Committee for Risk Assessment

RAC

Annex 1
Background document
to the 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

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to public consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

28 January 2019

CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2

Substance Name:

2-(4-tert-butylbenzyl)propionaldehyde

EC Number: 201-289-8

CAS Number: 80-54-6

Index Number: --

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Version number: 2.1 **Date: December 13, 2017**

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 2-(4-*TERT*-BUTYLBENZYL)PROPIONALDEHYDE

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PART A

1.PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Harmonised Classification and Labelling Proposal

Table 1: Substance identity

Substance name:	2-(4-tert-butylbenzyl)propionaldehyde
EC number:	201-289-8
CAS number:	80-54-6
Annex VI Index number:	--
Degree of purity:	≥ 99 %
Impurities:	Impurities are not considered relevant for the classification and labelling of the substance.

Table 2: The current Annex VI entry and the proposed harmonised classification

	CLP Regulation
Current entry in Annex VI, CLP Regulation	No classification
Current proposal for consideration by RAC	Classification Repr. 2, H361f Labelling GHS08 Wng, H361f
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Classification Repr. 2, H361f Labelling GHS08 Wng, H361f

1.2 Proposed harmonised classification and labelling based on CLP Regulation

Table 3: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
3.7.	Reproductive toxicity	GHS08, Repr. 2, H361f	none	none	

[1)] Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

No other endpoints have been discussed in this report.

Labelling:

Signal word:
Warning

Pictogramms:
GSH08

Hazard statements:
H361f: Suspected of damaging fertility.

Precautionary statements:
No subject for Annex entry.

Proposed notes assigned to an entry: none

1.3 History of the previous classification and labelling

2-(4-tert-butylbenzyl)propionaldehyde (Lysmeral) has a self-classification according to Regulation 1272/2008/EC (CLP) but is not listed in the Annex VI of the CLP regulation. Available data indicate that a classification for reproductive toxicity is warranted. In agreement with the German MSCA, BASF SE took the lead to submit the proposal for a CLH report for 2-(4-tert-butylbenzyl)propionaldehyde (Lysmeral) to the ECHA. A first CLH proposal on reproductive toxicity was already submitted by industry in 2014 which was, however, withdrawn as new and relevant information on this endpoint had to be generated. During the substance evaluation by the Competent Authority of Sweden under the Community rolling action plan (CoRAP) further information was required to clarify concerns regarding endocrine disrupting properties and developmental toxicity. This information requirement included to conduct an Extended one-generation reproductive toxicity study (EOGRTS) in rats, oral route (test method: OECD 443) with the extension of Cohort 1B to mate the F1 animals to produce a F2 generation, the 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. BASF SE resubmits an updated CLH report including the requested information on the EOGRTS and further relevant information on the proposed classification.

1.4 Short summary of the scientific justification for the CLH proposal

Toxicity to reproduction

Current classification: no classification in Annex VI of CLP

Proposed classification: Repr. 2, H361f

Lysmeral (2-(4-tert-butylbenzyl)propionaldehyde) has been found to induce testicular toxicity and spermatotoxicity when administered orally to rats and at higher dose levels to dogs. Infertility in rats due adverse effects of orally administered Lysmeral on the male reproductive system has been confirmed in feeding one-generation range-finding studies. Based on clear evidences from experimental animals, it is considered appropriate to classify Lysmeral for reproductive toxicity, i.e. adverse effects on fertility.

However, in determining the appropriate hazard category, the assessment of the relevance of the given hazard to humans needs to be taken into account.

Species specificity for Lysmeral induced testicular toxicity has been observed. Adverse effects of Lysmeral on the male reproductive system at a clearly defined threshold dose have been found in rats whereas no evidence for testicular toxicity was observed in the mouse and guinea pig. Considering non-rodent species, the dog has been shown to be susceptible towards Lysmeral induced testicular toxicity. In contrast, short-term oral exposure to rabbits did not indicate a potential of Lysmeral to induce testicular toxicity. Furthermore, in rhesus monkeys no indication of testicular toxicity, at doses causing testicular toxicity in the rats, was observed.

Based on the accordance in the testicular toxicity profile of Lysmeral, p-tert-benzaldehyde (TBB), p-tert-butyltoluene (TBT) and the shared metabolite para-tert-butylbenzoic acid (TBBA), the formation of the systemic TBBA intermediate represents a key metabolic event for Lysmeral induced testicular toxicity.

Species specificity for Lysmeral induced testicular toxicity is reflected by species dependent differences in the conversion of Lysmeral to TBBA in hepatocytes. TBBA formation in human hepatocytes is of low magnitude compared to rats and is comparable to levels found in the rabbit at toxicologically relevant doses, a species not sensitive to Lysmeral induced testicular toxicity.

Furthermore, a strong correlation has been established between the formation of TBBA-CoA conjugates in rat hepatocytes, disruption of lipid synthesis and testicular toxicity. The conjugation of TBBA with CoA represents the mode of action for Lysmeral induced testes toxicity and spermatotoxicity. In human hepatocytes, lower and transient concentrations due to a rapid decreases of TBBA-CoA conjugates strongly indicate, that testicular toxicity/spermatotoxicity is a species-specific effect with little relevance for humans.

Because of the properties of Lysmeral as fragrance material leading to palatability issues, substance administration needed to be performed via gavage or encapsulation. Dermal studies in rats represent the most appropriate route of administration, having regard to the likely route of human exposure as a fragrance material. These studies showed no testicular toxicity up to the limit dose. A prolonged human uptake of Lysmeral doses inducing systemic toxicity (testes toxicity or spermatotoxic effects) is highly unlikely.

Overall, a clear evidence for a species specificity and, if at all, a low human susceptibility concerning Lysmeral induced testicular toxicity raises doubt about the relevance of the effect for humans. Furthermore, evident reproductive toxicity has been observed after substance administration via gavage or encapsulation, representing a non-relevant form of application. In support, a prolonged human uptake of Lysmeral doses inducing systemic toxicity (testes toxicity or spermatotoxic effects) is highly unlikely, when compared to doses leading to rat testicular toxicity. Therefore, a classification as substance to be suspected of damaging fertility, i.e. category 2 (H361f) according to the CLP regulation (EC/1272/2008) is proposed**.**

Developmental toxicity has been observed at doses leading to evident maternal toxicity and is considered to be a secondary non-specific consequence of general systemic toxicity in the dams. Therefore, based on the present data, no classification concerning developmental toxicity is warranted.

1.5 Current harmonised classification and labelling

1.5.1 Current classification and labelling in Annex VI, Table 3.1 in the CLP Regulation

No classification.

1.6 Current self classification and labelling

1.6.1 Current self-classification and labelling based on the CLP Regulation criteria

Classification

Repr. 2, H361f

Labelling

GHS08, H361f, Warning

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.

