Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

2-(4-*tert*-butylbenzyl)propionaldehyde

EC Number: 201-289-8
CAS Number: 80-54-6

CLH-O-0000001412-86-259/F

Adopted

28 January 2019

28 January 2019

CLH-O-0000001412-86-259/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **2-(4-*tert*-butylbenzyl)propionaldehyde**

EC Number: **201-289-8**

CAS Number: **80-54-6**

The proposal was submitted by **Basf SE** and received by RAC on **15 December 2017.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

Basf SE has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **12 February 2018**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **13 April 2018**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Ralf Stahlmann**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **28 January 2019 via written procedure** by **consensus**.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitter's proposal	605-RST-VW-Y	2-(4-*tert*-butylbenzyl)propionaldehyde	201-289-8	80-54-6	Repr. 2	H361f	GHS08 Wng	H361f			
RAC opinion	605-RST-VW-Y	2-(4-*tert*-butylbenzyl)propionaldehyde	201-289-8	80-54-6	Repr. 1B	H360Fd	GHS08 Dgr	H360Fd			
Resulting Annex VI entry if agreed by COM	605-RST-VW-Y	2-(4-*tert*-butylbenzyl)propionaldehyde	201-289-8	80-54-6	Repr. 1B	H360Fd	GHS08 Dgr	H360Fd			

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

2-(4-*tert*-butylbenzyl)propionaldehyde (further referred to as Lysmeral in this opinion) is used as a fragrance in a wide number of industries. It has an intensive, radiant, floral odour with a typical 'lily-of-the-valley' note. As a component of fragrance mixtures, the uses include cosmetic/personal care products, washing/cleaning products, air care products, and biocidal products. In the final products, concentrations of up to 0.75% are used in washing/cleaning products and up to 1.42% in personal care products. The highest levels of Lysmeral have been determined in air care products (up to 10%).

Lysmeral has a self-classification as Repr. 2; H361f, according to Regulation (EC) 1272/2008 (CLP Regulation) but has no entry in Annex VI of the CLP Regulation. During substance evaluation, by the Swedish Competent Authority under the Community rolling action plan (CoRAP), further information was required to clarify concerns regarding endocrine disrupting properties and developmental toxicity. This included a requirement to conduct an extended one-generation reproductive toxicity study (EOGRTS; OECD TG 443) in rats, by the oral route, with the extension of Cohort 1B to mate the F1 animals to produce an F2 generation, Cohorts 2 and 3 to assess developmental neurotoxicity (DNT) and immunotoxicity (DIT), and additional examinations of acetyl cholinesterase activity in different compartments in parental animals and offspring.

Although Lysmeral is also self-classified for acute oral toxicity, skin irritation, and skin sensitisation, only reproductive toxicity was addressed in the CLH dossier.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

No human data on reproductive toxicity is available.

Animal Data

The dossier submitter (DS) presented two non-guideline, non-GLP, one-generation range finding studies in rats, one modified extended one-generation reproductive toxicity study (EOGRTS; OECD TG 443) in rats conducted under GLP conditions, as well as one GLP-compliant prenatal developmental toxicity (PNDT; OECD TG 414) study in rats. All four used oral dosage.

Fertility

In both range finding studies, impairment of male fertility (decreased reproductive organ weights, reduced sperm counts), combined with signs of general toxicity and changes in clinical parameters of the liver, was observed starting from doses of around 25 mg/kg bw/d. The DS considered the observed lack of pregnancies and lack of delivered offspring as related to spermatotoxic effects in male animals.

In the EOGRTS, encapsulated Lysmeral was administered to groups of 35 male and female Wistar rats in the control, low and mid dose groups and to 40 male and female rats in the high dose

group, as a homogeneous addition to the food. Nominal concentrations in capsules were 75, 230 and 750 ppm leading to 13, 41 and 133 ppm of the active ingredient in formulated capsules. Targeted doses were 1, 3 and 10 mg/kg bw/d. The overall mean doses of Lysmeral administered to the male and female Wistar rats, throughout all study phases and across all cohorts, were approximately 1.4, 4.5, and 15.1 mg/kg bw/d in the 75, 230, and 750 ppm dose groups, respectively. Thus, the targeted Lysmeral dose levels were achieved or exceeded. Control groups received plain diet or capsules without Lysmeral via the diet.

Changes in liver parameters (increased organ weights and gamma-GT levels) were seen in high dose animals. There was no evidence from clinical examinations or from gross and histopathology, that Lysmeral adversely affected the fertility or reproductive performance of the F0 and F1 parental animals, up to and including the administered nominal highest dose of 10 mg/kg bw/d. Oestrous cycle data, mating behaviour, conception, gestation, parturition, lactation and weaning, as well as sexual organ weights and gross and histopathological findings of these organs (specifically the differential ovarian follicle count) were comparable between all groups including controls, and ranged within the historical control data (HCD). A higher mean percentage of abnormal spermatozoa (9.8±13.2%) in the high dose F0 males compared to controls (6.3±0.6%) and the historical control range (6.0-6.6%) was observed. Although this was statistically non-significant, the high standard deviation indicates changes in some of the rats. However, there were no findings in sperm motility (86% versus 88% in controls) or sperm head counts in testes (108 mio/g versus 102 mio/g in controls) and epididymides (717 mio/g versus 723 mio/g in controls) of these animals. Further, the corresponding F1 offspring males did not show these effects.

Overall, the DS concluded that there were no indications of an impairment of fertility in the F0 and F1 generations at doses up to a level of approximately 15 mg/kg bw/d.

In a PNDT study, Lysmeral was administered via gavage in nominal doses of 0, 5, 15, and 45 mg/kg bw/d (leading to ingested doses of 0, 4.1, 12.7, and 40.7 mg/kg bw/d, respectively) to female Wistar rats from day 6 to day 20 *post coitum* (p.c.).

Maternal toxicity was observed starting at the mid dose level. Although no decrease in food consumption was detectable in mid dose animals, mean maternal weight gains significantly decreased on day 6-8 p.c., to about 56% below controls, but recovered during the study period. In high dose animals, a statistically significant mean body weight loss was observed on day 6-8 p.c., and the mean body weight gain over the entire treatment phase was found to be about 25% below controls. Furthermore, a statistically significant reduction of mean body weights on day 13-20 p.c. (about 7% below controls at study termination) was found. In line with that, the corrected body weight gain was statistically significantly lower (about 32% below control).

Increases in mean alanine aminotransferase levels (20-30% above control) and decreases in serum cholinesterase levels (20-45% below control) were found, starting from the mid dose. In the high dose group, mean glutamate dehydrogenase levels were found to be 79% above controls. Increases in absolute and relative liver weights (10% and 10-20% above controls, respectively) were found at all dose levels. However, due to the lack of changes in the respective clinical parameters, the DS considered only the liver weight changes in the mid and high dose group as adverse.

Gestational parameters such as number of corpora lutea, implantation sites, and pre-implantation loss were not influenced at any dose level. However, mean post-implantation losses (mainly early resorptions) were found to be significantly increased in the high dose group. In high dose animals, mean resorptions per dam were 15.1%, compared to 4.4% in controls. Subsequently, the mean number of live foetuses per dam was decreased (7.4 vs. 8.1 in controls).

These high dose findings were slightly below the HCD and the DS attributed them to maternal toxicity.

As supporting evidence, the DS summarised 15 repeated dose oral toxicity studies with focus on reproductive toxicity in different species, and one repeated dose dermal toxicity study in rats.

