increase with respect to the vehicle control (DMSO) level No significant reduction of the CBPI value	Reliability: 2 No short-term treatment	Limited	Santovito *et al*., 2018*
Mitotic abnormalities and micronuclei evaluated in DAPI stained cells: - Hep-2 cells (human epithelial cells from laryngeal carcinoma); - MRC-5 cells (human lung fibroblasts) Cell viability using CellTiter-Blue assay, after	BPA 0.44 nM, 4.4 nM, 4.4 µM (0.1 ng/mL, 1 ng/mL, 1 µg/mL) for 48 h; 1000 cells scored for each treatment	BPA (Sigma) purity ≥97% not reported in the study but available on the website of the company	Positive Slight (two-fold) increase in MN frequency from BPA 4.4 nM in both cell lines Mitotic index: - in Hep-2 cells, no effects;	Reliability: 3 No short-term treatment Proliferation of the cell population not determined; extremely low % of mitosis is indicative of a very	Low	Ramos *et al*., 2019*

48 h exposure In vitro comet assay (Table 5)*			- in MRC-5 cells, statistically significant increase Cytotoxicity: no effects on cell viability	low rate of cell division, which is not appropriate to measure MN formation Protocol of MN assay not reported; no positive control		
Micronucleus assay in Chinese hamster V79-derived cell lines expressing various human CYP enzymes Micronucleus assay in C3A cells (human hepatoma cell line, endogenously express various CYP enzymes,	1) Micronucleus assay in V79-derived cell lines: - BPA 0, 40, 80, 160 µM for 9 h + 15 h; (recovery period); - 2000 cells analysed for each treatment 2) Micronucleus assay in: - V79-Mz, V79-hCYP1A1 cells: BPA 0 to	BPA (99.6%), AccuStandard Inc.	1) Micronucleus assay (9 h + 15 h): - Negative in V79-Mz; - Positive in V79-hCYP1A1 cells and in V79-hCYP1B1 cells; - Cytotoxicity: statistically significant decrease	Reliability: 2 Micronucleus method poorly described No short-term treatment	Limited	Yu *et al.*, 2020

including CYP1A1, 1A2, 1B1, 2E1, 3A4, and phase II metabolic enzymes, such as UGTs and SULTs) 2000 cells analysed for each treatment Cytotoxicity: CCK-8 Assay γ-H2AX in V79-Mz, V79-hCYP1A1 cells and in C3A cells; analysis using In-Cell Western Blot Immunofluorescence staining of CENP-B of MN induced in C3A cells	80 µM for 24 h + 0 h ; with or without ABT; - C3A cells: BPA 0 to 80 µM for 72 h + 0 h; with or without ABT or 7-HF 3) Micronucleus assay in C3A cells: BPA 0 to 5 µM for 72 h + 0 h, with or without KET or PCP (phase II enzyme inhibitors), an inhibitor of UGT1 and SULT1, respectively Immunofluorescence staining of CENP-B was applied Cytotoxicity performed for each test using the		at the highest concentrations 2) Micronucleus assay (24 h + 0 h): - Negative in V79-Mz; - Positive in V79-hCYP1A1 cells, effect abrogated by ABT 2)Micronucleus assay (72 h + 0 h): - Positive in C3A cells, effect abrogated by ABT or 7-HF; - Cytotoxicity: statistically significant decrease at the highest concentrations 3)Micronucleus assay			

	same testing conditions of the MN assay or of γH2AX analysis γH2AX: BPA 0, 10, 20, 40, 80, 160 µM for 9 h; ABT (1-aminobenzotriazole a CYP inhibitor) or 7-HF (a selective CYP1A1 inhibitor) were added from 2 h ahead of test compound exposure to the end of cell culture BPA 0, 10, 20, 40, 80, 160 µM for 9 h; ABT (1-aminobenzotriazole a CYP inhibitor) or 7-HF (a selective CYP1A1		in C3A cells (72 h + 0 h): - Positive - Effects enhanced by KET or PCP; statistically significant increase of MN negative for CENP-B staining, (clastogenic mechanism) Cytotoxicity: statistically significant increase in cell viability from 2.5 µM γH2AX: - increase in V79-Mz, in V79-hCYP1A1 cells and in C3A cells (concentration			

	inhibitor) were added from 2 h ahead of test compound exposure to the end of cell culture		dependent); effect reduced by ABT or 7-HF - Effects enhanced by KET or PCP; statistically significant increase of MN negative for CENP-B staining, (clastogenic mechanism) Cytotoxicity: statistically significant increase in cell viability from 2.5 µM γH2AX: - increase in V79-Mz, in V79-hCYP1A1 cells and in C3A cells (concentration			

			dependent); effect reduced by ABT or 7-HF			

Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021)

In vitro DNA damage (comet assay)

Table 5: In vitro DNA damage (comet assay)

Test system/Test object	Exposure conditions (concentration / duration/metabolic activation)	Information on the characteristics of the test substance	Results	Reliability/ Comments	Relevance of the result	Reference
Alkaline comet assay MCF-7 (oestrogen receptor (ER) positive) and MDA-MB-231 (ER negative) γH2AX foci using immunofluorescence in MCF-7 cells	MCF-7 cells exposure: - BPA 0, 0.1 10, 100 µM for 3 h; - BPA 100 µM for 1, 3, 24 h MDA-MB-231 cells exposure: - BPA 100 µM for 3, 24 h; 30 cells analysed (10 cells/slide) Immunofluorescence in	BPA (Wako Pure Chemicals Industries, Ltd) purity ≥99% not reported in the study but available on the website of the company	Positive MCF-7: increased comet tail length after 3 h at 10, 100 µM and after all exposure times at 100 µM MDA-MB-231: increased comet tail length after 3 and 24 h exposure times at 100 µM No toxicity in comet assays Induction of γH2AX foci in MCF-7 cells (10 µM) ER-positive	Reliability: 2 Only 30 cells were Analysed No positive control	Limited	Iso *et al*., 2006[1]

	MCF-7 cells: BPA 10 µM for 3 h Without metabolic activation		MCF-7 cells are more sensitive than ER-negative MDA-MB-231 cells to BPA-induced DNA damage			
Alkaline comet assay in CHO-K1 cell line In vitro chromosomal aberrations (Table 3)*	BPA 0, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 mM; 1 h exposure Positive control: H2O2 200 cells were scored Quantification of DNA damage: a score of 0–3 (mean score value = mean comet points/cell, comet points) Cell viability (trypan blue) Without metabolic activation	BPA, purity > 99% (Tokyo Kasei Kogyo Co., Ltd)	Positive Increased DNA strand breaks only at the highest concentration tested (0.7 mM)	Reliability: 3 Non-standard method of DNA damage quantification Data of cytotoxicity not clearly reported	Low	Tayama *et al*., 20081*