2. JUSTIFICATION THAT ACTION IS needed at COMMUNITY level

2-(4-tert-butylbenzyl)propionaldehyde (Lysmeral) shows adverse effects on testes and fertility, i.e. reproductive toxicity. Harmonized classification and labelling for CMR and respiratory sensitiation is a Community-wide action under article 36(1) of CLP. 2-(4-tert-butylbenzyl)propionaldehyde (Lysmeral) is currently not listed in Annex VI of the CLP regulation. Repeated-dose toxicity and toxicokinetic data are presented for information as they provide relevant data for assessment of reproductive toxicity but no classification is discussed and proposed for these endpoints and other hazard classes.

PART B

SCIENTIFIC EVALUATION OF THE DATA

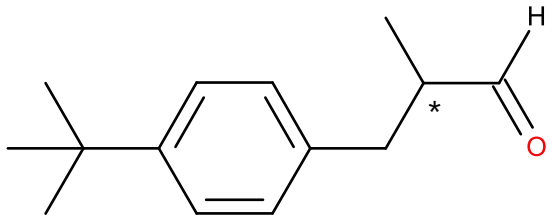
1. Identity of the substance

1.1 Name and other identifiers of the substance

Table 4: Substance identity

EC number:	201-289-8
EC name:	2-(4-tert-butylbenzyl)propionaldehyde
CAS number (EC inventory):	80-54-6
CAS number:	80-54-6
CAS name:	Benzenepropanal, 4-(1,1-dimethylethyl)-.alpha.-methyl-
IUPAC name:	3-(4-tert-butylphenyl)-2-methylpropanal
CLP Annex VI Index number:	--
Molecular formula:	$C_{14}H_{20}O$
Molecular weight range:	204.31 g/mol

Structural formula:



1.2 Composition of the substance

In the manufacturing process of the trade product Lysmeral Extra, the substance is obtained as racemic mixture (1:1) of the two enantiomers (2S)-3-(4-tert-butylphenyl)-2-methyl-propanal and (2R)-3-(4-tert-butylphenyl)-2-methyl-propanal, showing differences in their odour thresholds.

Table 5: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
• 2-(4-tert-butylbenzyl)propionaldehyde • EC no.: 201-289-8	--	>99 — <= 99.5 % (w/w)	--

Current Annex VI entry: No classification

Table 6: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
Non specified impurities	--	>=0.5 — < 1 % (w/w)	--

Current Annex VI entry: No classification

Table 7: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
No additives	--	--	--	--

Current Annex VI entry: Not applicable.

1.2.1 Composition of test material

Not applicable.

1.3 Physico-chemical properties

Table 8: Summary of physico - chemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	liquid	Ullmann (2003)	
Melting/freezing point	< -20°C	BASF AG (1991)	
Boiling point	279.5°C at 1013 hPa	BASF AG (1999)	
Relative density	0.94 at 25°C	BASF AG (2007)	
Vapour pressure	0.0025 hPa at 20°C	BASF AG (1999)	
Surface tension	based on chemical structure, no surface activity is predicted.	Expert judgement	Estimated
Water solubility	33 mg/l at 20°C	Givaudan-Roure (1995)	
Partition coefficient n-octanol/water	4.2 at 24°C	Givaudan-Roure (1995)	
Flash point	79°C	BASF (2009)	
Flammability	Flammability upon ignition derived from flash point. The substance has no pyrophoric properties and does not liberate flammable gases on contact with water.	Expert judgement	Estimated
Explosive properties	Non explosive	Expert judgement	Estimated
Self-ignition temperature	257°C	BASF (2009)	
Oxidising properties	No oxidizing properties	Expert judgement	Estimated
Granulometry	Substance is marketed or used in a non solid or granular form	Expert judgement	Estimated
Stability in organic solvents and identity of relevant degradation products	Stability of the substance is not considered as critical	Expert judgement	Estimated
Dissociation constant	Substance does not contain any ionic structure	Expert judgement	Estimated
Viscosity	12.3 mPa*s at 20°C	BASF (2009)	

2 MANUFACTURE AND USES

2.1 Manufacture

Not relevant for this dossier.

2.2 Identified uses

Lysmeral (2-(4-tert-butylbenzyl)propionaldehyde) is used as fragrance in a wide number of industries. It shows an intensive, radiant, floral odour with a typical lily-of-the-valley note. As a component of fragrance mixtures the main uses include cosmetic/personal care products and washing/cleaning products. Lysmeral may also be included as fragrance substance in air care products and biocidal products. No oral applications of Lysmeral are known and supported.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Not evaluated in this report.

4 HUMAN HEALTH HAZARD ASSESSMENT

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Non-human information

Quantitative data on the toxicokinetics of Lysmeral (2-(4-tert-butylbenzyl)propionaldehyde) are available from experimental animals (rat, mouse, rabbit, guinea pig, dog and rhesus monkey) and humans. Based on its physico-chemical properties, i.e. water solubility (33 mg/l at 20°C), partition coefficient log Pow (4.2 at 24°C), molecular weight (204 g/mol) and vapour pressure (0.25 Pa at 20°C), Lysmeral is considered to have a high bioavailability via the oral route and a limited bioavailability via the inhalation route. After acute and repeated oral and dermal administration of Lysmeral to experimental animals and humans there is clear evidence of systemic absorption. However, in humans only limited percutaneous absorption of Lysmeral is observed especially when compared to the rat.

After semiocclusive dermal application of [β-^{14}C]-Lysmeral (14.7 µCi or 11.37 mg test substance in 70% ethanol on 10 cm^2 back skin) on 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 radioactivity was below the detection limit in urine samples of later time points and in all faeces and blood plasma samples (Huntingdon Research Centre, 1994). Taking into account, that the chosen vehicle promotes dermal penetration of the applied test substance, these data indicate very limited percutaneous absorption of Lysmeral in humans.

In an in vitro study using human skin (acc. to OECD 428 and GLP), penetration of 14C-Lysmeral for 24 hours was assessed in different in-market cosmetic formulations (BASF 2016). The formulations consisted 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” based type of formulations.

Additional prolonged experiments were performed to characterize the total radioactive residues in the skin preparation 72 hours after application and to investigate the behavior of Lysmeral remaining on the skin surface after the 24 hours skin wash (Table 9).

More than 50% of the applied 14C-Lysmeral was recovered in a charcoal filter placed above the Franz cell, when applied in the alcoholic or “silicone in water” formulation. The skin washes contained a predominant fraction of 14C-Lysmeral when applied in the “oil in water” and “water in oil” formulation (48-56%), whereas lower fractions were found for the other vehicles (12-14%). Mean absorbed doses were 3.5 - 5.3% of the applied dose in the 24 hours absorption experiments and 5.0 - 7.8% of the applied dose in the 72 hours absorption experiments. Especially when applied in a hydroalcoholic and “oil in water” vehicle, no evident differences in the absorbed doses of 14C-Lysmeral were found 48 hours after removal. Only minor amounts of 14C-Lysmeral were associated with the skin after the exposure period. In the 24 hours experiments, these amounts were differentiated into dermis and epidermis compartments and accounted to 0.64 - 0.78% and 0.69 - 1.50% of the applied dose, respectively.

