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SCIENTIFIC OPINION

Re-assessment of the risks to public health related to the genotoxicity of styrene present in plastic food contact materials

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts

Abstract

The EFSA Panel on Food Contact Materials (FCM) was requested by the European Commission to re-evaluate the potential genotoxicity of styrene after oral exposure and its safety for use in plastic FCM with a specific migration limit (SML) of 40 μg/kg food. A rigorous assessment of the in vivo genotoxicity studies (i) provided by third parties, (ii) identified by a targeted literature search and (iii) reported in the 2019 IARC Monograph was performed. All studies were assessed for reliability and relevance and the results integrated in the weight of evidence. The results provided by reliable in vivo oral genotoxicity studies, covering different genetic endpoints and target tissues, including liver, the primary site of metabolism, demonstrated that the oral administration of styrene in mice and rats up to the maximum tolerated dose (300 and 500 mg/kg body weight (bw), respectively) did not induce genotoxic effects. The Panel concluded that there was no evidence that styrene is genotoxic following oral exposure. For substances demonstrated to be non-genotoxic, according to the EFSA Note for Guidance for FCM, an SML up to 50 μg/kg food would not be of safety concern. Consequently, the use of styrene in the manufacture of FCM respecting the SML of 40 μg/kg food proposed by the European Commission is not of safety concern.

KEYWORDS

CAS No 100-42-5, food contact materials, genotoxicity, oral exposure, safety assessment, styrene

CONTENTS

1 | INTRODUCTION

1.1 | Background and Terms of Reference

1.1.1 | Background

Styrene is authorised without a migration limit or other restrictions for the manufacture of plastic food contact materials (FCM) in accordance with Commission Regulation (EU) No 10/2011.[1] In 2019, the International Agency for Research on Cancer (IARC) published a monograph, which concluded that styrene and its primary metabolite styrene-7,8-oxide (SO) are classified in group 2A as 'probably carcinogenic to humans' (IARC, 2019).[2] As this conclusion could affect the existing authorisation of styrene, on 6 December 2018, the European Commission requested the European Food Safety Authority (EFSA) to re-evaluate whether the evidence examined by IARC could be of consequence to the safety of styrene in FCM.

On 9 September 2020, EFSA adopted an opinion addressing the mandate of the Commission, in which the Panel concluded that a concern for genotoxicity associated with oral exposure to styrene could not be excluded and that additional data were required for assessing the safety of styrene for its use in FCM.[3] The EFSA opinion also stated that a systematic review of genotoxicity and mechanistic data as well as comparative toxicokinetics to address potential species differences would be required. EFSA reported that the migration of styrene into foods packed in styrenic plastics was below 10 µg/kg for the majority of the foods but can reach 230 µg/kg (MAFF, 1983, 1994).

In the course of a Commission consultation on the use of styrene in and migration from FCM, the Commission received information that indicated that the level of migration from styrenic plastics into the majority of foods could be above 10 µg/kg. In addition, it was identified that the residual monomer concentration in styrene-based plastics may be up to 1000 mg/kg. The high monomer concentration explains another observation stemming from the consultation, namely that high migration may occur in food simulants and in some foods, even when the food or simulant is only mildly aggressive on the plastic.

In view of the remaining uncertainty regarding the genotoxicity of styrene and the lack of a migration limit combined with a high migration potential, the Commission is preparing a measure to lay down a migration limit of 40 ppb in foods, based on the guidance value established for styrene in drinking water (a tolerable daily intake (TDI))[4] by the World Health Organization (WHO). The WHO included an allocation factor of 10% to account for exposure from all sources. Such an allocation factor is routinely applied in the FCM domain and its use is indeed appropriate also in this case.

The WHO TDI, however, pre-dates the new scientific information originating from the 2019 IARC assessment and the 2020 EFSA evaluation, which raises questions about the potential genotoxicity of styrene. As it is based on a TDI and was not challenged before the new information, the specific migration limit (SML) of 40 ppb proposed by the European Commission would be adequate as a limit for use in the FCM domain. However, it should be ensured that this limit is indeed protective, in particular in view of the possible genotoxicity of styrene. Thus, it is necessary to further assess – in accordance with the terms of reference (ToR) provided on 5 May 2023 whether the use of styrene-based polymers would be safe, if an SML of 40 ppb were to be set.

1.1.2 | Terms of Reference

In accordance with Article 12(3) of Regulation (EC) No 1935/2004,[5] the European Commission requests EFSA to provide an opinion, which addresses the following points:

- Whether styrene is genotoxic following oral exposure and the relevance to human health, and,
- Whether the use of styrene if authorised in accordance with Article 5 of Regulation (EU) No 10/2011 subject to the above mentioned SML of 40 ppb, is in accordance with Article 12(3) of Regulation (EC) No 1935/2004.

To this purpose, EFSA shall take into account data provided by third parties during 2022 and 2023, which is already available to EFSA.

EFSA should ensure the opinion is conclusive on the above points. If needed EFSA shall thereto:

- Conclude on a lower SML or alternative restrictions under which the use could be considered safe, if any, if this would be needed to ensure Article 12(3) is met, and,

[1] O J L 12, 15.1.2011, p. 1.

[2] The evaluation was already made available as a report published in The Lancet journal in 2018. Carcinogenicity of quinoline, styrene and styrene-7,8-oxide, 2018. Lancet, vol.19. April 18, 2018. https://doi.org/10.1016/S1470-2045(18)30316-4.

[3] EFSA Journal, 18(10), 6247.

[4] Guidelines for drinking-water quality, 4th edition, Geneva, World Health Organization; 2022; p.465 (https://www.who.int/publications/i/item/9789240045064). Based on: WHO (2003), Styrene in Drinking-water. Background document for development of WHO Guidelines for Drinking-water Quality; assessment date 1993.

[5] O J L 338, 13.11.2004, p. 4.

- In view of remaining uncertainties or knowledge gaps, or if the available data would not be suitable to support the SML of 40 ppb, request additional data from the applicant in accordance with Article 10 of Regulation (EC) No 1935/2004[6].

In addition, should there be a risk for an inconclusive opinion due to insufficiency of available data over the toxicity of styrene, the Commission invites EFSA to explore all tools at its disposal for the collection of all relevant scientific data and studies, including the potential launch of new scientific studies to support the risk assessment.

With respect to the systematic review of genotoxicity and mechanistic data on which EFSA in its previous opinion concluded that it should be required, the Commission considers that such a review should be carried out only to the extent that EFSA considers it necessary for the purpose of addressing the mandate.

If, as part of its work on the mandate, the Panel would observe variations between its reasoning expressed in the 2019 IARC conclusion, these variations should be detailed and explained.

EFSA shall deliver its opinion within 15 months following reception of this mandate. In case EFSA would request data from third parties, launch a call for data, launch scientific studies or organise a public consultation on the resulting opinion, it may 'stop the clock' to accommodate the required time. However, the total time for the delivery of the opinion shall not exceed 30 months following the reception of the mandate.

1.2 | Interpretation of the Terms of Reference

It was deemed appropriate to report some explanations to further clarify the background and the ToRs of the mandate.

It should be clarified that also data related to human biomonitoring (HBM) and dietary exposure to styrene were included in the cluster of evidence supporting the re-evaluation of the potential genotoxicity of styrene for use in FCM.

The term 'toxicity' refers specifically to the genotoxic potential of styrene after oral exposure.

The studies focusing only on the genotoxic effect of the reactive metabolite SO were not considered in the assessment because its potential contribution to the in vivo genotoxicity of styrene is covered by the studies on the parent compound.

The term 'mechanistic' could be misleading, since it refers to the carcinogenicity of styrene, whereas the scope of the mandate is only to perform a systematic review of genotoxicity, metabolism and toxicokinetic data. EFSA considered genotoxicity relevant as toxicological endpoint per se (EFSA Scientific Committee, 2011).

The sentence 'if, as part of its work on the mandate, the Panel would observe variations between its reasoning expressed in the 2019 IARC conclusion, these variations should be detailed and explained' should be amended as 'if, as part of its work on the mandate, the Panel would observe variations since its reasoning expressed in the 2020 EFSA opinion, these variations should be detailed and explained'.

The adequacy of the SML of 40 ppb proposed by the Commission will be considered in the context of the requirements of the EFSA Note for Guidance for Food Contact Materials (EFSA CEF Panel, 2021).

2 | DATA AND METHODOLOGIES

2.1 | Data

The data taken into consideration for the re-assessment and included in this opinion originate from the following sources:

- Studies reported in the 2019 IARC Monograph (IARC, 2019).
- Studies provided by the US Styrenic Information and Research Center (SIRC):

  1. Combined *Pig-a*, micronucleus and comet study in B6C3F1 mice after oral administration of styrene. ILS Study Number 60952.00203. January 2023.
  2. Combined *Pig-a*, micronucleus and comet study in Fischer 344 rats after oral administration of styrene for 28 days. ILS Study Number 60952.00103. December 2022.
  3. Styrene: mammalian alkaline comet study in male Fischer 344 rats via oral gavage administration for 28 days. Inotiv Study Number 3940-2300099. October 2023.
  4. An oral gavage in vivo mutation assay of styrene at the *cII* locus in transgenic Big Blue® Haemizygous B6C3F1 mice. Charles River Project ID 20427171. March 2024.

- Studies collected through a targeted literature search (01 January 2018 to 01 October 2024). The outcome is reported below in Table 1.

[6]The Commission is aware that the Styrenics group of Plastics Europe is acting as the applicant.

TABLE 1 Outcome of the literature searches.

Records identified	3392
Title and abstract screening	Genotoxicity: 603 Toxicokinetics: 2001 Human exposure: 788
Full-text screening	Genotoxicity: 57 Toxicokinetics: 75 Human exposure: 156
Included after full-text screening	Genotoxicity: 9 Toxicokinetics: 8 Human exposure: 7
Cited in the opinion	Genotoxicity: 5 Toxicokinetics: 4 Human exposure: 5

It should be noted that data and references published before 1 January 2018 reported in the opinion have been added to further support the assessment. These studies are cited in the references' list of the opinion.

2.2 | Methodologies

The assessment was conducted in line with the principles laid down in Regulation (EC) No 1935/2004[7] on materials and articles intended to come into contact with food.

To establish the safety from ingestion of migrating substances, the toxicological data indicating the potential hazard and the likely human exposure data need to be combined. Exposure is estimated from studies on migration into food or food simulants and considering that a person may consume daily up to 1 kg of food in contact with the relevant FCM.

As a general rule, the greater the exposure through migration, the more toxicological data are required for the safety assessment of a substance. Currently, there are three tiers with different thresholds triggering the need for more toxicological information as follows:

a. In case of high migration (i.e. 5–60 mg/kg food), an extensive data set is needed.
b. In case of migration between 0.05 and 5 mg/kg food, a reduced data set may suffice.
c. In case of low migration (i.e. < 0.05 mg/kg food), only a limited data set is needed (at least two genotoxicity tests).

For this specific evaluation, given the proposed SML, only the assessment of genotoxicity is required.

Detailed information on the required data is available in the Scientific Committee on Food (SCF) guidelines (European Commission, 2001).

The assessment was conducted in line with the principles described in the EFSA Guidance on transparency in the scientific aspects of risk assessment (EFSA, 2009) and considering the relevant guidance documents from the EFSA Scientific Committee.

The protocols used for the risk assessment refer to standard EFSA guidance documents (EFSA, 2010, 2023). The assessment of the genotoxicity studies was based on a harmonised methodology reported in the 2023 EFSA Technical Report (EFSA, 2023). The core of the assessment of genotoxicity is reported in Section 3.1 of this opinion, whereas the assessment of the studies' reliability and relevance and their individual summaries are reported in Appendices B and C, respectively. The assessment of the toxicokinetics and human exposure data was performed narratively and is reported in Sections 3.2 and 3.3, respectively.

Details on the protocol used for the literature search are reported in Appendix A.

3 | ASSESSMENT

3.1 | Genotoxicity data

According to the ToR, the assessment of styrene genotoxicity focused on the potential genotoxic hazard associated with oral exposure to styrene. To this aim, publicly available and newly submitted in vivo oral genotoxicity studies were evaluated for reliability and relevance (EFSA, 2023) and their results integrated in a weight of evidence (WoE) approach. Data from in vitro studies and in vivo studies using non-oral routes considered in the 2019 IARC Monograph, and from HBM studies using genotoxicity biomarkers, were also assessed and integrated in the WoE as supporting information.

[7]Regulation (EC) No 1935/2004 of the European parliament and of the council of 27 October 2004 on materials and articles intended to come into contact with food and repealing Directives 80/590/EEC and 89/109/EEC. OJ L 338, 13.11.2004, pp. 4–17.

3.1.1 | In vitro genotoxicity studies (2019 IARC monograph)

A number of in vitro genotoxicity studies on styrene available in the scientific literature were evaluated in the IARC Monograph volume 121 (IARC, 2019) and already discussed in the previous Food Contact Materials, Enzymes and Processing Aids (CEP) Panel opinion on styrene for use in plastic FCM (EFSA CEP Panel, 2020). The main findings from these studies are summarised below and in Table 2.

The mutagenicity of styrene in the Ames test was evaluated by several research groups. Negative results, in the presence and absence of exogenous metabolic activation, were reported in several studies using *Salmonella* Typhimurium strains sensitive both to base pair substitutions (TA1535 and TA100) and to frameshift mutations (TA1537 and/or TA97, TA1538 and/or TA98) (Brams et al., 1987; Busk, 1979; De Flora, 1979; Florin et al., 1980; Stoltz & Whitey, 1977; Watabe et al., 1978; Zeiger et al., 1988). A positive response was only observed in the presence of metabolic activation in strain TA1535 (de Meester et al., 1977; Vainio et al., 1976) or in TA1535 and TA100 (de Meester et al., 1981), with negative results in strains TA1537, TA1538 and TA98 in the presence or absence of metabolic activation.

In mammalian cells, DNA strand breaks were detected following in vitro exposure to styrene in the absence of exogenous metabolic activation in human blood leucocytes (Laffon et al., 2003), human skin cells (Costa et al., 2006) as well as in primary rat (Sina et al., 1983) and mouse hepatocytes (Fontaine et al., 2004).

In mutagenicity assays in V79 cells (*hprt* locus), styrene was mutagenic in one study, using a perfused rat liver for metabolic activation (Beije & Jenssen, 1982). It was negative in another study only performed without metabolic activation (Loprieno et al., 1976).

Positive results were reported in micronucleus (MN) and/or chromosomal aberration (CA) assays in human peripheral blood lymphocytes in the absence of metabolic activation (Jantunen et al., 1986; Linnainmaa et al., 1978; Pohlová et al., 1984) and in Chinese hamster lung cells only with metabolic activation (Ishidate Jr. & Yoshikawa, 1980; Matsuoka et al., 1979). Positive results were also obtained in the absence of exogenous metabolic activation in sister-chromatid exchange assays in human peripheral blood lymphocytes (Bernardini et al., 2002; Chakrabarti et al., 1993; Lee & Norppa, 1995; Norppa et al., 1983) and in Chinese hamster ovary cells (de Raat, 1978) only in the presence of metabolic activation.

Other in vitro or non-mammalian studies included in the IARC Monograph (IARC, 2019) were not further considered, since not validated for human risk assessment (e.g. studies in yeast, plants, insects).

Overall, although most studies are old and with limited protocol (Table 2), the available evidence indicates that styrene is genotoxic in vitro. The mechanism(s) responsible for styrene genotoxicity is not fully elucidated. The strain specificity observed in the Ames test, with positive results only in the presence of metabolic activation and in strains sensitive to base pair substitutions, is consistent with the proposed role of SO in styrene genotoxicity (IARC, 2019), in view of the miscoding properties of SO-DNA adducts. However, the FCM Panel noted that, despite the uniformly positive response of SO in Ames tests with TA1535 and TA100, styrene mutagenicity was not clearly expressed under Ames test conditions, where the majority of the studies provided negative results. The Panel also noted that other mechanisms, including oxidative stress (Chakrabarti et al., 1993; Costa et al., 2006), are proposed to contribute to styrene genotoxicity in addition to DNA binding by SO (Sina et al., 1983). In this respect, the Panel noted that no studies have been conducted on S. typhimurium TA 102 or E. coli WP2 or WP2 (pKM101).

Concerning the metabolic activation, the available data point to the role of both exogenous and endogenous metabolism. Styrene induced DNA single strand breaks (ssb) in studies using metabolically proficient primary rodent hepatocytes (Fontaine et al., 2004; Sina et al., 1983) and human skin explants (Costa et al., 2006) as well as in human blood leucocytes, in which styrene activation was modulated by donor genotype (Laffon et al., 2003). Conversely, in rodent cell lines, the chromosome-damaging activity of styrene was mainly observed when the experiments were conducted in the presence of exogenous metabolic activation (S9 mix) (Beije & Jenssen, 1982; de Raat, 1978; Ishidate Jr. & Yoshikawa, 1980; Matsuoka et al., 1979). In human blood leucocytes, in contrast with rodent cell lines, chromosomal damage (structural aberrations, MN and sister chromatid exchanges (SCEs)) was induced by styrene mainly in the absence of exogenous metabolic activation (Bernardini et al., 2002; Chakrabarti et al., 1993; Lee & Norppa, 1995; Linnainmaa et al., 1978; Pohlová et al., 1984) and with higher efficiency in whole blood cultures compared to isolated leucocytes, suggesting the involvement of styrene oxidation by oxyhaemoglobin in whole blood cultures (Jantunen et al., 1986; Norppa et al., 1983).

TABLE 2 Summary of in vitro genotoxicity studies with styrene.

