Scientific Committee on Consumer Safety

SCCS

OPINION on Triphenyl phosphate

(CAS No. 204-112-2, EC No. 115-86-6)

The SCCS adopted this document
during the plenary on 27 March 2024

ACKNOWLEDGMENTS

SCCS members listed below are acknowledged for their valuable contribution to the finalisation of this Opinion.

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1. ABSTRACT

The SCCS concludes the following:

1. *In light of the data provided and taking under consideration the concerns related to potential endocrine disrupting properties of Triphenyl Phosphate, does the SCCS consider Triphenyl Phosphate safe when used as a plasticiser in nail products up to a maximum concentration of 5%?*

   Based on the currently available information, it is not possible for the SCCS to conclude on the safety of Triphenyl phosphate because the genotoxicity potential cannot be excluded.

2. *Alternatively, what is according to the SCCS the maximum concentration considered safe for use of Triphenyl Phosphate in nail products?*

   /

3. *Does the SCCS have any further scientific concerns with regard to the use of Triphenyl Phosphate in nail products?*

   The SCCS mandates do not address environmental aspects. Therefore, this assessment did not cover the safety of Triphenyl phosphate for the environment.

Keywords: SCCS, scientific opinion, triphenyl phosphate, preservative, Regulation 1223/2009, CAS No. 204-112-2, EC No. 115-86-6)

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on triphenyl phosphate (CAS No. 204-112-2, EC No. 115-86-6) preliminary version of 27 March 2024, SCCS/1664/24

About the Scientific Committees

Two independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems, which may pose an actual or potential threat.

These Committees are: the Scientific Committee on Consumer Safety (SCCS) and the Scientific Committee on Health, Environmental and Emerging Risks (SCHEER) and they are made up of scientists appointed in their personal capacity.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

The Committee shall provide Opinions on questions concerning health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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ISSN ISBN

Doi ND

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2. MANDATE FROM THE EUROPEAN COMMISSION

Background on substances with endocrine disrupting properties

On 7 November 2018, the Commission adopted the review[1] of Regulation (EC) No 1223/2009 on cosmetic products ('Cosmetics Regulation') regarding substances with endocrine disrupting (ED) properties. The review concluded that the Cosmetics Regulation provides the adequate tools to regulate the use of cosmetic substances that present a potential risk for human health, including when displaying ED properties.

The Cosmetics Regulation does not have explicit provisions on EDs. However, it provides a regulatory framework with a view to ensuring a high level of protection of human health. Environmental concerns that substances used in cosmetic products may raise are considered through the application of Regulation (EC) No 1907/2006 ('REACH Regulation').

In the review, the Commission commits to establishing a priority list of potential EDs not already covered by bans or restrictions in the Cosmetics Regulation for their subsequent safety assessment. A priority list of 28 potential EDs in cosmetics was consolidated in early 2019 based on input provided through a stakeholder consultation. The Commission carried out a public call for data in 2019[2] for 14 substances (Group A)[3] and a second call in 2021[4] for 10 substances (Group B)[5] in preparation of the safety assessment of these substances. Triphenyl Phosphate is one of the above-mentioned substances for which the call for data took place.

Background on Triphenyl Phosphate

Triphenyl Phosphate (CAS No. 204-112-2, EC No. 115-86-6) is included in the European database for information on cosmetic substances and ingredients (CosIng) with the reported function of 'plasticiser', meaning that it is used to soften or make supple various synthetic polymers that otherwise could not be easily deformed, spread or worked out.

Triphenyl Phosphate is used in nail products, including nail polishes, enamels or manicuring preparations, but it has additional functions as fire retardant and plasticizer in various industrial and other consumer materials. Currently, Triphenyl Phosphate is not regulated under the Cosmetic Regulation (EC) No. 1223/2009.

During the call for data, stakeholders submitted scientific evidence to demonstrate the safety of Triphenyl Phosphate as a plasticiser in nail products. The Commission requests the SCCS to carry out a safety assessment on Triphenyl Phosphate in view of the information provided.

Terms of reference

1. *In light of the data provided and taking under consideration the concerns related to potential endocrine disrupting properties of Triphenyl Phosphate, does the SCCS consider Triphenyl Phosphate safe when used as a plasticiser in nail products up to a maximum concentration of 5%?*

2. *Alternatively, what is according to the SCCS the maximum concentration considered safe for use of Triphenyl Phosphate in nail products?*

3. *Does the SCCS have any further scientific concerns with regard to the use of Triphenyl Phosphate in nail products?*

[1] https://ec.europa.eu/transparency/regdoc/rep/1/2018/EN/COM-2018-739-F1-EN-MAIN-PART-1.PDF
[2] https://ec.europa.eu/growth/content/call-data-ingredients-potential-endocrine-disrupting-properties-used-cosmetic%20products_en
[3] Benzophenone-3, kojic acid, 4-methylbenzylidene camphor, propylparaben, triclosan, Homosalate, octocrylene, triclocarban, butylated hydroxytoluene (BHT), benzophenone, homosalate, benzyl salicylate, genistein and daidzein
[4] https://ec.europa.eu/growth/content/call-data-ingredients-potential-endocrine-disrupting-properties-used-cosmetic-products-0_en
[5] Butylparaben, Methylparaben, Ethylhexyl Methoxycinnamate (EHMC)/Octylmethoxycinnamate (OMC)/Octinoxate, Benzophenone-1 (BP-1), Benzophenone-2 (BP-2), Benzophenone-4 (BP-4), Benzophenone-5 (BP-5), BHA/Butylated hydroxyanisole/tert-butyl-4-hydroxyanisole, Triphenyl Phosphate and Salicylic Acid

3. OPINION

In this Opinion, the abbreviation TPP will be used for triphenyl phosphate. In the public literature it is also abbreviated to TPHP.

3.1 CHEMICAL AND PHYSICAL SPECIFICATIONS

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

Triphenyl phosphate
Chemical class: organophosphates

3.1.1.2 Chemical names

Phenyl phosphate ($(PhO)_3PO$)
Phosphoric acid, triphenyl ester
Triphenoxyphosphine oxide
Triphenyl phosphate

IUPAC name
Triphenyl phosphate

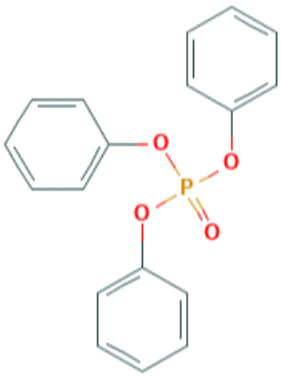
3.1.1.3 Trade names and abbreviations

Disflamoll TP
Celluflex TPP
TPP
TPHP
EVERFOS TP
(see PubChem for more names)

3.1.1.4 CAS / EC number

CAS: 115-86-6 EC: 204-112-2

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

$C_{18}H_{15}O_4P$ or $(C_6H_5)_3PO_4$

3.1.2 Physical form

Colourless crystalline solid with an aromatic, phenol-like odour

3.1.3 Molecular weight

326.3 g/mol

3.1.4 Purity, composition and substance codes

>99.6%

3.1.5 Impurities / accompanying contaminants

Phenol and esters

3.1.6 Solubility

Water: 1.9 mg/L at 20°C (practically insoluble, or insoluble),
Soluble in ethanol, acetone, ether, benzene, carbon tetrachloride, chloroform

3.1.7 Partition coefficient (Log Pow)

Log Kow: 4.63 at 20°C

3.1.8 Additional physical and chemical specifications

Melting point: 49-50 °C
Boiling point: BP: 245 °C at 11 mm Hg
Vapour pressure: 7.50 x 10^{-6} mmHg at 25°C
Density: 1.21 g/cm^3 at 50 °C
Viscosity: 7.8 cSt at 60^0C
UV / visible light spectrum: max 236 – 238 nm

3.1.9 Homogeneity and Stability

Stable under neutral and acidic conditions.

3.2 TOXICOKINETICS

The major metabolites of TPP are diphenyl phosphate (DPHP), monohydroxylated TPP and dihydroxylated TPP. These metabolites, as well as TPP, have been detected in the urine of humans in various cohort studies.

According to the applicant, TPP is expected to have a moderate oral absorption, based on its physicochemical parameters (i.e., low molecular weight of 226.29 g/mol, low water solubility of 1.9 mg/L and high log K_{OW} of 4.63).
In the absence of oral absorption data, the SCCS will apply its default 50% bioavailability to the NOAEL from oral studies.

Distribution
There are no studies available that specifically investigate the distribution of TPP within the body following absorption. Given the small molecular size and high lipophilicity, TPP is

expected to be distributed to highly perfused organs/tissues (i.e., liver, kidney) once absorbed. This is supported by evidence from available repeated dose toxicity studies, where liver and kidney appear to be the target organs. However, significant toxicity was not observed in these studies and the bioaccumulation potential of the substance is expected to be low considering the metabolism and bioconcentration (BCF) values determined in fish (ECHA 2021).

3.2.1 Dermal / percutaneous absorption

The absorption potential of TPP was evaluated in human volunteers (two cohorts; 26 volunteers) exposed via fingernail painting. Urine samples (n=411) were collected before and after application of a polish containing 0.97% w/w TPP and the metabolite diphenyl phosphate (DPH) was measured. Before application, the geometric mean of DPH for the control samples was 0.96 ng/mL. In a second part of the study, to determine relative contributions of inhalation and dermal exposure, 10 volunteers also painted their own nails or synthetic nails adhered to gloves on two separate occasions. Urine was collected for 24 hours following applications for metabolite analysis. The concentration of DPH was found to increase nearly seven-fold approximately 10–14 hours after fingernail painting (13.02 ng/mL). Urinary DPH was significantly diminished when the volunteers wore gloves, suggesting that the primary route of exposure was dermal (Mendelsohn *et al.*, 2016).

The *in vitro* percutaneous penetration of TPP was studied in human skin using Franz diffusion cells. Skin areas of 2.64 cm^2 were exposed, and the average volume of the receptor chamber was 16.6 mL. An aqueous solution composed of 0.9% sodium chloride, 5% bovine serum albumin, 40 mg/L hexamycin, and disodium phosphate buffer served as receptor fluid. The entire skin surface was covered with 1000 ng (0.001 mg) TPP in 500 µL ethanol: toluene (4:1) solution (i.e., 0.0002% of TPP). The diffusion cells were studied at 24, 48, and 72 hours after dosing and the donor cell wash, epidermis, dermis, and receptor fluid were analysed for the test substance content. The apparent flux was determined to be 0.093 ng cm^{-2} h^{-1}. The permeability coefficients (kp) were $0.92x10^{-4}$ cm h^{-1} and $3.3x10^{-4}$ cm h^{-1} for receptor only and receptor and dermis, respectively. The results suggested that TPP tended to build up in the upper layers of the skin tissues. Little TPP permeated the skin and reached the receptor fluid within 72 hours.
(Frederiksen *et al.*, 2018)

According to the applicant, the above (Frederiksen 2018) study suggests a very low absorption of TPP via the dermal route. Based on the information from the above studies and the limited quantitative data, a maximum dermal absorption of 10% is considered as a conservative value for the exposure assessment.

SCCS comment
The study by Frederiksen *et al* is not in accordance with the SCCS basic criteria for dermal absorption. The tested concentration (in a mixture of organophosphate esters) was much lower than the intended use concentration. Considering these aspects, the default dermal absorption of 50% will be used in this Opinion for the skin adjacent to the nails.

3.2.1 3.2.2 Other studies on toxicokinetics

Metabolism
An *in vitro* metabolism study was conducted with TPP using rat liver microsomes. TPP at 0.0004M in ethanol served as a substrate to determine the extent of microsomal decomposition with and without NADPH and/or other enzyme systems. Degradants were characterised by gas chromatography. The results showed that TPP was easily decomposed by the rat liver microsomal fraction without NADPH. The metabolic reactions were inhibited almost completely by SKF-525A and carbon monoxide in the absence of NADPH, whereas KCN, NaN3, dipyridyl and EDTA showed little effect. It was therefore concluded

that the mixed-function oxidase system in the microsomes plays a central role in the metabolism of TPP. The only major metabolite, obtained by hydrolysis, was diphenyl phosphate (DPH), which was not further decomposed by the microsomes. (Sasaki *et al.*, 1984)

In an *in vitro* metabolism study using human liver S9 fraction and microsomes, TPP was mainly transformed to a diester metabolite by *O*-dearylation and to a hydroxylated metabolite. (Van den Eede *et al.*, 2013)

The metabolism of TPP was investigated in a further *in vitro* study using primary human hepatocytes. The liver cells were incubated for up to 2 hours in media containing 20 µM TPP. Extracts of these materials were then analysed by liquid chromatography with mass spectrometry detection. DPH and mono and di- hydroxylated TPP proved to be the major metabolites. The final DPH concentrations corresponded to less than half of the depletion of TPP. Other metabolites, mainly sulphate and glucuronide conjugates, were formed at lower rates. The authors concluded to a low percentage of TPP depletion and slow hepatic clearance. (Van den Eede *et al.*, 2016)

3.3 EXPOSURE ASSESSMENT

3.3.1 Function and uses

Triphenyl phosphate has a function of 'plasticiser', meaning that it is used to soften or make supple various synthetic polymers that otherwise could not be easily deformed, spread or worked out. It is used in nail products, including nail polishes, enamels or manicuring preparations, but it has additional functions as fire retardant and plasticizer in various industrial and other consumer materials.

3.3.2 Calculation of SED/LED

The applicant stated that as TPP is intended to be used in nail polishes only, the dermal route is the major route of exposure. The exposure by inhalation is assumed to be an unlikely route of exposure due to the low vapour pressure and thus low volatility of TPP.

According to the applicant, consumer exposure to TPP via nail polish is expected to be extremely low when used as intended with limited contact to skin by careful application to the nail plate only. As exposure to an ingredient in a nail polish through the nail plate is limited (Brown *et al.*, 2009; Kreutz *et al.*, 2019; Thatai *et al.*, 2016), the systemic bioavailability via the nail plate may be considered as a negligible contribution compared to any contact with skin. This is because, for any amount of the substance that penetrates in the nail structure and then through the nail plate, there is a significant lag time for a substance to transverse through the nail to become systemically bioavailable (Brown *et al.*, 2009; Cutrín-Gómez *et al.*, 2018; Jackson, 2008; Kobayashi *et al.*, 2004; Kobayashi *et al.*, 1999; Kreutz *et al.*, 2019; Lee *et al.*, 2019; McAuley *et al.*, 2016; Mertin and Lippold, 1997; Palliyil *et al.*, 2013). This results in a negligible exposure on a daily basis compared to estimates of exposure to skin around the nail from an application where direct contact (unintentionally) occurs.
For evaluating potential exposure via the skin, estimates from consumer applications can be considered (e.g., RIVM, 2006; Danish EPA, 2008). Dermal exposure to skin around the nail from consumer nail polish applications has been reported in the literature with a P50 value of 40 mg per application, and a P95 value of 58 mg (Ficheux *et al.*, 2014). This is similar to a default value of 50 mg proposed as a value for risk assessment in RIVM Cosmetics fact sheet (Bremmer *et al.*, 2006) or 9% of an applied nail product being in contact with skin around the nail (Danish EPA, 2008). As consumers can sometimes also apply additionally a base-coat

and/or a top-coat, but with lower quantities of product use than the primary nail lacquer (Ficheux *et al.*, 2014), doubling the default value of 50 mg is a conservative total estimate for contact to nail polishes when accounting for additional coats applied in a single day.