In the 72 hours experiments, the residues of 14C-Lysmeral in the skin preparations were differentiated into an extractable and non-extractable portion. The non-extractable portion of Lysmeral in living skin is assumed to be bound to the skin matrix and therefore represent a non-absorbable fraction. Based on the findings from these experiments 20-40% of the fraction found in living skin was not extractable and therefore potentially not absorbable. For the “oil in water” formulation, the residues of 14C-Lysmeral in the stratum corneum and the remaining skin appeared to be higher directly after test substance removal when compared to the respective 72 hours experiment, however, the absorbed dose did not significantly differ between experiments (4.77 versus 4.97%). Furthermore, the overall comparability of fractions found in the tape strips, living skin and absorbed doses observed for other vehicles indicate, that the post application phase does not significantly alter the distribution of 14C-Lysmeral in these compartments.

Table 9: Summary of [14C]-Lysmeral penetration through and into human skin. Single topical application of target doses of 95.0 and 5.0 µg/cm² of test substance formulated in different test-substance preparations expressed as % of applied doses.

Vehicle		**EtOH 70%**		**Silicone in water**		**Water in oil**		**Oil in water**	
Exposure / Sampling		**24h/24h**	**24h/72h**	**24h/24h**	**24h/72h**	**24h/24h**	**24h/72h**	**24h/24h**	**24h/72h**
Dislodgeable dose	Charcoal filter	55.35	56.49	55.13	62.88	32.87	28.24	35.11	23.92
	Membrane washing after 24 hours	11.60	13.20	14.45	12.28	53.85	50.37	47.75	56.23
	Sum (membrane (24/72h) and donor chamber washings)	68.60	73.48	73.61	80.55	87.77	84.86	84.58	81.89
Dose associated to tape strips	Tape strips 1-20	4.32	4.26	4.37	2.17	3.26	1.67	5.40	0.56
Dose associated to remaining skin	Epidermal membrane	1.50	-	0.96	-	0.74	-	0.69	-
	Dermal membrane	0.71	-	0.64	-	0.73	-	0.78	-
	Skin residue	-	0.32	-	0.25	-	0.24	-	0.18
	Skin extract	-	1.31	-	0.71	-	0.50	-	0.28
Absorbed dose	Sum (receptor fluid, receptor chamber washing)	5.31	5.29	3.50	5.04	4.83	7.82	4.77	4.97
Total recovery		80.44	84.67	83.08	88.72	97.32	91.01	96.21	87.88

Overall, the percentage of systemically available Lysmeral has been calculated based on the absorbed dose and the test substance content in remaining skin (dermis+epidermis), substracted by the non-extractable/ non-systemically available fraction determined in the living skin (see Table 10). Accordingly, 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.

Table 10: Calculation of the percentage of non-extractable skin fraction and the overall percentage of potentially systemically available [14C]-Lysmeral. Calculation is based on the penetration data through and into human skin after single topical application in vitro.

% non-extractable skin fraction	Ethanol in Water	= (0.32) * 100 / (0.32 + 1.31) = 20%
	Silicone in Water	= (0.25) * 100 / (0.25 + 0.71) = 26%
	Water in Oil	= (0.24) * 100 / (0.24 + 0.50) = 32%
	Oil in Water	= (0.18) * 100 / (0.18 + 0.28) = 39%
% systemically available Lysmeral	Ethanol in Water	= 5.31+((1.5+0.71)*(100-20)%) = 7.08%
	Silicone in Water	= 3.5+((0.96+0.64)*(100-26)%) = 4.68%
	Water in Oil	= 4.83+((0.74+0.73)*(100-32)%) = 5.83%
	Oil in Water	= 4.77+((0.69+0.78)*(100-39)%) = 5.67%

In rats, occlusive dermal application of [β-^{14}C]-Lysmeral in 70% ethanol at a single dose of 6.8 mg/kg bw accounting for a topical concentration of 0.2 mg/cm^2 for a maximum of 6 hours (2 animals/time point i.e. 0.5, 1, 3, 6, 12, 24, 48, 72, 120 h sacrifice) good bioavailability and rapid urinary excretion was observed (Huntingdon Research Centre, 1995). 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. Two animals per time point each were examined for tissue distribution and remaining dose at the application site. 120 hours after application of the test substance, the remaining radioactivity in all tissues investigated – excluding the skin at the application site – amounted to 1.2% of the applied radioactivity. The maximum urinary excretion rate (1.95 µg equivalents/hour) was observed 6 - 12 hours after dermal application of [β-^{14}C]-Lysmeral. Dermal radioactivity concentrations were more persistent and declined with a half-life of approx. 152 hours compared to other tissues ranging from 10 - 93 hours (Table 11).

The highest concentration of the absorbed 14C radiolabel was recovered in the liver (Cmax = 15.6 µg/g tissue representing 0.826 % of given dose/g tissue). Overall, a relation of Tmax and the blood perfusion rate of a respective tissue is indicated based on the findings for highly perfused tissues (i.e. lungs, heart) and poorly perfused tissues (i.e. skin, fat).
Although pharmacokinetic variables were not reported for testes in this study, a maximum concentration in this tissue was determined to be 0.008% of the topically applied dose per gram tissue 1 hour post application.

The mean total proportion of dose in excreta and tissues was about 19%, which represents the apparent level of absorption of radioactivity into the systemic circulation. Overall, a distribution predominately to the liver has been observed after dermal administration and can be assumed for the oral route as well.

Table 11: Pharmacokinetic variables of radioactivity in whole blood, plasma and selected tissues after single dermal dose administration of [β-14C]- Lysmeral to rats. C_{max} and T_{max} represent the observed values, whereas AUC were estimated up to the last time point at which concentrations were above the quantification limit by the linear trapezoidal rule and extrapolated to infinity (AUC∞). Testing laboratory specific programs were used for data processing, i.e. "Radioactivity", "Fast Radioactivity" and KIN 5.1.

	C_{max} (µg /ml or g tissue)	**T_{max} (h)**	**AUC (µg * h/g)**	**AUC∞ (µg * h/g)**	**Terminal rate constant (h)**	**Terminal half-life (h)**
Fat	0.4	24	34.9	-	-	-
Heart	1.3	3	11.7	12.0	0.0644	11
Kidneys	0.6	6	15.6	15.8	0.0655	11
Large Intestines	3.0	12	76.2	86.9	0.0110*	63
Liver	15.6	6	337.5	345.3	0.0205	34
Lungs	0.3	1	7.8	8.6	0.0150*	46
Skin	0.9	24	74.8	183.2	0.0046*	152
Small intestine	2.8	12	75.3	77.9	0.0177*	39
Pancreas	0.5	12	19.4	-	-	-
Stomach	2.5	0.5	16.5	22.6	0.0075*	93
Plasma	0.5	1	6.2	8.9	0.0492*	14
Whole blood	0.4	1	4.6	6.4	0.0521	13

* Period used for the rate constant estimation was < 2-fold above half-life. Therefore, estimated half life and AUC∞ are regarded as approximations.