Test system	Dose (range and/or LEC/HTC)	Results	Reference
Ames test			
Reverse mutation *Salmonella* Typhimurium TA1535, TA1537, TA1538, TA98 and TA100	10^{-8} to 10^{-4} moles/plate (~1–10,400 μg/plate)	Positive in TA1535 at 10^{-8} and 10^{-7} moles/plate (not dose related), only with S9; toxic at 10^{-5} moles/plate and above equivocal in TA100 ± S9; negative in the other strains ± S9	Vainio et al. (1976)

(Continues)

TABLE 2 (Continued)

Test system	Dose (range and/or LEC/HTC)	Results	Reference
Reverse mutation *Salmonella* Typhimurium TA1535, TA1537, TA1538, TA98 and TA100	Up to 500 µg/mL	Negative ± S9	Stoltz and Whitey (1977)
Reverse mutation *Salmonella* Typhimurium TA1535, TA1537, TA1538, TA98 and TA100	1–100 µmoles/plate (104–10,400 µg/plate)	Positive in TA1535, only +S9 Negative in the other strains ± S9	de Meester et al. (1977)
Reverse mutation *Salmonella* Typhimurium TA1535, TA1537, TA1538, TA98 and TA100	10–500 µg/plate	Negative (only tested +S9)	Watabe et al. (1978)
Reverse mutation *Salmonella* Typhimurium TA1535, TA1537, TA1538, TA98 and TA100	Up to 10^{-6} moles/plate (104 µg/plate)	Negative ± S9	Busk (1979)
Reverse mutation *Salmonella* Typhimurium TA1535, TA1538, TA98 and TA100	Up to 250 µg/mL	Negative ± S9	De Flora (1979)
Reverse mutation *Salmonella* Typhimurium TA1535, TA1537, TA98 and TA100	Up to 312 µg/mL	Negative ± S9	Florin et al. (1980)
Reverse mutation *Salmonella* Typhimurium TA1535, TA1537, TA1538, TA98 and TA100	Up to 1000 µg/mL (vapour exposure)	Weakly positive (2- to 3-fold increase) in TA1535 and TA100, only +S9 Negative in the other strains ± S9	de Meester et al. (1981)
Reverse mutation *Salmonella* Typhimurium TA98 and TA100	Up to 500 µg/mL	Negative ± S9	Brams et al. (1987)
Reverse mutation *Salmonella* Typhimurium TA1535, TA1537, TA97, TA98 and TA100	Up to 1666 µg/plate	Negative ± S9	Zeiger et al. (1988)
DNA damage			
Primary rat hepatocytes (alkaline elution)	312 µg/mL	Positive (without S9)	Sina et al. (1983)
Isolated blood leucocytes from healthy donors (comet assay)	5 and 10 mM (521–1042 µg/mL)	Positive (without S9) Response modulated by CYP1A1, CYP2E1 and GSTP1 genotypes	Laffon et al. (2003)
Primary mouse hepatocytes (comet assay)	2.5–10 mM	Positive (without S9) Response attenuated by CYP inhibitor	Fontaine et al. (2004)
Human skin cells (comet assay)	10^2–10^6 ppm (vapour exposure)	Positive (without S9) With concurrent increase of oxidative stress biomarkers	Costa et al. (2006)
Gene mutation in mammalian cells			
V79 cells/*hprt* gene mutation test	1771 µg/mL	Negative (only tested without S9)	Loprieno et al. (1976)
V79 cells/*hprt* gene mutation test	6250 µg/mL	Positive (only with coincubation of cells with an isolated perfused rat liver as metabolic activation system)	Beije and Jenssen (1982)
Chromosomal damage			
CAs and MN in human whole blood lymphocytes	0.03% v:v	Positive (only tested without S9)	Linnainmaa et al. (1978)
CAs in human whole blood lymphocytes	Up to 0.5 mM (52 µg/mL)	Positive (only tested without S9)	Pohlová et al. (1984)
CAs in human lymphocytes (whole blood and isolated)	1–4 mM (isolated lymphocytes) 0.5–6 mM (whole blood lymphocytes)	Positive (only tested without S9) Greater response in whole-blood cultures	Jantunen et al. (1986)
CAs in Chinese hamster lung cells	250 µg/mL	Positive (only +S9)	Matsuoka et al. (1979)
CAs in Chinese hamster lung cells	100 µg/mL	Positive (only +S9)	Ishidate Jr. and Yoshikawa (1980)
Sister chromatid exchanges, Chinese hamster ovary cells	455 µg/mL	Negative ± S9 Positive only + S9 and in presence of an inhibitor of EH	De Raat (1978)

TABLE 2 (Continued)

Test system	Dose (range and/or LEC/HTC)	Results	Reference
Sister chromatid exchanges, human blood lymphocytes (whole blood and isolated)	0.5–4 mM	Positive (only tested without S9) Greater response in whole-blood cultures compared to isolated lymphocytes	Norppa et al. (1983)
Sister chromatid exchanges, human whole blood lymphocytes	1–1000 μM (1–1040 μg/mL)	Positive (only tested without S9)	Chakrabarti et al. (1993)
Sister chromatid exchanges, human whole blood lymphocytes	0.5–1.0 mM	Positive (only tested without S9)	Lee and Norppa (1995)
Sister chromatid exchanges, human whole blood lymphocytes	0.5 1.5 mM	Positive - S9 Greater response with GSTM1/GSTT1 *null* genotypes	Bernardini et al. (2002)

3.1.2 | In vivo genotoxicity studies (2019 IARC monograph)

The in vivo genotoxicity studies on styrene reported in the IARC Monograph were evaluated for their reliability and relevance according to the EFSA criteria (EFSA, 2023) and reported in Appendix B together with their main findings. A narrative description of these studies is presented in Appendix C.

Fifteen studies evaluated the induction of chromosomal damage in rodents, following the exposure to styrene by various routes.

Ten out of 15 studies reported in the IARC Monograph, considered of limited relevance, were included in the WoE according to the EFSA criteria (EFSA, 2023). Nine of these studies provided negative results, including two CA tests in mice with single (Loprieno et al., 1978) or repeated (Sbrana et al., 1983) oral administration, two MN assays by intraperitoneal (i.p.) administration in polychromatic erythrocytes (PCE) of mice (Sharief et al., 1986) and rats (Simula & Priestly, 1992) and five inhalation studies in mice and rats (CAs in rats, (Sinha et al., 1983); CAs and MN in mice and rats (Kligerman et al., 1993); MN in mice (Engelhardt et al., 2003); MN in rats (Gaté et al., 2012)). A single study (Simula & Priestly, 1992) was evaluated as positive for the induction of MN in PCE of mice following i.p. administration of styrene. Five studies were considered of low relevance: two due to insufficient reliability related to major limitations in the study protocol (Preston & Abernethy, 1993; Vodicka et al., 2001) and three due to the inconclusive or equivocal[8] results reported (Norppa, 1981; Norppa et al., 1980; Penttilä et al., 1980).

Four studies evaluated the in vivo DNA damaging activity of styrene by the alkaline comet assay. Three studies were considered of limited relevance: one showed positive results in several tissues (lymphocytes, bone marrow, liver and kidney) of mice treated by i.p. (Vaghef & Hellman, 1998), while negative results were obtained in leucocyte of rats (Kligerman et al., 1993) and rats (Gaté et al., 2012) following the inhalation exposure to styrene. One inhalation study (Vodicka et al., 2001) provided equivocal results and, hence, was considered of low relevance for this assessment.

Other in vivo studies included in the 2019 IARC Monograph, considered of less relevance, include a negative rat liver unscheduled DNA synthesis (UDS) (Clay, 2004) and SCEs tests in rats and mice with mixed results: positive results were observed in rat and mouse splenocytes cultured in vitro after in vivo i.p. administration of styrene (Simula & Priestly, 1992), in rat and mouse peripheral lymphocytes (Kligerman et al., 1993) and in mouse bone marrow and alveolar macrophages (Conner et al., 1980), after inhalation exposure to styrene; whereas, negative results were observed in mouse bone marrow after i.p. administration of styrene (Sharief et al., 1986). Low relevance, due to methodological limitations, was given to a study measuring DNA ssb by the DNA unwinding technique (Solveig Walles & Orsén, 1983).

3.1.3 | In vivo genotoxicity studies (available after the 2019 IARC monograph)

Combined *Pig-a*, micronucleus and comet study in B6C3F1 mice after oral administration of styrene

The study was provided by Plastics Europe on behalf of SIRC in 2023 (SIRC, 2023a) and published by Gollapudi (2023).

A combined *Pig-a*, micronucleus and comet assay was carried out in B6C3F1 mice treated with styrene by oral gavage for 29 consecutive days. The study was performed in compliance with good laboratory practices (GLP) principles, following the Organisation for Economic Co-operation and Development Testing Guidelines (OECD TG) 470 (*Pig-a*) (OECD, 2022a), 474 (MN) (OECD, 2016a) and 489 (in vivo comet assay) (OECD, 2016c).

[8]According to the EFSA Technical Report (EFSA, 2023), an equivocal result usually refers to a situation where not all the requirements for a clear positive result have been met. Whereas an inconclusive result could be considered one where no clear result was achieved, but this may have been a consequence of some limitation of the test or procedure. In this case, repeating the test under the correct conditions should produce a clear result.

Dose range-finding test

A 28-day dose range-finding study was performed in B6C3F1 mice. Styrene was administered by oral gavage at three doses (50, 250 or 500 mg/kg bw per day) to groups of male mice (8 animals/group). After 28 days of dosing, animals were euthanised and blood collected 7 or 15 min after the last dose administration. Blood was used for haematology assays, for clinical chemistry assays and for bioanalysis of styrene levels in plasma. Animals receiving 500 mg/kg bw per day showed mortality and adverse toxicity; surviving animals had their dose reduced to 350 mg/kg bw per day on days 7–28. Three additional mice received 350 mg/kg per day oral styrene for 21 days. Two animals, one in each groups receiving lower doses of 50 or 250 mg/kg bw per day, died and were noted to have acute pulmonary haemorrhage and chronic peribronchiolar inflammation, respectively. Animals that received 350 mg/kg per day for 21 days also showed adverse lesions in the liver (mild coagulative necrosis or minimal focal pigmentation) or lungs (minimal chronic peribronchial/periarterial inflammation). Due to these results, the doses of 350 and 500 mg/kg bw per day were considered to exceed the maximum tolerated dose (MTD) and supported setting a MTD of 300 mg/kg bw per day. The bioanalysis of plasma samples collected shortly (7 and 15 min) after the last administration showed styrene concentrations in plasma up to approximately 11 μg/mL in the 350/500 mg/kg bw group.

Main experiment

Based on the results of the range-finding study, styrene (99.95% pure, dissolved in corn oil) was administered by gavage at three dose levels (75, 150 or 300 mg/kg per day) to groups of eight male mice (6 evaluated) for 29 days. Control animals received the vehicle (corn oil) alone at 5 mL/kg bw per day. Positive control animals were administered with N-ethyl-N-nitrosourea (ENU, 51.7 mg/kg bw per day) for the first 3 days (days 1–3) and ethyl methanesulfonate (EMS, 150 mg/kg bw per day) for the last 3 days (days 27–29). Animals were sacrificed approximately 3 h after the final administration. Blood was collected to determine *Pig-a* mutant and MN frequencies by flow cytometry: at least 10^6 red blood cells (RBC) and reticulocytes (RET) were analysed for the *Pig-a* assay, and ~10,000 PCE for the MN test. Duodenum, glandular stomach, kidneys, liver and lungs were collected to assess for DNA damage using the alkaline comet assay, scoring 150 cells per tissue per animal. Hedgehogs (ghost cells) were tabulated to determine their frequency but evaluated separately from cells analysed for DNA migration.

Results

There was no evidence of toxicity of styrene at any dose level. *Pig-a* mutant and MN frequencies were not increased in animals treated with styrene compared to vehicle control animals, while a statistically significant increase was observed in the positive control groups. No decrease of the percentages of RETs, RBCs and PCEs was noted. Evidence of bone marrow exposure was provided by the results of plasma analysis (approximately 11 μg/mL at 350/500 mg/kg bw per day) carried out in the dose range-finding study after 28 daily administrations of 500/350 mg styrene/kg bw per day. No treatment-related increase in DNA damage (% Tail DNA) was observed in liver, lung, kidney and stomach tissues analysed by comet assay. For duodenum, a high baseline level of DNA damage was observed, with no response of the positive control group. The results of the comet test on duodenum were consequently considered as inconclusive. No treatment-related variation in hedgehogs' frequency was observed in any tissue.

The Panel considered the study as reliable without restrictions and the results of high relevance in all tissues, except for duodenum.

Combined *Pig-a*, micronucleus and comet study in Fischer 344 rats after oral administration of styrene for 28 days

The study was provided by Plastics Europe on behalf of SIRC in 2022 (SIRC, 2022) and published by Gollapudi in 2024 [RefID 3035].

A combined *Pig-a*, micronucleus and comet assay was carried out in Fischer 344 rats (7–8 weeks of age) treated with styrene by oral gavage for 29 consecutive days. The study was performed in compliance with GLP principles, following the OECD TGs 470 (*Pig-a*) (OECD, 2022a), 474 (MN) (OECD, 2016a) and 489 (in vivo comet assay) (OECD, 2016c).

Dose range-finding test

A 28-day dose range-finding study was performed in Fischer 344 rats. Styrene was administered by oral gavage at three doses (100, 500 and 1000 mg/kg bw per day for 28 days) to groups of male rats (8 animals/group). After 28 days of dosing, animals were euthanised and blood collected 7 or 15 min after the last dose administration. Blood was used for haematology assays, for clinical chemistry assays and for bioanalysis of styrene levels in plasma. Decreased final body weight (~ 10%) was observed in the 1000 mg/kg bw per day dose group. Decreased body weight gain of 28% and 92% was noted in the 500 and 1000 mg/kg bw per day dose groups, respectively. Increases in absolute and relative liver weights as well as absolute kidney weights in these two groups were also noted. At the highest dose of 1000 mg/kg bw per day, significant clinical signs of central nervous system depression were observed, such as uncoordinated movement and decreased movement

and lethargy. Due to these results, 1000 mg/kg was considered to exceed the MTD. The decreased body weight gain and organ weight changes in the 500 mg/kg bw per day group indicated an acceptable tolerability and was chosen as the top dose in the main study. The bioanalysis of plasma samples, collected shortly after the last administration (7 and 15 min) on day 28, showed styrene concentrations in plasma up to ~30 μg/mL in the 500 and 1000 mg/kg group.

Main experiment

Based on the results of the dose range-finding study, styrene (99.95% pure, dissolved in corn oil) was administered by gavage at three dose levels (100, 250 or 500 mg/kg bw per day) to groups of eight male animals (6 evaluated) for 29 days. Control animals received the vehicle (corn oil) alone at 5 mL/kg bw per day. Positive control animals were administered ENU (51.7 mg/kg bw per day) daily for the first 3 days (days 1–3) and EMS (150 mg/kg bw per day) for the last 3 days (days 27–29). Animals were sacrificed approximately 3–7 h following the final administration. As this sampling time was deemed not appropriate for the comet assay, an additional group of rats were subsequently dosed with the same vehicle and styrene doses to replicate the comet part of the study. The positive control group in this case received only EMS and all animals were sacrificed ~3 h after the final dose administration. Blood was collected to determine *Pig-a* mutant and MN frequencies by flow cytometry: at least 10^6 RBC and RET were analysed for the *Pig-a* assay and ~10,000 PCE for the MN test. Duodenum, glandular stomach, kidneys, liver and lungs were collected to assess for DNA damage using the alkaline comet assay, scoring 150 cells per tissue per animal. Hedgehogs (ghost cells) were tabulated to determine their frequency but evaluated separately from cells analysed for DNA migration.

Results

In rats from both the initial and the additional studies, orally dosed styrene caused a dose-dependent decrease in body weight gain of 24%, reaching statistical significance in animals receiving 500 mg/kg bw per day. Transient dose-related decreased movement was observed in the 250 and 500 mg/kg bw per day exposure groups. *Pig-a* mutant and MN frequencies were not increased in animals treated with styrene compared to vehicle control animals, while a statistically significant increase was observed in the positive control groups. No decrease of the percentage of RETs, RBCs and PCEs was noted. Evidence of bone marrow exposure was provided by the results of plasma analysis (approximately 30 μg/mL at both 500 and 1000 mg/kg bw per day) carried out in the dose range-finding study after 28 daily administrations of 500 or 1000 mg styrene/kg bw per day.

No treatment-related increase in DNA damage (% Tail DNA) was observed in liver and lung tissues analysed by the comet assay. For duodenum, stomach and kidney, a high baseline level of DNA damage was observed, with no response of the positive control group. For kidney, such a result was attributed to an outlier animal. However, when additional rats were included in the analysis, the response of the positive control was not statistically significant. Consequently, the results of the comet assay for duodenum, stomach and kidney were considered inconclusive. No treatment related variation in hedgehogs' frequency was observed in any tissue.

The Panel considered the study as reliable without restrictions. The results obtained for liver and lung are considered of high relevance, whereas the results obtained for duodenum, stomach and kidney are considered of low relevance due to their inconclusiveness.

Styrene: mammalian alkaline comet study in male Fischer 344 rats via oral gavage administration for 28 days

The study was provided by Plastics Europe on behalf of SIRC in 2023 (SIRC, 2023b).

Styrene (99.86% pure) was evaluated in the alkaline comet assay in male Fischer 344 rats following oral gavage administration for 28 consecutive days. The study was performed in compliance with GLP principles following the OECD TG 489 (OECD, 2016c).

Study protocol

Based on the toxicity findings obtained in a previous study (SIRC, 2022), three dose levels (100, 250 or 500 mg/kg bw per day) were administered to groups of eight male animals (6 evaluated). Control animals received the vehicle (corn oil) alone at 5 mL/kg bw per day. Positive control animals were administered with EMS (200 mg/kg bw per day) for 2 days. Animals were sacrificed 3–4 h after the last administration. DNA damage was evaluated by comet assay in liver, lung, kidney, duodenum and glandular stomach analysing 150 cells per animal. Hedgehogs (ghost cells) were tabulated to determine their frequency but evaluated separately from cells analysed for DNA migration.

Results

Treatment with styrene at doses up to 500 mg/kg bw per day had no effect on mortality, clinical signs, body weight and food consumption. No treatment-related variation in hedgehogs' frequency was observed in any tissue. No statistically significant and/or dose-related increase in DNA damage was observed in liver, lung and kidney of styrene-treated animals.

The study for liver and lung was considered by the Panel as reliable without restrictions and the results of high relevance.

The experiment in kidney was considered reliable with restrictions due to the low value of the % Tail DNA in negative control that was outside the range of historical control values. The results were considered by the Panel of limited relevance.

Statistically significant increases in % Tail DNA were observed in stomach samples of treated animals compared to the negative control group; such increases were not dose-related and were within the negative control historical range. The results were considered by the Panel equivocal and of limited relevance.

For duodenum, a high and highly variable baseline level of DNA damage was observed in the vehicle control group, well above the historical control limits, which was statistically significantly higher than in styrene-treated animals. The Panel considered the results as inconclusive and of low relevance.

In vivo mutagenicity assessment of styrene in MutaMouse liver and lung (Murata et al. (2023) 45:12. [RefID 2224])

The study was identified by a targeted literature search covering the timespan from 1 January 2018 to 1 October 2024.

Styrene was evaluated in a transgenic rodent mutation assay in MutaMice following oral gavage administration for 28 consecutive days. The study was performed following the OECD TG 488 (OECD, 2022b). No information on GLP compliance was provided.

Main study

In a dose-finding preliminary experiment, styrene (Tokyo Chemical Industries, purity 100%) was administered by gavage to MutaMice (mice harbouring the bacterial *lac Z* gene incorporated in a lambda phage) once a day for 2 weeks at the doses of 30, 100, 300 and 1000 mg/kg bw per day (maximum recommended dose in OECD TG 488 (OECD, 2022b)). Based on the results of the dose-finding assay, in which severe toxicity was observed at 1000 mg/kg bw per day, the doses of 75, 150 and 300 mg/kg bw per day were selected for the main experiment, in which styrene was administered by daily gavage for 28 consecutive days to male MutaMice (6–8 animals/group). Control animals received the vehicle alone (corn oil). ENU, as positive control, was administered by i.p. (100 mg/kg bw) for two consecutive days. Animals were sacrificed 3 days after the last administration. Mutations in the *lacZ* transgene were determined in DNA isolated from liver and lung of five animals.

Results

No clinical signs of toxicity were observed in the main experiment at any dose; gross pathological changes (liver darkening) were observed in three of eight animals at the high-dose group (300 mg/kg bw per day). The determination of *lacZ* mutants showed an extremely high mutant frequency, attributed to clonal expansion, in liver and lung of a single animal in the low-dose group. This animal was excluded and replaced with an additional treated mouse. Mean mutant frequencies in liver and lung of styrene-treated mice were not statistically different from vehicle control animals and were within the historical control range. A statistically significant increase in *lacZ* mutant frequency was observed in the positive control group.

The study was evaluated by the Panel as reliable without restrictions and the results of high relevance.

Oral gavage in vivo mutation assay of styrene at the *cII* locus in transgenic Big Blue® haemizygous B6C3F1 mice (SIRC, 2024)

The study was provided by Plastics Europe on behalf of SIRC in 2024 (SIRC, 2024) and published by Gollapudi in 2025.

Styrene (99.95% pure) was evaluated in the in vivo mutation assay at the *cII* Locus in the glandular stomach, duodenum, lung and liver from transgenic Big Blue® haemizygous B6C3F1 male mice following 28 consecutive days of administration by oral gavage and a 28-day non-dosing fixation period. The study was performed in compliance with GLP principles and following the OECD TG 488 (OECD, 2022b).

Main study

Based on the toxicity findings obtained in a previous study (SIRC, 2023a), three dose levels (75, 150 or 300 mg/kg bw per day) were administered to groups of eight males (only 5 animals evaluated). Control animals received the vehicle (corn oil) alone at 5 mL/kg bw per day. Positive control animals were administered with ENU (40 mg/kg bw per day) at days 1, 2 and 3.

Animals were sacrificed on study day 56, after 28 consecutive days of administration and a 28-day non-dosing fixation period. Kidneys, lungs, liver, duodenum, glandular stomach and testes were collected and frozen. Bone marrow was collected from both femurs of all surviving animals. DNA was isolated from frozen liver, lung, glandular stomach and duodenum samples from each of the five animals. A sixth animal was also processed in Group 1 for all tissue types, as an animal was determined to contain a jackpot mutation and was, therefore, excluded from group mean calculations for all tissue types. Isolated DNA from liver, lung, glandular stomach and duodenum was processed for the determination of total mutant frequency (*cII* mutant).