Assuming that the application of multiple coats of nail polishes all containing TPP at 5% results in 100 mg total exposure of nail polish to skin in a day, the resulting amount of TPP in contact with the skin is calculated as 100 mg x 5% = 5 mg/day. When accounting for skin penetration of 10%, and adjusting for bodyweight, the resulting systemic exposure is calculated as 0.008 mg/kg bw/day.

SCCS comments

Exposure through the nail plate.

The SCCS agrees that penetration through the nail plate and the subsequent systemic exposure can be considered extremely low. Penetration through the nails by pharmaceuticals (mainly anti-fungal agents) has generally been found to be insufficient to deliver the desired dosage. The nail plate is likely to act as a complex compact hydrophylic filter (Brown 2009). Nail permeability is independent of lipophilicity, but clearly decreases with increasing molecular weight (Kobayashi 2004). Flux rates through the nail plates were determined by Kobayashi 2004 for caffeine as 3.64 and by Brown 2009 for methylparaben as 6.05 and for Terbenafine (which has a molecular weight almost similar to TPP) as 0.55 microgram per cm2 per hour. In view of these studies, and considering that most of the TPP will remain lodged in the nail polish/lacquer polymers, it can be considered that only negligible amounts will penetrate the nail plate.
It is as yet unknown whether filing or sanding ('roughening') of the nails before application will lead to enhanced penetration of TPP.

Exposure through the skin adjacent to the nails.
As explained in 3.2.1, the SCCS will use a skin penetration of 50%. Therefore, a systemic exposure dose (SED) of 0.04 mg/kg bw/d will be used for MoS calculation.

Inhalation exposure
A few studies have attempted to assess the exposure to TPP via ambient air.
The concentration of TPP in bulk air in one nail salon was reported to be 43.7 ng/m^3 (Kim 2019).
A study using urine metabolites as a proxy for inhalation exposure in volunteers who applied nail polish on gloved fingers (to eliminate dermal exposure) showed no difference with controls (Mendelsohn 2016).
The USA National Institute for Occupational Safety and Health (NIOSH) measured TPP in the ambient air in 4 nail salons for 12 workers in nail salons. The geometric mean of TPP in air was 7.39 ng/m^3 (max 21.85 ng/m^3) (Fairfield-Estill 2021)
Based on this, the SCCS agrees that exposure from inhalation is also likely to be negligible.

In occupational settings, the 8-hr time weighted inhalation exposure limit for the inhalable fraction is 3 mg/m3 (OECD-SIDS 2002). The German Technical Guidelines of the Ministry of Labour and Social Affairs has set the limit in respirable air at 12.5 mg/m3 (TRGS-900, 2023).

3.4 TOXICOLOGICAL EVALUATION

In conformity with the ban on animal studies after 2013, the applicant has not submitted such data in its dossier. When such data were available from other sources, the SCCS took it into consideration for this Opinion.
According to ECHA 2021, the self-classification indicates hazard statements H400 and H410: very toxic to aquatic life.

3.4.1. Irritation and corrosivity

3.4.1.1 Skin irritation

Undiluted TPP was not irritating to the skin in several studies. See the Table below for an overview.

Table 1. Skin irritation studies (Adapted from CIR, 2018)

Test system	Exposure (Concentration/ Dose/Vehicle	Method	Results	Reference
3 New Zealand White rabbits; sex not reported	99.7% pure; 500 mg; in water	Dermal irritation/ corrosion study in accordance with OECD TG 404; test substance applied to shaved rabbit skin for 4 hours and semi-occlusive; test area = 6 cm^2	Not irritating	(OECD SIDS, 2002)
6 Albino rabbits; sex not reported	500 mg; concentration and vehicle not reported	Dermal irritation/ corrosion study; test substance applied to shaved intact and abraded skin for 24 hours and semi-occlusive	Not irritating	(OECD SIDS, 2002)
6 New Zealand White rabbits; 3/sex	50 mg/mL suspension in 1.0 mL/patch; 50% aqueous solution of polyethylene glycol	Dermal irritation/ corrosion study; test substance applied to shaved intact and abraded skin for 24 hours and occluded	Not irritating	(OECD SIDS, 2002)
25 male CF-1 mice	70% solution in alcohol	Dermal irritation study; semi-occluded patch for 24 to 72 hours; no further details provided	Not irritating	(Sutton *et al.*, 1960)

3.4.1.2 Mucous membrane irritation / eye irritation

According to the applicant, undiluted TPP is minimally irritating to the eye.

Table 2. Eye irritation studies (Adapted from CIR, 2018)

Test system	Exposure (Concentration/ Dose/Vehicle	Method	Results	Reference
3 New Zealand White rabbits; sex not specified	99.7% pure; 70 mg; neat	Ocular irritation study in accordance with OECD TG 405 ; test substance applied for 24 hours; eyes washed after 24 hours and examined for 7 days post-application	Not irritating; Mild reactions of the mucous membranes and the cornea observed immediately after exposure was considered mechanically induced effects	(OECD SIDS, 2002)
6 New Zealand White rabbits; 3/sex	100 mg; neat	Ocular irritation study according to the US FDA Hazardous Substances guideline; test substance was washed in 3/6 eyes after 30 seconds	Minimally irritating; Mild conjunctival effects (slight redness in all rabbits) observed 24 hours post instillation which cleared in all but 1 unwashed eye by 72 hours (remaining eye cleared by day 6); slight corneal opacity observed in 1 unwashed eye at 24 hours which cleared by 48 hours	(OECD SIDS, 2002)
9 albino rabbits; sex not specified	100 mg/eye; neat	Ocular irritation study; 3 eyes washed 4 seconds after instillation; eyes examined 24, 48, 72 hrs and 7 days post installation.	Minimally irritating; Mild conjunctival effects (slight redness 6/6, slight discharge 4/6) at 24 h ours in unwashed eyes which cleared by 72 hrs, no effects in washed eyes	(OECD SIDS, 2002)

3.4.2 Skin sensitisation

The skin sensitization potential of TPP was assessed based on a GLP compliant Guinea Pig Maximisation Test (GPMT). No evidence of skin sensitisation was observed following application of 75% test substance in arachis oil.

Magnusson & Kligman Guinea Pig Maximisation test
Guideline: OECD Test Guideline 406
Species/strain: Guinea pig/ Dunkin-Hartley (Hsd Poc:DH (SPF)
Group size: 5 male animals in the control, 10 male animals in the test groups
Test substance: Triphenyl phosphate
Purity Not specified
Batch F21022
Vehicle: (for intradermal) Arachis oil or FCA / 0.9% aqueous NaCI solution (1:1)
Induction: intradermal, 5% w/w in arachis oil or FCA / 0.9% saline (1:1)
Induction: epicutaneous, 75% w/w in arachis oil, occlusive
Challenge: 75 and 50% in arachis oil, epicutaneous, occlusive
Positive control: 2-Mercaptobenothiazole
GLP: Yes
Study period: 2001

Results: The challenge with the 75 and 50% w/w test substance on the flanks did not cause any skin reactions in either the control or test group animals. No data were available on the treatment related impact on body weight gain and signs of toxicity.
Conclusion: Under the conditions of the study, TPP did not trigger any skin reactions indicative of a skin sensitisation response.
(OECD SIDS, 2002)

In a non-validated mouse ear swelling test, dose-dependent elicitation responses were observed at concentrations of 3 or 10%. This study was not considered reliable because of an insufficient description of procedure & results and because of deviations from accepted procedures regarding challenge concentrations (OECD-SIDS 2002).

On the basis of the available results, TPP is not considered to have a skin sensitisation potential.

SCCS comment
The SCCS agrees with the Applicant's conclusion regarding the lack of skin sensitisation potential of TPP. This is also in view of the absence of any published reports that convincingly show sensitisation in humans.

3.4.3 Acute toxicity

3.4.3.1 Acute oral toxicity

Acute oral toxicity studies with TPP are available in the rat, mouse, and guinea pig. The database comprises six non-guideline studies, as summarised in the Table below. According to the applicant, with oral LC50 values consistently above 3000 mg/kg, the available information suggests that TPP has a low potential for acute toxicity.

Table 3. Acute oral toxicity studies (Adapted from CIR, 2018; ECHA, 2021)

Species	Exposure	Results	LD50 (mg/kg bw)	Reference
Rat	20000 mg/kg bw (25% aqueous solution); 5 animals/sex; Wistar rats via intragastric intubation	No premature deaths observed; gross examination revealed sporadic visceral haemorrhages	>20000	(OECD SIDS, 2002)
Rat	Maximum dose level 15800 mg/kg bw administered in corn oil; male and female Sprague Dawley rats via gastric intubation; Number of animals and concentration not reported	Mortality and systemic toxicity data not provided	10800	(OECD SIDS, 2002)
Rat	2500, 5000 mg/kg in 20% emulsion with gum Arabic; 5 animals/sex/group; gavage; strain not reported	No premature deaths and no clinical symptoms observed	5000	(OECD SIDS, 2002)

Rat	3000 mg/kg bw; 11 male Holtzman rats; Concentration and vehicle not reported	1 death recorded within a month of exposure, no clinical symptoms observed	>3000	(Sutton *et al.*, 1960)
Mouse	20% emulsion with gum Arabic; 2500, 5000 mg/kg; 5 animals/sex/group; gavage; strain not reported	Slight stupor observed; no premature deaths reported	>5000	(OECD SIDS, 2002)
Guinea pig	3000, 4000 mg/kg bw in corn oil; 5 male albino guinea pigs; Concentration not reported	No premature deaths and no clinical symptoms observed	>4000	(Sutton *et al.*, 1960)

3.4.3.2 Acute dermal toxicity

The acute dermal toxicity of triphenyl phosphate (purity not specified) was evaluated via the dermal route of exposure. A single dose of 10000 mg/kg bw was occlusively applied to the intact or abraded skin of 10 albino rabbits and the animals were observed for mortality and clinical signs for 14 days. Necropsy with gross pathological examinations were performed after sacrificing the animals at study Day 14.

No mortalities, clinical signs of systemic toxicity or skin irritation were observed at 10000 mg/kg bw.

Under the conditions of the study, the acute dermal LD_{50} of TPP was considered to be greater than 10000 mg/kg bw in rabbits (OECD SIDS, 2002).

The acute dermal toxicity of triphenyl phosphate (purity not specified) was evaluated in New Zealand White rabbits. A single dose of 7900 mg/kg bw was occlusively applied to the intact skin of male and female rabbits for 24 hours and the animals were observed for mortality, and clinical signs for 14 days. Necropsy with gross pathological examinations were performed after sacrificing the animals at study Day 14.

No mortalities, clinical signs of systemic toxicity or skin irritation were observed at 7900 mg/kg bw.

The acute dermal LD_{50} of TPP was determined to be greater than 7900 mg/kg bw in male and female rabbits (OECD SIDS 2002).

3.4.3.3 Acute inhalation toxicity

Triphenyl phosphate (purity not specified) was evaluated for acute inhalation toxicity in Wistar rats. The rats (5 males and 5 females) were exposed to the test substance in dust form for 1 hour at a nominal concentration of 200000 mg/m^3. The animals were observed for signs of toxicity during the exposure period.

No mortality and clinical signs of systemic toxicity were observed during the study. The acute inhalation LC_{50} of TPP in rats can be considered to be greater than 200000 mg/ m^3 (ref: OECD SIDS, 2002).

3.4.4 Repeated dose toxicity

The USA National Toxicology Program (NTP) conducted a 5-day study on triphenyl phosphate in rats (see 3.4.10, Special investigations). The goal of this study was to provide a rapid assessment of *in vivo* biological potency by evaluating a combination of traditional

toxicological endpoints and transcriptomics analysis to broadly query the biological space for any dose-related change. The report stated that as the LOEL for the study, cholinesterase inhibition appeared to be the most sensitive apical measure; further studies are warranted to assess cholinesterase effects at concentrations <55 mg/kg to obtain an accurate point of departure (NTP 2018).

3.4.4.1 Repeated dose (21-28 days) oral / dermal / inhalation toxicity

1.

Dermal

A dermal subacute toxicity of triphenyl phosphate (purity not specified) was investigated in a GLP compliant study similar to an OECD Test Guideline 410 in New Zealand White rabbits. Ten male and ten female animals per group were treated on clipped, intact (half of the animals) and abraded skin (half of the animals), 6 hours/day, five times/week for three weeks with doses of 0, 100 and 1000 mg/kg bw/day under open conditions for 21-23 days. Samples of the test substance were taken one week pre-test and on the last day of the study and sent to the sponsor for analytical confirmation of the test substance. The test substance was applied as a 50% (w/v) solution in ethanol. Control animals were treated with 2 mL/kg bw/day ethanol alone. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption. Haematological, biochemical, ophthalmological examination and urinalysis were performed. At termination of treatment, all animals were sacrificed and macroscopically examined, organs were weighed, and comprehensive histopathology was performed.

Results:

No mortalities, clinical signs, body weight and food consumption changes were observed during the study. No changes in haematological, biochemical, ophthalmological examination and urinalysis were observed up to high dose. Depression of acetyl cholinesterase in plasma, erythrocytes, and brain was observed in males and females of treated rabbits. No clinical or histological correlation was found. No quantitative data were reported for this endpoint. This effect was not considered as a toxicologically relevant effect.

Conclusion:

Under the conditions of the study, the NOAEL for TPP was established by the study authors at 1000 mg/kg bw/day in rabbits

(ref: OECD SIDS, 2002).

2.

Oral

The oral subacute toxicity of triphenyl phosphate (Disflamoll TP; purity: 99.6%) was investigated in a 28-days GLP compliant OECD Test Guideline 407 feeding study. Wistar rats (5/sex/group) were dosed daily via the diet at 0, 250, 1000 and 4000 ppm (equivalent to 23/39, 104/161 or 508/701 mg/kg bw/day in males/females) for 4 weeks. The doses or concentration of the dietary test substance preparations was analysed. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption at defined intervals. Samples for haematological and clinical-chemical examination were taken on day 29 of treatment. Neurobehavioral parameters, functional observational battery (FOB) was performed on day 26. At termination of treatment, all animals were sacrificed and macroscopically examined, organs were weighed, and comprehensive histopathology was performed.

Results:

The test substance was stable and homogenously distributed in the diet for the duration of use. No mortalities or clinical signs of toxicity were observed during the study. There were no remarkable changes in body weights or body weight gain in males at 23 mg/kg bw/day and females receiving up to high dose. At 104 mg/kg bw/day and 701 mg/kg bw/day body weight gain was reduced in males (without dose dependence). No toxicologically relevant changes in

food intake were observed up to 104 and 161 mg/kg bw/day in males and females, respectively.
There were no toxicologically relevant changes in red blood or in blood coagulation up to 508 and 701 mg/kg bw/day in male and females, respectively. A statistically increased mean for the monocytes was observed at 701 mg/kg bw/day in females, is considered incidental, because correlations to this finding are lacking. Mean ASAT activities decreased at 104 and 508 mg/kg bw/day in males. Mean cholesterol concentration increased in 508 mg/kg bw/day males.

No statistically significant changes were observed in the functional observation battery up to high dose. There was no indication of test substance related changes in motor activity up to high dose.

At necropsy, statistically significant increase in absolute and relative liver weights were observed in high dose males and females and the frequency of enlarged liver was increased in males. These findings correlated with minimal to slight hypertrophy/cytoplasmic change of periportal hepatocytes at 104 and 508 mg/kg bw/day in males and at 701 mg/kg bw/day in females. A swollen eosinophilic appearance with a homogenous dust like granulated cytoplasm was observed in the periportal and partly midzonal areas of hepatocytes. Taken together, indications of distinct changes in liver function were observed at 104 and 508 mg/kg bw/day in males and at 701 mg/kg bw/day in females. No toxicologically relevant organ weight differences, gross and histopathological findings was observed in the remaining organs up to high dose group. Under the conditions described the NOEL for test substance was established at 23 mg/kg bw/day for male and 161 mg/kg bw/day for female rats. The NOAEL was established at 23 mg/kg bw/day for male based on effects on body weights and 701 mg/kg bw/day for female rats.