The oral studies consisted of the following:

- one 90-d study (in rats);
- three 5-d studies (in rats);
- two single dose studies for 1, 2, 3, 4, or 14 days (one in mice, one in rats);
- three short term (5-d) single dose studies with focus on testicular toxicity (in mice, Guinea pigs, and rhesus monkeys);
- one 15-d screening study (in rabbits);
- five studies in Beagle dogs: one 9-week pilot study, two 90-d studies, one 14-d testicular toxicity screening study, and one 14-d follow up study.

The DS concluded that no effects on male reproductive organs, nor any general toxicity, were seen in seven of these studies: the two 90-d dog studies up to 44.6 mg/kg bw/d (males) and 200 mg/kg bw/d (females), the two studies with male mice up to 100 mg/kg bw/d, the 5-d Guinea pig study at 100 mg/kg bw/d (males only), the 5-d study with two male rhesus monkeys given 100 mg/kg bw/d Lysmeral with feed, and the 15-d screening study in male rabbits up to a dose of 300 mg/kg bw/d.

Findings from other studies are summarised in the table below.

Table: *Summary of repeated dose toxicity studies with adverse effects on male fertility*

Method, Duration of study, Route of exposure, Guideline, GLP status	**Species, Strain, Sex, No/ group**	**Test substance Vehicle, Dose levels, Duration of exposure**	**NOAELs, LOAELs**	**Results**
Dermal 5 days GLP (Givaudan, 1991a)	Rat 5 males per group	99.1% Lysmeral 0, 250, 500, 1000, 2000 mg/kg bw/d 6 h/day	NOAEL (general toxicity): 2000 mg/kg bw/d LOAEL (fertility): 2000 mg/kg bw/d	**2000 mg/ kg bw/d:** ↓ body weights, 2%; ↓ number of germ cells; ↑ number of degenerating germ cells (incl. giant cells) in epididymides (5/5); spermatocele (1/5); marked testicular atrophy; disorganisation of epithelial structure of seminiferous tubules
Oral 14 days (BASF, 2006a)	Rat 5 males per group	99.1% Lysmeral or Lysmerylic acid via gavage 50 mg/kg bw/d for 1, 2, 3, 4, or 14 consecutive days		**1 day:** slight to severe testicular atrophy (2/5 for Lysmeral, 3/5 for Lysmerylic acid) **≥ 2 days:** slight to severe testicular atrophy (5/5); diffuse tubular testicular degeneration; fine vacuolar change of pachytene spermatocytes up to apoptosis **14 days:** ↓ bwg (25% with Lysmeral, 20% with Lysmerylic acid); ↓ sperm motility; ↓ spermatid count in testes; ↓ sperm count in cauda epididymides; altered sperm morphology

Oral 5 days GLP (2/3 studies) (Givaudan, 1986b; Givaudan, 1991a; Newberne, 1990a)	Rat 5 or 8 males per group	Lysmeral via gavage 0, 25, 50, 100, 200, 400 mg/kg bw/d	LOAEL (general toxicity): 100 mg/kg bw/d (2/3 studies) 200 mg/kg bw/d (non-GLP study) NOAEL (male fertility): 25 mg/kg bw/d (2/3 studies) 50 mg/kg bw/d (non-GLP study)	**≥ 50 mg/kg bw/d:** ↓ body weights; macroscopic liver changes; degeneration and loss of seminiferous/ testicular tubule epithelium; minimal to marked atrophy of testes; degenerated germ cells; ↓ sperm counts **≥ 100 mg/kg bw/d:** ↓ kidney weights; ↓ testis weights; ↓ sizes of prostate and seminal vesicles
Oral 90 days OECD TG 408, GLP (Givaudan, 1986a)	Rat 14 males and 14 females per group Plus one high dose satellite group for 4 weeks post-treatment of 14 males and 14 females (recovery)	97.8% Lysmeral via gavage 0, 2, 5, 25, 50 mg/kg bw/d 5 d/week	LOAEL (general toxicity): 25 mg/kg bw/d NOAEL (male fertility): 25 mg/kg bw/d	**≥ 25 mg/kg bw/d:** ↑ rel. liver weights*, 21-45% (males) 59-75% (females); ↓ plasma ChE*, 30-70% (males and females); ↓ plasma cholesterol*, 40-70% (males and females); ↑ rel. adrenal glands weights*, 18-36% (females); hypertrophy of zona fasciculata (females) **50 mg/kg bw/d:** disturbed spermiogenesis** (3/14) and spermatogenesis (4/14); Sertoli cell-only tubules** (4/14); ↑ surface density of Leydig cells** (3/14); ↓ density of spermatozoa in epididymides** (11/14); nucleated cells in epididymides (14/14); spermatoceles in epididymides (9/14) **50 mg/kg bw/d - recovery:** disturbed spermatogenesis (4/14); Sertoli cell-only tubules (4/14); ↑ surface density of Leydig cells (1/14); ↓ density of spermatozoa in epididymides (5/14); nucleated cells in epididymides (9/14); spermatoceles in epididymides (11/14) *reversible in recovery group (4 week) ** also observed in single males of 0, 5, 25 mg/kg bw/d groups
Oral, pilot study 9 weeks (Givaudan, 1990a)	Dog (Beagle) 2 males	95% Lysmeral in gelatine capsules Subsequently increasing doses from 47 to 564 mg/kg bw/d over 9 weeks		**General toxicity:** occasional vomiting (2/2); diarrhoea (1/2); ↓ body weight; ↑ GLDH; ↑ ALAT; multifocal inflammation of the liver (2/2) **Male reproductive organs:** mild atrophy in seminiferous tubules; necrosis of germ cells; multinucleated giant cells in tubular lumen

Oral, screening 14 days GLP (BASF SE, 2008a)	Dog (Beagle) 4 males per group	99.1% Lysmeral in gelatine capsules 0, 40, 200, 1000 mg/kg bw/d high dose reduced to 500 mg/kg bw/d due to general toxicity	LOAEL (general toxicity): 200 mg/kg bw/d NOAEL (male fertility): 40 mg/kg bw/d	**40 mg/kg bw/d:** ↓ prostate size **200 mg/kg bw/d:** ↓ bwg and food efficiency (some animals); vomitus, soft faeces/diarrhoea (4/4); ↑ rel. liver weights, 30-40%; centrilobular hypertrophy of hepatocytes; ↑ activated partial thromboplastin time; ↑ serum Mg^{2+}, K^{+}, inorganic phosphate; ↓ ALAT; ↓ ASAT; 1 out of 4 males: massive diffuse degeneration of seminiferous tubules; hyperplasia of Leydig cells in the testes; aspermia and epithelial vacuolation in the epididymides; ↓ rel. testes weights/size; ↓ epididymides size 1 out of 4 males: slight one-sided and focal degeneration of seminiferous tubules (also seen in HCD) **1000/500 mg/kg bw/d:** ↓ bwg and food efficiency (some animals); vomitus, soft faeces/diarrhoea (4/4); ↑ rel. liver weights, 30-40%; centrilobular hypertrophy of hepatocytes; ↑ activated partial thromboplastin time; ↑ serum Mg^{2+}, K^{+}, inorganic phosphate; ↓ ALAT; ↓ ASAT; ↓ glucose; no adverse testicular effects
Oral, follow-up 14 days GLP (BASF SE, 2008b)	Dog (Beagle) 10 males per group	99.1% Lysmeral in gelatine capsules 0, 200 mg/kg bw/d		**General toxicity:** ↓ body weights; ↓ food consumption, > 25% vomitus (7/10); diarrhoea (4/10); ↑ rel. liver weights, 17%; centrilobular hypertrophy of hepatocytes; ↑ ALAT, 80%; ↑ ASAT, 310%; ↑ activated partial thromboplastin time, 10%; ↓ serum triglycerides, 35%; ↓ red blood cell count and haemoglobin, 5%; ↓ haematocrit, 10%; ↓ reticulocyte count; ↑ serum urea (45%); creatinine (25%); Ca^{2+} (5%); Mg^{2+} (20%) **Male reproductive organs:** ↓ prostate weights + minimal to moderate multifocal atrophy (3/10); ↓ rel. testes weights, ~25% (9/10); slight to severe degeneration of seminiferous tubules (9/10); unilateral ↓ testicular length or width (6/10); ↓ progressively motile spermatozoa + morphological alterations (9/10)