Alkaline comet assay HepG2 cells Cell viability: MTT test Bacterial reverse mutation assay (Table 1)*	BPA 0, 0.1, 1.0 and 10.0 µM for 4 and 24 h; 50 nuclei scored/treatment; at least 2 independent experiments; positive control: benzo[a]pyrene MTT test: 12.5, 25, 50, 100 µM for 24 h	BPA, purity >99% (Sigma-Aldrich)	Negative after 4 h of exposure Equivocal after 24 h exposure (no concentration related effect) No cytotoxicity was observed	Reliability: 2 Only 50 nuclei scored	Limited	Fic *et al*., 2013[1]*
Comet assay in rat INS-1 insulinoma cells Cell viability: Hoechst staining kit and trypan blue (apoptotic cells detection) Expression of nuclear	BPA 0, 25, 50, 100 µM for 24 h; or pretreatment with or without NAC (10 mM) for 1 h then BPA (100 µM) was added for 24 h; Without metabolic activation	BPA, purity 99% (Sigma-Aldrich)	Positive concentration related increase in tail DNA %, tail moment and tail length at 50 and 100 µM Significant decrease in tail DNA % in cells pre-treated with NAC No apoptotic cells	Reliability: 2 No positive control; results on cytotoxicity assessment are not reported	Limited	Xin *et al*., 2014

p53 and p-Chk2 (T68) proteins: western blotting Intracellular (ROS): DCFH-DA Glutathione (GSH): detection with ophthalaldehyde (OPT)	50 cells/slide were analysed; 3 experiments ROS and GSH analysis: BPA 0, 25, 50, 100 µM for 24 h ROS measurements also in cells pre-treated with NAC and exposed to 100 µM BPA		and 90% cell survival were used in comet assays (results are not shown) Increase of expression of DNA damage-associated proteins: p53 (from 50 µM) and p-Chk2 (at 100 µM) Levels of p53 are reduced by NAC pre-treatment Intracellular ROS: increase at 50 and 100 µM Decrease of ROS upon NAC pretreatment GSH: concentration related decrease			
Alkaline comet assay in CHO cells Cytotoxicity: MTT assay	BPA 0, 40, 80, 100 and 120 µM for 12 and 24 h; 100 cells were	BPA (purity 99%)2, was purchased from Tianjin Guangfu Fine Chemical	Positive Concentration related increase in (%) tail DNA from 80 µM with 12 h	Reliability: 2 No positive control	Limited	Xin *et al*., 2015*

Bacterial reverse mutation assay (Table 1) In vitro chromosomal aberration (Table 3) micronucleus assays (Table 4)*	analysed/sample. Without metabolic activation MTT assay: BPA 0, 40, 80, 100 and 120 µM for 12 and 24 h	Research Institute (Tianjin, China)	treatment, and at all tested concentrations after 24 h MTT assay: decrease in cell viability (but less than 50%) from 80 µM after 12 and 24 h			
Alkaline comet assay NIH3T3 cells (mouse embryonic fibroblast cell line) At least 100 nucleoids/sample Cytotoxicity: CCK-8 assay and LDH release	BPA 0, 2, 10 and 50 µM (0.4–11 µg/mL) for 24 h CCK-8 and LDH assays, ROS, 8-OHdG, γH2AX analysis: BPA 0, 2, 10 and 50 µM for 24 h At least 100 nucleoids of each sample were obtained in 3 independent experiments	BPA (Sigma-Aldrich) purity >97% not reported in the study but available on the website of the company	Positive increase tail DNA% at 50 µM Cytotoxicity: 80% cell survival at 50 µM γH2AX, ROS and 8-OHdG: increase at 50 µM	Reliability: 2 No positive control	Limited	Chen *et al*., 2016

Intracellular ROS: DCFHDA 8-OHdG: EpiQuick 8-OHdG DNA damage quantification direct kit γH2AX: immunofluorescence and western blot	without metabolic activation					
Alkaline comet assay in FRTL-5 rat immortalised thyrocyte cell line Cell proliferation (population doubling) Transcriptome analysis (microarray) Intracellular ROS: H2DCFDA	BPA 10^{-9} M for 6h, 48h, 96 h; 100 cells for each condition Transcriptome analysis and intracellular ROS: cells exposed for 1, 3, and 7 days to 10−9 M BPA Without metabolic activation	BPA (Sigma-Aldrich), purity ≥97% not reported in the study but available on the website of the company	Comet assay on BPA alone: Negative Intracellular ROS: statistically significant increase after 1 and 3 days exposure Transcriptome analysis: decreased expression of genes involved in DNA replication, recombination and repair (confirmed by RT-PCR) (after 3 and 7 days BPA exposure)	Reliability: 3 Comet assay: - one low concentration tested; - no positive control Small effects on transcription Large variations in DNA strand breaks in the comet assay	Low	Porreca *et al*., 2016

Comet assay MCF-7 cells (from human breast adenocarcinoma) Cell viability: CCK-8 assay Cell membrane damage: LDH ROS	BPA 0, 1, 10, 25, 50 µM; 24 h Positive control: tBHP (tert-butyl hydroperoxide); 300 cells from each sample were analysed Without metabolic activation CCK-8 assay: 0, 0.01, 0.1, 1, 10, 25, 50, 100 µM for 24 h LDH: 0, 1, 10, 25, 50, 100 µM for 24 h ROS: 0, 0.01, 0.1, 1, 10, 25, 50 µM for 24 h	BPA, purity > 98% (Tokyo Chemical Industry)	Positive Concentration dependent increase in % tail DNA from 10 µM Cell viability: at 1 µM increase in cell viability; inhibition of cell viability at concentrations from 10 µM (70%) to 100 µM (80%) Cell membrane damage: increase in LDH release in a concentration dependent manner from 10 µM ROS formation: concentration dependent increase in ROS levels No measurement at 50 µM, because of excessive cell death (90%)	Reliability: 3 Excessive toxicity at the analysed positive concentrations Results of positive control are not reported Comet methods are not described in detail	Low	Lei *et al*., 2017

Alkaline comet assay HepG2 cells Cytotoxicity: MTT assay Oxidative stress: intracellular ROS: DCFHDA in the same cells, also MDA and SOD	BPA from 10–8 to 10–6 mol/L (0.02–22.8 µg/mL) for 24 h MTT: BPA from 10–8 to 10–4 mol/L for 24 h ROS, MDA and SOD analysis: BPA from 10–8 to 10–4 mol/L for 6 h Positive control: H2O2	BPA purity > 99.8% (Sigma-Aldrich)	Positive Concentration related increase of tail DNA (%) MTT: concentration related increase of cytotoxicity; increase of ROS and MDA; decrease of SOD	Reliability: 2 No sufficient details on the comet method (e.g. number of cells analysed is not specified)	Limited	Li *et al*., 2017
Alkaline and neutral comet assay Human PBMC (3 donors) 450 cells/concentration Cytotoxicity using flow cytometry	Alkaline comet assay: - BPA 0.1, 1 and 10 µg/mL for 1 h; - 0.01, 0.1, 1 and 10 µg/mL for 4 h Neutral comet assay: - BPA 0.1, 1 and 10 µg/mL for 1 h	BPA, 99–99.5% purity (Sigma-Aldrich)	Positive Both alkaline and neutral comet DNA repair of DNA breaks: decrease at 60 min, but the repair was not complete after 120 min	Reliability: 2 unusual software for comet analysis No positive control	Limited	Mokra *et al*., 2017

	DNA repair: BPA at 10 µg/mL Without metabolic activation					
Alkaline comet assay and modified comet assay with Fpg enzyme in human peripheral blood lymphocytes	1 h exposure to BPA: 0.001 mM, 0.1 mM, 2.5 mM Three experiments	BPA (Sigma-Aldrich) Purity ≥97% not reported in the study but available on the website of the company	Positive Increase of % tail DNA, only at the first 2 concentrations tested With Fpg a higher increase of % tail DNA was observed at all concentrations, but not concentration related	Reliability: 3 Inadequate response of positive control; the use of hydrogen peroxide as positive control is not adequate for the comet + Fpg Number of cells scored in not specified	Low	Durovcova *et al*., 2018
Comet assay in human sperm cells	BPA 0, 1, 1.5, 2 and 3 µmol/L for 1 h Without metabolic	BPA (purity >99%, Sigma-Aldrich)	Negative No differences in % tail DNA between control samples and BPA-treated cells at all concentrations	Reliability: 3 Test not validated and not adequate for	Low	Sharma *et al*., 2018**