After oral administration of radiolabelled [β-^{14}C]-Lysmeral in a single dose of 25 and 100 mg/kg to 4 male lbm:RORO (SPF) rats per dose via gavage (Huntingdon Research Centre, 1995) a rapid absorption of the radioactive compound for both doses applied and proportionate plasma maximum concentration (C_{max}) has been observed (Table 12). In contrast, AUC was found to increase disproportionate to the dose applied which is interpreted to be indicative for a saturation of the renal clearance of Lysmeral acid metabolites, e.g. TBBA.

When compared to the dermal toxicokinetic study in rats described above, dermal administration of Lysmeral revealed 7 fold lower C_{max} plasma values compared to oral administration taking into account the doses applied (0.5 µg/g after dermal administration of 6.75 mg/kg bw Lysmeral vs. 14.3 µg/mL after oral administration of 25 mg/kg bw Lysmeral). This comparison demonstrates, that systemic bioavailability after dermal administration is considerably lower in rats compared to that after oral administration. Since rat skin is more permeable to dermally applied substances than human skin, and taking into account the results of a dermal penetration study in human volunteers, very limited percutaneous absorption and systemic bioavailability of Lysmeral is expected in humans.

Table 12: Pharmacokinetic variables of radioactivity in blood plasma after single oral dose administration of [β-

14C]- Lysmeral to rats. Tmax und Cmax represent the mean of Tmax and Cmax observed for single animals. AUC was estimated up to the last time at which concentrations were above the quantification limit by the linear trapezoidal rule. Testing laboratory specific programs were used for data processing, i.e. "Radioactivity", "Fast Radioactivity" and KIN 5.1; mg/kg bw (mg/kg body weight).

Dose (mg/kg bw)	**C_{max} (µg equivalents/ml)**	**T_{max} (h)**	**AUC $_{0-48}$ (µg x h/ml)**
25	14.3	3.5 ± 1.9	122
100	52	1.8 ± 0.5	937

Blood plasma kinetics of Lysmeral and Lysmerylic acid in rodents was studied in male Wistar rats (BASF SE 2006A) and male C57BL/6NCrl mice (BASF SE 2006B) after oral application of a single dose of 50 mg/kg bw of each Lysmeral and Lysmerylic acid by gavage. Blood was taken retroorbitally, 3 days before gavage, directly after the first oral application (i.e. 20 minutes for mice and 10 minutes for rats), as well as 2, 4, 8, and 24 hours after application and blood plasma was analysed for Lysmeral and Lysmerylic acid by HPLC/MS. After application of Lysmeral, no unchanged parent compound was detectable in any plasma sample of both rodent species.

In the male rat, Lysmerylic acid was detected in all plasma samples and highest plasma concentration was observed 4 hours after application of Lysmeral or directly after application of Lysmerylic acid (Table 13). In the male mouse, highest plasma concentration of Lysmerylic acid was observed directly after application of both Lysmeral and Lysmerylic acid.

Toxicokinetic parameters for Lysmerylic acid in rodents show no species difference, whereas some difference in the toxicokinetics after oral application of Lysmeral between rat and mouse can be observed regarding T_{max} and C_{max} (Table 13).

Table 13: Plasma kinetics of Lysmerylic acid in the male rat and male mouse after application of a single dose of 50 mg/kg bw/day of each Lysmeral and Lysmerylic acid by gavage (BASF SE 2006A and 2006B). Parameters have been derived using TopFit 2.0 (Heinzel. G. et al.; TopFit 2.0, Pharmacokinetic and Pharmacodynamic data analysis system for the PC; Gustav Fischer Verlag, Stuttgart, Jena, New York; 1993). The AUC was determined according to the linear trapezoidal rule.

Test substance	**Species**	**T_{max}**	**C_{max} (µg/g)**	**AUC_{0-24} (µg x h/g)**	**$T_{1/2}$**
Lysmeral	Rat	4 h	8.8	81.4	5.8 h
	Mouse	Directly after application, i.e. 20 minutes.	18.4	85.1	3.3 h
Lysmerylic acid	Rat	Directly after application i.e. 10 minutes.	29.4	89.3	3.6 h
	Mouse	Directly after application, i.e. 20 minutes.	22.1	106.7	4.0 h

Excretion of the expected urinary metabolites of Lysmeral, i.e. tert.-butyl benzoic acid (TBBA) and tert.-butyl benzoyl hippuric acid (TBHA) has been compared in the rat, mouse, guinea pig, dog and rhesus monkey (Roche 1985A). Urine was collected for 24 hours after the last bolus oral administration of Lysmeral for 5 consecutive days in the 5 species mentioned above. The doses ranged from 45 to 400 mg/kg bw/d differing between species (rat: 50-400 mg/kg bw/d; mouse, guinea pig, and rhesus monkey: 100 mg/kg bw/d; dog: 45 mg/kg bw/d). Urinalysis of tert-butylbenzoic acid (TBBA) and tert-butylhippuric acid (TBHA) was performed by GC/MS. In the control group of all species, no TBBA and TBHA were found. Considering the relation between TBBA and TBHA, the main urinary metabolite in orally treated rats, dogs and rhesus monkeys was found to be TBBA, whereas in the guinea pig and mouse TBHA resulting from glycine conjugation predominates (Table 14). Surprisingly in the rat, urinary TBHA amounts are very low compared to other rodent species in this study, thus glycine conjugation or urinary TBHA excretion might not occur in the same rate as it does in other rodents. The urinary TBBA amount in one of the two rhesus monkeys was found to be comparable to rat amounts, whereas the other monkey showed 2-3 fold lower TBBA amounts than the rat.

Table 14: Urinalysis of tert.-butyl benzoic acid (TBBA) and tert.-butyl benzoyl hippuric acid (TBHA) in five different species after application of comparable Lysmeral doses by gavage for five consecutive days (Roche 1985A). Data on urinary metabolites are given in mg metabolite/kg body weight and in % of the dose Lysmeral applied; mg/kg bw (mg/kg body weight).

Species	Gender	No. of animals per dose	Dose (mg/kg bw)	Urinary metabolite			
				TBBA		TBHA	
				mg/kg	% dose*	mg/kg	% dose**
Rat	Male	8	50	4.8	11%	<0.3	
	Male	no data	50	8.1	19%	<0.9	
	Male	8	100	5.9	7%	0.8	1%
	Female	no data	50	6.7	15%	<1.1	
Mouse	Male	5	100	<0.8		14.5	13%
Guinea pig	Male	5	100	<0.03		56	49%
Dog	Male	3	45	1.1	3%	0.6	1%
	Female	3	45	1.4	4%	0.5	1%
Rhesus Monkey	Male	2	100	2 – 10	3 - 11%	0.04 - 0.2	< 0.1%

*% TBBA = TBBA in urine (µM/kg) / applied dose (µM/kg)
**% TBHA = TBHA in urine (µM/kg) / applied dose (µM/kg)

Similarly, species specific differences in the urinary excretion of TBBA have been observed after oral application of p-tert-butylbenzaledhyde (TBB) or p-tert-butyltoluene (TBT).