The repeated dose toxicity studies showed similar liver effects as seen in the PNDT in rats, and in dogs, along with testicular toxicity and spermatotoxic effects mainly in rats. The LOAEL for fertility effects in rat repeated dose toxicity studies was 50 mg/kg bw/d. In dogs, toxicity to male reproductive organs was found at an oral dose of 200 mg/kg bw/d Lysmeral. The DS concluded that dogs seem less sensitive to testicular toxicity of Lysmeral compared to rats.

No testicular effects were seen in other species tested in short term (5 days) studies up to doses of 100 mg/kg bw/d (mouse, Guinea pig, and rhesus monkey), or 300 mg/kg bw/d (rabbits). The DS deemed these species "non-responders".

Toxicokinetics, Metabolism

The DS considered the testicular and spermatotoxicity of Lysmeral to be caused by a specific metabolite, p-tert-butylbenzoic acid (TBBA), and the formation of stable TBBA- coenzyme A (CoA) conjugates, which in turn would disrupt lipid synthesis by depletion of physiological CoA. This interferes with other cellular processes and leads to cellular toxicity. Formation of TBBA and corresponding stable TBBA-CoA levels was shown to be at least quantitatively species dependent.

The DS presented a number of *in vivo* and *in vitro* toxicokinetics and metabolism studies in humans and other species:

- one pilot study on excretion kinetics in one volunteer after dermal exposure;
- one excretion kinetics study in 5 volunteers after oral exposure;
- one comparative oral 5-d study to evaluate urinary metabolites in rats, mice, Guinea pigs, Beagle dogs, and rhesus monkeys;
- one comparative *in vitro* metabolism study in liver microsomes and hepatocytes of rats, mice, rabbits, and humans.

Based on the results from the *in vitro* metabolism study in liver microsomes and hepatocytes of different species, the DS proposed the following main metabolic pathway in hepatocytes and microsomes: Lysmeral -> Lysmerylic acid -> TBBA -> TBHA (p-tert-butyl-hippuric acid; only in rodents).

Lysmerylic acid was found to be the main metabolite in hepatocytes of all tested species, whereas TBBA was found most abundantly in rat hepatocytes compared to other species. In microsomes, Lysmeral may also be transformed to Lysmerol, which is metabolised to the corresponding glucuronide in all tested species. Other minor pathways in hepatocytes of all tested species also lead to glucuronides. To confirm these data, the DS presented results from studies evaluating the urinary excretion of metabolites in humans, rats, mice, Guinea pigs, Beagle dogs, and rhesus monkeys.

Excretion kinetics in humans were evaluated in studies aiming to develop a biomonitoring method and identify suitable biomarkers in urine.

In the dermal pilot study with one volunteer, urine samples were collected including all fractions voided up to 48 hours after using a Lysmeral-containing sunscreen (5 g containing 6.5 mg/g Lysmeral, total 32.5 mg). Peak levels of Lysmerol and Lysmerylic acid were excreted into the urine about 3–6 hours after application, whereas TBBA and TBHA appeared about 12 hours after application. Of the applied dermal dose, TBBA accounted for 0.67%, TBHA 0.04%, Lysmerol 0.02%, and Lysmerylic acid 0.012% as measured in the urine samples. In total, the Lysmeral-related urinary analytes assessed represented 0.75% of the applied dose.

In the oral follow-up study, 5 healthy subjects were orally dosed with 5.26 mg Lysmeral, dissolved in ethanol. Urine was collected immediately before and for 48 hours after administration in separate fractions. Peak levels of the Lysmeral metabolites Lysmerol, Lysmerylic acid, hydroxylated Lysmerylic acid, and TBBA were measured between 3 and 6 hours after application.

Urinary excretion was fast, with more than 90% of all measured Lysmeral metabolites excreted after 12 hours, and the excretion was found to be complete by 48 hours after intake. The sum of the four metabolites assessed in urine reflected about 16.5% of the applied dose with TBBA representing about 14.3% of the administered dose, followed by Lysmerol (1.82% of the dose). The fractions of hydroxy-Lysmerylic acid and Lysmerylic acid were 0.20% and 0.16% of the applied dose, respectively. Lysmeral itself was detectable after enzymatic deconjugation, but in very low amounts, i.e. < 0.003% of the dose applied.

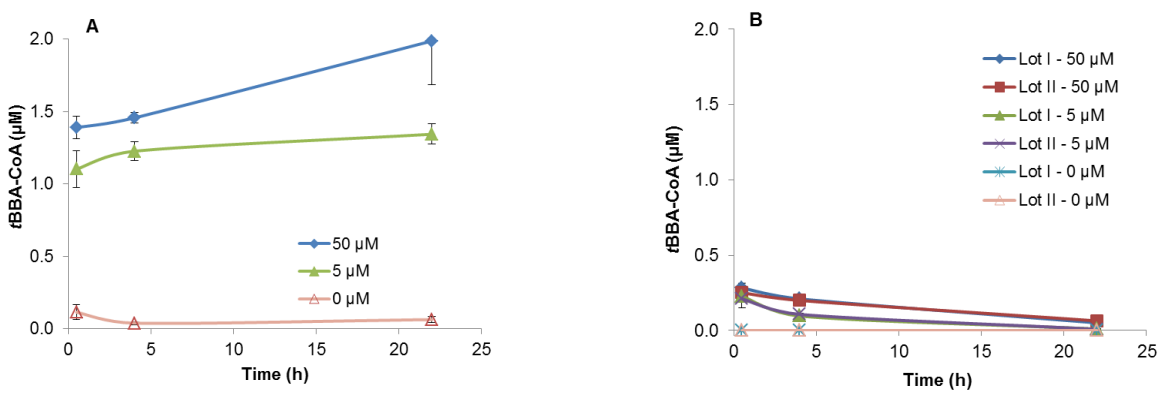
In animals, urinary metabolites (TBBA and TBHA) were analysed after oral application of Lysmeral via gavage for five consecutive days. In rats, TBBA accounted for 7-15% of the applied dose, whereas TBHA was merely detectable. In contrast, in mice and Guinea pigs urinary TBBA levels were low (< 1% of the applied dose) but TBHA levels high (13% in mice and 49% in Guinea pigs). In dogs, TBBA levels of 3-4% of the applied dose were found in urine, compared to TBHA levels of 1% of the applied dose. The two male rhesus monkeys tested showed different metabolic profiles: TBBA levels in one animal were as high as in rats (11%), while in the other animal these levels were comparable to the levels in dogs (3%). Levels of TBHA were below 0.1% of the applied dose in both animals.