Cell viability measured with a Nucleocounter NC 3000 In vivo comet assay (Table 8)**	activation Each concentration was scored in 3 independent experiments and 2 replicates of each experiment 600 cells were scored/concentration Cell viability: BPA from 0 to 5 µmol/L		tested Cell viability assay: concentrationdependent decrease in cell viability from 3 µmol/L (reduced cell viability to 60%)	cryopreserved samples		
Comet assay in human bronchial epithelial BEAS-2B cells Cytotoxicity: MTS assay after 24 h treatment γ-H2AX foci using immunofluorescence	BEAS-2B cells were exposed to BPA 200 µM for 24 h MTS assay: 12.5 to 200 µM; tests performed in triplicates and for at least 3 independent times Without metabolic	BPA (Sigma-Aldrich) purity ≥97% not reported in the study but available on the website of the company	Increase of DNA damage, but no quantitative data are reported MTS assay: - concentrationdependent cytotoxic effect; - cytotoxicity at 200 µM: 84.7 ± 2.1%; γ-H2AX: BPAinduced phosphorylation BPA-induced also	Reliability: 3 Only one concentration tested, which resulted in high cytotoxicity Comet assay results not reported in detail, (no quantitative data)	Low	George and Rupasinghe, 2018

Intracellular ROS: DCF proteins involved in the DNA damage response (p-ATM, p-ATR, p-Chk1, p-p53) using western blot	activation		phosphorylation of ATM/ATR complex and triggered Chk1 and p53 proteins Statistically significant increase of ROS	No positive control		
Comet assay in TM3 murine Leydig cells Cell viability: MTT assay Real-time cell growth kinetics [cellular index (CI)] Cell-cycle analysis (PI, FACS analysis)	BPA 0, 1, 10 and 100 µM for 3 h; cell viability analysed with trypan blue exclusion method; Positive control: doxorubicin; 250 nucleoids were analysed in each repetition (3 experiments) Without metabolic	BPA (Sigma-Aldrich) purity ≥97% not reported in the study but available on the website of the company	Negative No increase in damage index (DI) Cell viability was evaluated using trypan blue exclusion method, and only treatments with an index greater than 80% were considered (results not shown) Cell viability: statistically significant and concentrationrelated decrease	Reliability: 3 Results are reported as damage index (not a standard parameter)	Low	Gonçalves *et al*., 2018

Morphological analysis of cell death: chromatin staining with the Hoechst 33342 dye	activation BPA concentrations for MTT assay and real-time cell growth kinetics: 0, 0.5, 1, 5, 10, 50, 100, 250, 500 µM MTT assay exposure: 24 or 48 h Real-time cell growth kinetics: measurement every 30 min for 96 h Cell-cycle analysis, chromatin staining: BPA 0, 1, 10 and 100 µM for 24 or 48 h		from 5 and from 50 µM after 24 and 48 h exposure, respectively CI: TM3 cells exhibited a decrease in their CI after 34 h of exposure at concentrations from 10 µM BPA 100, 250 and 500 µM decreased CI within a few hours of exposure Cell-cycle analysis: BPA 100 µM induced an increase in the sub-G1 phase cell population No other effects induced in the distribution of TM3 cells in the G0 + G1, S, and G2 + M phases Morphological analysis of cell death: increase in chromatin staining			

			upon exposure to BPA 100 µM for 24 or 48 h			
Alkaline comet assay with repair enzymes [with DNA glycosylases, i.e. endonuclease III (Nth) and human 8-oxoguanine DNA glycosylase (hOGG1)] Oxidised purines and pyrimidines Human PBMC 300 comets from 2 independent experiments Cell viability: flow cytometry	BPA 0, 0.01, 0.1 and 1 µg/mL for 4 h and 0, 0.001, 0.01 and 0.1 µg/mL for 48 h Positive control: H2O2 (2 blood donors) Without metabolic activation	BPA, 99–99.5% purity (Sigma-Aldrich)	Positive After 4 h incubation: - statistically significant and concentrationdependent oxidative damage to purines (from 0.01 µg/mL) and to pyrimidines (from 0.1 µg/mL) After 48 h incubation: - concentration-dependent oxidative DNA damage to purines (from 0.001 µg/mL) and to pyrimidines from (0.01 µg/mL) Statistically significant differences for DNA damage between 4 h and 48 h exposure at the highest	Reliability: 2 No appropriate positive control unusual software for comet analysis	Limited	Mokra *et al*., 2018

			concentrations tested (0.01 and 0.1 µg/mL) Cell viability: no significant changes			
Alkaline comet assay (CometChip platform) in mouse embryonic fibroblasts (MEF) Analysis of γH2AX (immunofluorescence) In vitro chromosomal aberrations test (Table 3)*	BPA 150 µM for 24 and 48 h (24 h for γH2AX), or co-exposure with camptothecin (CPT) Data of 4 replicates, each with 1500 ± 300 comets Without metabolic activation	BPA (Sigma-Aldrich) purity ≥97% not reported in the study but available on the website of the company	Negative No significant increase in the % tail DNA No significant increase in the percentage of γH2AX-positive nuclei	Reliability: 3 No positive controls, no sufficient details on the methods applied; single concentration; cytotoxicity not evaluated	Low	Sonavane *et al*., 2018*
Comet assay in murine macrophage RAW264.7 cells Cell viability: MTT assay	BPA 0, 3, 10, 30, or 50 µM for 24 h; no positive control; a minimum of 50 cells/slide were analysed	BPA (Sigma-Aldrich) purity ≥97% not reported in the study but available on the website of the company	Positive Increase in tail moment and tail length in a concentrationdependent manner	Reliability: 2 No positive control	Limited	Huang *et al*., 2018*

Intracellular ROS level: semiquantitative DCFHDA fluorescence assay Assessment of the antioxidative enzymes activities: CAT, SOD, and GPx In vitro micronucleus assay (Table 4)*	MTT assay: BPA 0, 3, 10, 30, or 50 µM for 12 or 24 h DCFH-DA assay and assessment of antioxidative enzymes activities: - BPA 0, 3, 10, 30, or 50 µM for 24 h Without metabolic activation		starting from 10 µM of BPA Cytotoxicity: concentration- and time-dependent decrease of cell viability BPA-induced ROS generation and reduced antioxidative enzyme activities from 10 µM			
Comet assay and comet modified with FpG In cryopreserved: - Hep-2 cells (human	BPA 0.44 nM, 4.4 nM, 4.4 µM for 48 h; Hep-2 cells: 300 cells analysed for each treatment MRC-5 cells: 100 cells analysed for each	BPA (Sigma) purity ≥97% not reported in the study but available on the website of the company	Inconclusive	Reliability: 3 Comet assay is not validated and recommended for testing cryopreserved cell samples	Low	Ramos *et al*., 2019*

epithelial cells from laryngeal carcinoma); - MRC-5 cells (DNA damage responsive cell line, human lung fibroblasts) Cell viability: CellTiter-Blue assay In vitro micronucleus assay (Table 4)*	treatment Cell viability: BPA 0.44 nM, 4.4 nM, 4.4 µM, 48 h exposure in both Hep-2 and MRC-5 cells			No positive control		
Comet assay in sperm cells from Sprague Dawley rats Analysis: ROS, LPO, SOD In vivo comet assay	BPA 0, 1, 10, and 100 µg/L for 2 h No positive control Without metabolic activation	BPA (99% purity) Santa Cruz Biotechnology	Positive Increase of tail DNA% only at 100 µg/L BPA increased SOD, ROS, TBARS [thiobarbituric acid reactive substances (TBARS) as an index of LPO] only at 100	Reliability: 3 The study was performed following a nonstandard, neutral protocol and unusual evaluation	Low	Ullah *et al*., 2019**