After 5-day oral administration of 12.5 and 50 mg/kg bw/day p-tert-butylbenzaldehyde (TBB) or 25 and 100 mg/kg bw/day p-tert-butyltoluene (TBT) to rats, p-tert-butylbenzoic acid (TBBA) was identified as metabolite in the urine 24 hours after the last administration, but not the secondary metabolite p-tert-butylhippuric acid (TBHA) was found. Amounts in urine yielded 17.2 mg TBBA /kg bw after administration of TBT (100 mg/kg bw/d) and 12.7 mg TBBA/ kg bw after administration of TBB (50 mg/kg bw/ day), being approximately 2-3 fold higher compared to TBBA urine amounts observed after administration of comparable Lysmeral doses to rats. No further metabolites have been investigated and glucuronic acid conjugates could not be identified by the analytical method used (Givaudan 1982B).

On the occasion of different 5 day oral toxicity studies in mice, guinea pigs and dogs, 100 mg/kg bw/d TBB or TBT was administered for 5 days, urine was collected for 24 h after the last administration and analyzed for TBBA and TBHA by GC analysis.

After application of TBB, TBBA was determined as metabolite in urine samples of treated dogs, guinea pigs and as very low amounts in the urine of mice. However, higher TBHA amounts were found in urine samples of treated mice and guinea pigs compared to TBBA, whereas TBHA amounts tended to be lower in the urine of dogs than TBBA amounts (Givaudan 1985). A similar pattern was observed after application of TBT. TBBA was determined as metabolite in urine samples of treated dogs and at very low amounts in guinea pigs but not in mice. Higher TBHA amounts were found in urine samples of treated mice and guinea pigs compared to TBBA, whereas TBHA amounts were lower in the urine of dogs than respective TBBA amounts (Givaudan 1985).

The excretion kinetics of Lysmeral in humans was investigated in an explorative study in human volunteers to develop a human biomonitoring (HBM) method including identification of suitable biomarkers of exposure in human urine (Scherer 2016, Pluym 2016). As a pilot study, the preliminary analytical method was applied to an urine sample collected including all fractions voided up to 48 h after using a Lysmeral-containing sunscreen (5g containing 6.5 mg/g Lysmeral) by a volunteer (male nonsmoker, 65 years old). Further, 5 healthy subjects (3 females, 2 males) were orally dosed once with 5.26 mg Lysmeral, dissolved in ethanol and applied as a chocolate coated eatable waffle cup containing and approximately 20 mL coffee, milk or water, depending on the choice of the volunteers. Urine was collected immediately before and. for 48 h after administration (all urine voids completely collected in separate fractions with the time of voiding being free). Lysmeral associated metabolites Lysmerol, Lysmerylic acid, hydroxylated Lysmerylic acid and 4-tert-butylbenzoic acid (TBBA) were determined by UPLC-MS/MS (ultra-high pressure liquid chromatography combined with tandem mass spectrometry) method. Due to high variations concerning precision and accuracy during method validation the metabolite TBHA was not included as a reliable parameter for HBM and not followed up in the oral excretion kinetic study.

In the dermal pilot study with one volunteer, peak levels of Lysmerol and Lysmerylic acid were excreted into the urine about 3–6 h, whereas TBBA and TBHA appeared about 12 h after dermal application. TBBA represented 0.67% of the applied dermal dose, followed by TBHA (0.04 %), Lysmerol (0.02 %), and Lysmerylic acid (0.012 %). In total, the Lysmeral-related urinary analytes represented 0.75% of the dermally applied dose.

Oral uptake resulted in peak levels of the 4 metabolites between 3 and 6 h after application with Lysmerol and Lysmerylic acid appearing slightly earlier in the urine than the secondary metabolites hydroxyl-Lysmerylic acid and TBBA. A rapid urinary excretion was observed, since more than 90% of all measured Lysmeral metabolites were excreted after 12 h, and the excretion was found to be complete by 48 h after the oral intake. The sum of the 4 metabolites assessed in urine reflected about 16.5% of the applied dose. TBBA represented about 14.3% of the administered dose, followed by Lysmerol, yielding 1.82% of the dose. The urinary fraction of hydroxy-Lysmerylic acid and Lysmerylic acid was 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. Average times for peak excretion (t_{max}) were 2.2 h and 4.64 h for Lysmerol and TBBA and 3.1 h for both Lysmerylic acid and hydroxyl-Lysmerylic acid. The elimination half-lives ($t_{½}$) were found to be lower for the primary metabolites Lysmerol and Lysmerylic acid (1.19 h and 1.25 h, respectively) than for the secondary metabolites hydroxyl-Lysmerylic acid and TBBA (1.39 h and 1.40 h, respectively), showing that the primary metabolites are excreted more rapidly.

Based on the results obtained by this exploratory excretion kinetic study, urinary conversion factors (CF) were deduced to allow the back-calculation of absolute Lysmeral uptake doses from creatinine standardized urinary metabolite levels of spot urines samples of 40 adult volunteers from the general population. Back-calculation based on these CF resulted in median daily exposure doses of 224 µg/d Lysmeral (range: 67-2218 µg/d) using all metabolites or 140 µg/d (range: 12-2249 µg/d) using all Lysmeral specific metabolites (excluding TBBA as metabolite of potential Lysmeral independent exposure sources).

Besides the selective assessment of specific metabolites in animals and humans mentioned above, a detailed in vivo study to cover the full metabolic range of Lysmeral between species is not available. To close this gap, a comparative in vitro metabolism study has been performed in order to study relevant Lysmeral specific metabolic pathways in different species (BASF SE 2010). For this purpose liver microsomes and hepatocytes of male Han-Wistar rats, male CD1-mice, male white New Zealand rabbits and male humans were incubated with 14C-Lysmeral for 2 hours (microsomes) or 4 hours (hepatocytes) in triplicates at nominal substrate concentrations of 10, 50 and 100 µM. Human hepatocytes (purchased by CELSIS In Vitro Technologies, Baltimore) were pooled from 10 donors, cryopreserved and fully characterized concerning xenobiotic metabolizing enzyme activity (e.g. ECOD, UGT/ST activity). Post thaw viability was confirmed and cells were validated by incubation with a positive control (testosterone). The established positive control Testosterone was chosen to prove the validity of the test systems, the applied incubation conditions as well as the methodology. Testosterone is an appropriate positive control for in vitro-metabolism investigations, because in the Cytochrome P450 linked oxidation of Testosterone, specific oxidation sites can be attributed to specific isoenzyme activities. Animal cryopreserved hepatocytes have been purchased, characterized and validated accordingly. The viability for all test substance concentrations (0.1 – 500 µM) in cell cultures of all tested species (rats, mice, rabbits and humans) was assessed and reported to be > 85 %.

Metabolic profiles were detected and quantified by Radio-HPLC after appropriate work up procedures of received incubates. Structure elucidation of formed metabolites was performed from14C-Lysmeral incubates (100 µM) of liver microsomes and hepatocytes of rats and humans by LC/MS-analyses.