Results

No substance-related clinical observations were reported. Body weights and food consumption were unaffected by test substance administration. No statistically significant increase in mutant frequency at the *cII* gene was reported in lung, glandular stomach and duodenum of treated animals compared with the vehicle control animals. A statistically significant increase in mutant frequency in the liver at the dose levels of 75 mg/kg bw per day was reported. Two additional animals across all treatment groups were added and processed to re-evaluate the mutant frequency in liver. The results of the second analysis showed a statistically significant increase at doses of 75 and 300 mg/kg bw per day, but not at 150 mg/kg bw per day.

The Panel considered the results in liver as inconclusive, as the statistically significant increases in mutant frequencies observed were small (1.3-fold) and not dose-related. According to the OECD TG 488 (OECD, 2022b) criteria, the distribution of historical negative control data should be considered as additional evaluation criterium. In this respect, the Panel noted that no laboratory historical control database for Big Blue® B6C3F1 mice was available, as the data reported in the submitted study report were related to a different strain of mice (C57BL/6) (10–11 animals compared to the minimum recommended of 30 animals).

The study was considered by the Panel as reliable with restrictions. The results in lung, glandular stomach and duodenum were considered of limited relevance and the inconclusive results in liver of low relevance.

3.1.4 | Human biomonitoring studies applying genotoxicity biomarkers

The biomonitoring studies available in the scientific literature are related to the application of genotoxicity biomarkers in workers occupationally exposed to styrene. The large majority of the studies were carried out in the glass-reinforced plastics industry and evaluated DNA adducts, DNA strand breaks by comet assay, oxidative DNA damage, CA and MN. These studies were reviewed in the IARC Monograph (IARC, 2019) and briefly summarised in the previous CEP Panel opinion (EFSA CEP Panel, 2020); they are assessed in the present opinion together with newly published meta-analyses.

DNA damage

Twelve studies carried out in the 80s and in 90s on the determination of DNA adducts in groups of workers occupationally exposed to styrene were described. DNA adducts were detected by 32P-postlabelling analysis in DNA isolated from lymphocytes. A correlation between the concentrations of the adducts and the extent of styrene exposure was observed. Adducts at N2-guanine (Horvath et al., 1994; Rappaport et al., 1996), O6-deoxyguanosine (Vodicka et al., 1993; Vodicka et al., 1994; Vodicka et al., 1999; Vodicka & Hemminki, 1993) and N3-adenine (Mikes et al., 2010) were identified.

Positive results were reported in a number of studies on DNA damage evaluated by comet assay (Brenner et al., 1991; Buschini et al., 2003; Laffon et al., 2002; Shamy et al., 2002; Somorovská et al., 1999; Walles et al., 1993; Wongvijitsuk et al., 2011). The increase in DNA damage was correlated with the extent and the years of exposure to styrene. Some limitations were reported in these studies, such as small sample size, absence of control groups or of the analysis for relevant confounding factors, such as the smoking habits. Moreover, these positive results were not confirmed in other studies in which no significant differences in the levels of DNA damage were observed in exposed subjects compared with control groups (Costa et al., 2012; Godderis et al., 2006; Hanova et al., 2010; Teixeira et al., 2010; Vodicka et al., 2004). Few studies with negative results were reported on the evaluation of oxidative damage using the enzyme-modified version of the comet assay (Fracasso et al., 2009; Hanova et al., 2010; Somorovská et al., 1999). The studies measuring 8-hydroxy-2′-deoxyguanosine (8-OHdG) in DNA reported inconsistent results (Manini et al., 2009; Marczynski et al., 1997; Wongvijitsuk et al., 2011).

Gene mutations

Few studies reported on gene mutations in human subjects exposed to styrene (Bigbee et al., 1996; Compton-Quintana et al., 1993; Vodicka et al., 2001). The results of these studies were inconclusive due to some limitations, such as the co-exposure to other compounds and large inter-individual variability in the mutation frequency.

Chromosomal damage

The large majority of the HBM studies reported in the IARC Monograph are related to the evaluation of chromosomal damage. Thirty-nine studies reported inconsistent results on CAs or MN frequency. These studies, published starting from 1978 (Meretoja et al., 1978), reported results in various occupational settings with the use of different methods for genotoxicity endpoints and statistical analyses. Several studies of adequate size and study design described positive results on chromosomal biomarkers. The exposure to styrene was measured through the determination of styrene concentration in environmental and exhaled air, by urinalysis of styrene metabolites or by determination of styrene in blood. They showed that the exposure to high concentrations of styrene induced an increase in the frequency of CAs or of MN with different level of evidence. However, no correlation between the exposure indices and the frequency of chromosomal damage was reported in some of the studies (Andersson et al., 1980; Forni et al., 1988). The duration of exposure was not associated

with the induced chromosomal damage (Camurri et al., 1983; Tomanin et al., 1992). Also inconsistency between the results of CAs and MN frequency in the same groups of exposed subjects was observed (Nordenson & Beckman, 1984; Tomanin et al., 1992; Yager et al., 1993). In at least 10 studies reported in the IARC Monograph and considered of adequate size and study design, no increase of chromosomal endpoints was observed, including groups of workers exposed to concentration of styrene above 100 ppm. Other studies reported in the IARC Monograph were considered limited due to a number of factors, such as small sample size, lack of control group and co-exposure to other genotoxicants.

Two publications were retrieved by the targeted literature search carried out to address the current mandate covering the timespan from 1 January 2018 to 1 October 2024, not reported in the 2019 IARC Monograph. A study reported the application of the comet assay and MN test in buccal exfoliated cells from a group of workers occupationally exposed to styrene in glass reinforced plastic industry (Cavallo et al., 2018 [RefID 39]). No induction of DNA strand breaks nor of MN frequency was reported in exposed subjects. The study was considered inconclusive due to some limitations, such as the small sample size, experimental shortcomings and lack of control for possible confounding factors.

Another study showed an increase of MN frequency and of DNA damage evaluated by the comet assay in peripheral blood lymphocytes of a small group of workers from a polymer producing industry (Ladeira et al., 2020 [RefID 174]). However, the observed genotoxic effects could not be associated with the styrene exposure due to the reported co-exposure to other genotoxic substances, such as organic solvents.

Two meta-analyses of the published biomonitoring studies on the application of the MN assay (Collins & Moore, 2019 [RefID 232]) and the CA test (Collins & Moore, 2021 [RefID 80]) in the peripheral lymphocytes of occupationally exposed styrene workers were available. The objective of these meta-analyses was to explore whether, despite the limitations and the heterogeneity of the individual studies, a general trend for the induction of chromosomal damage associated with styrene exposure could be identified. The two meta-analyses were not available at the time of the 2019 IARC evaluation.

The meta-analysis of MN frequencies (Collins & Moore, 2019 [RefID 232]) considered 24 studies. In a first step, it selected those that used the cytokinesis block method (addition of cytochalasin B), i.e. 15 studies. Three further studies were excluded, because they were nested in wider studies considered separately. The meta-analysis was conducted on a final sample of 12 studies, including 516 styrene-exposed workers and 497 non-exposed subjects. All selected studies provided a mean and a standard deviation or standard error of MN frequency per 1000 cells and the estimates of styrene exposure. Two exposure categories were identified: A high styrene exposure when either the mean air concentration exceeded the American Conference of Governmental Industrial Hygienists (ACGIH) 2003 standard of 20 ppm styrene (87 mg/m^3) or 400 mg (mandelic acid (MA) + phenylglyoxylic acid (PGA))/g creatinine for internal exposures, and a low exposure when below both ACGIH standards. The mean differences of MN frequencies between workers exposed to styrene and control groups were calculated. The standardised mean difference was used as the summary statistic parameter. An overall increase of MN in exposed workers was observed. The results of the primary meta-analysis showed slight but statistically significant meta-mean differences between exposed and non-exposed workers: 1.19 (95% confidence interval (CI) 0.20–2.18). Higher styrene exposure was associated with higher standardised mean difference. The low styrene-exposed workers had a meta-mean difference of 0.44 (95% CI 0.93–1.82) compared to the high styrene-exposed workers of 1.79 (95% CI 0.38–3.21) in the random effects models. A significant heterogeneity was also reported in the meta-analysis. Overall, the data were found insufficient to support a conclusion that styrene exposure increases MN frequencies in styrene-exposed workers.

A similar meta-analysis was conducted on the available published studies using CAs as the measure of cytogenetic damage (Collins & Moore, 2021 [RefID 80]). The main criteria applied to select the studies were the use of the standard method analysing the aberrations in cells stimulated to divide in vitro, the data reporting with a mean and a standard deviation or standard error of the CAs per 100 cells without gaps and other non-standard events. Eighteen publications were considered in the meta-analysis, including 505 styrene-exposed workers and 532 control individuals. The standardised mean difference was applied as the summary statistic. The results of the meta-analysis showed that the styrene workers overall had a slight but statistically significant difference in CAs: meta-mean difference of 0.361 (95% CI: 0.084–0.807) in the random effects model. A lack of consistency across the studies was observed. No exposure response was reported: Studies with higher styrene exposures had lower mean standard differences compared to studies with lower styrene exposures. Hence, also for CA, the data were considered insufficient to support a conclusion that styrene exposure increases CA frequencies in styrene-exposed workers.

The results of the two meta-analyses showed a significant heterogeneity across the studies which prevented the Panel to conclude on a chromosomal damage associated with occupational exposure to styrene by inhalation.

The Panel noted several limitations in the biomonitoring studies using genotoxicity biomarkers, such as the large variability and uncertainties in the extent and profile of exposure, the possible co-exposure to other genotoxicants, the lack of consistency across studies, the absence of an exposure response and the lack of control for relevant confounding factors. Overall, taking also into account the conclusions of the two meta-analyses, the results of these biomonitoring studies in workers occupationally exposed to styrene by inhalation do not provide sufficient evidence to support an association between styrene exposure and genotoxic damage in humans. Moreover, these human biomonitoring studies do not provide additional relevant information for the assessment of the genotoxic hazard posed by oral exposure to styrene.

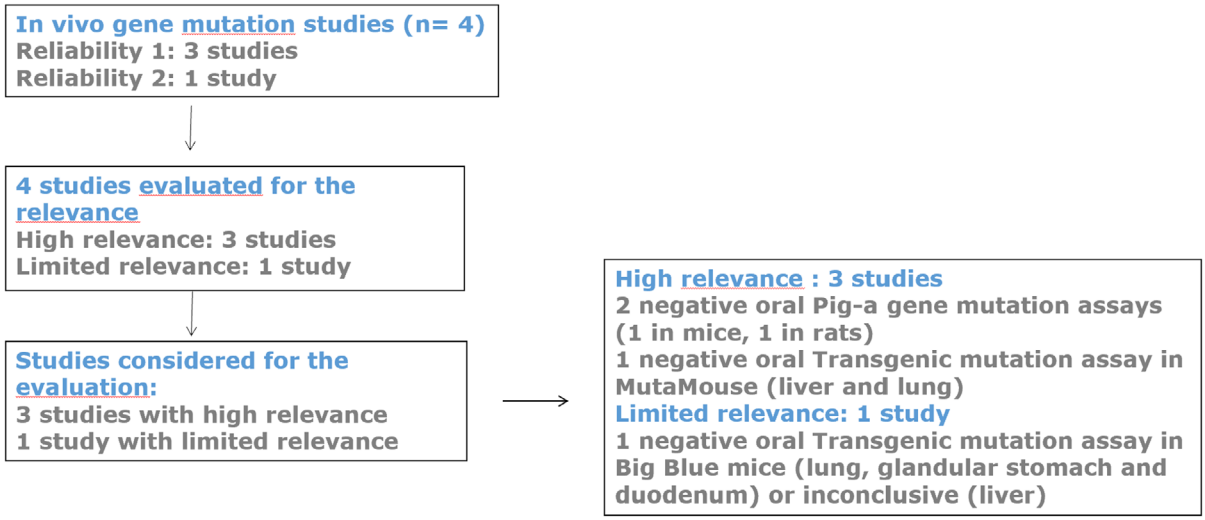
3.1.5 | Overall evaluation and conclusions on genotoxicity of styrene

The results of the in vivo genotoxicity studies on styrene described in the previous sections were integrated in a WoE approach, as recommended by the EFSA Scientific Committee (2011, 2017a, 2017b). To this aim, the study results addressing apical genotoxicity endpoints (gene mutation and chromosome damage) and primary DNA damage evaluated by the comet assay were grouped in lines of evidence. Only studies with results of high or limited relevance were taken into account (EFSA, 2023).

Gene mutation

Four studies evaluated the induction of gene mutations by styrene in various target tissues after oral administration (Flow chart 1). Negative results of high relevance were obtained in erythropoietic cells in two studies (*Pig-a* assays in mice and rats) in which the systemic exposure to styrene was demonstrated by analyses of plasma samples. In transgenic rodent models, negative results of high relevance were obtained in liver and lung in MutaMice. Negative results in lung, glandular stomach and duodenum, and inconclusive results in liver, all considered of limited relevance, were reported in a study in Big Blue® mice.

Overall, the evidence from the available studies does not indicate an in vivo mutagenic activity of styrene after oral exposure.

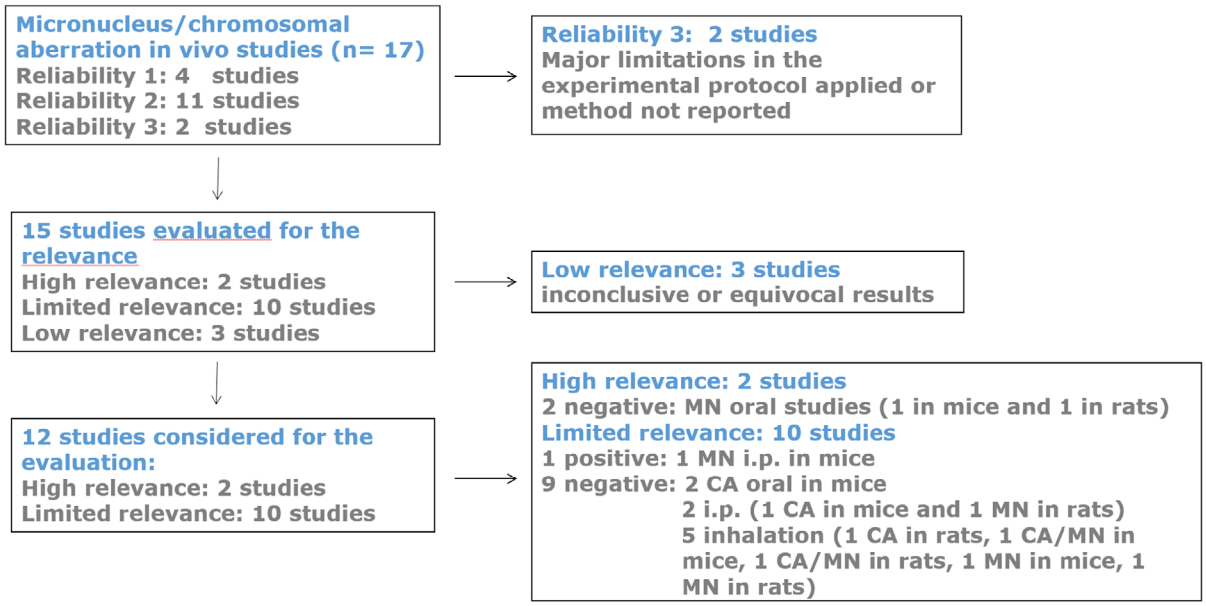


FLOW CHART 1 Summary of in vivo studies on gene mutations. According to the EFSA Technical Report (EFSA, 2023), the reliability of the studies is scored as: 1. Reliable without restriction; 2. Reliable with restrictions; 3. Not reliable; 4. Not assignable. The relevance is referred to the results of the individual genotoxicity studies.

Chromosome damage

Twelve out of seventeen studies presented results of high/limited relevance. These studies (four CA tests, six MN tests and two CA/MN tests) evaluated the in vivo clastogenic and/or aneugenic activity of styrene in rodents by various routes of administration (Flow chart 2). No induction of MN was observed in two highly relevant oral studies in mice and rats. Negative results were also reported in two oral CA tests of limited relevance. Negative results of limited relevance were obtained in five studies on CA and MN frequency in mice and rats exposed to styrene by the inhalational route and in two studies by i.p. administration, a non-physiological route of exposure (EFSA, 2023). A single positive result of limited relevance was reported in another i.p study in mice.

Overall, the Panel considered that the studies using physiological routes of exposure to styrene, including reliable oral studies in mice and rats, provided no evidence of structural or numerical chromosome damage.

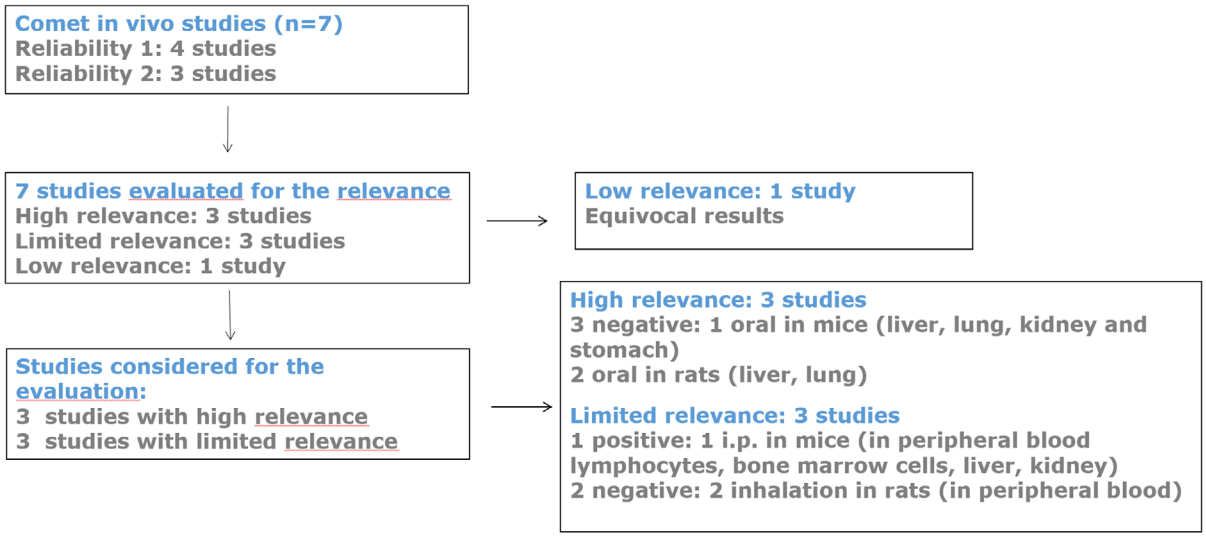


FLOW CHART 2 Summary of in vivo studies on chromosomal damage. According to the EFSA Technical Report (EFSA, 2023), the reliability of the studies is scored as: 1. Reliable without restriction; 2. reliable with restrictions; 3. not reliable; 4. not assignable. The relevance is referred to the results of the individual genotoxicity studies.

DNA damage (*in vivo* comet assay).

Six out of seven studies presented results of high/limited relevance. These studies (two in mice and four in rats) evaluated DNA damage in several tissues after exposure to styrene by various routes (Flow chart 3). Three highly relevant oral studies (one in mice and two in rats) did not show an increase in DNA damage in liver (three studies), lung (three studies), kidney (only two studies, in mice and rats) and stomach (negative in one study in mice; equivocal results, evaluated as of limited relevance, were observed in one study in rats). Negative results in peripheral blood were also obtained in two inhalational studies in rats of limited relevance. A positive result was reported in one study in mice evaluated as of limited relevance, in which an increase in primary DNA damage was observed in several tissues shortly after i.p. administration, a non-physiological route of exposure (EFSA, 2023).

Overall, the Panel considered that the studies using physiological routes of exposure to styrene, including reliable oral studies in mice and rats, provided no evidence of primary DNA damage.