To address the proposed mode of action (MoA), the DS summarised published *in vitro* data indicating an inhibitory capacity of TBBA on lipogenesis and gluconeogenesis in rat hepatocytes. Addition of glycine, which represents a relevant substrate to form the respective hippurate (TBHA), did not affect TBBA inhibition of lipogenesis in the cells. Furthermore, CoA, acetyl-CoA and citrate levels were decreased in these cells. A formation and accumulation of p-TBBA-CoA conjugates was suggested as the crucial step for toxic effects. The DS concluded that these findings underline the lack of efficient TBHA formation capacity observed in rats *in vivo*.

To underline species differences in the formation of TBBA-CoA conjugates, the DS presented a comparative *in vitro* study on the detection of CoA-conjugates in human and rat hepatocytes. Primary rat hepatocytes from Sprague-Dawley rats and two lots of primary human hepatocytes (Lot I: one single female donor; Lot II: pooled from five donors of different genders) were incubated with Lysmeral for 0.5-22 hours at doses of 5 and 50 µmol/L. Cells were lysated, and lysates were analysed for CoA conjugates by LC-HRMS. Results are plotted in the figure below (revised version provided by the DS during public consultation, now also containing data from controls).

As seen in the figure, the amount and the kinetics of TBBA-CoA formation differed between species. Lysmeral incubation for 0.5 hours resulted in approximately 5-fold lower TBBA-CoA levels in human hepatocytes compared to rat hepatocytes, and a decrease was found over time. In rat hepatocytes CoA conjugate levels increased over the time period observed.

Revised Figure 4 of the CLH report: TBBA-CoA conjugates detected in plated primary rat (A) or human (B) hepatocytes incubated with two different concentrations of Lysmeral and without test chemical as control. Plated hepatocytes were exposed to 0, 5 or 50 µM Lysmeral for 0.5, 4 and 22 h and Coenzyme A conjugates analysed by LC-HRMS. A representative experiment from > 10 experiments is shown for rat hepatocytes. Data from two experiments with human hepatocytes using two different lots are shown: Lot I, 1 female donor; Lot II, pooled human donors of mixed sex (5 donors).



In contrast, no differences in the kinetics of the endogenously formed octanoyl-CoA were observed in untreated rat and human hepatocytes, excluding the possibility of a general loss of CoA conjugation capabilities by culturing of human cells.

Dermal absorption in rats and humans

Lysmeral is used as a fragrance in cosmetics, personal care and household products, and the DS concluded that the most relevant physiological route of exposure is the dermal route. Data on the dermal absorption of Lysmeral from cream formulations are available: one dermal percutaneous absorption study in 3 volunteers, one dermal absorption study in rats, and one *in vitro* penetration study in human skin.

After semi-occlusive dermal application of 11.37 mg Lysmeral in 70% ethanol on 10 cm^2 back skin of 3 human volunteers for 6 hours, a mean of 1.4% (range 0.8-2.4%) of the applied dose was excreted in urine within 24 hours, whereas no substance was detected in urine samples of later time points nor in any of the faeces and blood plasma samples.

In the *in vitro* study using human skin (according to OECD TG 428 and GLP), penetration of Lysmeral was assessed in different formulations consisting of a hydro-alcoholic preparation with 1.9% Lysmeral in 70% ethanol, and 0.1% Lysmeral in "silicone in water", "water in oil" and "oil in water" mimicking cosmetic formulations. Penetration was assessed 24 hours after test substance application. The percentage of systemically available Lysmeral after skin application was calculated to be between 5 and 7%, with the highest values obtained for the hydroalcoholic vehicle.

In rats, occlusive dermal application of 0.2 mg/cm^2 Lysmeral in 70% ethanol for 6 hours lead to a mean total percentage of dose in excreta and tissues of about 19%. Up to 120 hours after application of Lysmeral, a mean cumulative total of 14.6% of the dose was excreted in urine, 0.8% was recovered in cage washings and 2.0% was excreted via faeces, whereas levels in expired air traps were not detectable.

As a general conclusion, the DS derived quantitative species differences in the formation of the presumed toxic metabolite TBBA and the formation of stable conjugates with CoA, with the rat being more susceptible to this metabolic path than other species, including humans. Effects on male fertility were seen in rats and dogs after oral administration of Lysmeral. Dermal administration of Lysmeral to rats also led to some effects on male reproductive organs, but only at doses above the limit dose of 1000 mg/kg bw/d. Assuming that due to the unpleasant smell and taste of the compound at higher concentrations, human oral intake is unlikely, the most relevant path of exposure for humans is the dermal route. Taking into account that dermal penetration through human skin is lower than through rat skin, the DS concluded that the effects on fertility seen in rats are of minor relevance for humans and proposed to classify Lysmeral as **Repr. 2; H361f**.

Development

The PNDT study mentioned in the fertility section above is summarised in the table below.

Table: *Summary of the prenatal developmental toxicity study in rats*

Method, Duration of study, Route of exposure, Guideline, GLP status	**Species, Strain, Sex, No/ group**	**Test substance Vehicle, Dose levels, Duration of exposure**	**NOAELs, LOAELs**
Prenatal developmental toxicity 20 days oral, gavage OECD TG 414, GLP (BASF, 2004)	Rat (Wistar) 25 females/group	98.1% Lysmeral in olive oil Nominal doses: 0, 5, 15, 45 mg/kg bw/d Actual intake: 0, 4.1, 12.7, 40.7 mg/kg bw/d Exposure: day 6 p.c. - day 20 p.c	LOAEL (maternal toxicity): 12.7 mg/kg bw/d NOAEL (developmental toxicity): 4.1 mg/kg bw/d

Results	**0 mg/kg bw/d**	**4.1 mg/kg bw/d**	**12.7 mg/kg bw/d**	**40.7 mg/kg bw/d**
Maternal toxicity	none	↑ rel. liver weights, 9%	↑ rel. liver weights, 11%; ↑ ALAT; ↓ serum and erythrocyte ChE	↑ rel. liver weights, 19%; ↑ ALAT; ↓ serum and erythrocyte ChE; ↓ food consumption on days 6-8 p.c., 18%; ↓ mean body weights on days 6-8 p.c.; ↓ mean body weights on days 13-20 p.c.; ↓ corrected mean bwg, ~32%
Gestational parameters	Post-implantation loss, 4.4±7.35%; mean no. of foetuses/live foetuses, 8.1±1.5	Post-implantation loss, 4.7±7.59%; mean no. of foetuses/live foetuses, 8.2±1.18	Post-implantation loss, 4.9±10.56%; mean no. of foetuses/live foetuses, 8.8±1.37	↑ mean post implantation loss, **15.1%* (±20.25%)** vs 4.4% in controls; ↓ mean no. of foetuses/live foetuses per dam (7.4±2.15; n.s.)
Mean foetal weights	3.6 ± 0.28 g	3.5 ± 0.20 g	**3.3 ± 0.17 g****	**2.9 ± 0.29 g****
Discolouration of foetal liver foetuses per litter in %	0	0	1.7	15.5
Misshapen sacral vertebra foetal incidence in %	2.1	0	2.7	12[#]
Supernumerary thoracic vertebra foetal incidence in %	1	2.1	10	14[#]
Unossified sternebrae, unchanged cartilage foetal incidence in %	3.1	8.5	13	46[#]

Incomplete ossification of pubis, cartilage present foetal incidence in %	0	0	1.8	5.4[#]
Total foetal variations foetal incidence in %	91	91	99	98
Malformations foetal incidence in %	0	0	0	1.8 (3/170) anasarca with small spleen; polydactyly due to supernumerary phalanx; cervical hemivertebra

#value outside the HCD until 2012
*p ≤ 0.05
**p≤0.01

The NOAELs for both maternal toxicity and prenatal developmental toxicity were 4.1 mg/kg bw/d. The DS considered the variations seen in the mid and high dose foetuses as secondary to the decreased foetal weights and maternal toxicity in these groups. Liver discolouration in some foetuses of the high and mid dose groups was in line with liver changes in respective dams. The small number of malformations observed in 3 of 170 foetuses showed no consistent pattern and occurred only at the highest dose, which also caused maternal toxicity. Furthermore, the DS reported a historical control range for foetal malformations of 0-2.7% (foetal incidence).