(Table 8)**			µg/L	of comets based on the analysis of microphotographs. No positive control		
Comet assay in Marc-145 cells (rhesus monkey embryo renal epithelial cells) Cytotoxicity: MTT and LDH assays Intracellular ROS levels: DCFH-DA Lipid peroxidation: - TBARS; - SOD activity and GSH content	BPA 10–6 to 10–3 M for 24 h; 50 cells from each of 6 independent experiments were analysed MTT assay: BPA 10–6 to 10–1 M for 24 h; DCFH-DA and TBARS assays: BPA 10–6 to 10–3 M for 24 h; SOD activity and GSH content: BPA 10–6 to 10–3 M for 24 h Without metabolic	BPA (purity > 99%) Sigma-Aldrich	Positive Increase in % tail DNA, tail length and tail moment (10–6 - 10–3 M); Cytotoxicity: concentrationrelated increase; excess of toxicity at 10–3 and 10–4 M BPADCFH-DA, TBARS assays: - concentrationrelated increase of ROS and lipid peroxidation; - SOD activity and GSH content: concentrationrelated decrease	Reliability: 2 No positive control	Limited	Yuan *et al.*, 2019

	activation					
Alkaline comet assay and Fpg modified comet assay RWPE-1 cells [human papilloma virus 18 (HPV18) immortalised, non-tumorigenic prostatic cell line] Cell viability: modified MTT assay and trypan blue exclusion Enzymatic and nonenzymatic antioxidants:	BPA 0, 45 µM (IC20) for 24 h 450 comets analysed/treatment; experiments in triplicates Cell viability: 0, 50, 100, 200, 300, 600 µM for 24 h Enzymatic and nonenzymatic antioxidants: BPA 0, 45 µM (IC20) for 24 h Without metabolic activation	BPA (>99% pure)	Positive Comet assay: increase (2.5-fold) in tail intensity (at IC20 BPA) Fpg modified comet: increase in tail intensity Cell viability: decrease in cell viability (IC20 45 µM) Enzymatic and nonenzymatic antioxidants: decrease in: - GPx1 and SOD activity (29% and 24% respectively); - TAOC levels (20%); increase in: - GR activity (4.5-fold); - total GSH level (30%)	Reliability: 2 One concentration tested No positive control No metabolic activation	Limited	Kose *et al.*, 2020

analysis of GPx, GR, SOD, GSH and TAOC levels						
Comet assay in HepG2 cells (human hepatocellular carcinoma cell line) Cell viability: MTT test SOS/umuC assay (Table 1)*	BPA 0, 1, 10, 100 and 1000 µg/L, for 4 and 24 h; 3 independent experiments; 50 nuclei analysed/treatment MTT test: BPA 0, 1, 10, 100 and 1000 µg/L, for 24 h	BPA (Sigma-Aldrich) purity >97% not reported in the study but available on the website of the company	Positive increase of % tail DNA from 10 µg/L at both 4 h and 24 h exposure MTT test: no effects on cell viability	Reliability: 2 Low number of nuclei analysed	Limited	Balabanič *et al*., 2021*

Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021)

In vivo chromosomal aberrations assay

Table 6: *In vivo* chromosomal aberrations assay (OECD TG 475 was considered for the evaluation of the reliability)

Test system/Test object	Exposure conditions (concentration/ duration/metabolic activation)	Information on the characteristics of the test substance	Results	Reliability/ Comments	Relevance of the result	Reference
Chromosomal aberration assay in bone marrow Swiss albino mice Six animals (3 females and 3 males)/group (control and BPAtreated animals) 100 metaphases were scored per animal Mitotic effects In vivo micronucleus	BPA 0, 10, 50 and 100 mg/kg bw; 2% gum acacia was used as the suspending medium for BPA Single oral dose administered by gavage Sampling of bone marrow at 6, 24, 48 and 72 h Cumulative dose level: 10 mg/kg bw for 5 consecutive days Sampling of the bone marrow 24 h after the	BPA, purity 98% (Loba Chemie, Mumbai, India)	Negative No significant increase of structural chromosomal aberrations Significant increases in the frequencies of gaps at all doses at 48 and 72 h sampling time and at 50 and 100 mg/kg bw at the 24 h sampling time C-mitotic effects through increases of mitotic indices and decrease in anaphase for both higher dose	Reliability: 2 Low number of animals/sex, but in total 6 animals/group Low number of metaphases scored, treatment with colchicine shorter (1.5 h) than recommended (5–6 h)	Limited	Naik and Vijayalaxmi, 2009[1]*

assay (Table 7)*	last administration of BPA		level at 24, 48 and 72 h sampling times			
Chromosomal aberration in bone marrow Holtzman rats Ten animals (5 females and 5 males)/group (control and BPA-treated animals) Analysis of 100 metaphases per animal In vivo micronucleus assay (Table 7)* and comet assay (Table 8)* Bacterial reverse	BPA 0, 2.4 µg, 10 µg, 5 mg and 50 mg/kg bw administered orally once a day for 6 consecutive days; BPA dissolved in distilled ethyl alcohol and diluted with sesame oil Sampling of the bone marrow 24 h after the last administration of BPA	BPA, ~99% purity (Sigma Chemical Company)	Positive Dose-related increase of structural chromosomal aberrations starting from 10 µg	Reliability: 2 Mitotic index as a measure of cytotoxicity not determined	Limited	Tiwari *et al*., 2012[1]*,**

mutation assay (Table 1)**						

Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021)

In vivo micronucleus assay

Table 7: *In vivo* micronucleus assay
(OECD TG 474 was considered for the evaluation of the reliability)

Test system/Test object	Exposure conditions (concentration/ duration/metabolic activation)	Information on the characteristics of the test substance	Results	Reliability/ Comments	Relevance of the result	Reference
Micronucleus assay Male ICR mice Peripheral blood reticulocytes (1000/animal analysed, 5 mice per group)	228 mg/kg bw of BPA dissolved in DMSO, once by gavage; controls received vehicle alone Peripheral blood collected at 24, 48 and 72 h after administration	BPA purity >99% (Tokyo Kasei Kogyo Co., Ltd)	Inconclusive (negative with no demonstration of bone marrow exposure) No increase of micronucleated reticulocytes at any sampling time Cytotoxicity was not evaluated	Reliability: 2 Single dose tested, although relatively high; 1000 scored reticulocytes/animal instead of 2000 as in OECD TG 474 (1997)	Low	Masuda *et al*., 2005[1]**