FLOW CHART 3 Summary of in vivo studies on comet assay. According to the EFSA Technical Report (EFSA, 2023), the reliability of the studies is scored as: 1. Reliable without restriction; 2. Reliable with restrictions; 3. Not reliable; 4. Not assignable. The relevance is referred to the results of the individual genotoxicity studies.

In conclusion, the available studies did not show an in vivo genotoxic activity of styrene administered by physiological routes (non i.p.). In particular, no evidence for genotoxic activity was provided by reliable oral studies addressing different endpoints (see Table 3 Summary of oral studies), in which styrene was administered up to the MTD for a period of 4 weeks.

TABLE 3 Summary of test results and evaluations of oral in vivo genotoxicity studies.

Study design	Test material	Results	Reliability/ relevance	Reference
In vivo gene mutation				
Transgenic mutation assay in MutaMouse (*lacZ* system) liver and lung 75, 150 or 300 mg/kg bw per day for 28 days	Styrene 100% pure	Negative	1/high	Murata et al. (2023)
Mammalian Erythrocyte *Pig-a* gene mutation assay in mice 75, 150, 300 mg/kg per day for 29 days	Styrene monomer (CAS RN 100-42-5) purity 99.95%	Negative	1/high	SIRC (2023a)
Mammalian Erythrocyte *Pig-a* gene mutation assay in rats 100, 250 or 500 mg/kg per day for 29 days	Styrene monomer (CAS RN 100-42-5) purity 99.95%	Negative	1/high	SIRC (2022)
Mutation Assay at the *cII* Locus in Transgenic Big Blue® Mice 75, 150 or 300 mg/kg bw per day for 28 days	Styrene monomer (CAS RN 100-42-5) purity 99.95%			SIRC (2024)
Liver Lung, glandular stomach and duodenum		Inconclusive Negative	2/limited	
In vivo micronuclei/chromosomal aberrations				
Mammalian bone marrow CA test in mice 500 and 1000 mg/kg bw Single administration	Styrene (Merck Darmstadt F.R.G.)	Negative	2/limited	Loprieno et al. (1978)
Mammalian bone marrow CA test in mice 500 mg/kg bw per day for 4 days 200 mg/kg bw per day for 70 days	Styrene (Merck Darmstadt F.R.G.)	Negative	2/limited	Sbrana et al. (1983)
Mammalian Erythrocyte MN Test in mice 75, 150, 300 mg/kg per day for 29 days	Styrene monomer (CAS RN 100-42-5) purity 99.95%	Negative	1/high	SIRC (2023a)
Mammalian Erythrocyte MN Test in rats 100, 250 or 500 mg/kg per day for 29 days	Styrene monomer (CAS RN 100-42-5) purity 99.95%	Negative	1/high	SIRC (2022)
In vivo DNA damage by comet assay				
Comet assay in mice 75, 150, 300 mg/kg per day for 29 days Liver, lung, kidney, stomach	Styrene monomer (CAS RN 100-42-5) purity 99.95%	Negative	1/high	SIRC (2023a)
Comet assay in rats 100, 250 or 500 mg/kg per day for 29 days Liver and lung	Styrene monomer (CAS RN 100-42-5) purity 99.95%	Negative	1/high	SIRC (2022)
Comet assay in rats 100, 250 or 500 mg/kg per day for 28 days	Styrene monomer (CAS RN 100-42-5) purity 99.86%			SIRC (2023b)
Liver and lung Kidney Stomach		Negative Negative Equivocal	1/high 2/limited 1/limited	

3.2 | Toxicokinetics data

3.2.1 | Introduction

The overall absorption, distribution, metabolism and excretion (ADME) properties of styrene have been extensively evaluated in humans and experimental animals, along with the available physiologically based kinetic (PBK) models. A summary of this evaluation is reported below, while the detailed version can be found in Annex A.

For the purpose of this opinion, data from the IARC evaluation (IARC, 2019) and additional relevant information retrieved from a targeted literature search (Section 2, Appendix A) were used for the characterisation of the toxicokinetic behaviour of styrene. Occupational inhalation is the primary source of exposure to styrene. Therefore, most of the published experimental studies available on styrene ADME have been carried out by inhalation, dermal exposure and only in few cases by oral administration. Available HBM data showed that in the general population, exposed by different routes, including via the diet, measurable levels of styrene are present in blood (Section 3.3).

3.2.2 | Absorption

After inhalation exposure, the available results have shown that styrene is rapidly and well absorbed both in experimental animals and humans, with an absorption rate higher in mice than in rats and humans. SO was detected in the nanomolar range in human blood. The overall experimental evidence suggests that the rate of uptake of styrene in

the upper respiratory tract is strictly related to the local metabolism. Following oral exposure, considering its physicochemical properties and available experimental animal data, a similar fast absorption of styrene in humans through the gastrointestinal tract can be anticipated. After oral administration in rats and mice, styrene reached peak concentrations in blood after 1–2 h. In the same experimental conditions, SO in blood was hardly or not detectable and rapidly cleared.

3.2.3 | Distribution

After absorption, styrene is widely distributed throughout the body in different tissues (e.g. liver, kidney, lung), depending on the route of exposure (inhalation, oral, i.p.). After inhalation exposure, a slow release of styrene from adipose tissue was shown both in animals and humans. The elimination half-lives of styrene in humans following inhalation exposure were estimated to be 40 min in blood and 2–4 days in adipose tissue, respectively.

3.2.4 | Metabolism

Human and experimental animal data indicate extensive styrene biotransformation, albeit with species-specific quantitative differences in both liver and lung. The primary isoenzymes responsible for the epoxidation of the side chain of styrene into SO are CYP 2E1, 2B6 and 2C8 in liver and CYP 2F1/F2, 2A13 and 2E1 in lung. In humans, the major further biotransformation is the hydrolysis of SO by epoxide hydrolase, a highly efficient first-pass metabolism that readily reduces the systemic bioavailability of the epoxide. The alternative conjugation of SO with glutathione (GSH), producing mercapturic acids, contributes little to the overall epoxide metabolism in humans. Urinary excretion data suggest that oxidation of the phenyl ring, further conjugated to glucuronic acid and sulfate, is only a minor route of excretion in humans exposed to styrene. Comparative in vitro metabolism studies have shown clear differences in the activity and/or affinity of styrene and its metabolites to cytochrome P450 (CYP450), epoxide hydrolase (EPHX1) and glutathione S-transferase in the liver and lung between species. Mice and humans have the highest and lowest capacity to form SO, respectively. Furthermore, humans have a greater capacity to further metabolise SO compared to rodents. Quantitative interspecies differences were identified in the aromatic ring hydroxylation to produce 4-vinylphenol (less than 1% in humans) and the further biotransformation in its reactive electrophilic products, mainly observed in mice.

The liver, followed by the lung (after inhalation exposure), are the primary human tissues involved in the biotransformation of styrene and SO. The findings on the role of extrahepatic metabolism, including at intestinal level (as the first site of contact by oral route), suggest a minor impact, if any, on styrene metabolism.

3.2.5 | Excretion

In humans after inhalation, most of the absorbed styrene (95%) is metabolised and excreted in the urine as MA (85%) and PGA (10%); the sum of these two metabolites is considered a quantitative biomarker of styrene exposure. A lower extent of formation of these metabolites has been reported in rodents, suggesting a species-specific difference in the impact of different pathways on the overall metabolism of styrene. Additional metabolites, produced by other minor pathways (e.g. PHEMA, phenylacetic acid and/or phenylaceturic acids) in humans, account for less than 5% of the urinary metabolites. A small fraction of styrene is eliminated unchanged in the urine. No data are available on the urinary excretion of SO.

3.2.6 | PBK modelling

Several multicompartment PBK models, developed based on different exposure scenarios (inhalation, oral, single and repeated exposure), support the main kinetic properties of styrene among species (mice, rats and humans).

Interspecies differences in metabolic capacity with very low concentrations of SO (around 0.5 ng/mL) predicted in human blood; saturation of styrene metabolism in humans at high concentrations of styrene in blood (as shown by a plateau of SO blood levels reached during the first 2 h), with metabolism limited by the rate of blood perfusion in the liver or other organs involved in styrene elimination; conjugation with GSH as a minor pathway in the styrene detoxification pathway in humans; and slow release of styrene from adipose tissue were confirmed by modelling inhalation styrene exposure.

Applying PBK models by the oral route, the saturation kinetics of styrene metabolism at high doses as well as a higher affinity of styrene for adipose tissue were confirmed. An estimation of SO in blood, simulating food intake in humans, resulted in C_{max} values in the nanomolar range. By applying forward dosimetry, based on an estimated oral intake for the general population, no accumulation in the liver, although with a slightly higher styrene concentration than in the blood, was predicted.

3.2.7 | Conclusions on the toxicokinetics of styrene

In summary, although the same metabolic pathways were identified in mice, rats and humans, they differed quantitatively: mice exhibited a higher rate of SO formation, along with the lowest detoxification capacity. The opposite was observed in humans, where highly efficient first-pass hepatic hydrolysis by epoxide hydrolase significantly reduces the systemic availability of the already low amount of epoxide formed in situ. Based on the relative contribution of different metabolic enzymes in extrahepatic tissues, including the gastrointestinal tract (as the first site of contact), the liver is considered as the primary tissue responsible for styrene metabolism after oral exposure. The tissue-specific metabolism of styrene suggests that in situ metabolism may be the key to toxicity compared to overall systemic metabolism and blood levels of styrene metabolites: local metabolism in a target tissue must be considered.

Based on the available evidence, the Panel concluded that the toxicokinetic behaviour of styrene was species- and tissue-specific. Toxicokinetic, in particular metabolic differences, are considered as one of the determinants in the different sensitivity of some species to its toxicity (e.g. highest in mice).

3.3 | Internal and external exposure data

3.3.1 | Internal exposure

3.3.1.1 | *Introduction*

Monitoring of internal exposure to chemicals, characterised by measuring concentrations of these chemicals and/or marker metabolites in humans (e.g. body fluids like blood, urine, breast milk or saliva), is often referred to as HBM. All routes of (external) exposure are aggregated inside the human body. Therefore, in general, the different contributions of exposure routes and/or sources cannot be distinguished by measuring concentrations of parent compounds and/or metabolites in, e.g. body fluids. However, in some cases specific external exposure, like, e.g. inhalation of tobacco smoke, can be determined if subpopulations (smokers/non-smokers) can be distinguished.

In general (if no protective measures are taken), occupational exposure to industrial chemicals is higher than dietary exposure. Consequently, urinary concentrations of exposure biomarkers are high enough to be measured. In case of styrene, the general population is monitored by measuring blood levels of styrene and, for practical reasons, industrial workers are monitored by measuring urinary concentrations of (marker) metabolites at different time intervals (e.g. pre or post working shifts). See also next section on exposure biomarkers.

3.3.1.2 | *Exposure biomarkers*

Reliable, sensitive and specific analytical methods exist for the HBM of occupational exposure to styrene (IARC, 2019). The relationship between air concentrations and biomarkers of exposure to styrene has been studied extensively. About 95% of the absorbed styrene is excreted in urine as MA and PGA (see Section 3.2.5). The kinetics of MA and PGA formation and elimination can be influenced by exposure to other solvents, including ethylbenzene, phenylglycol, some pharmaceuticals and alcoholic beverages, thus limiting the specificity of these biomarkers of styrene and SO exposure (IARC, 2019). The sum of the two excreted metabolites (MA + PGA) is less affected by these confounding factors and is, therefore, better suited for monitoring exposure to styrene and SO (IARC, 2019; Ong et al., 1994). However, these biomarkers are also present in urine of humans (workers) exposed to ethylbenzene and, consequently, are not specific for styrene exposure.

Biomonitoring of styrene in urine can be limited by the fact that less than 1% of absorbed styrene is eliminated unchanged in urine (NTP, 2008). Typical urinary styrene concentrations are in the range of 5–50 µg/L, i.e. more than 1000 times less than urinary MA + PGA concentrations.

After occupational exposure to styrene, traditionally the parent compound has not been measured in blood. Styrene concentrations in blood of workers have only been published in a few studies (IARC, 2019). Probably the combined determination of MA and PGA in urine of workers is of interest, because simultaneous exposure to styrene and ethylbenzene could be relevant in some working environments. On the other hand, styrene exposure of the general population is usually determined by measuring styrene in blood. However, in the fourth national report on human exposure to environmental chemicals (updated in 2021) of the United States Centers for Disease Control and Prevention (CDC), MA and PGA as well as phase-2 metabolites like phenylhydroxyethylmercapturic acids (PHEMAs) have also been measured in urine of the US general population in the period of 2005–2006 and 2011–2016 (CDC, 2021a).

Styrene-7,8-oxide levels in blood and urine of humans are usually too low to be measured. SO albumin and haemoglobin adducts are specific biomarkers for styrene and SO exposure. They correlate well with inhalation exposures in workers to these compounds. However, the biomarker measurements have some disadvantages, including low concentrations and short half-life and/or lifespan in blood. Furthermore, the analytical methods are not sufficiently sensitive for these adducts (IARC, 2019).

3.3.1.3 | *General population*

Most HBM data for the general population have been generated in the USA, Canada and Italy. Blood levels of styrene in the general population (children and adults) of the USA (until 2008), including one paper with data from Italy (Brugnone et al., 1993), were mentioned in the IARC Monograph (2019). By means of the full text screening, new HBM data from the USA (2009–2010) and data from Canada (2014–2015) are incorporated in an overview given in Table 4. With their national reports on human exposure to environmental chemicals, the US CDC provides an ongoing assessment of the exposure of the US population to styrene and other environmental chemicals by HBM, conducted on random samples of NHANES participants.

Canada provided the only data set with a differentiation between smokers and non-smokers. From this data set, it can be concluded that in 2014–2015 styrene blood concentrations in the subpopulation of smokers (P50 and P95, respectively, 0.08 and 0.19 µg/L) were almost twice those in the non-smokers (P50 and P95, respectively, 0.043 and 0.10 µg/L).

From the year 2001 onwards, the median styrene blood concentrations for the Canadian and the USA general population range from below the detection limit to 0.08 µg/L. P95 concentrations range from 0.08 to 0.2 µg/L. Over the last two decades, the styrene blood concentrations in the USA are rather stable.

Overall exposures to styrene may vary by region and season. Children enrolled in the School Health Initiative in Minneapolis (Minnesota) displayed seasonal variation in blood styrene with a range of median concentrations of 0.07–0.11 µg/L, thereby exceeding the national median (Sexton et al., 2005). The authors state that the relatively high styrene concentrations in children, compared to the NHANES data of that period, were unexpected. The source of the children's exposure was not known and the seasonal variation were also not explained.

TABLE 4 Concentrations of styrene in blood (μg/L) of general populations in Canada, Italy and the United States of America.

Population	Age (years)	Sample size	Blood styrene concentrations (μg/L) at different percentiles				Survey years	Reference
			50th	75th	90th	95th		
Canada	12–16	–	0.043	–	–	–	2014-'15	Cakmak et al. (2020) [RefID 1888]
Canada	17–79	–	0.048	–	–	–	2014-'15	Cakmak et al. (2020) [RefID 1888]
Canada	All ages	3632	0.051	–	–	–	2014-'15	Cakmak et al. (2020) [RefID 1888]
Canada	All ages|NS	–	0.043	–	–	–	2014-'15	Cakmak et al. (2020) [RefID 1888]
Canada	All ages|S	–	0.08	–	–	–	2014-'15	Cakmak et al. (2020) [RefID 1888]
Canada	12–79|NS	2050	–	–	–	0.10	2014-'15	Faure et al. (2020) [RefID 1927]
Canada	12–79|S	417	–	–	–	0.19	2014-'15	Faure et al. (2020) [RefID 1927]
USA	12–19	237	<0.03	0.037	0.063	0.081	2009-'10	CDC (2021b)
USA	20–59	723	<0.03	0.052	0.111	0.146	2009-'10	CDC (2021b)
USA	60+	360	<0.03	0.043	0.071	0.103	2009-'10	CDC (2021b)
USA	All ages	1320	<0.03	0.048	0.096	0.132	2009-'10	CDC (2021b)
USA	All ages	2719	<0.03	0.045	0.096	0.130	2007-'08	CDC (2021b)
USA	All ages	2808	<0.03	0.047	0.099	0.135	2005-'06	CDC (2021b)
USA	All ages	1245	<0.03	0.050	0.089	0.120	2003-'04	CDC (2021b)
USA	All ages	950	<0.03	0.080	0.130	0.200	2001-'02	CDC (2021b)
USA	20–59	1459	<0.03	0.055	0.109	0.151	2007-'08	CDC (2021b)
USA	20–59	1380	<0.03	0.051	0.110	0.150	2005-'06	CDC (2021b)
USA	20–59	1245	<0.03	0.050	0.089	0.120	2003-'04	CDC (2021b)
USA	20–59	950	<0.03	0.080	0.130	0.200	2001-'02	CDC (2021b)
Louisiana	15–60+	297	0.021	0.036	0.049	0.06	2002	Uddin et al. (2014)
Minnesota	6–10	103	0.07	0.18	0.74	0.85	2000 Feb	Sexton et al. (2005)
Minnesota	6–10	108	0.09	0.18	0.54	0.68	2000 May	Sexton et al. (2005)
Minnesota	6–10	54	0.09	0.10	0.11	0.11	2001 Feb	Sexton et al. (2005)
Minnesota	6–10	88	0.11	0.12	0.17	0.21	2001 May	Sexton et al. (2005)
USA	20–59	2476	0.021	NR	0.11	0.158	1999–2004	Su et al. (2011)
USA	20–59	624	0.041	NR	0.129	0.177	1988–1994	Su et al. (2011)
Italy	20–59	81	0.17	NR	NR	0.51	Prior to 1993	Brugnone et al. (1993)

Abbreviations: NR, not reported; NS, non-smokers; S, smokers.

Styrene was detected in breast milk from 12 healthy women from Baltimore (Maryland) who were breastfeeding at least 30 days postpartum. The average styrene concentration in the breast milk was 0.219 μg/L, with a range of 0.055–0.710 μg/L and a median of 0.129 μg/L (Blount et al., 2010). The median concentration in breast milk is slightly higher than the range of median blood styrene concentrations in Canada and USA.

3.3.1.4 | *Summary*

For the general population, the biomonitoring of styrene is usually performed in blood. Sufficient HBM data from Canada and USA are available. Only one set of relatively old HBM data (Brugnone et al., 1993) is available from a European country (Italy). With their national reports on human exposure, the US CDC provides an ongoing assessment of the exposure of the US population to styrene by means of HBM.

Over the last two decades, the P50 values for Canada and the USA range from below detection limit to 0.08 μg/L; P95 values are in the range of 0.08–0.2 μg/L. During this period, styrene blood concentrations in the US general population were rather stable.

3.3.2 | External exposure

3.3.2.1 | *Introduction*

Known sources of external exposure for the general population are food, tobacco smoking and indoor/outdoor air (IARC, 2019). Migration from consumer products (e.g. toys) has also been addressed in one scientific paper.

3.3.2.2 | *Dietary exposure*

In the 2020 EFSA opinion, the mean dietary exposure to styrene migrated from styrenic plastics is in the order of 0.1 μg/kg bw per day for adults; the 90th percentile is 0.17 μg/kg bw per day for children (EFSA CEP Panel, 2020). It is in the same range as exposure from styrene present in foods as such. Overall, the mean daily total dietary exposure to styrene in food (from migration and as a food component) was estimated by Cao et al. (2018 [RefID 2561]) as 0.16 μg/kg bw for adults (19–70 years), 0.31 μg/kg bw for children (3–8 years) and 0.38 μg/kg bw for toddlers (2–3 years).

In order to compare dietary exposure with total exposure, a paper from Banton et al. (2019 [RefID 1383]), already cited in the 2020 EFSA opinion, is also described shortly in this paragraph. Banton et al. (2019 [RefID 1383]) concluded that, in general, dietary exposures to styrene increased from infants to toddlers and subsequently decreased in older children and adults. The authors stated that the average dietary styrene intake calculated for toddlers to elderly by Cao et al. (2018 [RefID 2561]) is within the range of estimates published by other authors, i.e. from 0.38 (toddlers) to 0.16 (adults) and 0.12 (elderly) μg/kg bw per day.