The DS presented further information on developmental toxicity from the one-generation range-finding studies and the EOGRTS.

In the older range-finding study, no viable offspring were derived from animals treated with 1700 ppm (63 mg/kg bw/d) and 3400 ppm (120 mg/kg bw/d) microencapsulated Lysmeral. In the 1700 ppm group, the only pregnant female had only one implant which was resorbed. Due to the absence of offspring, these dose groups were not evaluated for general toxicity. In the 800 ppm (18.3-29.4 mg/kg bw/d) dose group, pup survival was slightly decreased for postnatal day 0 to 4 (94% versus 99% in controls), and no pup mortality was observed between postnatal day 4 and 21 in any dose groups with offspring. Overall, the DS considered the respective viability and lactation index as not affected by treatment. No effects on sex ratios were observed and pup necropsy revealed only sporadic and non-dose related findings, including post mortem autolysis, situs inversus, haemorrhagic thymus, dilated renal pelvis and a small kidney. For the 400 and 800 ppm dose groups, a significant reduction in birth weights (19% and 22% below controls, respectively) and pup weight at weaning (17% and 21% below controls, respectively) were recorded for male and female pups. Accordingly, the pup body weight gain was decreased by 16% and 21% below controls, respectively. Although liver weights were not affected in maternal animals of these dose groups, significant changes in clinical chemistry were observed (increases in liver enzyme levels). Additionally, food consumption and body weight gain were decreased in the higher dose group.

In the recent range finding study, there were no effects on live birth indices, but pup survival was decreased for postnatal days 0 to 4 (86% and 75% in the mid (10.6-11.9 mg/kg bw/d) and high dose (21.0-34.7 mg/kg bw/d) groups versus 95% in control group). No pup mortality was observed between postnatal day 4 and 21 in any dose group. In the high dose group, a decrease in the number of delivered pups per dam (4.0 versus 11.1 in controls) was observed. This was attributed to lower numbers in implantation sites due to decreased fertility indices. A significant reduction in birth weights (17% and 18% below controls, respectively) and pup weight at weaning (13-21% and 30-32% below controls, respectively) was recorded for mid and high dose pups. Accordingly, the pup body weight gain was decreased in these dose groups (13% and 33%

below controls, respectively). These dose levels were associated with impaired maternal body weight development and food consumption during premating phase, gestation and lactation, and resulted in changes of clinical chemistry and haematological parameters (increases in liver enzyme levels and decreases in other parameters such as serum protein and electrolyte levels). The developmental effects were therefore considered secondary to maternal toxicity by the DS.

In the EOGRTS, no post-implantation losses were observed for F0 and F1 generation animals up to the highest dose level tested (15.1 mg/kg bw/d). Decreased mean numbers of delivered F2 pups were associated with a lower number of implants in F1 females and not considered an independent finding. The live birth index was not affected in these or other treated animals. The pup body weight development was affected in high dose F1 and F2 offspring (about 14-16% less than controls after birth and with no recovery until weaning). Organ weight changes (brain, thymus and spleen) were observed at this dose and were considered to be secondary to the changes in body weight, rather than independent findings. No evident influence on postnatal pup survival during early lactation nor later was observed.

Blood thyroid hormone levels in offspring were not influenced by Lysmeral administration and the sex ratio of F1 and F2 pups was not affected. A decrease in the anogenital distance of the high dose F2 pups (but not F1 pups), and a slight increase of the anogenital indices of high-dose male and female F1 and F2 pups were considered as a secondary consequence to the lower pup body weights by the DS. A delay in preputial separation in the high-dose male F1 offspring was within the historical control range, and the DS attributed this to a general delay in the development of high-dose male F1 offspring. No effect of Lysmeral treatment for vaginal opening was noted. No signs of developmental neurotoxicity or immunotoxicity were observed.

Overall, developmental toxicity observed in the EOGRTS represented by reductions in pup body weights in the high dose F1 and F2 offspring was deemed secondary to maternal toxicity. This dose level resulted in adverse maternal liver effects, effects on food consumption, body weights, and clinical chemistry.

In conclusion, the DS proposed no classification of Lysmeral for developmental toxicity.

Lactation

The DS concluded that the available data did not allow to specifically assess the effects of Lysmeral on or via lactation. No human evidence indicating a hazard to babies during lactation, nor information on presence and concentration of Lysmeral or its metabolites in milk, is available and the reproductive toxicity studies did not provide clear evidence of adverse effect in the offspring due to milk transfer or effects on the milk quality. Based on currently available data, the DS concluded that classification for effects on or via lactation was not warranted.

Comments received during public consultation

Eight comments were received during public consultation: five Member State Competent Authorities (MSCAs), one individual, and two company downstream users.

Five MSCAs commented on fertility effects of Lysmeral. One of these requested thorough discussion at RAC to decide upon the appropriate category, the other four were of the opinion that Repr. 1B for fertility is justified. All of them questioned the proposed MoA as other MoAs were not ruled out (e.g. endocrine disruption by binding of Lysmeral to the oestrogen receptor). In addition, since the metabolism of Lysmeral in humans does not qualitatively differ from animal metabolism, the commenting MSCAs considered the relevance to humans not precluded.

The MSCAs also questioned the appropriateness of the chosen high dose and application method (microencapsulation) in the EOGRTS, stating that this dose might have been too low to induce

effects. One MSCA expressed concern that doses used in the testes toxicity studies performed with other species (mouse, rabbit, Guinea pig, rhesus monkey) than rats might have been too low and the duration of the studies too short to induce effects.

Three MSCAs commented on developmental effects. Two of them requested thorough evaluation of the maternal toxicity used by the DS to dismiss classification. One stated that the observed maternal toxicity was not severe enough, and that developmental effects (post-implantation loss, effects on pup bw, skeletal variations, anogenital distance, and neonatal acetylcholine esterase inhibition) may have been directly linked to Lysmeral. They therefore proposed to classify Lysmeral in Category 2 for developmental effects.

One MSCA also requested a calculation of the ED10 to determine if a specific concentration limit (SCL) would be required. The DS responded that the NOAEL for all effects, and independent of treatment duration, was set at 25 mg/kg bw/d in rats and the LOAEL therefore slightly above this value. These values fall within the boundaries for the medium potency group (4 mg/kg bw/d < ATE ≤ 400 mg/kg bw/d) and there is no justification for setting a lower SCL below the given generic concentration limit (GCL: 3%). There is also no evidence that ED10 values for sperm parameters and testes toxicity fall within the low potency group, and thus, an SCL above the GCL would not be justified.