Conclusion:
Under the conditions of the study, the NOAEL for TPP was established by the study authors at 250 ppm in males and 4000 ppm in females (i.e., 23 and 701 mg/kg bw/day in males and females, respectively)
(Ref: ECHA, 2021)

3.
In a 30-day study on neurotoxicity (see also 3.4.10 Special investigations) internal lesions on brain tissue due to TPP exposure were examined by a histopathological test. Weaned male mice were exposed to 0, 50 or 150 mg/kg bw/d by oral gavage. According to the authors, no remarkable neuronal lesions, were observed in the brain of the control group. Neurons in the CA2 region of the hippocampus of the high TPP dosed group were arranged neatly and tightly, but neural cell loss and karyopyknosis occurred in the DG region in the high dose group. The same phenomenon was evident in the cortex. Both treatment groups showed a microglial invasion. In the thalamus, the tissue was slightly edematous in the high-dose group, and blood vessels were slightly dilated and congested.
(Ref: Liu 2020)

SCCS comment on study 3
The number of animals in each dosing group in this study is not clear, and neither is number of animals subjected to histopathological examination:it seems to be one animal per group.

3.4.4.2 Sub-chronic (90 days) oral toxicity

Taken from ANSES 2019 and ECHA 2021):

1.
In an unpublished study report according to OECD 408 (Unpublished report: Van Otterdijk FM, 2015, summarized in the registration report), Wistar rats (10/sex/dose) were treated during 90 days with TPP for 90 consecutive days by dietary administration at dose levels of 0, 300, 1500 and 7500 ppm. The mean estimated dose over the study period was 0, 20, 105, and 583 mg/kg bw/d for males and 0, 22, 117, and 632 mg/kg bw/d for females. According to the authors (no other precision given):

- No treatment-related mortality occurred, and no toxicologically relevant clinical signs were noted;
- The magnitude of liver weight was increased at 7500 ppm (approximately 30 and 21% for males and females, respectively).
- Histopathological findings in the liver consisted of centrilobular hepatocellular hypertrophy of the liver in males at 1500 and 7500 ppm and in females at 7500 ppm, accompanied by enlargement and red brown discolouration of the liver and higher liver weight at necropsy at 7500 ppm;
- Changes in clinical biochemistry parameters consisted of higher total proteins and calcium levels in males at 7500 ppm, and higher cholesterol concentration in males and females at 7500 ppm, and in males also at 1500 ppm.
- Morphological findings in the thyroid gland consisted of increased incidence and/or severity of follicular cell hypertrophy in males at 1500 and 7500 ppm (up to slight degree), which might be secondary to the hepatocellular hypertrophy and is not considered to be adverse. Necropsy of males at 7500 ppm showed enlargement and higher weight of the thyroid gland.

Based on the liver effects, particularly centrilobular hypertrophy observed at 1500 ppm in line with the increase in liver weight at 7500 ppm, a no observed adverse effect level (NOAEL) of 20 and 22 mg/kg (for males and females respectively) was established. In the particular case of this dossier, the reporters considered that the centrilobular hypertrophy identified, as the first effect impacting the liver, is significative, especially when considering the other effects observed on rodents in fish.
(Ref: ANSES 2019, ECHA 2021).

2.
The subchronic oral toxicity of triphenyl phosphate (purity not specified) was investigated in a 1986 non-guideline dietary study in male rats. Sprague-Dawley rats (10 males/group) were fed a diet containing 0.25, 0.5, 0.75 or 1% (equivalent to 161, 345, 517, 711 mg/kg bw/day) of test substance for four months. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption. The neurotoxicity was assessed at the end of every month in open field, accelerating rotarod, forelimb grip strength and negative geotaxis examinations.
Results:
There were no toxicologically relevant clinical signs observed or deaths reported during the study. Change in the body weight was observed in animals at 0.5% (345 mg/kg bw/d). At the dose level of 0.5 to 1% slight but statistically significant reduction in growth rate was detected as the only change.
Only limited data are reported and several standard parameters of repeated dose toxicity such as organ weight measurement and histopathology of organs as well as haematology and clinical chemistry other than serum proteins were not determined.
Under the conditions of the study, the NOAEL for TPP was established by the study authors at 161 mg/kg bw/day in male rats.

Ref: Sobotka 1986

SCCS comment
The SCCS will use the NOAEL of 20 mg/kg bw/d, derived from the above-mentioned (1) 90-day guideline study for the calculation of the margin of safety (MoS).

3.4.4.3 Chronic (> 12 months) toxicity

/

3.4.5 Reproductive toxicity

3.4.5.1 Fertility and reproduction toxicity

Taken from ANSES 2019, based on Welsh et al 1987:
Fertility and developmental toxicity were examined in a dietary study in Sprague- Dawley rats at doses of 0, 166, 341, 516 or 690 mg/kg bw/day (Welsh *et al.* 1987). Forty males and 40 females per group were treated for 3 months. Upon completion of the subchronic phase of the experiment, animals receiving identical diets were cohabitated in a 1:1 sex-ratio in the afternoon. The following morning, females were examined for the presence of sperm. The day of finding sperm was designated as day 0 of gestation. The animals continued to receive the test diets throughout mating and gestation. On day 20 of gestation, dams were examined externally and then sacrificed by carbon dioxide asphyxiation.
Body weights were measured and food cups were weighed on days 7 and 14 and before cesarean sections on day 20 of gestation. Daily observations were made on the dams and any changes in the general appearance, health or behaviour of the animals were noted. A laparotomy was performed on each followed by an examination of the major organs. Ovaries were removed and examined for numbers of corpora lutea. Uterine blood vessels were clamped off and the entire gravid uterus was excised and weighed. The number and the position of fetuses (viable or dead) and resorption sites (early or late) were recorded. Fetuses were examined individually for gross abnormalities. For each fetus, uterine position, sex, weight and crown-rump were recorded. Runts were defined as any fetus weighing less than 70% of the average weight of the male or female controls. No significant signs of parental toxicity were detected. There were no effects on pregnancy rate, number of viable fetuses and implants, corpora lutea, implants, implantation efficiency, number of early and late deaths, or average percent resorbed. There were no significant differences between treated groups and controls in the incidence of specific sternebral variations or in the average number of sternebral variations per litter.
It should be noted that male and female pups from all treated groups tended to weigh more than their respective controls. However, the difference was significant only for males in the 341 and 690 mg/kg bw/day groups. Furthermore, all treated groups had significantly more fetuses exhibiting moderate hydroureter and enlarged ureters (in the region adjacent to the kidney) than the control group, but the incidence of these variations seems not related to dose since a greater proportion of fetuses were affected in the two lowest dose levels than in the two highest levels. The authors explained this by the fact that the reference incidence in the controls was also high and there was no clear dose response. The significance of these effects remains unclear.
(Ref: ANSES 2019)

3.4.5.2 Developmental Toxicity

1.

Taken from ANSES 2019, based on Unpublished report 2015-a:

A prenatal developmental toxicity study was conducted in rabbits by oral gavage. In a dose-range-finding study doses of 0, 83, 250 and 750 mg/kg bw/day TPP were administered. All females at 750 mg/kg bw/day died. Therefore, no litters at this dose level were available for fetal examination. At 250 mg/kg bw/day, one female (euthanized) died; showing no food intake and reduced faeces production during the last week, body weight loss, pale appearance and pale faeces production. Another female at 250 mg/kg bw/day was noted with reduced production of (pale) faeces and a pale appearance. These two females were the most sensitive to treatment based on data on body weight and food consumption. There were no fetal findings up to 250 mg/kg bw/day that were considered to be toxicologically relevant (raw data not available).

Based on this range-finding study, doses of 32, 80 and 200 mg/kg bw/day were selected for the main study. At these doses, the only sign of maternal toxicity reported was a reduction in body weights and (corrected) body weight gain at 200 mg/kg/day mainly due to a marked effect in two females. A reduction of faeces production and food consumption were also noted but without a dose- response relationship.

The premature loss of 1 litter (litter 78 with 11 dead foetuses) at 200 mg/kg bw/day was considered to be related to maternal toxicity. This animal showed severely reduced food consumption during the week prior to delivery (21 g /day on days 23-26 and 7 g/day on days 26-29 compared with 112 g/day at the start of the study). The only other dead foetuses in this study were one fetus in litter 23 at 32 mg/kg bw/day and one fetus in litter 70 at 200 mg/kg bw/day. A higher incidence of lungs with absent accessory lung lobe(s) was reported in the 200 mg/kg bw/day group. Only one foetus in the low-dose and control groups had this malformation, but the occurrence in the high-dose group was 3 (3) foetuses (litter) making a litter incidence rate of 1.6%. Furthermore, two of the dead foetuses from the prematurely delivered litter (litter 78) had also presented with the malformation, thus making the total 5(4) foetuses (litter). This increased the litter incidence rate to 2.4% which is above the historical control maximum of 1.7%. The historical control data from this laboratory consisted of 17 developmental studies with this strain in which in total 2787 (315) control fetuses (litters) were examined. In 10 of these studies fetuses with absent accessory lung lobe(s) were found, i.e. in total 20 (17) control fetuses (litter). The highest occurrence of this finding in the historical control data was 3 (3) rabbits (litters) from two studies. Therefore, the incidence rate in the 200 mg/kg bw/day group was slightly above the relevant historical control range thus ANSES considers this finding toxicologically relevant. In the high-dose group there was an increase in the following parameters (which could potentially indicate delayed development): unossified tarsals, metacarpals and pubis; these changes were slight, not of statistical significance and could often be explained by lower foetal body weights. There were no other findings of concern for developmental toxicity. A NOAEL for maternal and developmental toxicity could be set at 80 mg/kg bw/day.

(Ref: ANSES 2019)

2.

Gestational TPP exposure in mice

Guideline:	/
Species:	pregnant C57B1/6 mice
Exposure:	intraperitoneal triphenylphosphate on gestational day 8, 10, 12 and 14
Dose:	0, 5, 25 or 50 mg/kg bw/d
Timing:	Dams euthanized on GD19, fetuses euthanized after 1 hour in incubator
Target in dams:	Weight of organs. Tissue analysis of liver

Target in fetus: Weight, litter size, sex ratio, gross morphological defects, AGD
Year: 2018 (publication)

Teratogenic outcome was the second aim of this study. The first aim was to investigate whether TPP exposure results in Insulin-like growth factor signaling (see 3.4.12 Special investigations: metabolism).

According to the authors, maternal weight gains as well as maternal organ weights of each of the exposure groups showed no statistically significant treatment effects, suggesting that these doses of TPP are not acutely toxic to the dams.
The effects of increasing doses of TPP on resorptions, gross morphological defects, skeletal defects fetal weights, crown rump length, placental weights and anogenital index showed no statistical differences across all exposure groups, except for placental weights. A significant increase in placental weights of mice exposed to 25 mg/kg compared to the unexposed controls was found. When normalized to fetal weight, as measured by the fetus to placenta weight ratio, there was a significant decrease in this ratio in mice exposed to 25 mg/kg compared to unexposed controls, indicating that this increase in placenta size did not coincide with an increase in fetal weight.
The authors concluded that TPP does not cause overt structural developmental toxicity.
(Ref: Philbrook 2018)

3.
Two generation 28-day study in rats.

In the context of a larger flame-retardant program of the US National Toxicology Program (NTP) program the study was conducted to evaluate the short-term perinatal toxicity of Triphenyl phosphate (TPHP or TPP) and Isopropylated phenyl phosphate (IPP) as an initial step.

In their abstract, the authors state that currently there are no data to evaluate potential risk from exposure to either TPHP or IPP during fetal development. Their short-term perinatal studies in rats aim to provide preliminary toxicity data for TPP and IPP, including information on transfer to fetus/offspring and across the pup blood brain barrier. In separate experiments, TPP or IPP were administered via dosed feed at concentrations 0, 1000, 3000, 10,000, 15,000 or 30,000 ppm to time-mated Hsd:Sprague Dawley® SD® rats from gestation day (GD) 6 through postnatal day (PND) 28; offspring were provided dosed feed at the same concentration as their dam (PND28-PND 56). TPHP and IPP-related toxicity resulted in removal of both 30,000 ppm groups on GD12 and 15,000 ppm IPP group after parturition. Body weight and organ weights were impacted with exposure in remaining dams. Reproductive performance was perturbed at ≥10,000 ppm TPHP and all IPP exposure groups. In offspring, both TPP and IPP-related toxicity was noted in pups at ≥10,000 ppm as well as reduction in bodyweights, delays in pubertal endpoints, and/or reduced cholinesterase enzyme activity starting at 1000 ppm TPHP or IPP. Preliminary internal dose assessment indicated gestational and lactational transfer following exposure to TPHP or IPP. These findings demonstrate that offspring development is sensitive to 1000 ppm TPHP or IPP exposure.

According to the authors, dose selection was intended to sufficiently challenge exposed animals and capture dose-response information for maternal and pup toxicity including a high enough top-dose level to be conclusive for hazard classification. The authors state that due to effects observed in all exposure groups, a no observed adverse effect level (NOAEL) could not be determined in the current studies.
(Ref: Witchey 2023)

SCCS comment on study 3
The publication does not state the above-mentioned feed intakes in terms of intake of TPHP in mg/kg bw/d. However, from a graphical presentation of the food consumption it can be deduced that in the 1000 ppm administered dose feeding the exposure to TPP in the dams during gestation was approximately 80 mg/kg bw/d to approximately 200 mg/kg bw/d during lactation.
For the higher-administered dose groups the TPP intake was, respectively, in the order of 300 to 600 mg/kg bw/d, 1000 to 2000 mg/kg bw/d and 1500 to 3000 mg/kg bw/d.
The cholinesterases activity in the blood from dams decreased in a dose-dependent manner, reaching significance at ≥3000 ppm TPP, corresponding to an intake of approximately 300 (gestation phase) to 600 (lactation phase) mg/kg bw/d. The reduction in offspring cholinesterases activity only reached significance in the BChE of 15000ppm (i.e. 1500 to 3000 mg/kg bw/d) TPHP exposed females. In the offspring, in general a reduction of brain AChE and BChE activity occurred as the TPHP concentration increased.

SCCS overall comment
The SCCS accepts the NOAEL for maternal and developmental toxicity of 80 mg/kg bw/d, as set by ANSES.

3.4.6 Mutagenicity / genotoxicity

3.4.6.1 Mutagenicity / genotoxicity *in vitro*

Gene mutation (Ames test and mammalian cell gene mutation assays)
Several studies have been reported in the literature on gene mutation and are summarised in Table 4.

Table 4. Summary of gene mutation tests reported on TPP.