Additional screening of scientific papers only led to one paper on the dietary exposure assessment of styrene (Hwang et al., 2019 [RefID 2625]). The authors determined the migration of styrene from FCM into four food simulants (water, 4% acetic acid, 50% ethanol and n-heptane). The estimated daily intake (EDI) was calculated using these migration results, food consumption factors (CF) and food-type distribution factors (fT). The CF and fT were from the US FDA guidelines (US FDA, 2007). The authors used a daily food intake of 1.5 kg food per person (excluding water and drinks) and an average body weight of 60 kg. The EDI of styrene was calculated to be 0.614 μg/kg bw per day, which is somewhat higher than that estimated by EFSA CEP Panel (2020).

3.3.2.3 | *Other routes of exposure*

Only one paper (Banton et al., 2019 [RefID 1383]), already described in the 2020 EFSA opinion, quantified exposure from sources other than food, like active smoking, passive smoking (environmental tobacco smoke), inhalation (air) and mouthing (toys).

Regarding inhalation of tobacco smoke and contaminated air, Banton et al. (2019 [RefID 1383]) concluded on a central tendency[9] respiratory daily exposure of 0.1 μg/kg bw for adult non-smokers and of 0.28 μg/kg bw per day for smokers, with upper bound[10] values of 0.31 and 0.83 μg/kg bw per day, respectively. Excluding babies of less than 1 month in age, the highest exposure was derived for children of 1–2 years, due to higher inhalation rates per unit bw, with central tendency respiratory exposure of 0.36 μg/kg bw and an upper bound value of 1.1 μg/kg bw per day. For the general population, central tendency styrene intakes via inhalation ranged from about 0.10 to 0.44 μg/kg bw per day, upper-bound intakes

[9]These authors state that the term 'central tendency' refers to "statistical measures that characterise a central or typical value of a probability distribution. Depending upon data availability, arithmetic mean, 95% upper confidence limit on arithmetic mean, geometric mean, or median (50th percentile) may be used as estimates of central tendency".

[10]These authors state that the term 'upper bound' refers to "statistical measures that characterise a "reasonable maximum" for a population – a plausible upper limit to the true value of a quantity. Depending upon data availability, 90–95th percentiles or maximum values may be used as estimates of the upper bound".

from about 0.31 to 1.3 μg/kg bw per day. Taking other papers into consideration, it has been concluded by EFSA in 2020 that the daily exposure to styrene by inhalation is in the range of 0.1–0.6 μg/kg bw for adults.

Styrene-containing polymers are used in the manufacturing of children's toys. According to Banton et al. (2019 [RefID 1383]), the estimated daily exposures to styrene for children from mouthing of toys range from 4.7 to 50 ng/kg bw per day, i.e. more than an order of magnitude lower than dietary exposures.

3.3.2.4 | *Summary*

Important exposure sources throughout the different age groups are food and air (similar contribution). For adult smokers, inhalation (air plus tobacco smoke) is the major exposure route.

The (rounded) mean daily total dietary exposure to styrene in food (from migration and as a food component) was estimated as 0.2, 0.3 and 0.4 μg/kg bw per day for adults, children and for toddlers, respectively.

Maximum (rounded) total external exposure is 0.5 μg/kg bw per day for non-smoking adults and 1.0 μg/kg bw per day for adult smokers. Total exposures vary significantly for different age groups.

3.3.3 | Correlating internal and external exposures of the general population

3.3.3.1 | *Introduction*

In order to relate internal to external exposure, kinetic models (in particular PBK models) can be used for forward and reverse dosimetry. Most models have been developed for the exposure of styrene to rodents (mice and rats), in particular the inhalation of styrene (through lung compartment) has frequently been included. See also Section 3.2.6. Only one paper addressed the correlation of internal and external exposure of the general population, see next section.

3.3.3.2 | *Correlation based on kinetic models*

Most models developed for the external exposure to styrene in humans have simulated the fate of styrene in blood. Some models have focussed on the fate of SO in human blood. In particular one PBK model (Miura et al., 2019 [RefID 2014]) used blood styrene levels to predict the oral exposure of styrene in humans. Taking the P95 styrene blood level of the (general) adult population in the US in 2010 (0.132 μg/kg bw per day, see also Table 4), they predicted that the oral intake should have been 2.89 μg/kg bw per day, which is a factor 2.9 higher than the estimated total external exposure of 1.0 μg/kg bw per day (Banton et al., 2019 [RefID 1383]) and approximately 18 times higher than a mean dietary exposure for adults of 0.16 μg/kg bw per day (Cao et al., 2018 [RefID 2561]). The most likely reason for the overestimation of the oral dose is the fact that their predicted decline of plasma levels was monophasic (steep), whereas others have shown a slower elimination from the fat tissue, which leads to a (predicted) biphasic decline of styrene in blood. A biphasic decline of SO in blood of rats and humans has been shown by Csanády et al. (2003), Csanády et al. (1994), Filser and Gelbke (2016). Such a biphasic decline leads to higher C_{max} and C_{min} blood levels and, consequently, the extrapolated oral dose would have been lower when compared to the prediction of Miura et al. (2019 [RefID 2014]).

Miura et al. (2019 [RefID 2014]) also predicted that after multiple oral dosing, styrene concentrations in liver would be higher than in plasma, but the hepatic clearance was comparable to the plasma clearance, indicating that no accumulation occurs in the liver. Kabadi et al. (2019 [RefID 1321]) also used a PBK model to predict that after administration of a single oral dose to rats (300 mg/kg bw per day), styrene levels in blood and liver were comparable.

3.3.3.3 | *Summary*

Only one paper described reverse dosimetry by using a PBK model and an observed styrene blood level in humans to predict an oral dose (related to that blood level). The fact that they overestimated the oral dose could be due to the design of the model and/or the underestimation of other exposure sources (only oral intake was assumed) contributing to the styrene blood level used for reverse dosimetry.

3.3.4 | Conclusions on the exposure to styrene

For the general population, the biomonitoring of the parent compound (styrene) in blood is preferred. Median concentrations in Canada and USA range from below detection limit to 0.08 μg/L; P95 values are in the range of 0.08–0.2 μg/L. Sufficient HBM data (blood styrene levels) are available from USA, but only little from European countries.

Important exposure sources throughout different age groups are food and air (similar contribution). For smoking adults, inhalation (air plus tobacco smoke) is the major exposure route. The estimated rounded mean dietary exposure (from migration and as a food component) is 0.2 μg/kg bw per day for adults, 0.3 μg/kg bw per day for children and 0.4 μg/kg bw per day for toddlers. The estimated (rounded) total exposure is 0.5 μg/kg bw per day for non-smoking adults and 1.0 μg/kg bw per day for adult smokers.

Reverse dosimetry was used to estimate an oral dose based on a given styrene blood concentration. The overestimation of the oral dose could be due to the design of the model and/or the underestimation of other exposure sources contributing to the styrene blood level used for reverse dosimetry (only oral intake was assumed).

A faster elimination of styrene from blood compared to that from fat tissue has been reported and, depending on dose frequency, some accumulation in fat tissue could occur. No accumulation has been observed in the liver, although predicted concentrations in human livers were consistently higher than coinciding blood levels.

HBM data in the general population support the predicted kinetics (in particular metabolism and elimination) in humans after oral exposure.

4 | CONCLUSIONS

The Panel noted that styrene was shown to be genotoxic in some in vitro systems, mainly following exogenous or endogenous metabolic activation, pointing to the role of SO in styrene genotoxicity in vitro.

However, the results provided by reliable in vivo oral genotoxicity studies, covering different genetic endpoints and target tissues, including liver, the primary site of metabolism, demonstrated that the oral administration of styrene in mice and rats up to the maximum tolerated dose (300 and 500 mg/kg bw, respectively) did not induce genotoxic effects. These results indicated that, under the conditions of these studies, the internal exposure to styrene and its metabolites, including SO, was insufficient to elicit a genotoxic effect.

The Panel also noted that the conclusion of the absence of genotoxicity in vivo after oral administration to styrene may not apply to other routes of exposure, where different toxicokinetic and toxicodynamic factors may be involved.

Overall, considering the available data, the Panel concluded that there is no evidence that styrene is genotoxic following oral exposure. This conclusion was based on the extensive experimental evidence obtained in sensitive animal models orally exposed to styrene at doses up to more than five orders of magnitude higher than the human exposure resulting from the use of styrene in FCM.

For substances demonstrated to be non-genotoxic, according to the EFSA Note for Guidance for FCM, an SML up to 50 µg/kg food would not be of safety concern. Consequently, the use of styrene in the manufacture of FCM respecting the SML of 40 µg/kg food proposed by the European Commission is not of safety concern.

5 | DOCUMENTATION AS PROVIDED TO EFSA

1. Combined *Pig-a*, micronucleus, and comet study in B6C3F1 mice after oral administration of styrene. ILS Study Number 60952.00203. January 2023. Submitted by Plastics Europe on behalf of SIRC.
2. Combined *Pig-a*, micronucleus, and comet Study in Fischer 344 rats after oral administration of styrene. ILS Study Number 60952.00103. December 2022. Submitted by Plastics Europe on behalf of SIRC.
3. An oral gavage in vivo mutation assay of styrene at the cII locus in transgenic Big Blue® Haemizygous B6C3F1 mice. March 2024. Submitted by Plastics Europe on behalf of SIRC.
4. Additional data. Styrene: mammalian alkaline comet study in male Fischer 344 rats via oral gavage administration for 28 days. Inotiv study number 3940–2300099. October 2023. Submitted by Plastics Europe on behalf of SIRC.
5. Additional data. Annex I "Response to EFSA's request for additional information on *Pig-a* studies conducted in mice and rats orally exposed to styrene" (S. Dertinger, B.B. Gollapudi). October 2023. Submitted by Plastics Europe on behalf of SIRC.
6. Additional data. Annex II: "Assessment of the Oral Absorption of Styrene in Rats and Mice". October 2023. Submitted by Plastics Europe on behalf of SIRC.

ABBREVIATIONS

ACGIH	American Conference of Governmental Industrial Hygienists
ADME	absorption, distribution, metabolism and excretion
CA	chromosomal aberration
CDC	Centers for Disease Control and Prevention
CEF	Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEP	Food Contact Materials, Enzymes and Processing Aids
CF	consumption factor
CI	confidence interval
C_{max}	maximum plasma concentration
C_{min}	minimum plasma concentration
CYP450	cytochrome P450
EC	European Commission
EDI	estimated daily intake
EFSA	European Food Safety Authority

EMS	ethyl methanesulfonate
ENU	ethyl nitrosourea
FCM	food contact material
fT	food-type distribution factors
GLP	good laboratory practice
GSH	glutathione
GSTM1	glutathione-s-transferase Mu 1
GSTT1	glutathione-s-transferase theta 1
HBM	human biomonitoring
IARC	International Agency for Research on Cancer
i.p.	intraperitoneal
MA	mandelic acid
MF	mutation frequencies
MN	micronucleus/micronuclei
MTD	maximum tolerated dose
NCE	normochromatic erythrocytes
OECD	Organisation for Economic Co-operation and Development
PBK	physiologically based kinetic
PCE	polychromatic erythrocytes
PGA	phenylglyoxylic acid
PHEMAs	phenylhydroxyethylmercapturic acids
RBC	red blood cells
RET	reticulocytes
SCF	Scientific Committee on Food
SIRC	Styrenic Information and Research Center
SM	sperm morphology
SML	specific migration limit
SO	styrene-7,8-oxide
ssb	single strand breaks
TDI	tolerable daily intake
TG	test guideline
ToR	terms of reference
UDS	unscheduled DNA synthesis
USA	United States of America
WoE	weight of evidence
WG	working group
WHO	World Health Organisation
8-OHdG	8-hydroxy-2′-deoxyguanosine

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AMENDMENT
An editorial correction in the Panel Members' list was carried out that does not materially affect the contents or outcome of this scientific output. To avoid confusion, the original version of the output has been removed from the EFSA Journal, but is available on request.

REQUESTOR
European Commission

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

Methodology of the literature search

A.1 | Methods for gathering the evidence

Time span of evidence search

The evaluation dealt with evidence available from 1 January 2018 until 1 October 2024.

The searches were initially conducted on 6 March 2023, and subsequently updated on 4 and 5 July 2023 (included intestinal metabolism), 3 November 2023 (included dietary exposure), 4 March 2024, 16 April 2024 and 1 October 2024. These updates were designed to cover the entire period from 2018 and de-duplicate the results with previous retrieved records. To streamline this document, only the most recent search update performed on 1 October 2024 is reported in Annex B.

Information sources

Literature searches were conducted to retrieve relevant studies from the following bibliographic databases: PubMed (NLM), Scopus (Elsevier) and the Web of Science Core Collection (Clarivate), which includes the Science Citation Index-Expanded, Emerging Sources Citation Index, Current Chemical Reactions and Index Chemicus data sets. These databases were selected based on their comprehensive coverage, as the combination of these platforms encompasses a broad spectrum of scientific publications.

An open search strategy was used, including only the terms 'Styrene' and synonyms, with a view to capture as many records as possible.

The search strings proposed to be used for each database search are annexed in Annex B. The search strings incorporated both free-text terms and controlled vocabulary when available (e.g. MeSH terms). Terms were selected based on previous searches and the expertise of the members of the working group (WG). The searches aimed to retrieve records on styrene and genotoxicity, toxicokinetic and metabolism and human exposure (including HBM) studies. New terms were introduced in July and November 2023 to capture evidence on styrene and intestinal metabolism, as well as dietary exposure, respectively. The searches were restricted to papers published in English from 01 January 2018 to 01 October 2024.

The literature search methods and strategies used are based on standard EFSA guidelines (EFSA, 2010).

Type of evidence

Only primary research studies were considered for the assessment. Reviews were checked in case relevant primary studies were reported. Comments, letters to the editors, book chapters, poster and/or conference abstracts and PhD theses were excluded.

Management of the information

The records for each database were downloaded and imported into EndNote software (Clarivate) where de-duplication was performed, this process was completed using SR accelerator Deduplicator tool.[11]

A total of 3392 records were uploaded into the literature review software DistillerSR (DistillerSR Inc.): 603 on genotoxicity, 2001 records for toxicokinetics and 788 on HBM and dietary exposure.

A.2 | Methods for selecting the studies

Screening of titles and abstracts

The titles and, when available, the abstracts identified in the searches described in Annex B were screened for relevance to the general scope of the assessment by answering to the following questions:

1. Is the paper reporting information about genotoxicity and exposure to styrene in experimental systems and humans?
2. Is the paper reporting information about toxicokinetics and metabolism of styrene and SO?
3. Is the paper reporting information about human internal exposure to styrene and SO OR is it reporting information on the external exposure to Styrene, specifically dietary exposure or from FCM?

The screening of titles and abstract was performed by two reviewers working independently.

[11] https://sr-accelerator.com/#/deduplicator.

The possibility of an 'unclear' reply was foreseen at this stage as the unavailability of full text may have hampered the possibility to take an informed decision. In case of an 'unclear' reply the paper was considered as meeting the inclusion criteria.

The DistillerSR tool allowed the identification of potential disagreements between the two reviewers on study eligibility.

In case of disagreement between the two reviewers, the paper was automatically brought to the next screening phase, i.e. at the level of full text.

A check on a random sample of studies was performed during the initial stages of the work in order to ensure that there were no misinterpretations of the selection criteria. If misinterpretations were identified from this check or from direct reporting of the reviewers, then the selection criteria would be better explained.

Examining full-text reports for eligibility of studies

For records passing the first screening based on titles and abstracts, the full text underwent a second screening against the inclusion criteria by means of two reviewers working independently.

For screening the genotoxicity studies, the categorisation was made into different subgroups of genotoxicity endpoints or effects associated with gene mutation and/or CAs and/or DNA damage and/or about Mode of Action.

The possibility of an 'unclear' reply was no longer foreseen at this stage because all the information needed for taking a decision was available in the full text.

In case of disagreement, the two reviewers would discuss the paper in order to reach a common decision. If the disagreement persisted, the article was brought to the attention of the whole WG for discussion and agreement on a final decision.

A.3 | Availability of full text and language

Availability of the full text in English was a pre-requisite for an article to be included in the assessment.

The reviewers were thus asked to reply to these questions:

Is the full text available?
Is the full text in English?

If any answer was negative, the record would be excluded from the assessment. If both answers were 'Yes', the reviewers proceeded to the next question.

A.4 | Selection of oral studies

The reviewers would be asked to reply to the following question:

Is the paper reporting information on styrene and oral only or oral and inhalation exposure to styrene?

If the answer to the question was 'Yes', the reviewers were prompted to reply to the following question.

A.5 | Selection of the type of studies

The reviewers would be asked to reply to the following question:

Is the paper a primary or a secondary study?

If the answer to the question was 'primary', the reviewers were prompted to reply to the following question.

If the answer to the question was 'secondary', the record was excluded from the assessment; however, the reviews were checked whether they contained additional references of primary studies that were not been captured by the literature search.

A.6 | Selection of the endpoints of interest

In the first instance the reviewers were asked to confirm that the record relates to a study reporting information considered relevant to the review question:

1. Is the paper reporting information about effects associated with gene mutation and/or CAs and/or DNA damage and/or about Mode of Action? (genotoxicity)
2. Is the paper reporting information about absorption, distribution, metabolism and excretion? (toxicokinetics)

3. Is the paper reporting information about human internal exposure to styrene and SO or is it reporting information on the external exposure to styrene, specifically dietary exposure or from FCM? (HBM)

Primary studies that were not aimed at studying genotoxic effects associated with oral exposure to styrene were excluded at this step.

If the answer to the question was 'Yes', the reviewers can start evaluating the paper.

If the answer to the question was 'No', the record was excluded from the assessment.

With regard to genotoxicity, an additional screening of the reliability and relevance of the studies was done by experts in this field following the full-text screening (EFSA, 2023).

One source could report on more than one outcome of interest and each outcome was assessed separately.

A.7 | Method for assessing genotoxicity evidence

The evaluation of data quality for hazard/risk assessment includes the evaluation of reliability and relevance as recommended in the EFSA technical report published in 2023 (EFSA, 2023). In a first step, the reliability of the study was assessed based on Klimisch criteria. 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.

Genotoxicity studies evaluated as of high or limited relevance have been considered in a WoE approach. Genotoxicity studies evaluated as of low relevance have not been further considered in the assessment.

A.8 | Method for assessing evidence on toxicokinetics and internal/external exposure to styrene

Toxicokinetics and human exposure studies are evaluated and reported in the opinion narratively.

APPENDIX B

Tables of the in vivo genotoxicity studies assessment

The tables below report the assessment of reliability and relevance of the following studies:

- in vivo genotoxicity studies after oral exposure to styrene submitted by Plastics Europe on behalf of US SIRC.
- in vivo genotoxicity studies after oral exposure to styrene identified by the literature search (01 January 2018–01 October 2024).
- in vivo genotoxicity studies after oral, i.p. and inhalation exposure to styrene evaluated in the 2019 IARC Monograph (IARC, 2019).

The assessment is based on the criteria described in the 2023 EFSA Technical Report (EFSA, 2023).

Compliance with OECD TGs or GLP is only presented in the tables when this was reported in the study concerned (Tables B.1–B.4).

TABLE B.1 In vivo gene mutation (OECD TG 488 was considered for the evaluation of reliability).