One MSCA noted that the CLH report did not include two *in vitro* dermal absorption studies which were part of the SCCS (Scientific Committee on Consumer Safety) opinion (SCCS/1540/14). In these studies, percutaneous absorption and penetration was determined in excised skin of mini pigs and naked rats. Although absorption in mini pig skin was found to be much lower than in rat skin (> 5% compared to > 66%, respectively), it was also shown that penetration increased when Lysmeral was applied as real cream formulation rather than dissolved in ethanol (> 25.7% in mini pig skin). The SCCS concluded that the dermal absorption in human skin might be as high as 25%.

Furthermore, this MSCA stated differences and uncertainties in the *in vivo* rat and human dermal penetration studies.

The individual and the downstream users supported the assessments made by the DS and the proposal to classify Lysmeral as Repr. 2; H361f.

The DS provided additional information on the proposed MoA (see Additional Key Elements section).

Assessment and comparison with the classification criteria

No human data is available.

Fertility

The dossier included several repeated dose toxicity studies in rats and other species as well as four reproductive toxicity studies in rats. Lysmeral elicited adverse effects on male reproductive organs in rats and in dogs.

In the two one-generation range finding studies in rats via the oral route, male fertility was markedly affected at doses starting from 25 mg/kg bw/d. Findings are summarised in the table below.

Table: *Summary of findings in male rats in two one-generation range finding studies*

Method, Duration of study, Route of exposure, Guideline, GLP status	**Species, Strain, Sex, No/group**	**Test substance, Vehicle, Dose levels Duration of exposure**	**NOAELs, LOAELs**
One-generation range finding Oral, diet 12 weeks Non-TG, non-GLP (BASF SE, 2006c)	Rat (Wistar) 10 males and 10 females per group	30.7% Lysmeral in sunflower oil, microencapsulated in gelatin Nominal doses*: 0, 400, 800, 1700, 3400 ppm For dams adjusted to 0, 200, 400, 850, 1700 ppm during gestation and lactation Actual intake*: Males: 0, 14, 28, 62.6, 116.8 mg/kg bw/d Exposure: from 6 weeks prior mating to PND21 *doses and intake refer to pure substance	LOAEL (general toxicity, males): 28 mg/kg bw/d NOAEL (male fertility): 28 mg/kg bw/d
Results: **Mating indices:** 100, 100, 100, 80, 50%	**0 mg/kg bw/d:** Fertility index: 100% **14 mg/kg bw/d:** Fertility index: 100% **≥ 28 mg/kg bw/d**: ↑ rel. liver weights, 10-20% Fertility index: 100% **≥ 62.6 mg/kg bw/d**: ↑ ALAT, 20-45%; ↑ ALP, 30-55%; ↑ GLDH, 4-5-fold; ↓ rel. testes weights, 30-45%; ↓ rel. cauda epididymis weights, 30-40%; Diffuse testes degeneration (8/10) moderate to severe focal testes degeneration (2/10), aspermia of the epididymis (10/10); ↓ testicular spermatid heads (6 mio vs 121 mio in controls); ↓ epididymal sperm heads (2 mio vs 591 mio in controls); 0% motile sperm Fertility index: 10% **116.8 mg/kg bw/d**: ↓ food consumption, 15%; ↑ rel. kidney weights, 15%; ↑ gamma-GT, 100%; ↓ seminal vesicle weights, 10%; ↓ prostate weights, 20%; Minimal to slight hyperplasia of Leydig cells (9/10) Fertility index: 0%		
Method, Duration of study, Route of exposure, Guideline, GLP status	**Species, Strain, Sex, No/ group**	**Test substance Vehicle, Dose levels Duration of exposure**	**NOAELs, LOAELs**
One-generation range finding	Rat (Wistar)	17.7% Lysmeral in sunflower oil	LOAEL (general toxicity, males):

Oral, diet 8 weeks Non-TG, GLP (BASF SE, 2017b)	10 males and 10 females per group	microencapsulated in alginate; Nominal doses*: 0, 230, 750, 2300 ppm For dams adjusted to 0, 115, 375, 1150 ppm during lactation Actual intake*: males, pre-mating: 0, 2.8, 9.1, 27.5 mg/kg bw/d males, post-mating: 0, 2.3, 7.4, 25.1 mg/kg bw/d Exposure: from 2 weeks prior mating to PND21 *doses and intake refer to pure substance	27.5/25.1 mg/kg bw/d NOAEL (male fertility): 9.1/7.4 mg/kg bw/d
Results: **Mating indices:** 100, 100, 100, 90%	**0 mg/kg bw/d:** Fertility index: 100% **2.8/2.3 mg/kg bw/d:** ↑ rel. liver weights, < 10% Fertility index: 90% **9.1/7.4 mg/kg bw/d**: ↓ food consumption within week 1 of treatment, 7%; ↓ total protein; ↑ rel. liver weights, < 10% Fertility index: 100% **27.5/25.1 mg/kg bw/d**: ↓ bwg, 45-84%; ↓ food consumption within 1st week of treatment, 9%; ↓ total protein, albumin, globulin, cholesterol, triglycerides, sodium, calcium; ↑ ALAT, 26%; ↑ rel. liver weights, 14-30%, ↑ incidences of discolouration; ↓ rel. cauda epididymis weights, 19%; ↓ epididymis weights, 16%; ↓ seminal vesicle weights, 19%; Minimal to moderate tubular degeneration (3/10 vs 1/10 in controls); Minimal to moderate ductal atrophy in epididymis (8 /10); Slight to moderate oligospermia (6/10); Slight to moderate cellular debris (2/10); ↓ epididymal sperm heads (469 mio vs 674 mio in controls); Testicular spermatid head counts not affected (115 mio vs 124 mio in controls); 25% motile sperm; 72% abnormal sperm; Fertility index: 40%		

Effects on testes included reduced organ weights and degeneration. Spermatotoxic effects included reduced sperm counts and increased numbers of abnormal sperms resulting in markedly reduced fertility indices.

Doses eliciting adverse testicular effects and spermatotoxicity also lead to hepatotoxicity represented by increased organ weights and changes in clinical chemistry.

Similar effect patterns were observed in the repeated dose toxicity studies in rats and dogs, presented by the DS as supporting evidence. LOAELs for male fertility were 50 mg/kg bw/d in rats and 200 mg/kg bw/d in dogs.

After dermal application, testicular effects were observed in rats at doses of 2000 mg/kg bw/d (above the limit dose). No effects on fertility were seen in other species up to oral doses of 100 mg/kg bw/d in rhesus monkeys, mice and Guinea pigs, and 300 mg/kg bw/d in rabbits.

Based on a number of *in vivo* and *in vitro* toxicokinetic studies with Lysmeral there is evidence that species differences exist, but these differences are considered as quantitative rather than qualitative. The proposed MoA includes the formation of stable TBBA-CoA conjugates from the main metabolite TBBA, the amount of which was shown to be species dependent. High levels of stable TBBA-CoA have been measured in rat hepatocytes after incubation with Lysmeral, while levels in human hepatocytes were around 5 times lower. TBBA-CoA formation also occurs in rat testicular tissue, although to a much lesser extent than in hepatocytes. Dermal penetration studies in rats and humans showed that Lysmeral is absorbed via the dermal route in both species, although the amount absorbed may differ.