Concentrations	Strains	Method	Result	Reference
plate incorporation assay: 0, 50, 160, 500, 1600, 5000 µg/plate preincubation assay: 0, 50, 160, 500, 1600, 5000 µg/tube	*S. typhimurium* TA 1535, TA 1537, TA 98, TA 100 and TA 102	-/+ S9 mix OECD 471 GLP compliant	Negative	ECHA AMES
plate incorporation: 1 – 1000 µg/plate	*S. typhimurium* TA 98, TA 100, TA 1535, TA 1537, TA 1538	-/+ S9 mix OECD 471	Negative	OECD SIDS, 2002
plate test and preincubation test: 50 – 5000 µg/plate	*S. typhimurium* TA 98, TA 100	-/+ S9 mix OECD 471	Negative	OECD SIDS, 2002
preincubation assay: up to 10000 µg/plate	*S. typhimurium* TA 98, TA 100, TA 1535, TA 1537	-/+ S9 mix OECD 471	Negative	Zeiger *et al.*, 1987
-S9 mix: 3.13 - 50 µg/mL	Mouse lymphoma L5178Y cells	-/+ S9 mix OECD 476	Negative	ECHA 2021 OECD SIDS, 2002

+S9 mix: 6.25 - 75 µg/mL				

SCCS comment on gene mutations
Several *in vitro* experimental mutagenicity tests show that TPP is not mutagenic in bacterial cells or mammalian cells.

***In vitro* Chromosomal damage**

Study #1- chromosomal aberrations

The clastogenic potential of the test item was evaluated in a chromosome aberration test *in vitro* according to OECD TG 473. For the short treatment, Chinese hamster V79 cells were exposed in the absence of S9 mix during 4 hours to concentrations of 3.5, 7, 14, 17.5 and 21 µg/mL of the test item, media were changed and cultures from all concentrations were harvested after 18 hours of treatment. In addition, cultures exposed to 14, 17.5 and 21 µg/mL were harvested 30 hours after treatment. In the presence of S9 mix, cultures were exposed for 4 hours to concentrations of 10, 20, 40, 50 and 60 µg/mL. C None of the cultures treated with the test item in the presence and absence of S9 mix exhibited biologically relevant or statistically increased numbers of aberrant metaphases. The positive controls induced clastogenic effects and demonstrated the sensitivity of the test system and the activity of the used S9 mix. Based on this study, triphenyl phosphate was considered by the Applicant not to be clastogenic for mammalian cells *in vitro*.
(Ref: ECHA 2021)

SCCS comment on Study #1
The SCCS, after having examined the full study report, identified the following limitations:

- No information is provided about the cell cycle, which is particularly relevant to long exposure. In view of the average doubling time of V79 cells (usually at least 12-14 h; https://www.atcc.org/products/ccl-93), the time of harvesting might be too short to yield an adequate number of duplicated cells, harvest after 18h incubation might not be appropriate for V79 cells.
- For the 4h exposure and harvesting at 30h, the level of cytotoxicity observed at the concentrations tested (survival index of 34,2 or 30,6% corresponding to cytotoxicity 65-69%) do not adequately justify the top dose-selection for the micronucleus analysis.
- The current version of OECD TG 473 (adopted in 2016) recommends that at least 300 well-spread metaphases should be scored per concentration, but in this study, 200 metaphases were analysed. The study was in accordance with TG 473 at the time it was conducted, but according to current standards, the lower number of metaphases scored may imply that the test was not sensitive enough to detect a weak mutagenic effect.

Study #2 – Micronucleus test

In a study published in 2023 (after the submission of the applicant's dossier), the genotoxicity of TPP in several mammalian cell lines and its relevance to CYP/ sulfotransferase (SULT) activities were investigated (Xie *et al.*, 2023).
The results indicate that TPP induced micronucleus formation at ≥1 µM concentrations in a human hepatoma (C3A, endogenous CYPs being substantial) cell line, which was abolished by the CYPs inhibitor 1-aminobenzotriazole. In the cell line HepG2 (parental to C3A with lower CYP expression) TPP was inactive up to 10 µM, while pretreatment with ethanol (CYP2E1 inducer), PCB 126 (CYP1A inducer), or rifampicin (CYP3A inducer) led to micronucleus formation by TPP. In V79-Mz and V79-derived cells expressing human CYP1A1, TPP was

inactive (up to 32 µM), and in cells expressing human CYP1B1, 2B6 and 3A4 it induced micronucleus weakly (positive only at 32 µM).

Ref.**:** Xie 2023

SCCS comment on Study #2

Although the study by Xie *et al.*, 2023 is not fully compliant with OEDC TG 487, its experimental design to a great extent follows the recommendations for MN test without using cytochalasin B. Therefore, the SCCS analysed the study with due attention.

The cell lines established from human liver may show some variability in expression and activity of metabolising enzymes, however if they originate from well recognised cell repositories and were propagated under reproducible laboratory conditions, they may provide relevant results. In this study, the HepG2 cell line (human hepatoma) was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and the C3A cell line (human hepatoma) was purchased from the American Type Culture Collection (ATCC). The analysis of the response of C3A cells to TPP exposure indicates increased frequency of MN without S9. The results were supported by staining for centromere protein B (for which relevant positive control substances were used) and by staining for γ-H2AX.

The SCCS is of the opinion that the results suggest a clastogenic potential of TPP, i.e. TPP induced micronucleus formation at concentration >1 µM (0.33 µg/mL), with human CYP1A2 and 2E1 being major activating enzymes and SULT1A1 being involved in detoxification.

Study #3 – Micronucleus test

Syrian hamster embryonic fibroblast Cells (SHE) were tested in an *in vitro* mammalian cell micronucleus test after exposure to TPP at concentrations: 10^{-6} to 10^{-4} M (0.33 – 33 µg/mL). The exposure was for 5 h with harvesting after 6, 12, 18, 24, 30 h post exposure. Two thousand cells per concentration were scored for MN.

The following MN frequencies were calculated for different harvesting time:

- background: 14.45±4.3/2000 cells
- 18 h: 28.7±4/2000 cells after exposure to 16.3 µg/mL vs. 18±4.9/2000 cells in control
- 24 h: 17±4.5/2000 cells after exposure to 0.33 µg/mL vs. 17.5±2.1/2000 cells in controls (larger decreases at all other concentrations).

Ref.: Schmuck 1989, cited in OECD-SIDS 2002 and in ECHA 2021.

SCCS comment on Study #3

Although the reliability of the study is limited, the results indicate an equivocal response in SHE cells.

3.4.6.2 Mutagenicity / genotoxicity *in vivo*

/

SCCS overall comment on TPP genotoxicity testing

In the opinion of the SCCS, after analysis of the currently available data, TPP has been shown not to induce gene mutations. However, the evidence for the lack of induction of chromosomal damage is questionable and recent data from the study by Xie *et al.* (2023) raises concerns on TPP clastogenicity *in vitro*. Hence, genotoxicity concern cannot be excluded based on the available information.

The SCCS requested additional evidence via an *in vitro* study of TPP to exclude genotoxicity potential. This was not provided by the Applicant. Hence, the genotoxic potential of TPP cannot be excluded.

3.4.1 3.4.7 Carcinogenicity

Theiss *et al.* (1977) studied the occurrence of lung adenomas in strain A/St male mice, 6 to 8 weeks old, using doses of 80, 40, or 20 mg TPP/kg injected intraperitoneally 1, 3, and 18 times, respectively, into groups of 20 mice. TPP purity: 95-99.9%. Twenty-four weeks after the first injection, the animals were sacrificed, and the frequency of lung tumours was compared with that in the control group of 50 animals treated with tricaprylin (vehicle). The pulmonary adenoma response at the highest dose of TPP was not significantly greater than the response of the control mice. Positive control (urethane) induced tumors in mouse with 100% survival, attesting sensitivity of the biological model.

(Ref: Theiss 1977)

SCCS comment

This study is considered inadequate due to the low survival of animals in two of the three experimental groups and the short duration of the study.

Several recent research studies that used cancer cells were identified, implicating TPP in the carcinogenic process (Zhang, Huang, Huang, Zhang et al., 2023; Hong et al., 2022; Zhang and Song 2022; Kwon et al., 2022; Ye et al., 2022). In one study, animal experiments suggested that TPP treatment significantly enhanced tumour growth in the xenograft hepatocellular carcinoma mouse model (Ye et al., 2022). In the study by Zhang et al. (2023), the application of bioinformatics tools to a bladder cancer cohort indicated a strong correlation between TPP exposure and bladder cancer.

Overall, the SCCS regards that these studies, carried out in cancer cells or animal cancer models, do not provide sufficient evidence to draw a conclusion on carcinogenicity.

3.4.8 Photo-induced toxicity

According to the Applicant, no photo induced toxicity studies on TPP could be identified. However, considering its maximum UV-absorption spectrum of 233-241 nm², TPP is not expected to be phototoxic.

3.4.9 Human data

The applicant provided studies from the public literature containing information on presence or absence of endocrine activity of TPP in humans (Meeker 2010, Preston 2017, Tao 2021, Doherty 2019, Messerlian 2018). Because the studies are based on urine metabolites, which may originate from exposure to other organophosphates, these studies cannot be used for the risk assessment in this opinion.

3.4.10 Special investigations: Endocrine disruption (ED) properties

From the Applicant:
Relevant data for the assessment of the potential ED properties of TPP is available in the form of *in vitro* and *in vivo* studies. In addition, the US EPA ToxCast database was consulted for existing high throughput screening (HTS) results. A Table (with one minor text-edit by the SCCS) summarising the available scientific information is presented in Annex 1.

ANSES 2019 and ANSES/ECHA 2023 also performed evaluations of the potential endocrine active properties. While the ANSES/ECHA 2023 evaluation had a focus on environment, several studies were selected from its evaluation as being relevant for this opinion.

Level 1 Existing data and non-test information

According to the Applicant, no existing data and non-test information for TPP were identified from the literature.

From ANSES 2019 summary:
TPP is predicted to be a non-binder to the oestrogen receptor because of its cyclical structure without a hydroxyl or amino group according to OECD QSAR Toolbox Version 3.4.

Level 2 *In vitro* assays
From the Applicant:
A)
In vitro high throughput screening (HTS) assays from the US EPA ToxCast program:
TPP has been tested as part of the ToxCast program of US EPA, which currently contains 105 ED-specific *in vitro* high throughput screening (HTS) assays, addressing the **E** (estrogen), **A** (androgen), **T** (thyroid) and **S** (steroidogenesis) modalities and are being used as part of the US EPA's Endocrine Disruptor Screening Program (EDSP-21). TPP was found to be active in 21 (E=10; A=4; T=3; S=4) out of 58 ED relevant assays (accessed in August 2021) out of which 12 (57%) have been flagged as potentially 'false positive' by the automated analysis tool from the US EPA (ToxCast pipeline (tcpl) package[5]). A closer review of the dose response curves was performed for each of the modalities, showing that:

- Estrogen receptor (ER) assays - no or only very weak agonistic and no antagonistic activity
- Androgen receptor (AR) assays - no specific or significant agonistic or antagonistic activity
- Thyroid assays- not considered to have specific activity
- Steroidogenesis assays - only one out of 24 assay was truly active, which therefore does not fulfil the US EPA criterion for the interpretation of an active result

Overall, the applicant concluded that except for no or weak estrogenic activity, TPP does not have significant ED activity in the other *in vitro* HTS assays considered under the EDSP-21 screening program of US EPA (CompTox/EDSP-21, accessed Aug 2021).

B)
In vitro endocrine mechanistic assays identified in the public literature:
TPP was found to have weak estrogenic activity which was >90,000 to 1 x 10^8-fold less potent as compared to standard agonists. The EC_{50} of the ER agonistic assays were determined to range from 5.3 x 10^{-3} to 10 µM. TPP also showed weak anti-estrogenic activity with potency in the range of >900 to 700,000-fold lower compared to standard antagonist. AR receptor binding potential of TPP was reported to be 4000 times less potent compared to R1881 ligand. TPP showed anti-androgenic activity 600-fold lower than that of AR antagonist hydroxyflutamide (IC50 >10 µM).

TPP showed neither thyroid agonist nor antagonist properties in reporter gene assay and T-screen assay. Also, TPP was shown not to have thyroid transport protein transthyretin (TTR)-binding property. TPP was not concluded to have glucocorticoid agonistic activity but have weak antagonistic activity in the glucocorticoid pathways (75-fold lower than that of antagonist). TPP (10 µM) was not found to have agonistic or antagonistic activity towards progesterone and glucocorticoid receptors in luciferase and β- galactosidase reporter assays. Furthermore, TPP was reported to decrease the cell viability, induce oxidative stress, and disrupt the steroidogenesis in TM3 cells by altering the underlying gene expressions; however, these effects were observed at higher cytotoxic concentrations, i.e., ≥60 µg/mL (≥184 µM). TPP however showed PXR agonistic activity but at 7-9-fold less potent compared to rifampicin).

Not referenced by the Applicant, but included in ANSES 2019: Kojima 2013 (with results in agreement with Kojima 2016) and Liu 2012.

From ANSES 2019 summary:
EDSP21 screening database:
7 out of 8 assays showed no androgenic activity and 1/7 showed some activity but at cytotoxic concentrations. 8 out of 16 assays showed no oestrogenic activity but 8/16 showed some weak activity for oestrogenic effects but at high concentrations, 3/3 assays showed no thyroid related activity.
Androgenic activity:
Triphenyl phosphate in two assays showed a close to weak binding activity to the androgen receptor in micromolar concentrations, partly in cytotoxic concentrations. The overwhelming majority of assays showed no specific binding activity. Based on this information there is no indication of a specific androgenic potential of triphenyl phosphate.
Estrogenic activity:
TPP shows positive results in some of the estrogenicity screening assays in the micromolar range.

The SCCS identified a recent study (Wang, Lee, Hales 2023) in the context of a publication on transcriptomic investigations of expression of genes involved in steroidogenesis in KGN human ovarian granulosa cells. The study showed that TPP increased basal production of Estradiol and Progesterone (see section 3.4.12 Special Investigations: metabolism).

A steroidogenic effect of TPP was demonstrated in an *in vitro* study on a human adrenal cell line, indicating decrease of basal production of cortisol and aldosterone (Lee, Robaire, Hales 2023 - see 3.4.12 Special investigations: metabolism). The study also indicated an altered expression of rate-limiting enzymes involved in cholesterol and steroid biosynthesis. In a transcriptional assay, triphenyl phosphate had weak inhibitory effects on the GR-mediated transcriptional activity induced by hydrocortisone (Kojima 2016).

Level 3 *In vivo* assays providing data about selected endocrine mechanisms/ pathways

According to the Applicant, no Level 3 *in vivo* assays providing data about the selected ED mechanisms with TPP prior to 2013 could be identified.

The ANSES 2019 summary mentions a metabolic study on the impact of a perinatal exposure to TPP on type 2 diabetes onset and adipose accumulation in UCD-type 2 diabetes mellitus rats (Green *et al.*, 2017). For a short description see in this Opinion 3.4.12 - Special investigations: metabolism.

The SCCS identified from the public literature several recent publications which are a combination of *in-vivo* and *in-vitro* studies, with a focus on metabolic transcriptomics and

metabolomics data. These studies are briefly described in this opinion the section 3.4.12 and 3.4.13 - Special investigations: Metabolism (for a brief overview see Annex 2). Overall, these studies point to an effect on metabolism of glucose and lipids.

Level 4: *In vivo* assays providing data on adverse effects on endocrine relevant endpoints

According to the Applicant, three subacute repeated doses (ECHA, 2021; OECD SIDS, 2002; Sutton *et al.*,1960) and an OECD Test Guideline equivalent one generation reproductive and developmental toxicity study (Welsh et al, 1987) conducted before 2013 are available as Level 4 studies on TPP.
The Applicant concluded that based on the *in vivo* studies that were described in his dossier, the available range of OECD Level 4 *in vivo* studies do not provide any evidence that TPP exerts adverse human health effects via an endocrine mode of action.
The studies are briefly described in this Opinion in the paragraphs 3.4.4.1 and 3.4.5.1.