On the basis of these findings, metabolic pathways of Lysmeral in rodents and non-rodents are depicted in figure 1. In liver microsomes, an oxidation of 14C-Lysmeral to its corresponding carboxylic acid (M7-Lysmerylic acid) or a reduction to its corresponding alcohol (M9 - Lysmerol), further oxidized at the tert-butyl group to form a hydroxy-metabolite (M3), was observed. In hepatocytes, oxidation to Lysmerylic acid was confirmed and its further dehydrogenation (most probably by hydroxylation and dehydration) to (E)-3-(4-tert-Butyl-phenyl)-2-methyl-acrylic acid (M16) was observed. Putative decarboxylation of Lysmerylic acid, followed by oxidation to the propanoic acid derivative and beta-oxidation led to the identified metabolite p-tert-butyl-benzoic acid (TBBA -M15). This metabolite was conjugated with glycine to form p-tert-butyl-hippuric acid (TBHA -M12) in rodents. In addition to these metabolites, glucuronic acid conjugates of metabolites M3, M7, M9, and M16 were detected.

The qualitative evaluation of the metabolic profiles of different species largely confirmed in vivo findings. The low test concentration chosen (10µM) reflect plasma levels observed after oral administration of no adverse testicular effect levels of Lysmeral whereas 100 µM covers plasma levels obtained after doses exerting testicular toxicity. Cmax for Lysmeral metabolites in plasma were 14 µg/ml or approx. 70 µM (assuming the molecular weight for Lysmeral) after oral application of 25 mg/kg bw Lysmeral (Huntingdon Research Center, 1995). Oral application of 50 mg/kg bw Lysmeral yielded a Cmax of 9 µg/ml or approx. 40 µM Lysmerylic acid, i.e. the main metabolite (BASF SE 2006A). The Radio-HPLC chromatograms were used to assign ROI values (region of interest = integrated peak area under Radio-HPLC) of each characterized metabolite in order to receive relative amounts of each metabolite in the respective metabolic profile. These ROI values were used for comparison of the incubation concentrations and species tested. As summarized in Table 15, Lysmeral was metabolized nearly completely in the hepatocytes of all species whereas Lysmerylic acid (M7) was quantitatively the main metabolite. The metabolite M16 ((E)-3-(4-tert-Butyl-phenyl)-2-methyl-acrylic acid) was more pronounced in hepatocytes of rats than in hepatocytes of mice or humans (not detected in hepatocytes of rabbits). In line with findings in vivo, species differences in metabolic profiles were seen for M12, representing TBHA, which was more pronounced in mice (4.9 – 27.1 % ROI) than in rats (3.5 – 3.6 % ROI). TBHA was not detectable in incubates of hepatocytes of rabbits and humans.

In rat hepatocytes, an increase of TBBA (M15) levels was found, and the incubation with lower Lysmeral concentrations resulted in higher TBBA levels. When compared to other rodent or non-rodent animal species, rats showed the highest concentration of TBBA. Whereas this metabolite contributed to 8.3 – 29.3 % ROI in hepatocyte cultures of rats, it was ≤ 0.5 % ROI in mice, ≤ 2.0 % ROI in rabbits. The concentrations observed in humans were found to be approx. 4 fold lower than in rat hepatocytes for corresponding tested Lysmeral concentrations, ranging from 1.9 – 7.5 % ROI. Furthermore, the concentrations of TBBA observed in the human system were similar to those found in the rabbit system at the 50 µM and 100 µM doses (the doses most relevant to plasma levels obtained after doses exerting testicular toxicity). These quantitative differences in TBBA formation between human and rat hepatocytes were apparently less pronounced or absent in the 24 hour urine samples of rats and rhesus monkeys. However, the TBBA detected in the 24 hour urine represent a cumulative amount of the excreted metabolite, to which the concentrations in the supernatant of the hepatocyte cultures cannot be compared to. These concentrations in hepatocyte supernatants are seen as directly proportional to given plasma concentrations in vivo. Since no comparative in vitro data for rhesus monkey hepatocytes are available and only two individual animals have been assessed in the primate study, the inclusion of these data for the overall assessment is questionable. The plasma concentrations (i.e. C_{max}) represent a more relevant parameter in respect of the thresholded testicular toxicity observed for Lysmeral, the data from the comparative in vitro metabolism study in hepatocytes are considered to better demonstrate species differences in Lysmeral metabolism.

Figure 1: Metabolic pathway of ^{14}C-Lysmeral based on the metabolites found in the supernatant of in vitro metabolism studies using liver microsomes and hepatocytes. Ra= rat; Mu=mouse; Rb=rabbit; Hu=human**.**