The classification criteria for reproductive toxicity state that "*the classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate.*"

RAC considers the testicular toxicity and spermatotoxicity shown in two species (rats and dogs) relevant for humans despite a quantitatively different metabolism of the compound in different species. Effects were consistently observed in several repeated dose toxicity studies in rats and dogs and in the two one-generation range finding studies in rats. RAC considers that the doses used in other species (mice, Guinea pigs, rhesus monkeys) may have been too low, and exposure periods (5 days) too short, to induce testicular effects in these species. RAC hence considers data from these species not sufficient to deem them "non-responders". In addition, doses in the EOGRTS were chosen to certainly produce offspring and may therefore also have been too low to induce similar effects as seen in other studies. RAC considers the proposed MoA, although plausible, not sufficient to preclude relevance for humans. It is not clear how relevant mechanistic findings from *in vitro* tests in hepatocytes are for the effects seen on testes tissue. For example, severe atrophy were seen already after only 24 hours after exposure. Although TBBA-CoA-conjugates were also formed in rat testes tissue *ex vivo*, concentrations were approximately 100-fold lower than in hepatocytes. Therefore, a direct effect of Lysmeral on this tissue cannot be ruled out. Even though some quantitative differences have been shown between rats and humans, no mechanistic data is available for dogs, the second species in which testicular effects were observed after exposure to Lysmeral. To dismiss these effects, and downgrade the classification to Category 2, RAC would have needed stronger (mechanistic) evidence.

The elaboration by the DS on the unpleasant smell and taste, and therefore unlikely human oral exposure, and on the low dermal penetration through human skin are not relevant for classification which is based on the intrinsic hazardous properties of the substance. As supporting evidence, the metabolite considered to be responsible for the compound's testicular and sperm toxicity – TBBA – is classified as Repr. 1B H360F.

Taking all available information into account, **RAC considers classification of Lysmeral as Repr. 1B; H360F warranted**.

Developmental toxicity

Effects on pre- and postnatal development after exposure to Lysmeral in doses up to 45 mg/kg bw/d were shown in rats.

Findings from the two one-generation range finding studies in rats are summarised in the table below.

Table: *Summary of the findings in dams and foetues/pups in two one-generation range finding studies*

Method, Duration of study, Route of exposure, Guideline, GLP status	**Species, Strain, Sex, No/group**	**Test substance, Vehicle, Dose levels Duration of exposure**	**NOAELs, LOAELs**
One-generation range finding Oral, diet 12 weeks Non-TG, non-GLP (BASF SE, 2006c)	Rat (Wistar) 10 males and 10 females per group	30.7% Lysmeral in sunflower oil, microencapsulated in gelatin Nominal doses*: 0, 400, 800, 1700, 3400 ppm For dams adjusted to 0, 200, 400, 850, 1700 ppm during gestation and lactation Actual intake*: Females: 0, 10-15, 18.3-29.4, 62.7, 123.2 mg/kg bw/d Exposure: from 6 weeks prior mating to PND21 *doses and intake refer to pure substance	LOAEL (general toxicity, females): 18.3-29.4 mg/kg bw/d NOAEL (female fertility): 18.3-29.4 mg/kg bw/d
Results: **Mating indices:** 100, 100, 100, 80, 50%	**0 mg/kg bw/d:** Mean implantation sites: 9.9; Mean post implantation loss: 5.1±9.27%; Mean pups delivered: 9.4±3.95; Number of litters: 10 Fertility index: 100% **≥ 10-15 mg/kg bw/d:** ↓ ChE, 50-60%; ↑ gamma-GT, 2-8-fold; Mean implantation sites: 8.5; Mean post implantation loss: **16.2±30.3%**; Mean pups delivered: 8.7±1.41; Number of litters: 9 Fertility index: 100% **18.3-29.4 mg/kg bw/d:** ↓ bwg 10-30% before/during mating; ~10% during gestation/lactation; ↓ food consumption, 20% during lactation; ↑ GLDH, 5-75%; Mean implantation sites: 8.8; Mean post implantation loss: **11.1±10.16%**; Mean pups delivered: 7.9±2.23; Number of litters: 10 Fertility index: 100% **62.7 mg/kg bw/d**:** Mean implantation sites: 1; Mean post implantation loss: 100%; Mean pups delivered: 0; Number of litters: 0		

	Fertility index: 13% **123.3 mg/kg bw/d**:** Mean implantation sites: 0; Mean pups delivered: 0; Number of litters: 0 Fertility index: 0% ** general toxicity was not evaluated due to absence of offspring		
Method, Duration of study, Route of exposure, Guideline, GLP status	**Species, Strain, Sex, No/ group**	**Test substance Vehicle, Dose levels Duration of exposure**	**NOAELs, LOAELs**
One-generation range finding Oral, diet 8 weeks Non-TG, GLP (BASF SE, 2017b)	Rat (Wistar) 10 males and 10 females per group	17.7% Lysmeral in sunflower oil microencapsulated in alginate; Nominal doses*: 0, 230, 750, 2300 ppm For dams adjusted to 0, 115, 375, 1150 ppm during lactation Actual intake*: females, premating/gestation: 0, 3.3-3.6, 10.6-11.9, 30.6-34.7 mg/kg bw/d females, lactation: 0, 3.7, 10.7, 21.0 mg/kg bw/d Exposure: from 2 weeks prior mating to PND21 *doses and intake refer to pure substance	LOAEL (general toxicity, females): 10.6-11.9 mg/kg bw/d NOAEL (female fertility): 10.6-11.9 mg/kg bw/d
	0 mg/kg bw/d: Mean implantation sites: 11.5; Mean post implantation loss: 3.8±6.85%; Mean pups delivered: 11.1±1.91; Number of litters: 10 Fertility index: 100% **3.3-3.7 mg/kg bw/d:** Mean implantation sites: 11.8; Mean post implantation loss: 3.9±6.29%; Mean pups delivered: 11.3±1.66; Number of litters: 9 Fertility index: 90% **10.6-11.9 mg/kg bw/d:** ↓ bwg and body weights during premating and gestation; ↓ body weights, ~10% during lactation, recovery at end of lactation; ↓ food consumption, during week 1 and 2 of lactation; ↓ triglycerides, sodium, calcium; ↑ ASAT, 23-47%; ↑ gamma-GT, 9-24-fold; Mean implantation sites: 10.1; Mean post implantation loss: 3.7±7.77%; Mean pups delivered: 9.7±2.36; Number of litters: 10 Fertility index: 100% **21.0-34.7 mg/kg bw/d:** ↓ bwg 32-59% during premating and gestation; ↓ body weights, ~10% during lactation, recovery at end of lactation;		

	↓ total protein, albumin, globulin, cholesterol, triglycerides, sodium, calcium, creatinine, total bilirubin, chloride, inorganic phosphate; ↑ ASAT, 23-47%; ↑ gamma-GT, 9-24-fold; ↓ food consumption, 14% during 1st week, 44-48% during lactation; Mean implantation sites: 4.5; Mean post implantation loss: **16.7±23.57%**; Mean pups delivered: 4.0±3.16; Number of litters: 4 Fertility index: 44%

In the first one-generation range finding study, post-implantation loss was increased starting from the dose of 10-15 mg/kg bw/d. Starting at the same dose, pup weights were significantly reduced at birth and at weaning, down to 22% below controls. In maternal animals, this and higher doses were associated with decreased choline esterase levels (50-60% below controls) and two- to eight-fold increased gamma-GT levels compared to controls, but liver weights were not affected. At the lowest dose, mean post-implantation loss showed a high variation (16.2±30.3%) and was higher than at the next dose level of 18.3-29.4 mg/kg bw/d (11.1±10.16%). At this dose level, maternal toxicity additionally consisted of a decreased body weight gain (up to 30% before and during mating, and around 10% during gestation and lactation), a decreased food consumption (-20% during lactation), and increased GLDH levels (up to 75% above control levels). Starting from the dose level of 62.7 mg/kg bw/d, there was only one implantation site, and general toxicity was not assessed due to lack of offspring. Taking into account the developmental effects and the maternal toxicity seen, RAC does not consider it clear that the developmental effects seen at these doses, in particular at 10-15 mg/kg bw/d, are secondary non-specific consequences of the maternal toxicity observed at 10-15 and 18.3-29.4 mg/kg bw/d.