Bacterial reverse mutation assay (Table 1)**				No positive control		
Micronucleus assay in bone marrow Male mice (102/ElxC3H/El)F1 (5 animals per group)	BPA 0, 0.002, 0.02 and 0.2 mg/kg bw oral gavage on 2 days Cells collected 24 h after last administration 2000 polychromatic erythrocytes (PCE) were scored per animal	BPA (Sigma-Aldrich) purity >97% not reported in the study but available on the website of the company	Inconclusive (negative with no demonstration of bone marrow exposure) No induction of micronuclei in the bone marrow polychromatic erythrocytes	Reliability: 2 No positive control; very low doses applied	Low	Pacchierotti *et al*., 2008[1]
Cytogenetic analyses of oocytes and zygotes in female C57Bl/6 mice Assessment of meiotic delay in spermatocytes by	Acute exposure: 0.2 or 20 mg/kg Sub-acute exposure: 0.04 mg/kg for 7 days by gavage Sub-chronic exposure: 0.5 mg/L for 7 weeks in drinking water 0.2 mg/kg bw starting on day 8 after BrdU,	BPA (Sigma-Aldrich)	Negative No significant induction of hyperploidy or polyploidy in oocytes and zygotes in any treatment condition No delay of meiotic divisions No induction of hyperploidy or	Reliability: 2 This study was adequately planned, performed and reported, even though specific guidelines for the effects in germ cells are not available	Limited	Pacchierotti *et al*., 2008[1]

BrdU incorporation and aneuploidy in epididymal sperm by multicolor FISH in male 102/ElxC3H/El)F1 mice (5 mice per dose)	for 6 consecutive days BPA 0, 0.002, 0.02 and 0.2 mg/kg for 6 consecutive days		polyploidy in epididymal sperms	No positive control Very low doses for the analysis of sperm aneuploidy		
Micronucleus in bone marrow Swiss albino mice Six animals (3 females and 3 males)/group (control and BPAtreated animals);	BPA 0, 10, 50 and 100 mg/kg bw; 2% gum acacia was used as the suspending medium for BPA Single oral dose administered by gavage sampling of bone marrow at 6, 24, 48 and 72 h Cumulative dose level: 10 mg/kg bw for 5 consecutive days	BPA purity 98% (Loba Chemie, Mumbai, India)	Negative No significant decrease of PCE/NCE ratio Significant increase of gaps and C-mitoses	Reliability: 2 Low number of animals/sex in each group, but in total 6 animals/group	Limited	Naik and Vijayalaxmi, 20091*

2000 PCE/anima l In vivo chromoso mal aberration (Table 6)*	Sampling of the bone marrow 24 h after the last administration of BPA					
Micronucle us in bone marrow Male Sprague Dawley rats 8 rats/group (control and BPA-treated animals) In vivo comet assay (Table 8)*	BPA 0, 200 mg/kg bw per day for 10 days Orally via drinking water Bone marrow processed at the end of treatment	BPA (Sigma-Aldrich) purity >97% not reported in the study but available on the website of the company	Inconclusive (negative with no demonstration of bone marrow exposure) No data on bone marrow toxicity are reported	Reliability: 2 Exposure of the bone marrow not demonstrated Single dose tested No positive control	Low	De Flora *et al*., 2011[1]*
Micronucle us in bone marrow Holtzman rats Ten animals (5	BPA 0, 2.4 µg, 10 µg, 5 mg and 50 mg/kg bw per day administered orally for 6 consecutive days	BPA, ~99% purity (Sigma Chemical Company)	Positive Dose-related increase of MN-PCE starting from 10 µg/kg bw per day	Reliability: 2 Inappropriate staining	Limited	Tiwari *et al*., 2012[1]*,**

females and 5 males)/group (control and BPA-treated animals) In vivo chromosomal aberration (Table 6)* Comet assay (Table 8)* Bacterial reverse mutation assay (Table 1)**	Sampling of the bone marrow 24 h after the last administration of BPA Analysis of 2000 PCE					
Micronucleus test in peripheral blood reticulocytes and in bone marrow of	BPA 5, 10, or 20 mg/kg bw per day for 2 weeks in drinking water Animals were sacrificed 24 h after the end of treatment	BPA, no information on purity or the supplier company	Positive in reticulocytes at 10 and 20 mg/kg bw after 2 weeks of exposure Negative in reticulocytes after 1 week of treatment	Reliability: 2 No criteria for scoring micronuclei were described No positive control	Low No information on source and purity of BPA	Gajowik *et al*., 2013*

Pzh:Sfis female mice No. of animals/group: 9 in control, 6 in BPA 5 mg/kg bw, 8 in BPA 10 mg/kg bw, 6 in BPA 20 mg/kg bw; 1000 reticulocytes or PCE were scored In vivo comet assay (Table 8)*	Blood was collected at 1 and 2 weeks of exposure		Negative in bone marrow			
Micronucleus test in bone marrow cells Adult male Wistar	Oral administration of 5 µg, 50 µg and 100 µg BPA/100 g bw once a day for 90 days, sacrifice and sampling of bone	BPA (<99% pure) purchased from Sigma-Aldrich, diluted in olive oil	Positive Increases (2–3-fold at the highest dose) in the frequency of	Reliability: 3 Major limitation in data presentation and analysis: low number of	Low	Srivastava and Gupta, 2016 [

albino rats Ten animals per group	marrow on the 91th day		micronuclei in polychromatic erythrocytes and normochromatic erythrocytes Statistical significance of the difference with negative controls not determined No decrease in PCE/NCE ratio	scored cells per animal lack of historical control data		
Micronucleus test in bone marrow Male Swiss albino mice, 10 animals/group; analysis of 2000 PCE/animal In vivo comet assay (Table 8)*	50 mg/kg bw, orally once a day for 28 days Sampling of the bone marrow at the end of treatment	BPA, purity ≥ 99%, (Sigma-Aldrich)	Positive Increase in the mean values of MNPCEs (66.40 ± 9.94 vs 10.40 ± 2.96) Cytotoxic (reduction in the ratio of PCE/NCE compared to control)	Reliability: 2 No positive control only one dose	Limited	Fawzy *et al*., 2018*

Micronucleus test in bone marrow Male Wistar rats; 6 animals/group Analysis of 2000 PCE for MN scoring and of 200 cells for PCE/NCE Ratio Lipid peroxidation: serum level of malondialdehyde (MDA) (8-OHdG) in urine In vivo comet assay	0, 50 and 100 µg/kg bw per day, 4 weeks, by gavage Sampling at the end of treatment	BPA (Sigma-Aldrich) purity >97% not reported in the study but available on the website of the company	Positive Significant dose-related increase (up to 3-fold) in the mean values of MNPCEs compared with control Cytotoxic (a weak statistically significant decrease in PCE/NCE ratio); dose-related increase of MDA in blood and of urinary 8-OHdG levels	Reliability: 2 No positive control only 2 doses	Limited	Panpatil *et al*., 2020*

(Table 8)*						

Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021)

In vivo DNA damage

Table 8: *In vivo* DNA damage (comet assay, OECD TG 489 was considered for the evaluation of the reliability)

Test system/Test object	Exposure conditions (concentration/ duration/metabolic activation)	Information on the characteristics of the test substance	Results	Reliability/ Comments	Relevance of the result	Reference
Comet assay in peripheral blood lymphocytes Sprague Dawley rats 8 rats/group (control and BPA-treated animals); 100 nuclei were scored	200 mg/kg bw for 10 consecutive days, orally via drinking water Sampling at the end of treatment	BPA (Sigma-Aldrich) purity >97% not reported in the study but available on the website of the company	Negative	Reliability: 2 Tail moment, used as only parameter to report the results for the comet assay, is not recommended by the Comet international Committee; single dose tested; no positive control	Limited	De Flora *et al*., 2011[1]*