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/ relevance of the results	Reference
Oral exposure						
Transgenic mutation assay in MutaMouse (lacZ system) (6–8 males/group); OECD TG compliance: TG 488, 2022b	Daily administration by gavage of 75, 150 or 300 mg styrene/kg bw for 28 days. Vehicle corn oil Positive control: ENU (100 mg/kg bw twice 24 h by i.p.) Sacrifice 3 days after last administration Mutations in the lacZ transgene determined in DNA isolated from liver and lung of five animals (50,000 scored plaques/ animal)	Styrene 100% pure (Tokyo Chemical Industry Co., Ltd. (Tokyo))	Negative A single animal in the low dose group displayed extremely high mutation frequencies (MF) in liver and lung, attributed to clonal expansion; it was replaced by an additional animal for mutation analysis in this group. When the outlier animal was excluded, mean group MF in liver and lung DNA was not statistically different in styrene treated mice and vehicle control animals, and within the range of historical control data. Mutant sequencing to confirm the clonal expansion in the outlier mice was not performed. Toxicity: In the preliminary dose range finding experiment carried out for 2 weeks, high toxicity and lethality were observed at 1000 mg/kg bw per day (> MTD). In the main experiment, at the highest tested dose (300 mg/kg bw per day) there were no clinical signs of toxicity but gross pathological changes in the liver (liver darkening) in 3/8 animals.	1 reliable without restrictions	High/High	Murata et al. (2023) [RefID 2224]

(Continues)

TABLE B.1 (Continued)

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/ relevance of the results	Reference
Mammalian Erythrocyte *Pig-a* gene mutation assay 8 male B6C3F1 mice (8–12 weeks of age)/group (6 analysed) Repeated dose 29 day oral (gavage) study OECD TG compliance: TG 470, 2022 GLP compliance: yes	75, 150, 300 mg/kg per day Positive control: ENU (51.7 mg/kg bw daily for the first 3 days)	Styrene monomer (CAS RN 100-42-5) purity 99.95% (Lyondell Chemical Company (Houston, TX, USA))	Negative Toxicity: no decrease in % PCE. Clinical signs: No signs of toxicity up to the top dose (300 mg/kg bw per day) Bioanalysis of styrene in plasma: 10.9 µg/mL at 500/350 mg/kg bw per day (15 min post last day of treatment in range-finding test)	1 reliable	High/High	SIRC (2023a) (also available as publication by Gollapudi (2023) [RefID 2204])
Mammalian Erythrocyte *Pig-a* gene mutation assay 8 male Fisher 344 rats (8–12 weeks of age)/group (6 analysed) Repeated dose 29-day oral (gavage) study OECD TG compliance: TG 470, 2022 GLP compliance: yes	100, 250, or 500 mg/kg per day Positive control: ENU (51.7 mg/kg bw daily for the first 3 days)	Styrene monomer (CAS RN 100–42-5) purity 99.95% (Lyondell Chemical Company (Houston, TX, USA))	Negative Toxicity: no decrease in % PCE. Clinical signs: Transient dose-related observations of decreased movement in the 250 and 500 mg/kg bw per day exposure groups. Dose-dependent decrease in body weight gain, statistically significant in animals receiving 500 mg/kg bw per day. Bioanalysis of styrene in plasma: 28.3 µg/mL at 500 mg/kg bw and 29.6 µg/mL at 1000 mg/kg bw per day (15 min post last day of treatment in range-finding test)	1 reliable	High/High	SIRC (2022) (also available as publication by Gollapudi (2024) [RefID 3035])
Mutation Assay at the *cII* Locus in transgenic Big Blue® Mice 8 male transgenic Big Blue® haemizygous B6C3F1 male mice (9 weeks of age)/group (5 analysed) Repeated dose 28-day oral (gavage) study Analysis of mutant frequency in liver, lung, glandular stomach and duodenum OECD TG compliance: TG 488, 2022b GLP compliance: yes	75, 150 or 300 mg/kg bw per day Positive control: ENU (40 mg/kg bw/day at day 1, 2 and 3)	Styrene monomer (CAS RN 100–42-5) purity 99.95% (Lyondell Chemical Company (Houston, TX, USA))	Negative in lung, glandular stomach and duodenum Inconclusive in liver Statistically significant increase of mutant frequency at two doses. No dose response. No laboratory historical control database for Big Blue® B6C3F1 mice Clinical signs: No test substance-related clinical observations. Bioavailability of the test substance not measured in this study but based on the results of a previous dose-range finding study.	2 reliable with restrictions (limited number of historical control animals of different mouse strain)	High/Limited High/Low	SIRC (2024) (also available as publication by Gollapudi et al., 2025)

TABLE B.2 In vivo CAs/MN test (OECD TG 474 and TG 475 were considered for the evaluation of reliability of MN and CA tests, respectively).

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/relevance of the results	Reference
Oral exposure						
Mammalian bone marrow CA test 4 CD-1 mice/group 9 animals/control group	500 and 1000 mg/kg bw dissolved in corn oil Single treatment by gavage Sampling 24h after the treatment 100 metaphases scored/animal Positive control: Mitomycin C 3.5 and 14 mg/kg by i.p.	Styrene (Merck Darmstadt F.R.G.)	Negative Toxicity: 2 out of 4 animals died at 1000 mg/kg bw	2 reliable with restrictions This study was performed before the establishment of the OECD TG 475 A limited protocol was applied with low number of animals/group 100 metaphases scored/animal	High/Limited because of the protocol limitations	Loprieno et al. (1978)
Mammalian bone marrow CA test Repeated treatment 4 days of treatment: 6 male CD-1 mice/group 3 animals/control group 70 days of treatment: 7 male CD-1 mice/group 3 animals/control group	Repeated treatment: 500 mg/kg bw day dissolved in corn oil, 4 days of treatment Subchronic treatment: 200 mg/kg bw day dissolved in corn oil, 70 days of treatment Positive control: Cyclophosphamide 10, 20 and 40 mg/kg by single treatment i.p. Sampling 24h after the last treatment	Styrene (Merck Darmstadt F.R.G.)	Negative Toxicity: Mitotic indexes not affected	2 reliable with restrictions This study was performed before the establishment of the OECD TG 475 A limited protocol was applied with low number of animals/group 100 metaphases scored/animal	High/Limited because of the protocol limitations	Sbrana et al. (1983)
Mammalian Erythrocyte MN Test 8 male B6C3F1 mice (8–12 weeks of age)/group (6 analysed) Repeated dose 29-day oral (gavage) study OECD TG compliance: TG 474, 2016a GLP compliance: yes	75, 150, 300 mg/kg per day	Styrene monomer (CAS RN 100–42-5) purity 99.95% (Lyondell Chemical Company (Houston, TX, USA))	Negative No increase in %MN-PCE Toxicity: no decrease in % PCE. Clinical signs: No signs of toxicity up to the top dose (300 mg/kg bw) Bioanalysis of styrene in plasma: 10.9 µg/Ml at 500/350 mg/kg bw (15 min post last dose day 28 in range-finding test)	1 reliable	High/High	SIRC (2023a) (also available as publication by Gollapudi, 2023 [RefID 2204])

(Continues)

TABLE B.2 (Continued)

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/relevance of the results	Reference
Mammalian Erythrocyte MN Test 8 male Fisher 344 rats (8–12 weeks of age)/group (6 analysed) Repeated dose 29-day oral (gavage) study OECD TG compliance: TG 474, 2016a GLP compliance: yes	100, 250, or 500 mg/kg per day	Styrene monomer (CAS RN 100–42-5) purity 99.95% (Lyondell Chemical Company (Houston, TX, USA))	Negative No increase in %MN-PCE Toxicity: no decrease in % PCE. Clinical signs: Transient dose-related observations of decreased movement in the 250 and 500 mg/kg/day exposure groups. Dose-dependent decrease in body weight gain, statistically significant in animals receiving 500 mg/kg/day. Bioanalysis of styrene in plasma: 28.3 µg/mL at 500 mg/kg bw and 29.6 µg/mL at 1000 mg/kg bw (15 min post last dose day 28 in range-finding test)	1 reliable	High/High	SIRC (2022) (also available as publication by Gollapudi, 2024 [RefID 3035])
Intraperitoneal injection						
Mammalian erythrocyte MN test 4 male Chinese hamsters (two in the control group)	1000 mg/kg bw by single i.p. injection Vehicle olive oil Sampling 30 h after treatment MN scored in 2000 PCE and 2000 NCE/animal No positive control	Styrene > 99% pure (Fluka AG/Buchs SG)	Inconclusive No increase of MN with no evidence of bone marrow exposure Toxicity: Non-statistically significant decrease in the PCE/NCE ratio in styrene treated animals	2 reliable with restrictions This study was performed before the establishment of the OECD TG 474. The study results are adequately reported, but a limited protocol was applied, with few animals (4 treated and 2 vehicle control animals), a single dose and sampling time, and no positive control.	High/Low because of the protocol limitations, the non-oral route of exposure and inconclusive results	Penttilä et al. (1980)

TABLE B.2 (Continued)

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/relevance of the results	Reference
Mammalian Erythrocyte MN Test 4 male C57BL/6 mice/group 13 animals/control group	250, 500, 1000 or 1500 mg/kg bw dissolved in olive oil Single treatment by i.p. Sampling 30 h after the treatment 2000 PCEs scored/animal No positive control	Styrene 99% pure (Fluka AG (Buchs SG, Switzerland)) further purified by distillation	Equivocal Increase of PCE with MN statistically significant at 250 and 1000 mg/kg bw No increase of normochromatic erythrocytes (NCE) with MN. No dose–response Toxicity: 2 out of 4 animals died at 1500 mg/kg bw. Statistically significant dose related decrease of PCE/NCE ratio	2 reliable with restrictions This study was performed before the establishment of the OECD TG 475 Low number of animals and 2000 PCEs scored/animal instead of 4000. Only aggregated data for the whole cellular population analysed were presented, in contrast with OECD TG 474 recommendations.	High/Low because of the protocol limitations, the non-oral route of exposure and equivocal results	Norppa (1981)
Mammalian bone marrow CA assay in male C57B1/6 mice (4 animals/dose). Mice were implanted subcutaneously with BrdUrd tablet 30 min before treatment for parallel SCE determination (reported separately under Table B.4) Other tests (Table B.4)	50, 100, 250, 500, 750 and 1000 mg/kg bw, by single i.p. injection Vehicle corn oil Positive control cyclophosphamide (50 mg/kg bw, by i.p.) Sampling time 16 h after treatment CAs scored in 100 M1 metaphases/animal; mitotic index estimated in 1000 cells/animal	Styrene >99% pure (Aldrich Chem. co.)	Negative Toxicity: Styrene administration was highly toxic at the two highest doses, with 1 and 3 animals dead out of 4 treated; there was no effect on replication index but a strong decrease in the mitotic index.	2 reliable with restrictions The CA assay was performed with a limited protocol: 4 animals, 100 metaphases scored; the BrdUrd differentiation of M1 is not required in the OECD TG 475, but it is considered not to affect the reliability of the assay.	High/Limited because of the protocol limitations and the non-oral route of exposure.	Sharief et al. (1986)
Mammalian Erythrocyte MN Test 10 male LACA Swiss mice/group Other tests (Table B.4)	150, 300, 450, 600 mg/kg bw dissolved in peanut oil Single treatment by i.p. Sampling 30, 48 and 72 h after the treatment 1000 PCE scored/animal Positive control: cyclophosphamide 1.25, 2.5, 5, 10 and 20 mg/kg bw by i.p.	Styrene of reagent grade quality (Ajax Chemicals)	Positive Increase of PCE with MN statistically significant at 600 mg/kg bw only 48 h after treatment. Toxicity: statistically significant decrease of PCE/NCE ratio 600 mg/kg only 48 h after treatment	2 reliable with restrictions 1000 PCE scored/animal Instead of 4000, as recommended by OECD TG 474	High/Limited because of the protocol limitations and the non-oral route of exposure	Simula and Priestly (1992)

(Continues)

TABLE B.2 (Continued)

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/relevance of the results	Reference
Mammalian Erythrocyte MN Test Group of male Porton rats: 4 (300 mg/kg); 7 (750 and 1500 mg/kg); 5 (3000 mg/kg) and 8 control animals Other tests (Table B.4)	300, 750, 1500, 3000 mg/kg bw dissolved in peanut oil Single treatment by i.p. Sampling 30, 48 and 72 h after the treatment 1000 PCE scored/animal Positive control: cyclophosphamide 1.25, 2.5, 5, 10 and 20 mg/kg bw by i.p.	Styrene of reagent grade quality (Ajax Chemicals)	Negative Toxicity: statistically significant decrease of PCE/NCE ratio at 3000 mg/kg 30 h after treatment, dose-related decrease of PCE/NCE ratio from 750 mg/kg 72 h after treatment.	2 reliable with restrictions Low number of animals for some dose group. 1000 PCE scored/animal Instead of 4000, as recommended by OECD TG 474	High/Limited because of the protocol limitations and the non-oral route of exposure	Simula and Priestly (1992)
Inhalation exposure						
Mammalian bone marrow CA test Male Chinese hamsters (4 per treated group, 3 control animals)	300 ppm for 4 days or 3 weeks (6 h daily, 5 days/week) 100 bone marrow metaphases were analysed per animal No positive control	Styrene >99% pure (Fluka AG/Buchs SG)	Inconclusive No increase of CA with no evidence of bone marrow exposure	2 reliable with restrictions This study was performed before the establishment of the OECD TG 475, applying a limited protocol with few animals (4 treated and 3 control animals) and scored metaphases (100 instead 200/animal), with no positive control and a limited presentation of data (only group means).	High/Low because of the inconclusive results, the protocol limitations and the non-oral route of exposure	Norppa et al. (1980)
Mammalian bone marrow CA test Sprague–Dawley rats (4 males and 4 females per group)	600 and 1000 ppm 6 h/day, 5 days a week, for a period of 1 year CAs were scored in 100 metaphases per animal and the mitotic index determined in 1000 cells per animal No positive control	Styrene 99.5%, pure stabilised with 5 ppm tertiary butyl catechol (Michigan Division, Dow Chemical U.S.A., Midland, MI)	Negative Toxicity: At 600 ppm clearance of styrene saturated At the end of treatment period, the mitotic indices were not significantly different in rats exposed to styrene compared to unexposed control animals.	2 reliable with restrictions This study was performed before the establishment of the OECD TG 475, applying a limited protocol with few scored metaphases (~100 instead of at least 200/animal) and no positive control	High/Limited because of the protocol limitations and the non-oral route of exposure	Sinha et al. (1983)

TABLE B.2 (Continued)

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/relevance of the results	Reference
CAs in splenic lymphocytes and in lung MN test in splenic lymphocytes and In NCE in peripheral blood 6 female B6C3F1 mice/group Other tests (Table B.4)	125, 250 and 500 ppm (nominal concentrations) (actual conc. 124 ± 4, 245 ± 9, 491 ± 5 ppm) Exposure 6h/day for 14 days Sampling 24 h after the treatment 100 metaphases scored/ animal No positive control	Styrene (purity 99.9%) (Shell Canada Ltd.) Vaporised at room temperature mixed with conditioned air. Delivered in the exposure chambers	Negative No cytotoxicity evaluated as replication index in peripheral lymphocytes, lung cells and splenic lymphocytes. Target cell exposure demonstrated by positive results obtained with SCE	2 reliable with restrictions Use of non-validated protocols (CA in lung cells and CA and MN in splenic cells) 100 metaphases scored/ animal instead of 300 as recommended by OECD TG 4751000/2000 NCE instead of 4000 as recommended by OECD TG 474	High/Limited because of the protocol limitations and the non-oral route of exposure	Kligerman et al. (1993)
CAs and MN in peripheral blood lymphocytes 6 female Fischer 344 rats/ group MN frequency in bone-marrow NCE In vivo comet assay (Table B.3) Other tests (Table B.4)	125, 250 and 500 ppm (nominal concentrations) (actual concentration 120 ± 4, 251 ± 6, 489 ± 12 ppm) Exposure 6 h/day for 14 days Sampling 24 h after the treatment 100 metaphases scored/ animal No positive control	Styrene (purity 99.9%) (Shell Canada Ltd.) Vaporised at room temperature mixed with conditioned air. Delivered in the exposure chambers 125, 250, 500 ppm Nominal conc. (actual conc. 120 ± 4, 251 ± 6, 489 ± 12 ppm) Exposure 6 h/day for 14 days	Negative No cytotoxicity evaluated as replication index in peripheral lymphocytes, lung cells and splenic lymphocytes. Target cell exposure demonstrated by positive results with SCE	2 reliable with restrictions 100 metaphases scored/ animal instead of 300 as recommended by OECD TG 475 1000/2000 NCE instead of 4000 as recommended by OECD TG 474	High/Limited because of the protocol limitations and the non-oral route of exposure	Kligerman et al. (1993)

(Continues)

TABLE B.2 (Continued)

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/relevance of the results	Reference
CA assay in rat peripheral blood lymphocytes Male Fischer 344 rats (6 animals/group) Other tests (Table B.4)	150, 500 or 1500 ppm 6 h/day, 5 days a week, for 4 weeks Positive control: ethylene oxide 150 ppm Sacrifice 1, 2, 3 and 4 weeks after initiation of treatment Structural CAs scored in 25 M1 metaphases/animal after in vitro mitogen stimulation of lymphocytes and 68 h culture in presence of BrdUrd for the parallel determination of SCE (reported separately under Table B.4)	No information on the substance purity was given	Negative Ethylene oxide (150 ppm), evaluated in parallel as positive control, was also negative	3 not reliable This study did not follow any specific TG. The study was performed with a limited protocol (25 cells/animal), which prevented using animals as statistical units. The positive control ethylene oxide produced an insufficient response, which does not allow to evaluate the adequacy of the ex vivo protocol applied to detect genotoxic effects arising from in vivo exposure	High/Low because of the insufficient reliability	Preston and Abernethy (1993)
Mammalian Erythrocyte MN Test 6 male NMRI mice/group In vivo comet assay (Table B.3) In vivo DNA adducts (Table B.4)	750 and 1500 mg/m^3 (actual concentration: 747 ± 38 and 1630 ± 54 mg/m^3) Appropriate amount of liquid styrene was delivered by micropump through calibrated glass capillary into the mixing bottle and evaporated. Inhalation performed in dynamic chamber for 1, 3, 7 or 21 days (6 h/day and 7 days/week). No positive control	Styrene (purity not reported)	Equivocal Statistically significant increase of MN in the bone marrow at 1500 mg/m^3 after 7 days of exposure (twice higher MN frequency than in control animals) No significant increase after 21 days and at 750 mg/m^3. Styrene conc. in blood: 7.44 ± 3.21 mg/mL at 1500 mg/m^3 after 21 days The study was repeated with negative results (Engelhardt et al., 2003)	3 not reliable The method is not reported	High/Low because of the equivocal results insufficient reliability and the non-oral route of exposure	Vodicka et al. (2001)

TABLE B.2 (Continued)

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/relevance of the results	Reference
Mammalian erythrocyte MN test Male NMRI mice (5 animals/group) OECD TG compliance: TG 474, 1997a GLP compliance: yes	750 and 1500 mg/m^3 styrene (~115 and 350 ppm), 6 h/day Positive control: cyclophosphamide (20 mg/kg bw, by i.p.) Sampling after treatment for 1, 3, 7, 14 or 21 consecutive days MN were scored in 2000 PCE per animal independently in two laboratories; the number of NCE (with or without MN) in the same microscopic fields was also recorded by one laboratory	Styrene > 99% pure (from BASF)	Negative Toxicity: At the high dose inhalation of styrene was lethal in 7/35 mice. Clinical signs of toxicity and reduced body weight gain were observed in the high dose group. The PCE/NCE ratio was not affects by treatments	1 reliable without restrictions Note: this study was aimed to elucidate the equivocal results previously reported under the same experimental conditions by Vodicka et al. (2001), who co-authored the study	High/Limited because of the non-oral route of exposure	Engelhardt et al. (2003)
Mammalian erythrocyte MN test Male Fischer 344 rats (6 per group) OECD TG compliance: TG 474, 1997a In vivo comet (Table B.3)	Exposure for 6 h/day, 5 days/week, for 4 weeks to (75, 300 or 1000 ppm styrene Positive control: ENU (40 mg/kg bw, by i.p.) Samplings at the end of 3rd and last (20th) day of exposure MN analysed by flow cytometry in RET Blood levels of styrene and SO were determined just before the end of treatment	Styrene (purity ≥99%) Sigma Aldrich (Saint Quentin Fallavier, France)	Negative Toxicity: The percentage of RET was significantly decreased at all doses at the first sampling time Blood concentration of styrene and SO in animals exposed to 1000 ppm styrene were 85.5 ± 11.3 µg/g of blood and ~0.37 µg/g blood, respectively	1 reliable without restrictions	High/Limited because of the non-oral route of exposure	Gaté et al. (2012)

TABLE B.3 In vivo DNA damage by comet assay (OECD TG 489 was considered for the evaluation of reliability).