In the second one-generation range finding study, post-implantation loss was increased up to 4-fold compared to controls at the dose of 21.0-34.7 mg/kg bw/d, which was the highest dose tested. This dose level was associated with a decrease in maternal body weight gain of up to 59% during premating and gestation, and a decrease in food consumption of up to 48% during lactation. Accordingly, body weights were decreased (10-16% below controls) from gestation day 14 into lactation, but had recovered at the end of lactation. Clinical chemistry parameters were also altered (see table above), but not liver weights. In contrast to the first study, in this study post-implantation loss was not affected at a slightly lower dose of 10.6-11.9 mg/kg bw/d (3.7±7.77% vs. 3.8±6.85% in controls).

In this second one-generation range finding study, pup survival was decreased on postnatal days 0 to 4 in the high and mid dose groups (75% and 86%, respectively, compared to 99% and 95% in the low dose and control groups, respectively). Furthermore, and similar to the first range finding study, a significant decrease in pup birth weights (17% and 18% below controls in the mid and high dose groups, respectively) and pup weights at weaning (up to 21% and 32% below controls, respectively) were observed in these dose groups. Again, according to RAC it is not considered clear that the effects on pup survival and pup body weight development at 10.6-11.9 mg/kg bw/d are secondary, non-specific consequences of the maternal toxicity observed.

In the EOGRTS, the mean number of implantation sites in the F1 generation was statistically significantly reduced in the highest dose group (mean dose of 15.1 mg/kg bw/d administered to male and female rats throughout the whole study). The number of implantation sites were 10.5±2.13 per dam, compared to 12.3±1.82 in controls. Consequently, F1 high dose dams delivered statistically significantly less pups (10.1±2.19 vs. 12.0±2.06 in controls). In the F0 generation high dose group, these parameters were not affected. Mean post-implantation loss was slightly, but not statistically significantly, increased in F0 and F1 dams starting from the

lowest dose level. However, these changes were not dose-dependent and showed high variations. RAC notes that in this study, dose levels were chosen with the aim to produce enough viable offspring for the additional cohorts, and they are considered too low to induce the same effect on post-implantation loss as was seen in the range finding studies where higher doses were used.

Maternal toxicity in F0 and F1 high dose dams consisted of increased ALAT (up to 30% above controls) and glutamate dehydrogenase levels (79% above controls), decreased choline esterase levels (down to 45% below controls), and increased relative liver weights (up to 28% above controls) with associated histopathology. Mean maternal body weight change during gestation was slightly, but statistically significantly, decreased in both high dose F0 and F1 dams (12 and 11% below controls, respectively), and mean maternal food consumption in these dams was slightly decreased during lactation (5 and 12% below controls, respectively). Accordingly, body weights at gestation day 20 and lactation day 14 were somewhat lower (4-8%) than in controls. Body weights of high dose F1 and F2 pups were decreased to 16% below controls at birth and did not recover until weaning, when pup body weights were still decreased (10% below controls). Decreased pup weights were associated with decreased organ weights (brain, thymus, spleen). Pup survival was not affected. A statistically significantly reduced anogenital distance was observed in F2 offspring (2.97/1.49 mm in males and females vs 3.08/1.55 mm in controls, respectively), but not in the F1 offspring (3.01/1.47 mm in males and females vs. 3.08/1.48 mmm in controls, respectively).

RAC also consulted the full study report, and found no correlation between individual maternal weight loss and the respective pup weights. Therefore, RAC considers the effects on pup body weights not secondary to maternal toxicity, and thus relevant for classification.

In the prenatal developmental toxicity study, developmental effects were observed in the mid and high dose groups of nominal 15 and 45 mg/kg bw/d, respectively (12.7 and 40.7 mg/kg bw/d effective doses). These consisted mainly of skeletal variations (delayed ossification and supernumerary ribs), post-implantation loss and decreased foetal weights. For the skeletal variations, only the incidences in the high dose group were outside the extended historical control range until 2012. Mean foetal weights were statistically significantly reduced in the mid and high dose groups, but at the mid dose the reduction was only slight (8% below controls). Post-implantation loss was increased only in the high dose group with a high variation (15.1±20.25% vs. 4.4±7.35% in controls). Malformations were observed at the top dose in 3 out of 170 foetuses (1.8%), but without a consistent pattern and at an incidence within the historical control range (0 – 2.7%). Maternal toxicity in the mid and high dose groups consisted of increased relative liver weights (11 and 19% above controls, respectively) and increased ALAT and choline esterase levels. The level of maternal toxicity was more marked at the high dose, with also a decrease in maternal food consumption (by 18%) on gestation days 6 to 8, resulting in body weight loss on these days. Mean body weights in this group were also decreased on gestation days 13 to 20, leading to a 25% decreased body weight gain over the treatment period as compared to controls. The corrected mean body weight gain was 32% below controls. As only the incidences for skeletal variations in the high dose group were outside the HCD, and malformations were also observed in this group only, RAC considers these findings *per se* not enough to warrant classification.

The CLP criteria states that: "*Developmental effects which occur even in the presence of maternal toxicity are considered to be evidence of developmental toxicity, unless it can be unequivocally demonstrated on a case-by-case basis that the developmental effects are secondary to maternal toxicity.*" (CLP Regulation, Annex I, 3.7.2.4.2)

Effects on post-implantation loss were seen consistently in several studies, albeit with a high variation. These effects were associated with doses also leading to clinical chemistry changes indicative of maternal liver toxicity; however, only in some cases these were accompanied by changes in liver weight and liver histopathology, or by markedly reduced maternal body weights

or food consumption. Effects on pup body weights were also consistently observed; starting from a dose of 10-15 mg/kg bw/d, i.e. doses without marked maternal toxicity.

In the PNDT study, skeletal variations at an incidence outside the range of the extended historical control data were only observed at the high dose of 40.7 mg/kg bw/d, and are likely secondary to the marked maternal toxicity and decreased foetal weights at this dose. Malformations observed in this high dose group lacked a consistent pattern and occurred at a very low incidence inside the historical control range. Hence, RAC does not consider these effects as relevant for classification.

However, the effects on post-implantation loss and pup body weights are considered to warrant classification, as RAC considers these not unequivocally attributable to the maternal toxicity seen in the studies. Therefore, **RAC considers classification of Lysmeral as Repr. 2; H361d warranted**.

Regarding lactation, since no data concerning effects on or via lactation are available, RAC considers classification of Lysmeral for lactation effects not warranted.

In conclusion, **RAC considers an overall classification of Lysmeral as Repr. 1B, H360Fd warranted**.

ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).