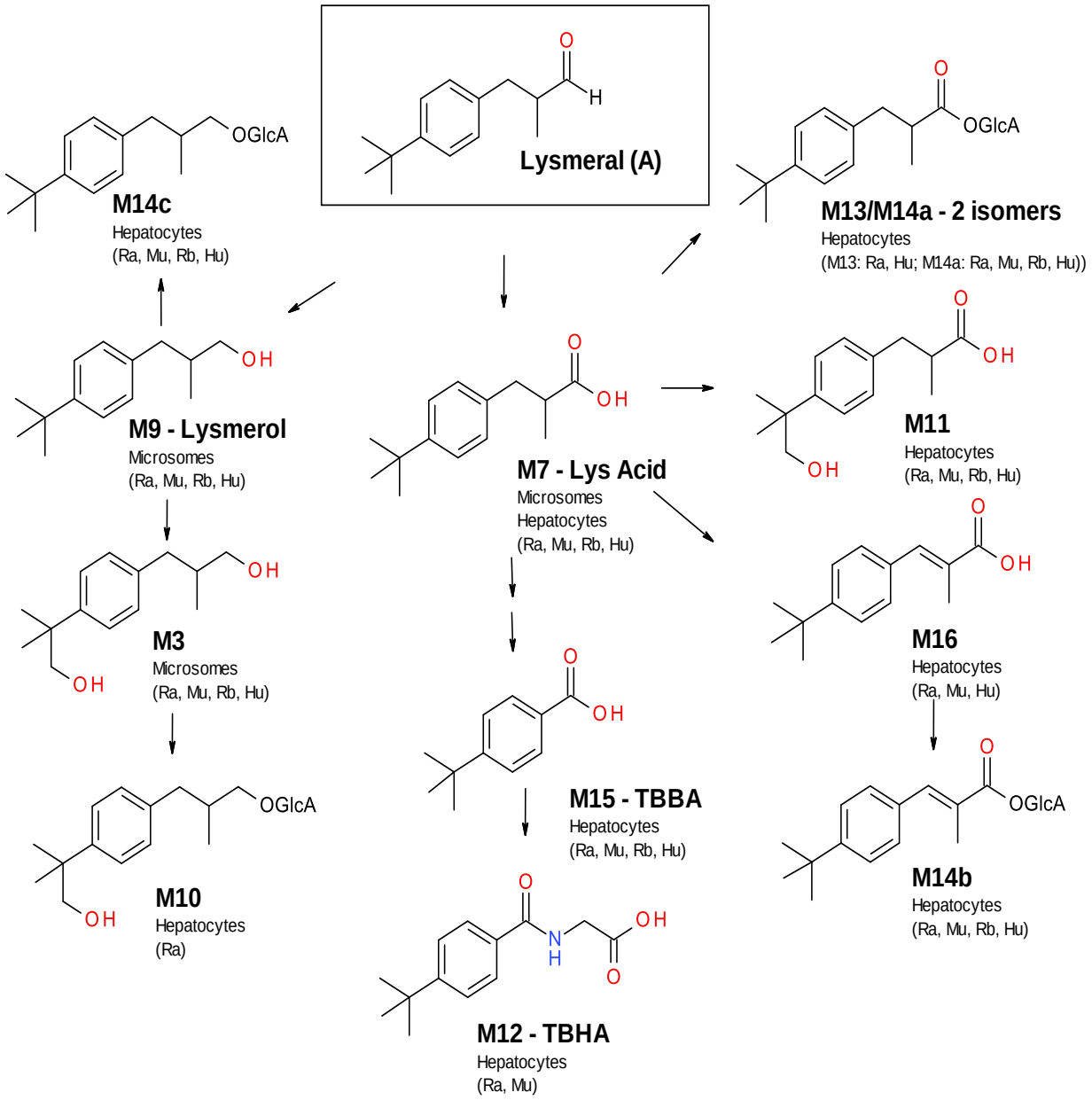
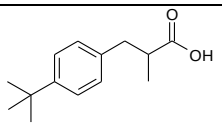
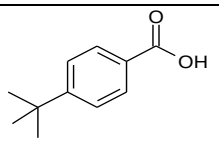
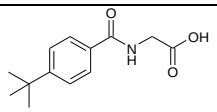
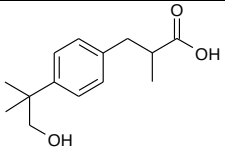
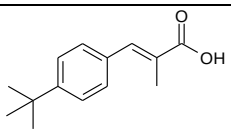
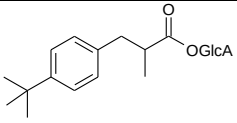
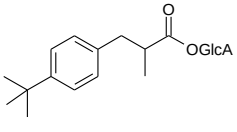
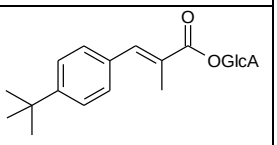
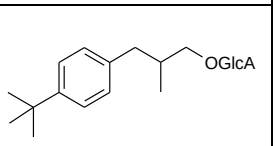
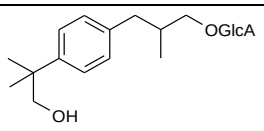


Table 15: Summary of detected metabolites in hepatocytes of different species after application of 10, 50 or 100 µM 14C-Lysmeral (BASF SE 2010) for 4 hours.

			ROI [%=rel. peak area] after incubation with 10, 50, 100 µM Lysmeral			
Metabolite(s)	**Retention time [min]**	**Structure[1)]**	**Rat**	**Mouse**	**Rabbit**	**Human**
Lysmeral	35.5-35.7		- -	- -	0.8 0.8 0.3	-
Lysmerylic acid M7	31.5-31.8		27.2 60.1 64.6	62.8 74.2 82.8	84.8 92.4 95	74.4 82.9 88.1
TBBA M15	28.7-29.0		29.3 12.7 8.3	- 0.5 -	1.3 2 1.3	7.5 3.1 1.9
TBHA M12	24.4-24.6		3.6 - 3.5	27.1 8.7 4.9	-	-
Hydroxy-lysmerlyic acid M11	23.6-24.0		8.5 7.5 8.1	4.4 6.1 4.9	13.1 3.4 2.4	8.8 6.8 5.5
(E)-3-(4-tert-Butyl-phenyl)-2-methyl-acrylic acid M16	32.4-32.5		24.9 15.5 10.7	5.8 9.7 7.4	-	3.4 2.7 1.4
Glucuronide M13	24.5-24.8		- 1.3 -	-	-	1.2 1.3 -
Glucuronide M14	25.9-26.4	  	4.6 2.8 4.8	- 0.7 -	- 1.4 1	4.7 2.7 3.1
Glucuronide M10	20.3-20.5		1.8 - -	-	-	-

			ROI [%=rel. peak area] after incubation with 10, 50, 100 µM Lysmeral			
Metabolite(s)	**Retention time [min]**	**Structure[1)]**	**Rat**	**Mouse**	**Rabbit**	**Human**

[1)]: structure elucidation was performed from the incubation in rat and human hepatocytes (100 µM)

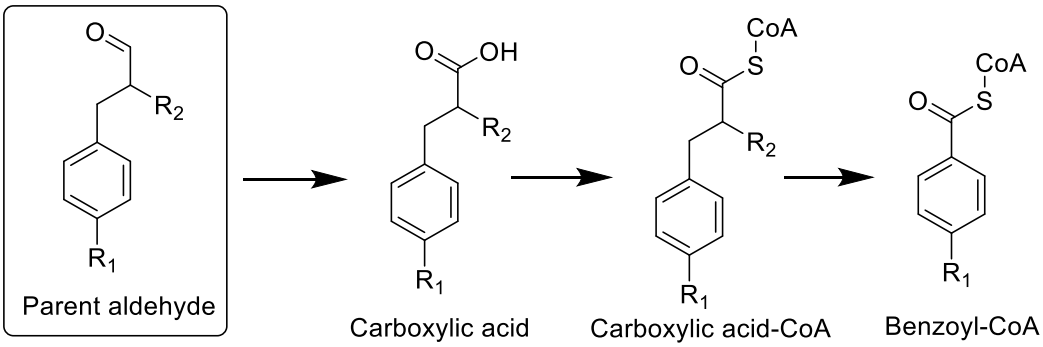
- not detectable

In vitro data in hepatocytes indicate an inhibititory capacity of TBBA on hepatic lipogenesis and gluconeogenesis (McCune et al. 1982). Addition of glycine, which represents a relevant substrate to form the respective hippurate (TBHA), did not affect TBBA inhibition of lipogenesis in the rat cells. These in vitro findings underline the lack of efficient TBHA formation capacity observed in rats in vivo. Furthermore, coenzyme A (CoA), acetyl-CoA and citrate levels were decreased in these cells. A formation and accumulation of p-tert.-benzoyl-CoA conjugates was suggested by the authors, although this could not be confirmed by the analytical methods used in the study at that time.