From ANSES 2019 summary:
The following *in vivo* studies were considered, with conclusions similar to those by the applicant and also described in section 3.4.4.1, 3.4.4.2, 3.4.5.1 and 3.4.5.2:
- 28-day repeated dose toxicity study (rat) – Liver effects only, no effects on any other organs or tissues (including reproductive) (ECHA 2021).
- 90-day repeated dose toxicity study (rat) – Liver effects and increase in thyroid weights, no effect on reproductive organs or tissues (Unpublished report 2015 –b).
- One-generation reproductive toxicity study (rat) – No effects on fertility (Walsh 1987).
- Prenatal developmental toxicity study (rabbit) – No effects on fertility or development (Unpublished report 2015-a).

From ANSES/ECHA 2023
Relevant academic studies published from 2018 to June 2022 were retrieved. Endocrine relevant endpoints related to Human health were not evaluated by FR-MSCA in the context of this SEV.

Level 5: *In vivo* assays providing more comprehensive data on adverse effects on ED related endpoints over more extensive parts of the life cycle of the organism

From ANSES 2019 summary:
No level five data is available, but a 'modified one- generation reproductive toxicity study' is currently being undertaken under the auspices of the US NT.

From the Applicant:
No OECD Level 5 *in vivo* assays conducted prior to 2013 were identified for TPP.

For studies in humans see 3.4.9: these studies cannot be used for risk assessment in the context of this Opinion.

Overall Conclusion of the Applicant on Endocrine Activity:
For the ED assessment in humans, the available weight of evidence combining results of *in vitro* screens from ToxCast and *in vivo* testing (repeated dose and reproduction and developmental toxicity studies) shows no evidence of adverse effects of TPP due to an endocrine-related mechanism and no human health-related EATS-mediated adversity or endocrine activity.
The available weight of evidence, combining results of *in vitro* screens from ToxCast, *in vitro* and *in vivo* mechanistic testing together with *in vivo* repeated dose toxicity studies with TPP, suggests that there is weak agonistic and/or antagonistic activity in estrogen and androgen

receptor binding assays with a potency in the range of >9 x 10^2 to 1 x 10^8-fold lower compared to standard agonist and/or antagonists. However, TPP did not show any activity on thyroid and progesterone receptors. Similarly, TPP did not show any glucocorticoid activity however it was shown to have antagonistic activity which was about 75 times less potent than the known antagonist.
The effects described in the available studies appear to be limited in rodents and do not allow to draw definitive conclusions on potential hazards of TPP on human health. Nevertheless, these data are insufficient to conclude that TPP is an endocrine disruptor according to the OECD conceptual framework for testing and assessment of endocrine disruptors (data allow to reach the level 3 of the OECD conceptual framework on endocrine disruptor (ANSES, 2019; OECD, 2012).
Taken together and in the absence of the clear evidence for co-relating any of the adverse effects with the ED activity, which is a requirement as per the WHO definition, TPP is not considered to pose a hazard due to endocrine disrupting properties. Further, the selected POD for risk assessment is concluded to be protective of the observed adverse effects on gonads and/or the reproductive parameters.

ANSES 2019 and 2023

Based on the studies that were available for its report, ANSES (ANSES 2019) concluded that the effects described in the available studies appear to be limited in rodents and do not allow to draw definitive conclusions on potential hazards of TPP on human health. Environmental data show endocrine disruptor potential of TPP. Nevertheless, these data are insufficient to conclude that TPP is an endocrine disruptor according to the OECD conceptual framework for testing and assessment of endocrine disruptors (data allow to reach the level 3 of the OECD conceptual framework on endocrine disruptor (OECD 2012)).
In the ANSES/ECHA evaluation of 2023 (ANSES/ECHA 2023), it was concluded that TPP shows endocrine activity on non-target organisms with adverse effects on fertility and reproduction in academic studies. Endocrine relevant endpoints related to Human health were not evaluated in the context of this SEV.

SCCS overall comment on ED activity

In addition to the studies that were used by the Applicant to assess an ED modality, more *in vitro and in vivo* toxicity studies were identified by the SCCS.
In level 2 *in vitro* assays, some estrogenic activity was observed in a few of the studies. This estrogenic activity was also demonstrated in a recent study on KGN human ovarian granulosa cells. In addition, that study showed a stimulation of secretion of progesterone.
The submitted studies do not indicate an androgenic potential of TPP. Although a short-term *in vivo* study in mice (see section 3.4.13) noted a decreased serum testosterone level, it cannot be derived from that study whether this could be attributed to an anti-androgenic effect.

No level 3 *in vivo* studies were submitted. From the published literature, the SCCS identified several recent *in vivo* and *in vitro* studies, with a focus on metabolic transcriptomic assays (see section 3.4.12 and 3.4.13 - Special investigations; a brief overview can be found in Annex 2). The studies point towards an effect of TPP on changes in glucose and lipid metabolism mainly taking place in the liver.
However, due to the design of the studies and the reporting of the results, no conclusion could be drawn on an exposure dose that could be used for a point of departure for risk assessment in this Opinion.

Two *in vitro* studies indicated a steroidogenic effect of TPP: one study showed a decrease of basal production of cortisol and aldosterone and one study with a transcriptomic assay

indicated weak inhibitory effects on the GR-mediated transcriptional activity induced by hydrocortisone.

In level 4 *in vivo* (OECD TG408, 421/422 and 443) studies, estrogenic effects were not observed.

Based on the available data regarding thyroid and thyroid hormones, the T modality did not seemed to be affected. Although some scattered effects were observed, including increased follicular cell hypertrophy (most likely due to hepatocellular hypertrophy) in males in the 90-day repeated dose toxicity, the results were not considered sufficient to establish effects on T modality.

From the *in vivo* studies that included weight gain as parameter, an obesogenic effect of TPP cannot be clearly established.

The Applicant provided studies from the published literature containing information on the presence or absence of endocrine activity of TPP in humans. Because the studies are based on urine metabolites, which may also originate from exposure to other organophosphates, these studies cannot be used for the risk assessment in this Opinion.

3.4.11 Special investigations: neurotoxicity and immunotoxicity.

Neurotoxicity

1

The neurotoxicity of TPP was investigated as part of a subchronic dietary toxicity study in male rats. Sprague-Dawley rats (10 males/group) were fed a diet containing 0.25, 0.5, 0.75 or 1% TPP (equivalent to 161, 345, 517, 711 mg/kg bw/day) for four months (see also 3.4.4.2). At the end of each month, evaluations of motility, exploratory behaviour, balance and general motor coordination, and muscular strength were performed.
No significant neurobehavioral changes (open field, accelerating rotarod, forelimb grip strength and negative geotaxis examinations) were observed. From the study results, a NOEL of TPP for neurotoxicity can be derived at 711 mg/kg bw/day in rats.

(Sobotka 1986)

2

In their review of the studies on neurotoxicity, ANSES (ANSES 2019) concluded that a decrease of cholinesterase activity has been reported, but no other neurotoxicity effect has been recorded in these (old) studies. However, the relevance of the available data to assess the delayed neuropathy of TPP is questioned due to the few endpoints assessed and the too short duration of assays in neurotoxicity studies available.

3

The USA National Toxicology Program (NTP) conducted a short-term study (4 days) on triphenylphosphate in male Harlan Sprague Dawley rats. The goal of this study was to provide a rapid assessment of *in vivo* biological potency by evaluating a combination of traditional toxicological endpoints and transcriptomics analysis to broadly query biological space for any dose-related change. Exposure to TPP dissolved in corn oil was once daily for 4 days by oral gavage; on Day 5, animals were sacrificed. TPP (>99%) was tested at six doses: 0, 55, 110, 220, 441, and 881 mg/kg body weight.
Cholinesterase inhibition was observed at all doses including the lowest tested dose of 55 mg/kg. The report stated that as the LOEL for the study, cholinesterase inhibition appeared to be the most sensitive apical measure; cholinesterase inhibition was so marked at all doses

that a BMD value could not be determined due to poor model fit. Further studies are warranted to assess cholinesterase effects at concentrations <55 mg/kg to obtain an accurate point of departure.

In the same study, transcriptional changes in the liver following TPHP exposure occurred at dose levels below that for which changes in circulating cholinesterase and cholesterol levels were observed. The most sensitively affected gene sets for which a reliable BMDL could be estimated were cellular polysaccharide biosynthetic process and oligodendrocyte development, both with a BMDL median value of 11 mg/kg. Fourteen Gene Ontology Biological Processes were potently affected below the lower limit of extrapolation from the dose curve (BMD < 18.3 mg/kg). This finding suggests that further testing at doses lower than 55 mg/kg would be useful toward refining estimates of the transcriptional point of departure.

(Ref: NTP 2018)

4

In a 30-day study weaned male mice were exposed to 0, 50, or 150 mg/kg TPP daily by oral gavage for 30 days (see also 3.4.4.1 Repeated dose (21-28 days) oral toxicity). The blood brain barrier permeability of TPP and its metabolite diphenyl phosphate (DPP) in the brain, and TPP induced metabolomic and transcriptomic changes of the brain were investigated. The number of exposed animals and the numbers used for sampling are not clearly specified in the publication.

According to the authors, untargeted metabolomic results showed that the changed level of glutamic acid, N-acetyl CoA metabolites, and organic acid in the brain of treated mice, suggest that amino acid and lipid metabolism was interfered. RNA-seq data indicated that neuronal transcription processes and cell apoptosis pathway (forkhead box (FOXO), and mitogen-activated protein kinase (MAPK) signaling pathways) were significantly affected by TPP exposure. RT-PCR showed proinflammation cytokine tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6)) levels were increased, while antioxidant genes including nuclear factor-E2-related factor 2 (Nrf2), heme oxygenase1 (HO-1) and superoxide dismutase (SOD1) decreased. These results suggest that TPP could cause a degree of neurotoxicity by inducing neuroinflammation and neuronal apoptosis, which are related to oxidative stress. The potential implications for neurophysiology and behavioral regulation cannot be ignored.

(Ref: Liu 2020)

SCCS comment

The SCCS agrees with ANSES that inhibition of cholinesterase has been observed, but that the studies do not indicate overt neurotoxicity. Inhibition of cholinesterase activity was also noted in a recent 28-day developmental toxicity study in rats from an oral exposure to 300 mg/kg bw/d and higher. (See 3.4.5.2: Developmental Toxicity – ref: Witchey 2023).

Immunotoxicity

1

The potential for immunotoxicity of TPP was investigated in a 120-day non-guideline study in rats. Spartan Sprague-Dawley rats (10 males/group) were fed a diet containing 0, 0.25, 0.5, 0.75 or 1% (equivalent to 0, 161, 345, 517, 711 mg/kg bw/day) of test substance. Immunization (initial, secondary, and tertiary) with sheep red blood cells in rats was performed at 60 (initial), 81 (secondary), and 102 (tertiary) days. At termination of treatment, all animals were sacrificed and macroscopically examined. Lymphoid organs were weighed, total protein analysis and electrophoretic analyses of serum proteins were performed, and comprehensive immuno-histochemical evaluation of spleen, thymus and lymph nodes using immunoperoxidase staining was conducted.

Reduced growth rate was observed at 711 mg/kg bw/day. Lymphoid organ weights varied in a non-dose dependent manner, and no significant changes were found in these organs and lymph nodes during histopathologic examinations. No significant effects were reported in

serum protein. There was an increase in the levels of alpha- and beta-globulin in male and female rats but effects were similar at all dose levels, relative to the control group. There were no significant differences between animals immunized with sheep red blood cells and nonimmunized animals. Non-dose-dependent variation was found in the humoral immune response to sheep red blood cells rats. Under the conditions of the study, the NOAEL for immunotoxicity was set at 711 mg/kg bw/day.

(Ref: OECD SIDS, 2002)

2

The immunotoxicity of TPP was also assessed in rabbits in a 3-week repeated dermal toxicity test (see 3.4.4.1). New Zealand White rabbits (10/sex/dose) were dosed topically at 0, 100 and 1000 mg/kg bw/day in 50% solution in ethanol. At the end of study, gross and microscopic effects on the spleen, thymus, or lymph nodes were recorded. There were no effects on the immune function parameters.

Ref: (OECD SIDS, 2002)

3

An in-vitro study with 12.5, 25 and 50 microMol TPP on a mouse-derived macrophages cell line showed induction of macrophages accompanied by upregulation of mRNA for inflammatory mediators.

(Lin, 2023)

3.4.12 Special investigations: metabolism (with transcriptomics)

(For a brief overview, see Annex 2)

In vitro

1

In the context of the EU Horizon 2020 projects, *in vitro* assays measuring key events linked to hepatic steatosis, such as lipid accumulation, mitochondrial dysfunction, gene expression, were applied in human hepatocellular carcinoma cells, HepG2, treated with 0.1, 5, 10 or 25 µM of TPP for 24 h. Cytotoxic effects were observed only at the highest concentration. TPP induced lipid accumulation in a dose-dependent manner and affected mRNA levels of lipid metabolism-related genes, namely induced DGAT2 and SCD1 and SREBP-1c (up to 2-fold inductions) involved in triglyceride or lipid synthesis, but did not affect the expression of CPT1α, ACLY, APOB, and ACCA. In addition, TPP decreased the cellular ATP production at 10 µM by 25–40 %; for TMPP, the effects were already observed at 2 µM. This suggests compromised ATP production in mitochondria, which may further propagate steatosis in liver cells. In silico analysis suggested that TPP formed a conventional hydrogen bond with Ser 342 and pi-donor hydrogen bonds with Ser 247 of the active binding sites of selected receptors, PPARγ and PXR, respectively. Overall, TPP was considered to induce lipid accumulation and enhance hepatic steatosis.

(Ref: Negi, 2021)

2

(Also referenced in ANSES 2019)

In-vitro study on cultured adipocytes.

The main objectives of this study were to assess the *in vitro* effect of TPhP and its metabolite diphenyl phosphate (DPhP) on the adipogenic differentiation of 3T3-L1 cells, as well as glucose uptake and lipolysis in differentiated 3T3-L1 adipocytes. TPhP increased pre-adipocyte proliferation and subsequent adipogenic differentiation of 3T3-L1 cells, co- inciding with increased transcription in the CEBP and PPARG pathway. Treatment of mature adipocytes with TPhP increased the basal- and insulin stimulated- uptake of the glucose analog 2-[N(-7-nitrobenz-2-oxa1, 3- diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG). This effect was

ablated by inhibition of PI3K, a member of the insulin signaling pathway. DPhP had no significant effect on cell proliferation and, compared to TPhP, a weak- er effect on adipogenic differentiation and on 2-NBDG uptake. Both TPhP and DPhT significantly enhanced the isoproterenol-induced lipolysis, most likely by increasing the expression of lipolytic genes during and after differentiation. This *in vitro* study suggests that TPhP increases adipogenic differentiation, glucose uptake, and lipolysis in 3T3-L1 cells through endocrine and noradrenergic mechanisms.

(Ref: Cano-Sancho 2017)

3

Short-term NTP study in rats.

In the abovementioned NTP 2018 study (see 3.4.11, Neurotoxicity) the most sensitive apical endpoint for which a BMD could be determined was HDL cholesterol with a BMDL (BMD) of 39 (79) mg/kg. Dose-dependent increases in absolute and relative liver weight [48 (136) mg/kg and 71 (103) mg/kg] and cholesterol [90 (142) mg/kg] for BMDL (BMD) were the next most sensitive apical endpoint changes.

Ref: NTP 2018

4

Hepatotoxicity of TPP was investigated in a culture of human L02 liver cells, exposed to 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M. According to the authors, transcriptomic analysis showed that TPP exposure markedly affected cell apoptosis, oncogene activation, REDOX homeostasis, DNA damage and repair. Metabolomic analysis verified that the related metabolic pathways, such as glycolysis, citrate cycle, oxydative phosphorylation, lipid and protein metabolism were also significantly disrupted.