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/ relevance of the results	Reference
Oral exposure						
Comet assay in liver, lung, kidney, stomach and duodenum 8 male B6C3F1 mice (8–12 weeks of age)/group (6 analysed) Repeated dose 29-day oral (gavage) study OECD TG compliance: TG 489, 2016 GLP compliance: yes	75, 150, 300 mg/kg/day per 29 days Positive control EMS (150 mg/kg bw) for the last 3 days	Styrene monomer (CAS RN 100-42-5) purity 99.95% (Lyondell Chemical Company (Houston, TX, USA))	Negative (liver, lung, kidney and stomach) Inconclusive (duodenum) due the high baseline values with high interindividual variability Clinical signs: No signs of toxicity up to the top dose (300 mg/kg bw) Bioanalysis of styrene in plasma: 10.9 µg/mL at 500/350 mg/kg bw (15 min post last dose day 28 in range-finding test)	1 reliable without restriction 3 Not reliable Negative response of positive control	High/High High/Low	SIRC (2023a)
Comet assay in liver, lung, kidney, stomach and duodenum, 8 male Fisher 344 rats (8–12 weeks of age)/group (6 analysed) Repeated dose 29-day oral (gavage) study OECD TG compliance: TG 489, 2016 GLP compliance: yes	100, 250, or 500 mg/kg/day per 29 days Positive control EMS(150 mg/kg bw) for the last 3 days	Styrene monomer (CAS RN 100-42-5) purity 99.95% (Lyondell Chemical Company (Houston, TX, USA))	Negative (liver, lung) Inconclusive (duodenum and stomach) due the high baseline values with high interindividual variability Inconclusive (kidney) due to the presence of an outlier control and the resulting low significance of the response of the positive control Clinical signs: Transient dose-related observations of decreased movement in the 250 and 500 mg/kg/day exposure groups. Dose-dependent decrease in body weight gain, statistically significant in animals receiving 500 mg/kg/day. Bioanalysis of styrene in plasma: 28.3 µg/mL at 500 mg/kg bw and 29.6 µg/mL at 1000 mg/kg bw (15 min post last dose day 28 in range-finding test)	1 reliable without restriction 3 Not reliable Negative response of positive control animals 3 Not reliable Due to low significance of the response of the positive control	High/High High/Low High/Low	SIRC (2022)

TABLE B.3 (Continued)

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/ relevance of the results	Reference
Comet assay in liver, lung, kidney, stomach and duodenum 8 male Fisher 344 rats (8–12 weeks of age)/group (6 evaluated) Repeated dose 28-day oral (gavage) study OECD TG compliance: TG 489, 2016 GLP compliance: yes	100, 250, or 500 mg/kg/day per 28 days Positive control EMS (150 mg/kg bw) for the last 3 days	Styrene monomer (CAS RN 100-42-5) purity 99.86% (Lyondell Chemical Company)	Negative (liver, lung) Equivocal (stomach) Statistically significant increase in the % Tail DNA in all three treatment groups relative to the concurrent vehicle control group. No dose response. Negative (kidney) Decrease of % Tail DNA compared to the control values at the highest dose tested. Inconclusive (duodenum) Statistically significant decrease in the % Tail DNA in all three treatment groups relative to the concurrent vehicle control group. High interindividual variability in the control and treatment groups. Clinical signs: No signs of toxicity were observed. No effect on the mean absolute body weight. Significant decrease in the mean body weight gain of Groups 2 (100 mg/kg) and 3 (250 mg/kg) from day 8 to 15 and Group 4 (500 mg/kg) from day 1 to 8.	1 reliable without restrictions 1 reliable without restrictions 2 reliable with restrictions High % Tail DNA in vehicle control animals (outside the range of the historical control animals) 3 not reliable High %tail DNA in vehicle control animals (largely outside the range of the historical control animals)	High/High High/Limited High/Limited High/Low	SIRC (2023b)
Intraperitoneal injection						
Comet assay in peripheral blood lymphocytes, bone marrow cells, liver and kidney cells 6 female C57BL/6 mice/group	100, 250, 350 or 500 mg/kg bw dissolved in corn oil Single i.p. injection Sampling time: 4 and 16 h after the treatment 50 comets per slide (200–300 images/treatment) Cyclophosphamide 150 mg/kg bw by i.p.	Styrene (Fluka, Buchs, Switzerland)	Positive Dose related increase of tail moment in all the tissues tested	2 reliable with restrictions Low number of analysed cells per animal Unusual software used for the comet analysis (tail moment, tail inertia)	High/Limited because of the protocol limitations and the non-oral route of exposure	Vaghef and Hellman (1998)

(Continues)

TABLE B.3 (Continued)

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/ relevance of the results	Reference
Inhalation exposure						
Comet assay in peripheral blood lymphocytes 6 female Fischer 344 rats/ group In vivo CA and MN assay (Table B.2)	125, 250 and 500 ppm (nominal concentrations) (actual conc. 120 ± 4, 251 ± 6, 489 ± 12 ppm) Vaporised at room temperature mixed with conditioned air. Delivered in the exposure chambers Exposure 6 h/day for 14 days Sampling 24 h after the treatment Tail length and density evaluated in 50 cells/animal No positive control	Styrene (purity 99.9%) (Shell Canada Ltd.)	Negative Target cell exposure demonstrated by positive results with SCE	2 reliable with restriction Experimental protocol not reported cited method by Singh et al. (1988)	High/Limited because of the protocol limitations and the non-oral route of exposure	Kligerman et al. (1993)
Comet assay in peripheral blood lymphocytes, bone marrow cells and liver cells 6 male NMRI mice/group Endonuclease III to detect oxidised pyrimidines and apurinic/ apyrimidic sites. In vivo MN assay (Table B.2) In vivo DNA adducts (Table B.4)	750 and 1500 mg/m^3 Appropriate amount of liquid styrene was delivered by micropump through calibrated glass capillary into the mixing bottle and evaporated. Inhalation performed in dynamic chamber for 1, 3, 7 or 21 days (6 h/day and 7 days/week). 50 cells analysed/slide %DNA in tail No positive control	Styrene (purity not reported)	Equivocal in bone marrow and lymphocytes Negative in liver cells (data not shown) Bone marrow: statistically significant increase in the endonuclease III-sensitive sites at 1500 mg/m^3 after 21 days of inhalation Lymphocytes: significant increase of DNA-SB at 1500 mg/m^3 after 7 days of inhalation. Styrene conc. in blood: 7.44 ± 3.21 mg/mL at 1500 mg/m^3 after 21 days	2 reliable with restriction Too low number of analysed cells per animal Aggregated mean data analysed (instead of animal median)	High/Low because of equivocal results, the protocol limitations and the non-oral route of exposure	Vodicka et al. (2001)

TABLE B.3 (Continued)

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/ relevance of the results	Reference
Comet assay in blood cells Male Fischer 344 rats (6 animals/ group) In vivo MN assay (Table B.2)	75, 300 or 1000 ppm 6 h/day, 5 days/week, for 4 weeks to styrene Positive control: ENU (40 mg/kg bw, by i.p.) Samplings at the end of 3rd and last (20th) day of exposure DNA damage measured in leukocytes by comet assay ±Fpg	Styrene (purity ≥ 99%) from Sigma Aldrich (Saint Quentin Fallavier, France)	Negative (equivocal +Fpg) At the end of treatment the number of circulating leukocytes was lower in animals exposed to the highest styrene concentration (1000 ppm). In comet assays without Fpg, there were no significant increase in the percentage of Tail DNA in circulating leukocytes of exposed animals as compared to untreated control animals at both sampling times. In the presence of Fpg, a significant increase of DNA strand breaks was observed in animals exposed to styrene, only on day 3rd and with no relationship with the dose.	1 reliable without restrictions	High/Limited because of the non-oral route of exposure	Gaté et al. (2012)

TABLE B.4 Other in vivo assays.

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/relevance of the results	Reference
Intraperitoneal injection						
DNA single strand breaks (ssb) by DNA unwinding in kidney, liver, lung, testis, brain 3–17 NMRI male mice/group	1.7–10 mmol/kg Sampling at 1, 4 and 24 h after dosing	Styrene (Fluka, Buchs, Switzerland) Dissolved in Tween 80	Positive In liver, lung, testis and brain: the maximum level of ssb detected at 4 h, decrease at 24 h. In kidney the maximum level of ssb detected at 2 h and decrease at 24 h. Dose-related increase of ssb 4 h after dosing.	3 not reliable	Low/Low	Solveig Walles and Orsén (1983)

(Continues)

TABLE B.4 (Continued)

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/relevance of the results	Reference
SCE assay in bone marrow cells Male C57B1/6 mice (4 animals/dose) implanted subcutaneously with BrdUrd tablet 30 min before treatment In vivo CA assay (Table B.2)	50, 100, 250, 500, 750 and 1000 mg/kg bw, by single i.p. injection Vehicle corn oil Positive control: cyclophosphamide (15 mg/kg bw, by i.p.) Sampling 23.5 h after treatment SCEs determined in 30 M2 metaphases/animal replicative index determined in 200 metaphases/animal	Styrene > 99% pure (Aldrich Chem. co.)	Negative Toxicity: Styrene administration was highly toxic at the two highest doses, with 2 and 3 animals dead out of 4 treated; there was no effect on replication index but a strong decrease in the mitotic index.	2 reliable with restrictions There is no OECD TG for the in vivo SCE assay, but it is noted that the assay was performed with a limited protocol, with 4 animals per dose	Limited/Limited because of the non-oral route of exposure, limited protocol and the indicator endpoint analysed	Sharief et al. (1986)
SCE assay Cultured splenocytes 5 male LACA Swiss mice/group In vivo MN assay (Table B.2)	150, 300, 450, 600 mg/kg bw Single i.p. injection Sampling 24 and 48 h after the treatment Analysis of 20 metaphases spreads/animal containing a minimum of 38 sister-chromatids. Cytotoxicity determined by the replicative index (RI) Positive control: cyclophosphamide 1.25, 2.5, 5, 10 and 20 mg/kg bw by i.p.	Styrene of reagent grade quality (Ajax Chemicals) Dissolved in peanut oil	Positive Weak but statistically significant increase of SCE/chrom at 450 mg/kg (lethal to 1/5 animal) No decrease in replicative index in splenocytes	2 reliable with restrictions Low number of metaphases analysed	Limited/Limited	Simula and Priestly (1992)
SCE assay in cultured splenocytes 5 male Porton rats/group In vivo MN assay (Table B.2)	375, 750, 1500, 3000 mg/kg bw by single i.p. injection Sampling 24 and 48 h after the treatment Analysis of 20 metaphases spreads/animal containing	Styrene of reagent grade quality (Ajax Chemicals) Dissolved in peanut oil	Positive Statistically significant increase of SCE/chrom at 750 and 1500 mg/kg bw A single result available at 3000 mg/kg bw (lethal to 4/5 animals) No decrease in replicative index in splenocytes	2 reliable with restrictions Low number of metaphases analysed	Limited/Limited	Simula and Priestly (1992)

TABLE B.4 (Continued)

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/relevance of the results	Reference
Inhalation exposure						
SCE assay 6 male mice BDF1/group of hepatectomised and no- hepatectomised animals Bone marrow, alveolar macrophages Regenerated liver in hepatectomised animals	104 ± 7.5, 387 ± 38, 591 ± 46 and 922 ± 52 (SD) ppm. 6 h/day for 4 days 922 ± 52 (SD) ppm 6 h/day for 1 day 922 ± 52 (SD) ppm 6 h/day for 2 days No positive control	Styrene (purity 99%) (Aldrich)	Positive Statistically significant increase of SCE/cell in all the tissues tested starting from 387 ppm.	2 reliable with restrictions No OECD TG available Low number of cells scored	Limited/Limited	Conner et al. (1980)
SCE in lung, spleen and peripheral blood lymphocytes 4–6 female B6C3F1 mice/group In vivo CA/MN test (Table B.2) In vivo comet assay (Table B.3)	125, 250 and 500 ppm (nominal concentrations) (actual conc. 124 ± 4, 245 ± 9, 491 ± 5 ppm) Exposure 6 h/day for 14 days Sampling 24 h after the treatment Ex-vivo analysis 50 metaphases scored No positive control animals	Styrene (purity 99.9%) (Shell Canada Ltd.) Vaporised at room temperature mixed with conditioned air. Delivered in the exposure chambers	Positive Weak dose-related increase of the frequency of SCE in lung, spleen and peripheral blood lymphocytes (the slope of the curve is small and similar in all the tissues examined) Statistically significant increase of high frequency SCE at 250 and 500 ppm No toxicity reported	2 reliable with restrictions No OECD TG available	Limited/Limited	Kligerman et al. (1993)
SCE in peripheral blood lymphocytes 5 female Fischer 344 rats/ group In vivo CA/MN test (Table B.2) In vivo comet assay (Table B.3)	125, 250 and 500 ppm (nominal concentrations) (actual conc. 120 ± 4, 251 ± 6, 489 ± 12 ppm) Exposure 6 h/day for 14 days Ex-vivo No positive control 50 metaphases scored	Styrene (purity 99.9%) (Shell Canada Ltd.) Vaporised at room temperature mixed with conditioned air. Delivered in the exposure chambers	Positive Dose-related increase of the frequency of SCE Statistically significant increase of high frequency at 250 and 500 ppm No toxicity reported	2 reliable with restrictions No OECD TG available	Limited/Limited	Kligerman et al. (1993)

(Continues)

TABLE B.4 (Continued)

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/relevance of the results	Reference
SCE assays in peripheral blood lymphocytes Male Fischer 344 rats (6 animals/group) CA assay (Table B.2)	Inhalational exposure to 50, 500 or 1000 ppm 6 h/day, 5 days a week, for 4 weeks Positive control: ethylene oxide (150 ppm) Samplings 1, 2, 3 and 4 weeks after initiation of treatment SCE scored in 25 M2 metaphases/animal after in vitro mitogen stimulation of lymphocytes and 68 h culture in presence of BrdUrd	Styrene (no further details given)	Negative The positive control ethylene oxide was also negative (weeks 1 and 2) or equivocal (weeks 3 and 4)	3 not reliable This study did not follow any specific TG. The study was performed with a limited protocol (25 cells/animal), which prevented using animals as statistical units. The positive control produced an insufficient response, which does not allow to evaluate the adequacy of the ex vivo protocol applied to detect genotoxic effects arising from in vivo exposure.	Limited/Low because of the insufficient reliability	Preston and Abernethy (1993)
DNA adducts (7- guanine and 1-adenine) with 32P-postlabelling in lung 6 male NMRI mice/group In vivo MN assay (Table B.2) In vivo comet assay (Table B.3)	750 and 1500 mg/m^3 (actual concentration: 747 ± 38 and 1630 ± 54 mg/m^3) Appropriate amount of liquid styrene was delivered by micropump through calibrated glass capillary into the mixing bottle and evaporated. Inhalation performed in dynamic chamber for 1, 3, 7 or 21 days (6 h/day and 7 days/week).	Styrene (purity not reported)	Positive Dose related increase at 1500 mg/m^3 Significant correlation between styrene concentrations in blood and 7-substituted guanines and 1-SO-adenines	2 reliable with restrictions Low number of animals (1–4 mice/group) analysed at the different experimental points.	Limited/Limited	Vodicka et al. (2001)

TABLE B.4 (Continued)

Test system/test object	Exposure conditions	Information on the characteristics of the test substance	Results	Reliability/comments	Relevance of the test system/relevance of the results	Reference
UDS assay in liver Female CD-1 mice (5/group) OECD TG compliance: TG 486, 1997	Inhalational exposure to 125 and 250 ppm for 6 h Positive control: N-DMA 10 mg/kg bw, by i.p. Sampling 2 and 16 h after the end of treatment Nuclear and net grain counts were determined in 100 hepatocytes/animal in 3 animals/dose/sampling time	Styrene (> 99% pure) from Shell Chem. Co.	Negative Toxicity: No clinical signs were observed in treated animals. In a preliminary range finding experiment, mild microscopic findings (glycogen deposition and inflammatory cell infiltration) were observed 4 days after 250 ppm exposure	1 reliable without restrictions	Limited/Limited because of the indicator endpoint and the non-oral route of exposure	Clay (2004)

APPENDIX C

Summaries of the in vivo genotoxicity studies (2019 IARC Monograph)

Loprieno et al. (1978)

The induction of chromosomal damage was investigated in bone marrow of mice after oral exposure to styrene (purity not reported). Male and female CD-1 mice (4 animals/treated group; 9 animals/control group) were treated by gavage with a single dose of styrene at 500 and 1000 mg/kg bw dissolved in corn oil. Two out of 4 animals dosed with 1000 mg/kg bw died after the treatment. Animals were sacrificed 24 h after the exposure. One hundred metaphases were scored per animal. No increase of structural CAs was observed at any dose tested. The study was considered reliable with restrictions because a limited protocol was applied with low number of animals per group, 100 metaphases scored per animal. This study was performed before the establishment of the OECD TG 475 (OECD, 2016b). The results were considered of limited relevance.

Penttilä et al. (1980)

In this study styrene was evaluated in a MN test in Chinese hamster bone marrow. Styrene (> 99% pure), dissolved in olive oil, was administered once by i.p. injection to four male animals at the dose of 1000 mg/kg bw. Two control animals received the vehicle alone. The study was terminated 30 h after treatment. The PCE/NCE was not statistically significantly lower in styrene treated animals than in vehicle control animals. The incidences of MN, evaluated in 2000 PCE and 2000 NCE per animal, were similar in animals receiving styrene and in concurrent vehicle control animals, as well as in negative control data from previous experiments. No evidence of bone marrow exposure was provided.

The study was considered reliable with restrictions because a limited protocol was applied, with few animals (4 treated and 2 vehicle control animals), a single dose and sampling time and no positive control. The study was performed before the establishment of the OECD TG 474. The results were considered inconclusive and consequently of low relevance.

Norppa et al. (1980)

This study evaluated the in vivo clastogenic activity of inhaled styrene in a CA assay in Chinese hamster bone marrow. Animals (4 males/group) were exposed to 300 ppm styrene (> 99% pure) in an exposure chamber for 4 days (6 h/day) or 3 weeks (6 h/day, 5 days/week), with or without concurrent oral administration of ethanol (15% v:v in drinking water). Control animals (3 animals) were exposed to air flow and/or received plain water. Structural CAs were evaluated in 100 metaphases per animal. No increase of CAs (including or excluding gaps) was observed in bone marrow cells of styrene treated animals (without and with co-administration of ethanol) compared to control animals. This study was considered reliable with restrictions because a limited protocol was applied with few animals (4 treated and 3 control animals) and scored metaphases (100 instead 200/animal), with no positive control and a limited presentation of data (only group means). The study was performed before the establishment of the OECD TG 474. The results were considered inconclusive and consequently of low relevance.

Conner et al. (1980)

The induction of SCE by styrene in alveolar macrophages, bone marrow and regenerating liver cells was evaluated in mice. Hepatectomised and no-hepatectomised BDF1 male mice (6 animals/group) were exposed by inhalation to styrene (purity 99%) concentrations of 104, 387, 591 and 922 ppm for 6 h per day for 4 days. Groups of mice were also exposed at the highest concentration 922 ppm for 1 and for 2 days. After 4 days treatment, a statistically significant increase in SCE/cell was reported in all tissues tested starting from 387 ppm. No significant effect, with the exception of an increase of regenerating liver cells in hepatectomised mice, was detected after 1 day of exposure at 922 ppm. No difference was observed among the different cell types studied or between hepatectomised mice and no-hepatectomised mice. This study was considered reliable with restrictions because a low number of cells were scored and no data on positive control were reported. No OECD test guideline is available on in vivo SCE. The positive results were considered of limited relevance and only considered as supporting evidence in the WoE.