In vivo micronucleus assay (Table 7)*						
Comet assay in peripheral whole blood cells of Wistar rats (6 animals/group BPAtreated animals; 5 animals in the control group; 3 animals in the positive control group)	0, 125 and 250 mg/kg bw; oral administration (gavage) for 4 weeks Positive control: MMS (i.p., sampling after 24 h); 50 cells were analysed on each replicated slide	BPA purity > 99% (Merkolab Chemistry)	Positive Increase of both tail length and tail moment at 250 mg/kg bw	Reliability: 3 Inappropriate presentation and evaluation of results Group mean tail length and tail moment values, rather than the means of animal median values (OECD TG 489) Sampling time, and frequency of administrations not stated	Low	Ulutaş *et al*., 2011[1]
Comet assay in blood lymphocytes Holtzman rats	2.4 µg, 10 µg, 5 mg and 50 mg/kg bw per day administered once a day	BPA, ~99% purity (Sigma Chemical Co.)	Positive Dose-related increase starting from 10 µg/kg bw per day	Reliability: 2 Inappropriate sampling time Low number of nucleoids scored	Limited	Tiwari *et al*., 2012[1]*,**

Ten animals (5 females and 5 males)/group (control and BPAtreated animals); analysis of 50 nucleoids/animal Plasma concentrations of 8-hydroxydeoxyguanosine (8-OHdG), lipid peroxidation (MDA) and glutathione activity In vivo micronucleus	for 6 consecutive days Sampling 24 h after the last administration of BPA		Significant increase in plasma concentration of 8-OHdG only at 50 mg/kg bw per day Dose-related increase of MDA and decrease of glutathione in liver Inconsistent results of 8-OHdG with comet assay			

assay (Table 7)* chromosomal aberrations assay (Table 6)* Bacterial reverse mutation assay (Table 1)**						
Comet assay in bone marrow, spleen, liver and kidney and germ cells Male Pzh:SFIS mice; 5 animals/group; 100 cells were analysed	0, 5, 10, 20 or 40 mg/kg bw Orally in drinking water Daily for 2 weeks Animals were sacrificed 24 h after the last treatment	BPA, no information on purity or the supplier company	Positive Increases of DNA tail moment in bone marrow, spleen, kidney and lung cells at any dose level without a clear dose response No increase of tail moment was detected in liver cells In sperm cells increase of	Reliability: 3 No information on purity; drinking water consumption (containing BPA) not measured, inadequate sampling time, poor study report; tail moment, used as only parameter to report the results for the	Low	Dobrzyńska and Radzikowska, 2013[1]

			tail moment: at all doses 24 h after the end of exposure; at the 2 highest doses 5 weeks after the end of treatment	comet assay, is not recommended by the Comet International Committees		
Alkaline comet assay in epididymal sperm of Holtzman rats In vivo dominant lethal mutations in male rats (Table 9)*	Oral gavage of 10 µg/kg bw and 5 mg/kg bw BPA dissolved in ethyl alcohol and diluted in sesame oil, for 6 consecutive day	BPA ~99% purity (Sigma Chemical Co.)	Positive Significant increase in the sperm DNA damage at 5 mg/kg bw	Reliability: 3 Comet assay is not considered appropriate to measure DNA strand breaks in mature germ cells due to the high and variable background levels in DNA damage in this cell type (OECD TG 489); moreover, the sampling time, i.e. 8 weeks after last treatment, is	Low	Tiwari and Vanage, 2013[1]*

				inappropriate for in vivo comet assay		
Comet assay in lung, spleen, kidneys, liver and bone marrow of Pzh:Sfis female mice No. of animals/group 9 in control, 6 in BPA 5 mg/kg bw, 8 in BPA 10 mg/kg bw; 6 in BPA 20 mg/kg bw 100 nucleoids scored/animal	BPA 5, 10, or 20 mg/kg bw/day for 2 weeks in drinking water Sampling 24 h after the end of treatment	BPA, no information on purity or the supplier company	Positive in lung at 5 and 10 mg/kg Negative in spleen, kidneys, liver and bone marrow	Reliability: 2 Inappropriate sampling time, tail moment, used as only parameter to report the results for the comet assay, is not recommended by the Comet International Committees	Low No information on source and purity of BPA	Gajowik *et al*., 2013*

In vivo micronucleus assay (Table 7)*						
Alkaline comet assay in brain cells of KM male mice; (11 animals/group); 200 cells for each group analysed	BPA 0.5, 50 and 5000 µg/kg bw (daily dose, diluted in tea oil, by gavage) for 8 weeks After 8 weeks of exposure, mice were sacrificed and the brain samples were immediately removed The tail DNA%, tail length and tail moment were measured using CASP comet analysis software Based on the DNA percentage of the tail intensity, the damage	BPA from Sigma-Aldrich (HPLC grade) purity >97% not reported in the study but available on the website of the company	Positive Significant increase of damaged cells from 23.0% in the control group to 47.3%, 66.6% and 72.5% in the low-, medium and high-exposed groups Severity of DNA damage, expressed as arbitrary units (AUs), increased with AUs of 0.28 in the control to AUs of 0.59, 0.96 and 1.28 in the low, medium and highly exposed	Reliability: 2 DNA damage was evaluated using arbitrary units and considering the distribution of DNA damage in the cell population analysed (n = 440), rather than using median animals data as the statistical unit, as recommended in OECD TG 489	Limited	Zhou *et al*., 2017

	level was divided into 5 grades Arbitrary units computed with the score of DNA damage in analysed cells were used to express the DNA damage		groups, respectively			
Comet assay in liver female Wistar rats; (7 animals/group) Serum biochemical analysis: ALT, ALP, TP, Alb, GGT, TC, Triglycerides, HDL; LDL	7 animals/group: control (corn oil) BPA 10 mg/kg bw; daily administration via gavage for 30 days Sampling at the end of treatment	BPA (Sigma-Aldrich) purity >97% not reported in the study but available on the website of the company	Positive: increase of tail DNA % BPA-induced: - increase of ALT, ALP, GGT, TC, LDL, MDA, caspase-3; - decrease of Alb, TP, GPx, SOD, CCYPR450 Histopathological analyses showed deleterious hepatic changes ranging from hepatocytes' vacuolisation and eccentric	Reliability: 3 Use of frozen tissues; without a positive control; a single dose applied; toxic effects in liver	Low	Abdel-Rahman et al., 2018

Hepatic antioxidants and lipid peroxidation level: GPx, SOD, MDA CYPR450 (ELISA) Histopathology Immunohistochemical evaluation of caspase-3			nuclei to focal necrosis and fibrosis			
Comet assay in liver of Sprague Dawley rats of either sex; 7 animals/group Serum analysis: ALT, ALP, AST, bilirubin	BPA, 25 mg/kg by i.p. negative control group; vehicle control group (10% DMSO in olive oil) Sampling: 4 weeks after the treatment	BPA, no information on purity or the supplier company	Positive increase of tail DNA % 28.35 ± 1.2 vs 0.01 ± 0.005 BPA-induced: - increase of WBC, ALT, AST, ALP, bilirubin, H2O2, nitrite - decrease of RBC, platelets, Hb, albumin,	Reliability: 3 Limitations: - a single administration by i.p. and comet, analysis after 4 weeks; - unusual software used for the comet analysis;	Low A single administration by i.p. No information on source and purity of BPA	Kazmi *et al*., 2018