(Ref: Wang, Li 2020)

5

Transcription of genes involved in hepatic glucose and lipid metabolism was also examined within a study in mice with once daily oral dosing of 25 and 50 mg/kg bw during 5 weeks.

According to the authors, TPP induced AdipoR1 in males while it reduced the expression of AdipoR1 and –R2 in females.

The picture for the up- or downregulation of genes related to glucose and lipid metabolism was mixed. See for a further description on glucose and lipid metabolism paragraph 3.4.12 Special investigations: metabolism – *in vivo*.

(Ref: Wang, Le 2020)

6

In vitro study on HepG2 cells.

The study investigated the adverse effects of triphenylphosphate (TPHP), OH-TPHP, and DPHP in HepG2 cells in terms of cell proliferation, lactate dehydrogenase release, reactive oxygen species generation, and mitochondrial membrane potential. Transcriptomic changes were measured using RNA sequencing, and bioinformatics characteristics including biological functions, signal pathways and protein- protein interaction were analysed to explore the potential molecular mechanisms. According to the authors, the order of cytotoxicity was OH-TPHP> TPHP> DPHP. The prioritized biological functions changes induced by TPHP and OH-TPHP were correlated with lipid metabolism. Significant lipid accumulation was observed as confirmed by increased total cholesterol and triglycerides contents, and enhanced oil red O staining.

Enrichment of PPARα/γ and down-stream genes suggested the participation of PPARs signal pathway in lipid metabolism disorder. In addition, TPHP and OH-TPHP induced endoplasmic reticulum stress (ERS), which was further confirmed by an ERS inhibitor experiment.

According to the authors, PPARs signal pathway and endoplasmic reticulum stress may be involved in the lipid metabolism disorder induced by TPHP and OH-TPHP.

The authors stated that regarding the complex metabolic responses to TPHP in organisms, the exposure doses selected in this study (0, 40 and 80 µM), are generally higher than the *in vivo* concentrations of TPHP and its hydroxylated form.
(ref: An, Jiang, Tang 2023)

7

In vitro study on KGN ovarian granulosa cells.
It was hypothesized that OPEs alter the steroidogenic ability of kGN ovarian granulosa cells by dysregulating the expression of transcripts involved in steroid and cholesterol biosynthesis. KGN cells were exposed for 48 hours to 1 of 5 OPEs (1-50µM): triphenyl phosphate (TPHP), tris(methylphenyl) phosphate (TMPP), isopropylated triphenyl phosphate (IPPP), tert-butylphenyl diphenyl phosphate (BPDP), and tributoxyethyl phosphate (TBOEP), or to a polybrominated diphenyl ether flame retardant (BDE-47), in the presence or absence of Bu_2cAMP. OPEs increased the basal production of progesterone (P4) and 17β-estradiol (E2) in a dose-dependent manner) and had either no effect or inhibited Bu_2cAMP-stimulated P4 and E2 synthesis.
Quantitative real-time polymerase chain reaction analyses revealed that OPEs (≥5µM) increased the basal expression of critical genes (STAR, CYP11A1, CYP19A1, HSD3B2, and NR5A1) involved in steroidogenesis; upon stimulation, the expression of all genes tested was downregulated. An overall inhibition in cholesterol biosynthesis was induced by OPEs, characterized by a downregulation in HMGCR and SREBF2 expression. TBOEP consistently showed the least effect. The authors concluded that OPEs perturbed steroidogenesis in KGN granulosa cells by targeting the expression of steroidogenic enzymes and cholesterol transporters.
(ref: Wang, Lee, Hales 2023)

8

In vitro and *in vivo* study on placental trophoblast cells.
In a study using the trophoblast cell line JEG-3, it was found that exposure to 33µM triphenylphosphate (TPhP) altered gene and protein expression in the tryptophan metabolism pathway, inhibited the tryptophan-serotonin pathway, and activated the tryptophan-kynurenine pathway. TPhP was found to induce oxidative stress by activating monoamine oxidase A (MAOA), promoting inflammatory factors including nuclear factor kappa-B (NFκB), interleukin-6, and tumor necrosis factor α. The NFκB inhibitor sulfasalazine could alleviate the effects of TPhP on tryptophan metabolism disturbance. The MAOA inhibitor clorgyline or the antioxidant N-acetylcysteine can mitigate oxidative stress and eliminate TPhP-induced inflammatory factors and tryptophan metabolism disturbances. Accordig to the authors, the data suggest that TPhP disturbed tryptophan metabolism by activating NFκB through MAOA-mediated oxidative stress.
For the *in vivo* component of this study see the *In vivo* section further below.
(ref: Lu, Hong, Zhang 2023)

9

In vitro study on human adrenal cells.
A high-content screening approach was used to elucidate the effects of organophosphate esters (OPEs) on H295R human adrenal cell phenotypic endpoints and function. The effects of 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), a legacy brominated flame retardant, on H295R cell cytotoxicity, oxidative stress, mitochondria, lysosomes, and lipid droplets were compared with those of 6 OPEs (including triphenylphosphate [TPP]). Most OPEs reduced oxidative stress, increased the numbers of mitochondria, decreased lysosomes, and increased lipid droplets.

Two potency ranking approaches (lowest benchmark concentration/administered equivalent dose methods and Toxicological Prioritization Index analyses), showed that the triaryl-OPEs (including triphenyl phosphate [TPP]) were more potent than BDE-47.
The basal production of cortisol and aldosterone was increased by IPPP but decreased by TPP or TMPP exposure; the response to forskolin (a steroidogenic inducer) was not affected by these OPEs. All 3 triaryl OPEs (including TPP) altered the expression of rate-limiting enzymes involved in cholesterol and steroid biosynthesis; CYP11B1 and CYP11B2 were the most prominently affected targets. The OPE chemical-specific effects on cortisol and aldosterone production was best explained by alterations in steroidogenic acute regulatory protein (STAR) expression.

The authors included an administered equivalent dose (AED) analysis for phenotypic endpoints. To estimate the AEDs (mg/kg body weight/day), *in vitro* to *in vivo* extrapolation modelling was done based on the benchmark concentrations (BMCs) and the steady-state concentration for each compound. The AED analyses predicted that the bioactive doses of TPP were in the order of 0.1 – 1 mg/kg bw/d for cytotoxicity and oxidative stress; for total area of lipid droplets this was approximately 0.05 mg/kg/day.
(ref: Li, Robaire, Hales 2023)

10
In vitro study on mouse spermatocytes.
Mouse spermatocyte GC-2spd (GC-2) cells were selected as an *in vitro* model. The impact of oxidative stress, mitochondrial impairment, DNA damage, cell apoptosis and the related molecular mechanisms were investigated using high content screening (HCS) system.
According to the authors, the study indicated that cell viability was decreased significantly in a dose-dependent manner after triphenylphosphate (TPhP) treatment with the half lethal concentration (LC_{50}) at 105.8, 61.61 and 53.23 μM for 24, 48 and 72 h. A concentration-related apoptosis occurrence was observed in GC-2 cells after TPhP exposure for 48 h. In addition, the elevated intracellular reactive oxygen species (ROS) and the total antioxidant capacity (T-AOC) was also observed after exposing to 6, 30 and 60 μM of TPhP.
The authors stated that, based on the enhancement of pH2AX protein and alteration of nuclear morphology or DNA content, DNA damage might be induced by higher concentration of TPhP treatment. Simultaneously, alteration of mitochondrial structure, enhancement of mitochondrial membrane potential (MMP), reduction of cellular adenosine triphosphate (ATP) content, altered expression of Bcl-2 family proteins, release of cytochrome c and increase of caspase-3 and caspase-9 activity demonstrated that caspase-3 dependent mitochondrial pathway might play a key role in the process of GC-2 cell apoptosis. According to the authors, these results showed that TPhP was a mitochondrial toxicant and apoptotic inducer, which might trigger alike responses in human spermatogenic cells and that therefore the potential reproductive toxicity of TPhP could not be ignored.
(ref: Feng, Shi, Li 2023)

11
In vitro study on human liver cell line.
In an *in vitro* study human cultured liver cell line L02 cells were exposed to various concentrations (0-400 μM) of either TPP or its major metabolite DPHP.
For RNA-seq analysis the cells were treated with 50 μM TPP to screen differentially expressed genes.
For the glucose uptake assay the L02 cells were treated with TPP (or DPHP) and cultured in the presence or absence of insulin followed by exposure to2-deoxy-d- glucose. After lysis, fluorescence intensities were measured. For a glycogen synthesis assay L02 cells were exposed to TPP with or without insulin. After lysis glycogen levels were measured. According to the authors insulin stimulated glucose uptake was decreased by exposure to 10, 50 and

100 µM TPP. Insulin-stimulated glycogen synthesis was also decreased at these doses. Co-exposure to the ER stress antagonist restored glucose uptake and glycogen synthesis.
For a TR-PCR assay, after exposure to TPP the mRNA levels of target genes were quantified. According to the authors, among the enriched pathways from TPP exposure the aminoacyl biosynthesis, steroid biosynthesis, insulin resistance and MAPK signaling pathways were the most prominent.
(ref: Yue, Sun, Duan 2023)

12

In vitro study on mouse Leydig cell line.
(taken from ANSES 2019)
In a Leydig cell line TM3, a significant induction of oxidative stress and a reduction in expression of genes related to testosterone synthesis was retrieved, however this was only observed at the high and moderately cytotoxic TPP concentration of 60 µg/mL (according to about 180 µM) (see Tegethoff report, 2017 quoting results from Chen *et al.*, 2015). In order to determine the effects of several OPFRs on testosterone production (which is synthetized mainly by Leydig cells in the testis), Schang *et al.*, 2016, studied the *in vitro* effects of OPFRs, including TPP, and of BDE-47, on MA-10 mouse Leydig tumors cells. The results showed that TPP significantly reduced MA-10 cell mitochondrial activity, significantly increased superoxide production, and had no effect on basal progesterone production nor on steroidogenesis (Schang, Robaire, and Hales 2016).

(ref: ANSES 2019)

In vivo

1

(Also referenced in ANSES 2019)
A single dose metabolic study on the impact of a perinatal exposure to TPP on type 2 diabetes onset and adipose accumulation was studied in UCD-type 2 diabetes mellitus rats. This rat model mimicks the pathophysiology and progression of type 2 diabetes mellitus (T2DM) in humans. TPP 170 microgram/day was administered to pregnant (GD) 8.5 to PND 21. This study highlights that perinatal exposure to TPP triggers metabolic disturbances characterized by enhanced weight gain and enhanced adiposity connected with enhanced plasma levels of leptin, the hormone of satiety, and possibly with leptin resistance explaining enhanced food intake.
No significant differences were observed in the length of gestation, litter size, sex ratio, or the body weight of the dams or pups at weaning. This suggests that exposure to TPP was not overtly toxic with respect to these parameters.

(Ref: Green, 2017)

2

In an *in vivo* study, mice with or without a high-fructose and high-fat (HFF) diet were exposed to 10 µg/kg bw/day) or 1000 µg/kg bw/day of TPP, for 12 weeks. The HFF diet was used to construct an obesity model. Compared with the controls, mice on the normal diet and receiving TPP 10 µg/kg bw/day showed changes in blood cholesterol and triglyceride levels. The 1000 µg/kg bw/d TPP exposure caused liver function and glucose sensitivity abnormalities, induced liver histopathological damage and lipid accumulation and also impaired the biological function of the mouse liver. TPP activated the protein expression related to immune and lipid metabolism. In summary, the authors stated that subchronic dietary exposure to TPP in the presence or absence of a HFF diet can induce the immune system damage and lipid metabolism disorders in mouse liver, inducing the potential health risk associated with infectious disease, cardiovascular disease and endocrine system.

(Ref: Cui, 2022)

3

In mice (both female and male) orally administrated with different doses of TPP during pubertal, endpoints such as fasting insulin and glucose, glucose intolerance, lipid and glucose metabolism in both liver and skeletal muscle were investigated. Animals (10 males, 10 females per group) received for 5 weeks once a day by gavage with olive oil (control group) 25 and 50 mg/kg bw TPP.

The authors reported that there were no differences in weight and liver gain. The 50 mg TPP dose increased the serum glucose level, cholesterol, triglycerides and slightly decreased the LDL-cholesterol. The 50 mg TPP decreased serum adiponectin in females and increased it in males.

In addition, the study showed a difference between males and females regarding the up- or downregulation of the two adiponectin receptors (AipoR1/R2) in adolescent mice after TPP treatment.

(Ref: Wang, Le 2020)

4

The effects of *in utero* and lactational exposure to TPP on obesity, non-alcoholic fatty liver disease and diabetes in adult male mice were investigated *in vivo*. In one part of the experiment, pregnant mice were exposed from gestational day 6 to lactational day 21, with different concentrations of TPP corn oil solutions (0, 10, 100, and 1000 microgram/kg bw) by oral gavage every morning. The authors stated that in the male offspring they found the promotional effects of TPHP exposure (10, 100, and 1000 microgram/kg bw on body weight in the treatment groups.

In the second part of the experiment, primigravida pregnant ICR mice (six pregnant mice per group) were administrated with corn oil solution or TPP corn oil solution (1000 microgram/kg bw, the dose that had the most significant effects on body weight) from gestational day 6 to lactational day 21 by oral gavage. After weaning at 21-day old, male offspring were divided into two groups (six mice per group and one mouse from each dam), provided with either a low-fat diet (LFD: 10% calories from fat) or a high-fat diet (HFD) for 10 weeks. Body weights were measured weekly, and blood samples were collected at 14 weeks.

In the low fat and the high fat diet group, 1000 microgram/kg bw increased the body weight, liver weight and gonadal fat weight. It also had an impact on the glucose tolerance test, insulin level, gut microbiome and expression of genes involved in lipid metabolism.

(Ref: Wang, Yan 2019)

5

A study investigated the effect of neonatal TPP (and also DPP) exposure in mice.

After delivery from primigravida ICR mice, foster females were randomly assigned eight newborns to ensure the growth of pups. The neonatal pups were subcutaneously injected on post-natal days 1 -10 with TPP and DPP. Dosing of TPP was subcutaneous with 2 or with 200 microgram in corn oil. Ovarian histopathology (at day 19), estradiol levels (at 12 weeks, glucose tolerance (10 weeks) and serum metabolomes (at 12 weeks) were analysed.

In an apparently separate uterotrophic assay, female ICR mice or SD rats (17-days-old) that had not undergone any previous treatment were subcutaneously injected once daily for three consecutive days with solutions of corn oil (control), 200 or 600 mg/kg TPP, 200 or 600 mg/kg DPP, or 100 mg/kg ethinyloestradiol.

According to the authors, the results showed that neonatal exposure to TPP has no negative effects on uterine weight, glucose tolerance and serum estradiol levels. Metabolomics analyses revealed a dose- and sex-specific response of adult mice to TPP and DPP exposures. The authors state that although the findings showed perturbations of metabolic profiles

induced by neonatal TPP (or DPP) exposure, the underlying mechanisms of action for these changes remain unknown.

(Ref: Wang, Zhu 2018)

6

Gestational TPP exposure in mice

In a gestational study in C57Bl/6 mice, dams were exposed to TPP on gestational days (GD) 8, 10, 12, and 14 to 0, 5, 25, or 50 mg/kg bw via the intraperitoneal route.

Dams were euthanized on GD19, their fetuses euthanized after 1 hour in an incubator.