Norppa (1981)

The study investigated the chromosomal damage induced by styrene in mice after i.p. exposure applying the MN test. Male C57BL/6 mice (4 animals/group) were treated by i.p. with a single dose of styrene (purity 99% further purified by distillation) at 250, 500, 1000 or 1500 mg/kg bw) dissolved in olive oil. A significant increase in micronucleated PCE was observed at 250 and 1000 mg/kg bw (maximum increase 2 folds with respect to the control value) 30 h after the end of treatment. No dose–response was observed. No increase in normochromatic cells with MN was reported. The highest tested dose of styrene (1500 mg/kg bw) was lethal to 2 out of 4 mice of the group. The study showed an equivocal increase of chromosomal damage, evaluated as MN frequency, in treated animals, with no dose response and at highly toxic doses.

This study was considered reliable with restrictions because a low number of animals was tested, with less scored cells than recommended, i.e. 2000 PCE scored/animal instead of 4000. The study was performed before the establishment of the OECD TG 474. The equivocal result obtained was considered of low relevance.

Sbrana et al. (1983)

The clastogenic damage induced by styrene (purity not reported) was investigated in bone marrow cells of CD1 male mice after either a 4-day treatment course with daily oral doses of 500 mg/kg or a 70-day course with daily oral doses of 200 mg/kg. Animals were sacrificed 24 h after the last treatment. Gaps, breaks and exchanges were scored in 100 metaphases/animal. Mitotic index, evaluated as the number of mitosis on 2000 cells per animal, was not affected by the prolonged exposure. Blood concentration of styrene at different times after repeated oral doses of 200 mg/kg was evaluated. Styrene was shown to be rapidly absorbed reaching peak blood concentrations of 10 μg/mL within 1 h. Styrene concentration then decreased with a half-life of 36 + 3 min and was detectable up to 8 h. No differences were observed between styrene kinetics in blood on days 1 and 70, the two curves being superimposable. On both the first and last days of treatment attempts were made to detect SO in blood. This metabolite was never detectable at a LOD of 10 μg/mL.

No increase of structural CA was reported at any dose and condition of treatment. The study was considered reliable with restrictions because a limited protocol was applied with a low number of animals/group and only 100 metaphases scored/animal. This study was performed before the establishment of the OECD TG 475. The results were considered of limited relevance.

Kligerman et al. (1993)

The study investigated the genotoxic effects induced by styrene (purity 99.9%) in mice and rats after inhalation exposure. Female B6C3F1 mice (4–6 animals/group) and Fischer 344 rats (5 animals/group) were exposed to styrene at nominal concentrations of 125, 250 and 500 ppm for 6 h per day for 14 consecutive days. Twenty four hours after the exposure peripheral blood, spleen and lung were collected from mice and cells were cultured for the analysis of CAs (spleen and lung), MN applying the cytokinesis block method (spleen and blood) and SCE (all target tissues). In rats peripheral blood lymphocytes were collected and cultured for the analysis of CAs, MN, SCEs and DNA ssb by comet assay. No statistically significant increase in CA, MN or DNA damage was reported in any tissue analysed in mice and rats. No cytotoxicity was reported evaluated as replication index in peripheral lymphocytes, lung cells and splenic lymphocytes.

A statistically significant and concentration related increase of SCE frequency was observed in all cell types in mice and rats. The extent of the increase of SCE was low and similar in all the tissues evaluated.

The study was considered reliable with restrictions for the limitations in the experimental protocol (100 metaphases scored/animal instead of 300 as recommended by OECD TG 475, 1000/2000 NCE instead of 4000 as recommended by OECD TG 474). The results were considered of limited relevance.

Sinha et al. (1983)

The clastogenic effect of long-term inhalation of styrene was investigated in Sprague–Dawley rats (4 males and 4 females) exposed 6h/day, 5 days a week, for a period of 1 year to 600 and 1000 ppm styrene (99.5% pure, stabilised with 5 ppm tertiary butyl catechol). At the end of treatment period, animals were sacrificed and the mitotic indices and incidences of CAs determined in 1000 cells and 100 metaphases per animal, respectively. No increase in structural CAs, including and excluding gaps, and no significant differences in mitotic index was observed in rats exposed to styrene compared to unexposed control animals. No positive control was included in the study, performed before the establishment of the OECD TG 475. The study was considered reliable with restrictions. The results were considered of limited relevance.

Solveig Walles and Orsén (1983)

The study evaluated the induction of DNA ssb by styrene (purity not reported), applying the DNA unwinding technique. Male NMRI mice were injected by i.p. with styrene at 1.7–10.1 mmol/kg. DNA damage was evaluated at 1, 4 and 24 h after dosing in different tissues (liver, lung, kidney, testis and brain). The maximum level of ssb was detected at 4 h in liver, lung, testis and brain. In kidney the maximum increase of ssb was observed 2 h after dosing. A decrease at 24 h was detected in all tissues analysed. The study was considered not reliable because the test system was not validated for genotoxicity assessment as unable to distinguish ssb arising from toxicity. The positive results were considered of low relevance.

Sharief et al. (1986)

Styrene (> 99% pure, dissolved in corn oil) was given by single i.p. injection at 50–1000 mg/kg bw to male C57B1/6 mice (4 animals/dose). Mice were implanted subcutaneously with Bromo-deoxyuridine (BrdUrd) tablet 30 min before treatment for sister chromatid differentiation and cell replication kinetics. CAs were scored in 100 M1 metaphases/animal, harvested 16 h after tablet implantation, and the mitotic index estimated in 1000 cells/animal. SCEs were determined in 30 M2 metaphases harvested 24 h after BrdUrd implantation, and the replication index determined in 200 metaphases. The protocol of this

study, entailing chromatid differentiation by BrdUrd labelling, did not follow any specific test guideline. Styrene administration was highly toxic at the two highest doses (750 and 1000 mg/kg bw), with 1/2 and 3/3 animals dead out of 4 treated in the SCE and CA tests, respectively; there was no effect on replication index but a strong decrease in the mitotic index. Structural CAs and SCE were not increased at any dose compared to untreated control animals (naïve of implanted with BrdUrd) and solvent control (implanted with BrdUrd). The CA assay was performed with a limited protocol: 4 animals, 100 metaphases scored. The study was considered reliable with restrictions and the results of limited relevance.

Simula and Priestly (1992)

The comparison of the genotoxic effects induced by styrene between rat and mice was evaluated applying three assays: bone marrow MN, sperm morphology (SM) and SCE. For this purpose, styrene was administered by a single i.p. dose to male LACA Swiss at 150, 300, 450, 600 mg/kg bw and to Porton rats at 300, 750, 1500, 3000 mg/kg bw. Sampling was carried out at 30, 48 and 72 h after the treatment.

The experimental protocols applied had some limitations: 1000 PCE/animal were scored for the MN test and the analysis of 20 metaphases spreads/animal containing a minimum of 38 sister-chromatids was performed for SCEs assay.

Styrene produced weak genotoxic responses in all assays in mice and only in the SM and SCEs in rats. In mice a statistically significant increase of PCE with MN was detected in bone marrow at 600 mg/kg bw only 48 h after treatment associated with a statistically significant decrease of PCE/NCE ratio. Weak but statistically significant increase of SCE/chromosome in mouse splenocytes was reported at the dose of 450 mg/kg, which was lethal to 1/5 animal. Negative results were reported for MN test in rats. Statistically significant decrease of PCE/NCE ratio at 3000 mg/kg 30 h after treatment was described and a dose-related decrease of PCE/NCE ratio from 750 mg/kg 72 h after treatment. In rats statistically significant increases of SCE/chromosome at 750 and 1500 mg/kg bw were reported. A single result was available at 3000 mg/kg bw (lethal to 4/5 animal). In the SM assay, a small but significant increase in the proportion of abnormal sperm was observed at 400 mg/kg bw (2.8-fold) and 1000 mg/kg bw (1.6-fold) in mice and rats respectively.

The MN test was considered reliable with restrictions due to limitations in the experimental protocol applied (1000 PCE scored/animal instead of 4000, as recommended in the OECD TG 474). The study was performed before the establishment of the OECD TG 474. The results of the MN assay were considered of limited relevance. No OECD TG is available for the in vivo SCE; the results in this assay were considered of limited relevance, as used as supporting information only. Low relevance was given to the results of the SM assay, which is not used for genotoxic risk assessment.

Preston and Abernethy (1993)

In this ex vivo study male Fischer 344 rats (6 animals/group) were exposed by inhalation to 50, 500 or 1000 ppm styrene (purity not reported) 6 h/day, 5 days a week, for 4 weeks. CA and SCE were evaluated in peripheral blood lymphocytes collected 1, 2, 3 and 4 weeks after initiation of treatment. Structural CAs were scored in 25 M1 metaphases/animal and SCE in 25 M2 metaphases/animal after in vitro mitogen stimulation of lymphocytes and 68 h of culture in presence of BrdUrd. Ethylene oxide (150 ppm) was tested in parallel as positive control. The protocol of this study, entailing chromatid differentiation by BrdUrd labelling, did not follow any specific test guideline. The results obtained did not show significant differences in CAs/cell and SCE/cell in any of the treatment group compared with the air control group, at any sampling time. A negative (CA) or equivocal (SCE) response was however also observed with the positive control ethylene oxide. The study was considered not reliable and the results of low relevance.

Vaghef and Hellman (1998)

The study evaluated by alkaline comet assay the induction of DNA strand breaks by styrene (purity not reported) in mice after a single i.p. injection. Groups of six female C57BL/6 mice were treated at 100, 250, 350 or 500 mg/kg bw 4 and 16 h before sacrifice. A dose-related increase of DNA damage, expressed as tail moment, was reported 4 h after treatment in different target tissues (lymphocytes, liver, bone marrow and kidney), with lymphocytes and liver cells as the most sensitive. A decrease of the tail moment was observed in all tissues, with the exception of the bone marrow, after 16 h of exposure. The study was considered reliable with restrictions due to some limitations in the experimental protocol (low number of analysed cells per animal, unusual software used for comet assay analysis). The results were considered of limited relevance.

Vodicka et al. (2001)

The ability of styrene (purity not reported) to induce genotoxic effects after inhalation exposure was investigated in male NMRI mice (6 animals/group) exposed at concentrations of 750 and 1500 mg/m^3 for 1, 3, 7 and 21 days (6 h/day, 7 days/week). A statistically significant increase of MN frequency (twice higher than in control animals) was observed at 1500 mg/m^3 of styrene only after 7 days of exposure. After 21 days of inhalation no significant difference between the control group and the two exposed groups was observed. DNA ssb were evaluated in lymphocytes, bone marrow and liver cells applying comet assay with the use of the enzyme endonuclease III (endo III). A statistically significant increase of DNA ssb was reported in bone marrow after 21 days at the two concentrations tested without dose response and in lymphocytes after 7 days of exposure at 1500 mg/m^3 only with the use of endo III. No increase of DNA ssb was reported in liver. In lung cells

the formation of SO induced 7-SO-guanines and 1-SO-adenines in DNA was analysed by the 32P-postlabelling technique with HPLC separation and on-line radioisotope detection. An increase of 7-SO-guanines and 1-SO-adenines, correlated with styrene concentration in blood, was observed. The experimental protocol for the MN assay was not reported and the study was considered not reliable and the equivocal results of low relevance. The comet study was considered reliable with restrictions due to the low number of analysed cells per animal and the use of aggregated mean data, instead of median, for the evaluation, and the equivocal results obtained of low relevance. Data on DNA adducts were considered of limited relevance, only to be used as supporting evidence in the WoE.

Engelhardt et al. (2003)

In this study, the induction of MN in bone marrow of male NMRI mice exposed to styrene (99% pure) by inhalation was investigated in two laboratories. The study was performed to elucidate the equivocal results previously reported under the same experimental conditions by Vodicka et al. (2001), who co-authored the study. Mice were exposed 6 h/day for 1, 3, 7, 14 or 21 days. MN were scored in 2000 PCE per animal in each of two laboratories; the number of NCE (with or without MN) in the same microscopic fields was also recorded by one laboratory. At the high dose inhalation of styrene was lethal in 7/35 mice; clinical signs of toxicity and reduced body weight gain were observed in the high dose group. The PCE/NCE ratio was not affected by treatments. The number of micronucleated PCE in treated mice was not significantly increased above that of concurrent negative control and was always in the range of the historical negative control at all exposure intervals. The study was considered reliable without restrictions, and the results of limited relevance because of the non-oral route of exposure.

Clay (2004)

The DNA damaging activity of inhaled styrene (purity > 99%) was evaluated in a liver unscheduled DNA synthesis assay in female CD-1 mice. Animals (5/group) were exposed for 6 h to 125 and 250 ppm styrene (MTD identified in a preliminary toxicity range-finding experiment) and sacrificed 2 or 16 h thereafter. Nuclear and net grain counts were determined in 100 hepatocytes per animal. No clinical signs were observed in treated animals in the main experiment. There were no significant increases, compared with the vehicle control, in mean net nuclear grain count or in percentage of cells in repair at either dose level or time point investigated.

The study was performed following the OECD TG 486 (OECD, 1997b) recommendations and was considered reliable without restrictions. The negative results were considered of limited relevance because of the use of an indicator endpoint and of the non-oral route of exposure.

Gaté et al. (2012)

MN were analysed in RET by flow cytometry and DNA strand breaks in leukocytes by comet assay ±Fpg (formamidopyrimidine-DNA glycosylase) in male Fischer 344 rats (6 per group) exposed to styrene (75, 300 or 1000 ppm) or SO (SO, 25, 50 or 75 ppm) by inhalation 6 h/day, 5 days/week, for 4 weeks. Blood samples were taken at the end of 3rd and last (20th) day of treatment. Blood levels of styrene and SO were determined at the end of 4 weeks treatment. At the end of the treatment the number of circulating leukocytes was lower in animals exposed to SO and to the highest styrene concentration (1000 ppm). There were no significant changes in the frequency of micronucleated RET, and no increases in the percentage of Tail DNA in circulating leukocytes in comet assays without Fpg, in exposed animals as compared to untreated control animals at both sampling times and with both styrene and SO. In the presence of Fpg, a significant increase of DNA strand breaks was observed in animals exposed to styrene (but not to SO), only on day 3rd and with no relationship with the dose. Blood concentration of SO in animals exposed to 1000 ppm styrene (0.37 μg/g blood) was between those measured in animals exposed to 25 and 50 ppm SO. The experimental protocol applied for the MN test and for comet assay were considered reliable without restrictions, and the results of limited relevance because of the non-oral route of exposure.

APPENDIX D

Clarification regarding the assessments by EFSA and the DHC

The Panel is aware that the Dutch Health Council (DHC) recently addressed the classification for hazardous properties of styrene in the context of occupational setting (link to the advisory report). The proposed classification of styrene as Muta 2 is mainly based on the results of epidemiological studies in occupationally exposed individuals (exposed by inhalation), supported by in vitro data with human cells. After assessing all the studies, the DHC did not specifically address oral exposure for their proposed classification because this route was not considered relevant for workers' exposure.

For what concerns EFSA's assessment, to give a consolidated answer to the EC mandate and ToRs reported in section 1 of this opinion, the Panel has reviewed all genotoxicity studies considered in the IARC Monograph volume 121 (2019 IARC), published after the IARC Monograph retrieved through a literature search, and the genotoxicity oral studies performed in line with Good Laboratory Practices and OECD test guidelines submitted by the industry. A thorough assessment of the reliability and relevance of all experimental evidence and their integration in a weight of evidence (WoE) approach were performed as recommended by the EFSA's guidance documents (EFSA Scientific Committee 2011, 2017a, 2017b). To this aim, the study results addressing apical genotoxicity endpoints (gene mutation and chromosome damage) and primary DNA damage evaluated by the comet assay were grouped in lines of evidence and only relevant studies were taken into account (EFSA, 2023). Data from in vitro studies and in vivo studies using non-oral routes considered in the 2019 IARC Monograph, and from human biomonitoring studies using genotoxicity biomarkers, were also assessed and integrated in the WoE as supporting information.

Evidence on the genotoxicity mechanism of action (MoA) was taken into account and integrated in the assessment in section 3.1.1 of this opinion. Overall, although most studies are old and with limited protocol (Table 2), the available evidence indicates that styrene is genotoxic in vitro. The mechanism(s) responsible for styrene genotoxicity is not fully elucidated. The strain specificity observed in the Ames test, with positive results only in the presence of metabolic activation and in strains sensitive to base pair substitutions, is consistent with the proposed role of SO in styrene genotoxicity (IARC, 2019), in view of the miscoding properties of SO-DNA adducts. However, the FCM Panel noted that, despite the uniformly positive response of SO in Ames tests with TA1535 and TA100, styrene mutagenicity was not clearly expressed under the standard Ames test protocol, where the majority of the studies provided negative results. The Panel also noted that other mechanisms, including oxidative stress (Chakrabarti et al., 1993; Costa et al., 2006), are proposed to contribute to styrene genotoxicity in addition to DNA binding by SO (Sina et al., 1983). In this respect the Panel noted that no studies have been conducted on S. typhimurium TA 102 or E. coli WP2 or WP2 (pKM101).

Information on SO genotoxicity have been taken into account in the discussion on the in vitro genotoxicity MoA of styrene, where also other mechanisms, different from SO-mediated DNA damage may be implicated.

Evidence on the carcinogenicity MoA was not considered in this opinion as the 2019 IARC studies on carcinogenicity of styrene and SO have been already assessed in the 2020 EFSA opinion. In addition, according to the EFSA note for guidance for substances to be used in plastic FCMs, only data on the genotoxicity potential is required for the evaluation of the chemical substance migrating up to 50 ppb (corresponding to the first tier defined by the SCF). The 2011 EFSA SC opinion on genotoxicity testing strategies considers evidence of genotoxicity as an adverse effect per se, separate from carcinogenicity.

This assessment considered the potential in vivo genotoxic effect of styrene and the integrated effects of styrene and the whole spectrum of metabolites formed in relevant biological models (the whole mammal). It is acknowledged that styrene primary metabolite, SO, is genotoxic; however, unlike for other routes of exposure (such as inhalation), it is not considered relevant for dietary exposure as it is further metabolised and rapidly eliminated from the organism. This is based on an extensive analysis and review of the toxicokinetic properties of styrene and SO and included in this opinion (Section 3.2 and Appendix A).

The toxicokinetic behaviour of styrene is species- and tissue-specific. In particular, metabolic differences are considered as one of the determinants in the different sensitivity of some species to its toxicity (e.g. highest in mice). The liver, followed by the lung (after inhalation exposure), is the primary human tissue responsible for the biotransformation of styrene and SO after oral exposure. Comparative in vitro metabolism studies have shown clear differences in the activity and/or affinity of styrene and its metabolites to cytochrome P450 (CYP450), epoxide hydrolase (EPHX1) and glutathione S-transferase in the liver and lung between species. Although the same metabolic pathways were identified in mice, rats and humans, they differed quantitatively: mice exhibited a higher rate of SO formation, along with the lowest detoxification capacity. The opposite was observed in humans, where highly efficient first-pass hepatic hydrolysis of SO by epoxide hydrolase significantly reduces the systemic availability of the already low amount of the epoxide (SO) formed in situ. The findings on the role of extrahepatic metabolism, including at gastrointestinal level (as the first site of contact by oral route), suggest a minor impact, if any, on styrene metabolism.

The Panel emphasises that the overall conclusion of this opinion based on the scientific evidence provided by reliable animal oral studies in which no significant genotoxic effects were observed following repeated exposure up to high, maximum tolerated doses, only concerns the genotoxic hazard posed by the oral exposure to styrene. Considering that rodents for their metabolic profile are expected to be more susceptible than humans to the potential genotoxic effects of styrene, the EFSA Panel concluded that the available experimental evidence rules out any remaining uncertainties concerning the genotoxicity associated with oral exposure to styrene.

Given the differences in context, sources and routes of exposure (i.e. oral exposure via food and inhalation exposure at the workplace, respectively), conclusions of both evaluations cannot be directly compared. When taking into account these distinctions, the conclusions of the EFSA Panel and the DHC Subcommittee are not conflicting.

ANNEXES

Annex A. Toxicokinetic data
Annex B. Key terms and search strings used in the targeted literature search
Annex C. Outcome of the Public Consultation