Analysis of antioxidant effects: CAT, POD, SOD, GSH Lipid peroxidation assay, hydrogen peroxide assay, nitrite assay Liver histopathology			CAT, POD, SOD, GSH, 'Histopathological examination of BPAtreated animals revealedintense hepatic cytoplasm inflammation, centrilobular necrosis, cellular hypertrophy, fatty degeneration, vacuolisation, steatosis and distortion of portal vein'	- the results reported using the different parameters (tail length, % of DNA in tail, tail moment) are not consistent; - the value of % of DNA in tail in controls is extremely low with respect to the data reported in the scientific literature; - high liver toxicity		
Comet assay in liver of Male Swiss albino mice (10 animals/group); images of 50 randomly	BPA dissolved in ethanol and diluted in corn oil by gavage at 50 mg/kg bw, once a day for 28 successive days	BPA (≥ 99 %) Sigma-Aldrich	Positive Mean tail length, tail moment and % tail DNA were significantly increased ($p < 0.05$) in liver of BPA-treated mice Increase of AST, ALT,	Reliability: 3 Major deviation from OECD TG 489: -too low number of analysed cells per animal -aggregated mean	Low	Elhamalawy *et al*., 2018

selected nuclei/ experimental group Analysis of liver toxicity markers (AST and ALT) and liver histopathology			marked histopathological alteration in liver of BPAtreated animals 'congestion of the hepatic blood vessels as well as marked vacuolar degeneration of the hepatocytes with many necrotic cells'	data analysed (instead of animal median) -no positive control - too high liver toxicity associated with treatment		
Alkaline comet assay in liver, kidney, testes, urinary bladder, colon and lungs cells CD-1 male mice (5 mice/group) In vitro comet assay	Gavage 0, 125, 250 and 500 mg/kg bw BPA (maximum tolerated dose) as suspensions in corn oil prepared by ultrasonication 2 doses (24 h apart) Animals were sacrificed 3 h after 2nd dose 200 cells analysed/mice (100 cells per gel and 2 gels per mouse)	BPA (purity >99%, Sigma-Aldrich)	Negative None of the tissues showed an effect of BPA except in testicular cells, in which an increased level of DNA strand breaks ($p < 0.01$ compared with control group) was observed at the lowest dose only	Reliability: 1 This study basically followed the OECD TG 489	High	Sharma *et al.*, 2018**

(Table 5)**						
Comet assay in liver and testes of male Swiss albino mice Male Swiss albino mice, 10 animals/group 50 nuclei/group were analysed In vivo micronucleus assay (Table 7)*	50 mg/kg bw, orally once a day for 28 days Sampling at the end of treatment	BPA, purity ≥ 99% (Sigma-Aldrich)	Positive Increase ($p \leq 0.05$) in the mean values of tail length, percentage of tail DNA and Olive tail moment in liver and testes Histopathological examination hepatocyte vacuolar degeneration with many necrotic cells Defective spermatogenesis characterised by severe necrosis and loss of the spermatogonial layers with multiple spermatid giant cells formation in most of the seminiferous tubules	Reliability: 3 No positive control, low number of nucleoids analysed, toxic effects observed in liver and testes, a single dose applied The standard alkaline comet assay applied is not considered appropriate to measure DNA strand breaks in mature germ cells	Low	Fawzy *et al*., 2018*

			and a congestion of the interstitial blood vessels			
Comet assay in heart of Wistar rats; 20 animals/group	BPA dissolved in corn oil 30 mg/kg bw per day injected subcutaneously (SC) 6 days/week for 4 weeks Sacrifice at the end of treatment	BPA Sigma-Aldrich; purity >97% not reported in the study but available on the website of the company	Positive Increase tail DNA % (6.88 vs 1.67) Histopathological changes: focal disruption of cardiomyocytes with some nuclear changes, such as karyolysis and pyknosis and sarcoplasmic vacuolisation The mitochondria appeared swollen and deranged with different sizes and shapes	Reliability: 3 Single dose; no positive control; inadequate cell preparation for comet assay; high toxicity	Low route of administration: subcutaneous	Amin *et al*., 2019
Comet assay in testes of Sprague Dawley	BPA (50 mg/kg bw) injected intraperitoneal on alternate days for 21 days	BPA analytical grade (Merck KGaA); purity >97% not reported in	Positive Histopathology: 'BPA caused significant damage	Reliability: 3 Single dose; no positive controls; an	Low BPA was administered by i.p.	Majid *et al*., 2019

rats; 7 rats/group Histopathology Antioxidant enzymes: CAT, SOD, GSH, POD, NO	Sacrifice 24 h after the end of treatment	the study but available on the website of the company	and abrasions to seminiferous tubules with low cellular density' BPA-induced: - decrease of body weight, epididymis and testes weight, testosterone, FSH, LH, CAT, SOD, GSH, POD; - decrease of sperm count, viability, motility - increase of estradiol	unusual software for the comet analysis used; the comet presented in the microphotographs are of low quality The standard alkaline comet assay applied is not considered appropriate to measure DNA strand breaks in mature germ cells		

Comet assay (neutral) on spermatozoa of Sprague Dawley rats (7 per group) 100 scored cells per animal In vitro comet assay (Table 5)**	Animals treated by gavage with 5, 25 and 50 mg BPA/kg bw per day for 28 days and sacrificed on day 29th, control received the vehicle alone (0.1% ethanol)	BPA (99% purity) from Santa Cruz Biotechnology	Positive Both tail moment and % tail DNA were significantly ($p < 0.05$) increased in the BPA 50 mg/kg bw per day group compared to vehicle controls, while no significant difference with controls was observed in the BPA 5 and 25 mg/kg bw per day groups	Reliability: 3 The study was performed following a non-standard, neutral protocol and unusual evaluation of comets based on the analysis of microphotographs No detailed information on data analysis is provided (e.g. the use of median vs mean as individual animal descriptor) No positive control	Low	Ullah *et al*., 2019**
Comet assay in testes of offspring of BPA treated	Animals were randomly divided into 7 groups. One	BPA (purity 99%, Sigma)	Positive The results obtained showed significantly increased Olive tail	Reliability: 3 The results obtained showed significantly	Low	Zhang *et al*., 2019

mice (pregnant Kumming mice, 20 in each group)	group served as control, the others received BPA in drinking water at 0.05, 0.5, 5, 10, 20 or 50 mg/kg bw per day, for 40 days from gestation day 0 to lactation day 21. F1 male mice were sacrificed at weaning (post-natal day 21) and DNA damage in testes evaluated by comet assay		moment (OTM) in testes cells of F1 animals treated with 5, 10, 20 and 50 mg/kg bw per day, compared with the control group ($p < 0.05$).	increased Olive tail moment (OTM) in testes cells of F1 animals treated with 5, 10, 20 and 50 mg/kg bw per day, compared with the control group ($p < 0.05$).		
Alkaline comet assay in thyroid tissue Male albino rats 20 rats/group	BPA dissolved in corn oil 200 mg/kg bw per day (1/20 of the oral LD50) for 35 days Sacrifice 24 h after the last administration	BPA (99.5% purity) was obtained from Sigma-Aldrich Co.	Positive % tail DNA 4 times increase compared with control level The histopathological examinations of thyroid	Reliability: 3 Only one dose level No positive control Comet method poorly described The	Low	Mohammed *et al.*, 2020

Biochemical investigation of MPO activity, GSH, SOD activity and MDA			gland showed severe congestion of interstitial blood capillaries, severe lymphocytic infiltration associated with variablesized follicles, most of which contain scanty colloid secretion, and some are atrophied in BPA group Significant induction of MPO activity and MDA concentration associated with significant decreases of SOD activity and GSH concentration in the thyroid gland of BPA group	microphotographs of comets are of low quality High toxicity		