Samples extracted from both maternal and fetal liver were assessed for mRNA transcript levels of Insulin growth factor (Igf) and various genes involved in the Igf signaling pathway (Igf1r, Insr: receptors for Igf1 and insulin), and Igf1/insulin signaling molecules).

Maternal Igf1 levels were significantly decreased in maternal liver samples extracted from dams exposed to 5 mg/kg compared to unexposed controls. While a significant increase in Igf1 transcript levels were detected in TPH exposed fetal livers, there was no corresponding statistically significant increase in Igf1 protein levels in fetal livers.

In maternal liver samples, 5, 25 and 50 mg/kg gestational TPH exposure resulted in a statistically significant decrease in transcript levels of Igf1 and Irs2 (insulin receptor substrate). No difference was detected in transcript levels of levels of Igf1 receptor, Insulin receptor or Igf binding protein 1.

In fetal livers, a significant treatment effect was detected for the transcript levels of all genes measured in the Igf signaling pathway. An increase in transcript levels was detected in livers of the 5 mg and 25 mg exposure groups, but not in the 50 mg exposed group.

(ref: Philbrook, 2018)

7

In vivo study on the placenta of pregnant mice.

In the context of the abovementioned *in vitro* transcriptomic study in placental trophoblasts (Lu, Hong Zhang 2023) two female mice and one male were placed together to mate. Female mice were considered pregnant when a vaginal plug was observed; this was counted as embryonic day 0 (E0). Afterward, dams were randomly selected for each group: control group (corn oil); low-dose treatment group (0.5 mg/kg TPP); middle-dose treatment group (1 mg/kg TPP); high-dose treatment group (2 mg/kg TPP).

Starting from E0, pregnant mice were given 0.1 mL/20 g body weight TPP (0, 0.5, 1, and 2 mg/kg) by gavage at 9 am daily. Considering that the serotonin need for fetal neurodevelopment was mainly from tryptophan-serotonin metabolism in the placentae until E14.5, on day E12, six dams were selected from each group and dissected, and placental samples were collected for further analysis. One placenta was collected from each dam as one replicate.

According to the authors, consistent with the results of their concomitant cellular experiments, TPP induced oxidative stress, promoted the secretion of inflammatory factors, and altered the expression of tryptophan metabolic enzymes. The result of targeted tryptophan metabolomic analysis showed that TPP reduced the level of tryptophan in the placenta and affected the levels of metabolites along the tryptophan-serotonin or tryptophane-kynurenine pathways to varying degrees.

(ref: Lu, Hong, Zhang 2023)

8

In vivo study in mice on insulin, glucose and liver.

In the context of an abovementioned *in vitro* study (Yue, Sun, Duan 2023), four groups of 10 mice each were dosed by gavage daily for 8 weeks with corn oil (control) or 40 or 80 mg/kg bw TPP, or 80 mg/kg bw TPP plus 200 mg/kg bw 4-PBA. Blood was collected from mice of

fasted overnight or 2 h after refeeding for the fasting glucose and postprandial blood glucose levels analysis. For the insulin tolerance test (ITT), after fasting for 6 h, the mice were intraperitoneally injected with 0.75 U/kg insulin and blood collected at designed time points. At the end of the exposure period, mice were sacrificed and the livers dissected.
Exposure to triphenyl phosphate induced nuclear enlargement, cytoplasmatic vacuolation, congestion of central vein and blood sinusoids and inflammatory cell infiltration. The expression of XBP1, CHOP and ATF4 in the liver were all upregulated, indicating that liver endoplasmic reticulum (ER) stress occurred under TPP exposure *in vivo*.
A significant increase of post-prandial blood glucose level was observed in both 40 mg/kg and 80 mg/ kg of TPP-treated mice, whereas the fasting blood glucose levels remained unchanged According to the authors, the results indicate that TPP disrupted hepatic insulin sensitivity in mice. Compared with the control group, the AUC value of blood glucose levels for TPP treated group was increased significantly, indicating the insulin tolerance was impaired by TPP exposure. Both the postprandial blood glucose level and insulin sensitivity were all restored partially by the ER stress inhibitor 4-PBA.

(ref: Yue, Sun, Duan 2023)

9

In vivo study in mice.
Five weeks old male mice (n=35) received 100, 300 mg/kg/bw oral exposure to TPP and TCEP daily for 35 days. The body and testis weights decreased in 300 mg/kg TPP and TCEP treated groups. Hepatic malondialdehyde (MDA) contents increased significantly in both TPP treated groups, while the contents of glutathione (GSH) decreased significantly in 300 mg/kg TPP and both TCEP treated groups. In addition, the hepatic activities of antioxidant enzymes including glutathione peroxidase (GPX), catalase (CAT) and glutathione S-transferase (GST) as well as their related gene expression were affected by TPP or TECP exposure. On the other hand, 300 mg/kg of TPP or TECP treatment resulted in histopathological damage and the decrease of testicular testosterone levels. Moreover, the expression of main genes related to testosterone synthesis including steroidogenic acute regulatory protein (StAR), low-density lipoprotein receptor (LDL-R), cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc) and cytochrome P450 17α-hydroxysteroid dehydrogenase (P450-17α) in the testes also decreased after the exposure to 300 mg/kg TPP or TCEP for 35 days. According to the authors, combined with the effects on physiology, histopathology and the expression of genes, TPP and TCEP can induce oxidative stress and endocrine disruption in mice.

(ref: Chen, Jin, Wu 2015)

3.4.13 Special investigations: other

1.
Combined *in vivo* (mice) and *in vitro* study on mouse Leydig cell line.
In an *in vitro* study mouse Leydig TM3 cells were exposed to 0, 50, 100 and 200 μM triphenyl phosphate (TPHP).
In the same study, C57BL/6J male mice were exposed to 0, 5, 50, and 200 mg/kg B.W. of TPHP for 30 d by gavage.
The authors reported that after the third week of TPHP exposure, the body weight of mice in the 200 mg/kg B.W. group was significantly lower than that in the control group. HE staining of the testes from the control group showed no significant structural abnormalities in the seminiferous tubules and Leydig cells. However, in the TPHP-treated groups, the germ cells in the seminiferous tubules are loose arrangement with reduced layers were observed, sperm in the lumen of seminiferous tubules and Leydig cells in the interstitial connective tissue were decreased. TPP treatment significantly decreased sperm count in cauda epididymidis compared with the control group. The result is consistent with the decreased sperm density in epididymal tubules of the cauda epididymidis. Abnormal sperm morphology, such as deformed heads, folded and/or twisted tails, was observed in the TPP-treated groups. The

levels of serum testosterone were markedly decreased in the 50 and in the 200 mg/kg bw group compared with the control group.

According to the authors, TPP can cause apoptosis in testicular Leydig cells and TM3 cells. Moreover, TPHP disrupted mitochondrial ultrastructure of testicular Leydig cells and TM3 cells, reduced healthy mitochondria content and depressed mitochondrial membrane potential of TM3 cells.
Pretreatment with the mitochondrial fusion promoter M1 alleviated the above changes and further mitigated TM3 cells apoptosis and testosterone levels decreased.
According to the authors, the results showed that TPP induced testes damage, including spermatogenesis disorders and testosterone synthesis inhibition. The authors summarized that the data revealed that apoptosis is a specific mechanism for TPP-induced male reproductive toxicity, and that ROS-mediated mitochondrial fusion inhibition is responsible for Leydig cells apoptosis caused by TPP.
(ref: Wang, Xu, Zhao 2023)

2.
In vitro study on MCF7R cells, hepatoma HuH-7 cells, HEC-BCRP cells, HepaRG cells and primary human hepatocytes.
A series of *in vitro* experiments with these different cultured cells and 7 common organophosphates, including TPP, investigated the effects on the main drug transporters involved in pharmacokinetics. According to the authors, the data show that transporters may be targeted by some OPFRs, including TPP, with possible consequences in terms of inhibition of hormone transport and endocrine disruptive effects.
In a prediction model for *in vivo* inhibition of transporter activity, TPP was not predicted to inhibit the activity of drug transporters at plasma concentrations expected from environmental or dietary exposure.
(ref: Tastet 2023)

SCCS overall comments on metabolism studies
It appears that the main target organ of TTP is the liver, where TPP is known to interfere with the metabolism. This is in line with the liver effects that were observed in the 90-day guideline study, from which a NOAEL of 20 mg/kg bw/d could be derived (see 3.4.4.2).
A few studies reported some metabolic consequences after exposure to TPP, such as cortisol decrease *in vitro* (human cell line).
The above-mentioned published non-guideline studies also point towards an effect of TPP on body weight, modulated through changes in glucose and lipid metabolism mainly taking place in the liver. However, due to the design of the studies and the reporting of the results, no conclusions could be drawn on an exposure dose that could be used for a different point of departure for risk assessment in this Opinion.

There are several other studies addressing the effects of TPP on metabolism, but these investigations are based on exposure to mixtures with other compounds or based on metabolites that may have originated from sources other than TPP. Because the observed effects cannot directly be linked to TPP, these studies were not considered in this Opinion.

3.5 SAFETY EVALUATION (INCLUDING CALCULATION OF THE MOS)

Because of the concern over genotoxicity, the MoS calculation is not included.

3.6 DISCUSSION

Exposure

The applicant has based the calculation of the dermal exposure on study in the publicly available literature. However, the study was not in accordance with the SCCS basic criteria for dermal absorption. The tested concentration (in a mixture of organophosphate esters) was much lower than the intended use concentration. Considering these aspects, the default dermal absorption of 50% was used in this opinion for the skin adjacent to the nails.
In view of the low vapour pressure and the publicly available studies, the SCCS regarded the exposure by inhalation as negligible.

Toxicological Evaluation

Irritation and corrosivity

Several studies indicate absence of skin irritating potential and a mildly irritating potential to the eyes.

Skin sensitisation

A guideline-compliant study and the absence of reports on sensitisation in humans indicate that the risk of sensitisation is negligible.

Acute toxicity

The acute dermal LD50 of TPP was determined to be greater than 7900 mg/kg bw and the acute inhalation LC50 of TPP in rats can considered to be greater than 200000 mg/ m3. No clinical signs of systemic toxicity were observed in the underlying studies.

Repeated dose toxicity

Because organophosphate (OP) esters can inhibit acetylcholinesterase (AChE), TPP has been investigated for neurotoxicity. More recent studies could not confirm the neurotoxicity that was found in the older studies, possibly because there was contamination of TPP with other neurotoxic organophosphate esters. In the short term (4-day) NTP study in rats and the 28-day 2-generation developmental toxicity study, cholinesterase inhibition was observed at doses equal or above 55 mg/kg bw/d and, respectively, 100 mg/kg bw/d. These doses are above those that show effects on the liver. The *in vivo* studies, including a 90-day study up to about 600 mg/kg bw day, do not indicate overt neurotoxicity.

The 28-day developmental toxicity study in rats (see 3.4.5.2, ref: Witchey 2013) showed an increase in relative liver weight of the dams at TPH exposure at and above approximately 300 mg/kg bw/d.
Based on the liver effects reported in the 90-day guideline study in rats, centrilobular hypertrophy observed at 1500 ppm in line with the increase in liver weight at 7500 ppm, a no observed adverse effect level (NOAEL) of 20 and 22 mg/kg (for males and females respectively) can be derived. A NOAEL of 20 mg/kg bw/d can be used as point of departure for the safety evaluation.

Reproductive toxicity

The ANSES 2019 report derived for maternal and developmental toxicity a NOAEL of 80 mg/kg bw/d. While ANSES 2019 stated that the available studies indicate that TPP do not have overt developmental toxic properties, the recent 28-day 2-generation study points towards perturbed reproductive performance at ≥1000 mg/kg bw/d. In offspring, TPP-related toxicity was noted in pups at ≥1000 mg/kg bw/d

Mutagenicity / genotoxicity

After analysis of the currently available data, TPP has been shown not to induce gene mutations. However, the evidence for the lack of induction of chromosomal damage is questionable and recent data from the study by Xie *et al.* (2023) raise concerns on TPP clastogenicity *in vitro*.

The SCCS also requested additional evidence via an *in vitro* study of TPP to exclude a genotoxicity potential. This was not provided by the Applicant. Hence, the genotoxic potential of TPP cannot be excluded based on the currently available information. Safety assessment of TPP will only be possible if genotoxicity potential could be excluded through further evidence.

Carcinogenicity

Several recent research studies that used cancer cells were identified in the public literature, implicating TPP in the carcinogenic process.
Overall, the SCCS regards that these studies, carried out in cancer cells or animal cancer models, do not provide sufficient evidence to draw a conclusion on carcinogenicity.

Human data

The human data are based on urinary metabolites resulting from exposure to a combination of several organophosphates and can therefore not be used for risk assessment in the context of this opinion.

Endocrine effects

In addition to the studies that were used by the Applicant to assess an ED modality, further *in vitro and in vivo* toxicity studies were noticed by the SCCS.
In level 2 *in vitro* assays, some estrogenic activity was observed in a few of the studies. This estrogenic activity was also demonstrated in a recent study on KGN human ovarian granulosa cells. In addition, that study showed a stimulation of secretion of progesterone.
The submitted studies do not indicate an androgenic potential of TPP; although a short-term *in vivo* study in mice (see section 3.4.13) noted a decreased serum testosterone level, it cannot be derived from that study whether this could be attributed to an anti-androgenic effect.
No level 3 *in vivo* studies were submitted. From the public literature the SCCS identified several recent publications which are a combination of in-vivo and in-vitro studies, with a focus on metabolic transcriptomic assays (see section 3.4.12 and 3.4.13 - Special investigations; a brief overview is in Annex 2). The studies point towards an effect of TPP on changes in glucose and lipid metabolism mainly taking place in the liver.
However, the design of the studies and the reporting of the results do not allow drawing conclusions on an exposure dose that could be used for a point of departure for risk assessment in this opinion.

Two *in vitro* studies indicate a steroidogenic effect of TPP: one study showing a decrease of basal production of cortisol and aldosterone and one study with a transcriptomic assay indicating weak inhibitory effects on the GR-mediated transcriptional activity induced by hydrocortisone.

In level 4 *in vivo* (OECD TG408, 421/422 and 443) studies, estrogenic effects were not observed.

Based on the available data regarding thyroid and thyroid hormones, the T modality was not clearly affected. Although some scattered effects were observed, including increased follicular

cell hypertrophy (most likely due to hepatocellular hypertrophy) in males in the 90-days repeated dose toxicity, the results were not considered sufficient to establish a T modality.

From the *in vivo* studies that included weight gain as parameter, an obesogenic effect of TPP cannot clearly be established. The 28-day developmental toxicity study (see 3.4.5.2) indicated in dams a decrease in weight gain at and above 1000 mg/kg bw/d.

The Applicant provided studies from the public literature containing information on the presence or absence of endocrine activity of TPP in humans. Because the studies are based on urine metabolites, which may originate from exposure to other organophosphates, these studies cannot be used for the risk assessment in this Opinion.

Other special investigations

Recently several non-guideline studies appeared in the public literature. These studies are *in vitro*, *in vivo* and combinations of this, addressing a variety of endpoints including transcriptomic data.

It appears that the main target organ is the liver, where TPP is known to interfere with the metabolism. This is in line with the liver effects that were observed in the 90-day guideline study, from which a NOAEL of 20 mg/kg bw/d could be derived (see 3.4.4.2).

The published non-guideline studies also point towards an effect of TPP on body weight, modulated through changes in glucose and lipid metabolism mainly taking place in the liver. However, the design of these studies and the reporting of the results do not allow drawing conclusions on an exposure dose that could be used for a point of departure for risk assessment in this Opinion.

There are several other studies addressing the effects of TPP on metabolism, but these investigations are based on exposure to mixtures with other compounds or based on metabolites that may have originated from other sources than TPP. Because the observed effects cannot directly be linked to TPP, these studies were not considered for this opinion's purpose.

4. CONCLUSION

1. *In the light of the data provided and taking under consideration the concerns related to potential endocrine disrupting properties of Triphenyl Phosphate, does the SCCS consider Triphenyl Phosphate safe when used as a plasticiser in nail products up to a maximum concentration of 5%?*

   Based on the currently available information, it is not possible for the SCCS to conclude on the safety of Triphenyl phosphate because the genotoxicity potential cannot be excluded.

2. *Alternatively, what is according to the SCCS the maximum concentration considered safe for use of Triphenyl Phosphate in nail products?*

   /

3. *Does the SCCS have any further scientific concerns with regard to the use of Triphenyl Phosphate in nail products?*

   The SCCS mandate does not address environmental aspects. Therefore, this assessment did not cover the safety of Triphenyl phosphate for the environment.

5. MINORITY OPINION

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7. GLOSSARY OF TERMS

See SCCS/1647/22, 12th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – Appendix 15 - from page 158.

8. LIST OF ABBREVIATIONS

See SCCS/1647/22, 12th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – Appendix 15 - from page 158.

ANNEX 1

Applicant's overview of studies on endocrine disruption properties.

Studies available	Cell line / species doses / duration	Results	Reference
Level 1: Existing data and non-test information (not related to a specific receptor)			
No data available			
Level 2: *In vitro* mechanistic assays			
***In vitro* high throughput screening (HTS) assays**	Various human and rat cell lines / from 0.0005 to 200 µM / 0.5 to 80 hours	TPP was found to be active in 21 (E=10; A=4; T=3; S=4) out of 58 ED relevant assays. However, 12 (57%) have been flagged as potentially 'false positive' by the automated analysis tool from the US EPA.	CompTox/EDSP-21 (accessed August 2021)
***In vitro*, (anti)estrogenic activity**	Uterine cytosol preparation / 20 hours	TPP was found to be a non-binder to the ER at doses up to > 10-4 M (>100 µM)	(Blair *et al.*, 2000)
***In vitro* luciferase reporter gene expression assay / estrogenic activity**	α-positive human breast cancer cell line (MCF-7) cells and human ovarian cancer cell line BG1Luc luciferase assay	TPP increased proliferation in a dose-dependent manner (at concentrations that were about six orders of magnitude higher when compared to that of E2). TPP was found to exhibit significant estrogenic activity in both BG1Luc and MCF-7 assays with an EC50 of 4.7and 2.2 µM, respectively.	(Bittner *et al.*, 2014)
***In vitro* luciferase reporter gene expression assay / (anti)estrogenic activity**	Chinese Hamster Ovary cell line (CHO-K1) and yeast two-hybrid reporter assay and cell proliferation assay using α-positive MCF-7 cells / 24 hours	TPP was found to have potent estrogenic activity with the relative effective concentration (REC20) value of 0.27 and 0.65 µM in dual luciferase reporter and yeast two-hybrid assay indicating estrogenic activity. However, TPP did not exert any antiestrogenic activity	(Zhang *et al.*, 2014)
***In vitro*, gene reporter and cell proliferation assay / (anti)estrogenic activity**	Yeast two-hybrid reporter gene and MVLN ERE-luciferase reporter gene assay and the MCF7 cell proliferation assay / 1.5 × 104 cells/ 72 hours	TPP showed agonistic activity in MVLN ERE-luciferase reporter gene assay (>700,000 fold lower than E2) and agonistic activity in the MCF7 cell proliferation assay (at >90,000 fold lower than E2) and antiestrogenic activity in yeast two-hybrid reporter gene assay (>80,000 fold lower than E2)	(Ji *et al.*, 2020)
***in vitro* estrogen α(ERα) activity**	COS-1 cells	TPP was not found to have agonistic or antagonistic activity towards ERα receptor	(Honkakoski *et al.*, 2004)

***In vitro,* luciferase reporter gene expression assay / (anti)estrogenic activity**	CHO-K1 and COS-7 cell lines / 3× 10-5 M/24 hours	Metabolites 3-Hydroxylphenyl diphenyl phosphate (HO-m-TPP) and 4-Hydroxylphenyl diphenyl phosphate (HO-p-TPP) showed ERα and ERβ mediated estrogenic activity with REC20 values for ERα and ERβ agonistic activity were, 4.6 µM and 7.3 µM for TPP, 1.7 µM and 3.8 µM for HO-m-TPP, 0.29 µM and 0.30 µM for HO-p-TPP. HO-m-TPP and HO-p-TPP showed no antagonistic ERα activity but inhibited ER-β mediated estrogenic activity. RIC20 values for ERβ antagonistic activity were >1 ×10-5 µM for TPP, 15 µM for HO-m-TPP, 5.4 µM for HO-p-TPP. Overall, TPP was concluded to have ERα as well as ERβ estrogen agonistic activity (>100,000 fold lower than E2) and ERβ antagonistic activity (>900-fold lower concentration than tamoxifen)	(Kojima *et al.*, 2016)
***In vitro,* flow-cytometric proliferation assay / (anti)estrogenic activity**	α-positive MCF-7 cells	TPP increased cell proliferation. The relative proliferative potency (RPP) was found to be 9x10-9 however, potency was many orders of magnitude lower when compared to that of E2. The EC20 in MCF-7 cells were 88 µM for TPP versus 8 x 10-6 µM for E2. Overall, TPP was shown to induce cell proliferation and exhibit estrogenic activity (1 x 108 fold lower than that of E2.)	(Krivoshiev *et al.*, 2016)
***In vitro,* AR receptor binding assay / (anti)androgenic activity**	Recombinant AR protein of rats expressed in *Escherichia coli*	TPP showed an IC50 of 15 µM, RBA of 0.021 and logRBA of -1.69 to the androgen receptor suggesting that TPP showed moderate binding (4000 times less potent than R1881) towards androgenic receptors.	(Fang H *et al.*, 2003)
***In vitro,* luciferase reporter gene assay / (anti)androgenic activity**	CHO-K1 and COS-7 cell lines / 30 µM / 24 hours	Metabolites of TPP ((HO-*m*-TPP and HO-*p*-TPP) did not show agonist androgenic activity. However, TPP and its hydroxy metabolites (HO-*m*-TPP and HO-*p*-TPP) were found to have some antagonist activity at high concentrations (RIC20 – 650-fold lower than that of AR antagonist hydroxyflutamide), suggesting that TPP showed slight anti-androgenic activity at higher concentrations.	(Kojima *et al.*, 2016)
***In vitro* androgenic activity**	COS-1 cells	TPP was shown to decrease the AR activity by 40–50%. TPP also reduced the testosterone-induced AR-dependent activity by 30–40%. TPP	(Honkakoski *et al.*, 2004)

		was shown to inhibit AR-dependent activity	
***In vitro* luciferase reporter gene assay and T-screen cell proliferation assay thyroid hormone-disrupting activity**	Thyroid receptor β (TRβ) in CHO-K1 cells and T-screen cell proliferation assay in GH3 cell	TPP showed no agonistic or antagonistic activity in both assays (data not shown).	(Zhang *et al.*, 2016)
***In vitro* thyroid transport protein transthyretin (TTR)-binding potency**	Human TTR-binding potency bioassay	TPP was shown to have IC50 >25 µM and determined not to have a binding potential to the TTR	(Weiss *et al.*, 2015)
***In vitro* luciferase reporter gene expression assay / (anti) progesterone activity**	MA-10 mouse Leydig tumour cells	TPP showed no significant effect on basal progesterone production nor on steroidogenesis up to 10 µM. Overall, TPP had no effect on Leydig cell survival and steroidogenesis	(Schang *et al.*, 2016)
***In vitro* luciferase reporter gene assay / glucocorticoid receptor activity**	CHO-K1 and COS-7 cell lines / 30 µM / 24 hours	TPP and its metabolites HO-*m*-TPHP and HO-*p*-TPHP showed slight antagonist glucocorticoid activity (induced by 3×10-8 M of hydrocortisone) with RIC20 values of 4.3 to 12 µM suggesting glucocorticoid antagonistic activity of HO-*p*-TPHP (75-fold lower than that of RU-486). TPP and its metabolites showed to have no agonistic activity but have slight antagonistic activity.	(Kojima *et al.*, 2016)
***in vitro,* β-galactosidase reporter assay/ Glucocorticoid (GR) activity**	COS-1 cells / 10 µM	TPP was shown to inhibit human GR by 20% in the absence of agonist. TPP was not found to affect GR activity	(Honkakoski *et al.*, 2004)
***In vitro* β-galactosidase reporter assay, Progesterone-induced receptor (PR) activity**	COS-1 cells/ 10 µM	TPP was not shown to affect human PR activity. TPP was not shown to have agonistic or antagonistic activity towards PR receptor	(Honkakoski *et al.*, 2004)
***in vitro* steroidogenesis disruption in TM3 cells**	Leydig cell line TM3 cells / 24 hours / 61 or 184 µM	TPP decreased the cell viabilities significantly at 60 µg/mL, being 41.4% lower than that of the control group. TPP has the potential to decrease cell viability, induce oxidative stress and disrupt the steroidogenesis in TM3 cells; however, these effects were observed at higher	(Chen *et al.*, 2015)

		cytotoxic concentrations, i.e., ≥60 µg/mL (≥184 µM)	
***vitro* luciferase reporter gene expression assay / (anti) pregnane activity**	CHO-K1 and COS-7 cell lines / 30 µM /24 hours	TPP and its metabolites HO-*m*-TPHP and HO-*p*-TPHP showed agonistic pregnane activity with REC20 values in the range of 2.2 to 4.7 µM (7-15 fold lower compared to rifampicin)	(Kojima *et al.*, 2016)
***In vitro* adipogenicity, CEBP and PPARG pathway**	3T3-L1 cells	TPP was shown to increase pre-adipocyte proliferation (at the low concentration 0.1 µM; $p < 0.001$) and adipogenic differentiation of 3T3-L1 cells along with induced transcription in the CEBP and PPARG pathway. Short term exposure of TPP at concentration of 50 µM, enhanced insulin-stimulated GLUT4 translocation. TPP was concluded to mimic or enhance the effects of insulin signalling on adipocyte glucose uptake	(Cano-Sancho *et al.*, 2017)
Level 3: *In vivo* mechanistic assays			
No level-3 *in vivo* mechanistic assays could be identified			
Level 4: *In vivo* assays providing data on ED adversity			
Subacute toxicity study (OECD TG 407; GLP)	Rat / 0, 250, 1000 and 4000 ppm. Equivalent to 23/39, 104/161 or 508/701 mg/kg bw/day in males/females / 28 days	TPP-related effects in liver were observed at dose levels of 1000 ppm and above in males. No effects on any other organs or tissues (including reproductive). The NOAEL was established at 23 mg/kg bw/day for male based on effects on body weights and liver and at 701 mg/kg bw/day for female rats.	(ECHA, 2021)
Subacute toxicity study (No guideline followed; non-GLP)	Rat (male)/ 0, 0.1 or 0.5% in feed (equivalent to 0, 70 and 350 mg/kg bw/day / 35 days	Slight decrease in body weight gain and increase of liver weights was observed at a level of 0.5% (i.e., 350 mg/kg bw/day) in the diet. No effects on any other organs or tissues (including reproductive). The NOAEL was established at 70 mg/kg bw/day	(Sutton *et al.*, 1960)
Subacute dermal toxicity study (Similar to OECD TG, 410; GLP)	Rabbit/ 100 and 1000 mg/kg bw/day / 21-23 days	No treatment-related effects were observed up to highest tested dose of 1000 mg/kg bw/day.	(OECD SIDS, 2002)
Subacute toxicity study (OECD TG 407; GLP)	Rat / 0, 250, 1000 and 4000 ppm. Equivalent to 23/39, 104/161 or 508/701 mg/kg bw/day in	TPP-related effects in liver were observed at dose levels of 1000 ppm and above in males. No effects on any other organs or tissues (including reproductive). The NOAEL was established at 23 mg/kg bw/day for male based on effects on body weights	(ECHA, 2021)

	males/females / 28 days	and liver and at 701 mg/kg bw/day for female rats.	
One-generation reproductive toxicity study (Similar to OECD TG 415; non GLP)	Rat/ 0.25, 0.5, 0.75 or 1 % in feed (equivalent to 166, 341, 516, 690 mg/kg bw/day) / 4 months	No findings indicating adverse effects on fertility or the development of the foetus up to the highest tested dose of 690 mg/kg bw/day	(Welsh *et al.*, 1987)
Level 5: *In vivo* assays providing more comprehensive data on adverse effects on ED related endpoints over more extensive parts of the life cycle of the organism			
No Level 5 *in vivo* mechanistic assays could be identified.			

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ANNEX 2

Additional studies with TPP, identified by the SCCS and commented in Section 3.4.12

IN VITRO		
HepG2 cells	Measuring key events linked to hepatic steatosis	Negi 2021
Human LO2 liver cells	Transcriptomic study and metabolomic analysis	Wang, Li 2020
HepG2 cells	Multiple parameters to explore molecular mechanisms.	An, Jiang Tan 2023
KGN ovarian granulosa cells	Expression of transcripts involved in steroids and cholesterol synthesis.	Wang, Lee, Hales 2023
Placental trophoblast cells	Gene and protein expression in tryptophan metabolism pathway. Also *in vivo* component: see below.	Hong, Zhang 2023
Human adrenal cells	High-content screening approach and potency ranking on several parameters, including expression of enzymes involved in cholesterol and steroid biosynthesis. Also production of cortisol and aldosterone.	Li, Robaire, Hales 2023
Mouse CG-2 spermatocytes	High-content screening system. Multiple parameters, including mitochondrial toxicity and apoptosis induction.	Feng, Shi, Li 2023
Human LO2 liver cells	Several parameters, including insulin-stimulated glucose uptake and glycogen.	Yue, Sun, Duan 2023
MCFR7, HuH-7, HEC-BCRP, HEPARG and primary human hepatocytes	Focus on drug transporters in pharmacokinetics.	Tastet 2023
IN VIVO		
Mice with or without high-fructose and high-fat diet	Several parameters, including cholesterol and triglyceride levels, glucose sensitivity, lipid accumulation.	Cui 2022
Mice	Fasting insulin and glucose, glucose intolerance, lipid and glucose metabolism.	Wang, Le 2020
Mice	Exposure of pregnant mice, with focus on male offspring, study on glucose tolerance, body weight and liver weight.	Wang, Yan 2019
Mice	Neonatal mice, subcutaneous. Several parameters, including glucose tolerance and serum estradiol, as well as separate	Wang, Zhu 2018

	uterotrophic assay and metabolomics analysis.	
Mice	Study on transcripts in maternal and fetal liver tissues, with focus on insulin growth factor.	Philbrook 2018
Mice	*In vivo* component of abovementioned *in vitro* study. Study on tryptophan metabolic pathway in the placenta.	Lu, Hong, Zhang 2023
Mice	*In vivo* component of abovementioned *in vitro* study. Study on insulin tolerance and gene expression and glycogen in liver.	Yue, Sun, Duan 2023
Mice	Several parameters, mainly enzyme activity and gene expression in liver and testicular tissues.	Chen, Jin, Wu 2015
Mice, combined *in vitro* (Leydig TM3 cells) and *in vivo*.	Study on apoptosis and sperm in testis and epidydimis. *In vivo* also serum testosterone.	Wang, Zhu, Zhao 2023

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