Alkaline comet assay in testes Male juvenile Sprague Dawley (SD) rats (7 animals/group) Sperm DNA damage was evaluated by the comet and Halo assays using duplicate slides; apoptosis in testes cells was quantified using TUNEL assay, and	Gavage 8 weeks BPA (100 mg/kg bw per day) daily/5 days per week by gavage for 8 consecutive weeks Animals were sacrificed after 8 weeks	BPA (Sigma-Aldrich) Purity >99% not reported in the study but available on the website of the company	Negative All comet assay parameters (tail length, Olive tail moment and % DNA in the tail) and the nuclear diffusion factor in Halo assay, were slightly but not significantly increased in testes cells of BPA-treated rats compared with controls TUNEL-positive cells and per cent of 8-OHdG positive areas in testicular tissue were also slightly but non-significantly increased in BPA-treated rats	Reliability: 3 The standard alkaline comet assay applied (OECD TG 489) is not considered appropriate to measure DNA strand breaks in mature germ cells Other test methods (Halo and immunohistochemical determination of 8-OHdG) are not standardised and/or validated for regulatory use For all end-points, only a single dose was tested Sampling time not	Low	Sahu *et al*., 2020

testicular levels of 8-OHdG were determined by immunohistochemistry				specified No positive control		
Comet assay on whole brain cells from KM mice of F1 and F2 (8 male and 8 female)	Pregnant mice (F0) were orally dosed with BPA dissolved in tea oil at 0.5, 50, 5000 µg/kg bw per day from gestational day 1 until weaning (post-natal day 21). Then, the first generation (F1) of mice were used to generate the F2 DNA damage in brain cells was evaluated by comet assay in mice from both F1 and F2	BPA (purity: 98 %) Sigma-Aldrich	Equivocal DNA damage, expressed as arbitrary units, was slightly (less than twofold) increased in the F1male mice at the lowest dose and in females at the intermediate dose. No effect of BPA exposure was observed in the F2 mice	Reliability: 3 The study protocol is only shortly described The presentation and interpretation of the results is inadequate No positive control	Low	Zhang *et al*., 2020

Comet assay in blood liver and kidney Male Wistar rats (WNIN) 6 animals/group 50 nuclei/slides were scored Lipid peroxidation: serum level of malondialdehyde (MDA) 8-Hydroxy-2□-deoxyguanosine (8-OHdG) in urine collected 24 h before	0, 50, and 100 µg/kg, per oral (gavage) for a period of 4 weeks Sampling at the end of treatment	BPA, (Sigma-Aldrich) purity >97% not reported in the study but available on the website of the company	Positive A weak but statistically significant and doserelated increase of tail length in liver In kidney increase of DNA damage observed only at the dose of 50 µg/kg Comet parameters are not reported for blood cells Dose-related increase of MDA in serum and of 8-OHdG levels in urine	Reliability: 2 Low number of nucleoids analysed No positive controls	Limited	Panpatil *et al*., 2020*

the sacrifice In vivo micronucleus assay (Table 7)*						
Evaluation of sperm DNA damage by alkaline comet and DNA ladder assays Male Sprague Dawley rats (groups of 7 animals) ROS, Catalase, POD and SOD, GSH, Lipid peroxidation, TBARS, hydrogen peroxide,	BPA diluted in 10% DMSO was injected intraperitoneally at 25 mg/kg bw on alternate days for 30 days	BPA, no information on purity or the supplier company	Positive Significant ($p < 0.01$) increase of all comet parameters in BPA-treated animals compared with vehicle controls Electrophoresis on agarose gel showed extensive DNA fragmentation in testes of BPA-treated rats Significant increase in ROS level and decreased levels of CAT, GSH SOD and POD	Reliability: 3 The standard alkaline comet assay applied (OECD TG 489) is not considered appropriate to measure DNA strand breaks in mature germ cells The comet protocol is shortly described, with no information on the number of analysed sperm cells	Low For insufficient reliability and lack of information on test item purity	Zahra *et al*., 2020

nitrite assay, AOPP			in the testis of BPA-treated group	per animal; sampling time not specified; cytotoxicity not evaluated; no positive control The DNA ladder assay is a biochemical method not validated for genotoxicity assessment		

Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021)

In vivo dominant lethal assay

Table 9: *In vivo* dominant lethal assay (OECD TG 478 was considered for the evaluation of the reliability)

Test system/Test object	Exposure conditions (concentration/ duration/metabolic activation)	Information on the characteristics of the test substance	Results	Reliability/ Comments	Relevance of the result	Reference
Dominant lethal test with male Holtzman	Rats treated by oral gavage with BPA dissolved in ethyl	BPA ~99% purity (Sigma Chemical Co.)	Positive Significant decrease in	Reliability: 2 No positive control	Limited	Tiwari and Vanage, 2013[1]*

rats (7 per group) Each treated male was mated with 2 females per week over a period of 8 weeks; the mated females were sacrificed on 15th day of gestation and uterine content examined In vivo comet assay in rat epididymal sperm (Table 8)*	alcohol and diluted in sesame oil, at dose levels of 10 µg/kg bw and 5 mg/kg bw once a day for 6 consecutive days Negative controls were treated with vehicle		total implants/female and live implants/female, in females mated with males treated with 5.0 mg BPA/kg bw the fourth week and sixth week after treatment	No negative historical control Limited study design, with less analysable total implants and resorptions than recommended (OECD TG 478)		

Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021)

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Abbreviations

7-HF	7-hydroxyflavone
8-OHdG	8-hydroxy-2′-deoxyguanosine
ABT	1-Aminobenzotriazole
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AOPP	Advanced oxidation protein products
ATM	Ataxia-telangiectasia mutated
AU	Arbitrary units
BPA	Bisphenol A
BrdU	5-bromo-2-deoxyuridine
Bw	Body weight
CA	Chromosomal aberrations
CAT	Catalase
ChE	Cholinesterase
CHO cells	Chinese hamster ovary cells
CI	Cellular index
CYPR450	Cytochrome P450 reductase
DCF	Dichlorofluorescein
DMSO	Dimethylsulphoxide
EM	Electron microscopy
ER	Oestrogen receptor
FACS	Fluorescence activated cell sorting
FISH	Fluorescence in situ hybridisation
Fpg	Formamide pyrimidine glycosylase
GGT	Gamma glutamyl transferase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
Hb	Haemoglobin
HDL	High-density lipoprotein cholesterol
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vascular endothelial cells
i.p.	Intraperitoneal
KET	Ketoconazole
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein cholesterol
MDA	Malondialdehyde
MMC	Mitomycin
MMS	Methyl methane sulfonate
MPO	Myeloperoxidase
NAC	N-Acetyl-L-cysteine
NO	Nitric oxide
OECD	Organisation for Economic Co-operation and Developement
OTM	Olive tail moment
PBMC	Human peripheral blood mononuclear cells
PCE	Polychromatic erythrocytes
PCP	Pentachlorophenol
PHA	Phytohemagglutinin
POD	Peroxidase

RBC	Red blood cells
ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction
SCE	Sister chromatid exchange
SD	Sprague Dawley
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TC	Total cholesterol
TG	Test guideline
TP	Total protein
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
WBC	White blood cells
WGS	Whole genome sequencing

Technical Information

Aneugen: is a substance that causes a daughter cell to have an abnormal number of chromosomes or aneuploidy.

Clastogen: is a mutagenic agent that disturbs normal DNA related processes or directly causes DNA strand breakages, thus causing the deletion, insertion, or rearrangement of entire chromosome sections. These processes are a form of mutagenesis which if left unrepaired, or improperly repaired, can lead to cancer.