In a very recent study, a sensitive method in detecting specific CoA conjugates has been applied in plated rat and human primary hepatocytes by using high resolution mass spectrometry linked to liquid chromatography (LC-HRMS), (Givaudan 2017; Laue et al. 2017). Plateable primary male rat hepatocytes (Sprague-Dawley, Grade P pooled cryopreserved hepatocytes) were seeded and incubated at a density of 450,000 cells/mL in 0.25 mL seeding medium (WEM supplemented with fetal bovine serum, Dexamethasone, penicillin-streptomycin, insulin, GlutaMAX^{TM} and HEPES) on 48-well plates coated with collagen. After an attachment period of 4-5 hours, cells were kept in medium without serum (WEM supplemented with penicillin, streptomycin, insulin, hydrocortisone). The primary human hepatocytes were cultivated in a comparable manner. Two different lots (HMCPMS, lot no. HU1824 (single donor, female) and HMCPP5, lot no. HPP1870744 (5 donor, mixed gender)) were plated and incubated at a density of 700,000 cells/mL in 0.20 mL of the same seeding medium using collagen coated 48-well plates. After an attachment period of 5 hours, cells were kept in culturing medium without serum and including ITS+ (insulin, transferrin, selenium complex, BSA, and linoleic acid).

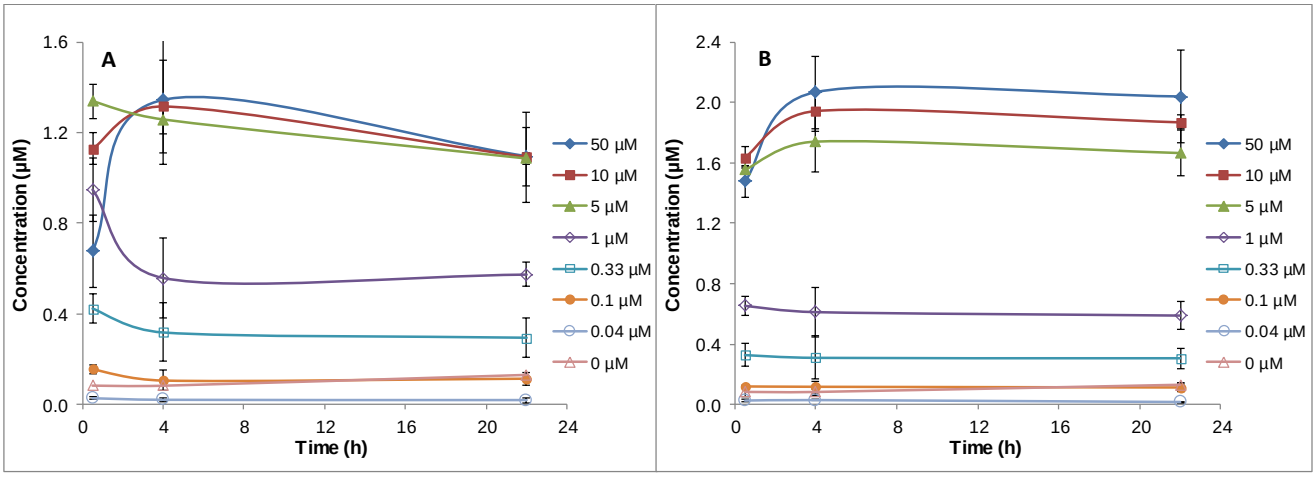
The rat and human hepatocytes were incubated with Lysmeral, Lysmeral-like materials and their relevant metabolites for 0.5-22 h at 5 and 50 µM. For analysis 2 µM decanoyl CoA and 0.2 µM n-heptadecanoyl CoA was added as internal standard, the supernatant of the cell lysates was loaded onto solid phase extraction cartridges and eluted sequentially with 50 µL acetonitrile and 50 µL water and the CoA conjugates were analysed with LC-HRMS on a Dionex UltiMate 3000 RS HPLC system coupled to a Q-Exactive orbitrap mass spectrometer with electrospray ionization (ESI) in both positive and negative ionization mode. CoA, acetyl-CoA, benzoyl-CoA, TBBA-CoA, and octanoyl-CoA served as calibration standards and the limit of detection with this method was 0.005 µM for CoA conjugates. Two types of CoA conjugates were detected with this analytical approach (Figure 2): Carboxylic acid CoA conjugates (i.e. the conjugate formed from an acid or oxidized aldehyde without degradation of the carbon side chain), or benzoyl-CoA conjugates (referring to the CoA-conjugate of a benzoic acid formed from the test chemical).

Figure 2: Structural features of the CoA conjugates, detected by LC-HRMS (Givaudan 2017)



As postulated initially by Cune et al., the recent study demonstrated, that TBBA and Lysmeral is rapidly and dose dependently transformed by rat hepatocytes to TBBA-CoA and an accumulation to stable levels occurs within 0.5-4 hours (Figure 3). This stabilization over time indicates, that TBBA - once conjugated to CoA - is not rapidly and/or quantitatively transferred to secondary acceptors such as glycine. Physiological CoA conjugate levels such as oleoyl-CoA, palmitoyl-CoA or arachidonoyl-CoA were clearly below (<0.1 µM) the TBBA-CoA levels (1-2 µM), which indicates hepatotoxicity due to a competitive inhibition of other CoA dependent cellular processes. In contrast to TBBA-CoA levels, the direct CoA conjugate of Lysmerylic acid is only transiently formed at low levels within 0.5-4 hours and not detectable after 22 hours incubation with Lysmeral. In cultures incubated with 50 µM benzoic acid, only negligible amounts of benzoyl-CoA were found after 0.5 hours incubation but benzoyl-glycine (hippuric acid) was formed. In line with the in vivo metabolism data in rats, Lysmeral or TBBA treated hepatocytes formed no TBBA-glycine conjugates.

Figure 3: TBBA-CoA conjugates detected in plated primary rat hepatocytes incubated with different concentrations of (A) TBBA and (B) Lysmeral. Plated hepatocytes were exposed to 0-50 µM TBBA and Lysmeral for 0.5, 4 and 22 h and Coenzyme A conjugates analyzed by LC-HRMS.



Plated human hepatocytes were incubated with Lysmeral and TBBA under identical conditions as rat hepatocytes, except for a slight adjustment of seeding density to account for small difference in cell size and slightly differences in media to provide optimal culture conditions. The amount and the kinetics of TBBA-CoA formation was fundamentally different between human and rat hepatocytes. Lysmeral incubation for 0.5 hours resulted in approx. 5 fold lower TBBA-CoA levels compared to rat hepatocytes and a strong decrease was found over time (Figure 4). No differences in the kinetics of the octanoyl-CoA (i.e. an endogenously formed and the most prominent CoA conjugate) was observed in untreated rat and human hepatocytes, excluding the possibility of a general loss of CoA conjugation capabilities by cell culturing over time in human cells. Findings for the two human hepatocyte lots tested were highly comparable which supports absence of effects due to a donor or sex differences. In human hepatocytes, amounts and kinetics of Lysmerylic acid-CoA were comparable to TBBA-CoA and resemble Lysmerylic acid-CoA formation in rat hepatocytes, whereas a sustained accumulation of TBBA-CoA was a unique finding only seen in rat hepatocytes. Furthermore, similar results were obtained after incubation of rat and human hepatocytes with TBBA (Figure 5). Lower levels of TBBA-CoA were detected in human compared to rat hepatocytes at 0.5 hours of incubation and a rapid and almost complete decrease of TBBA-CoA was observed within 22 hours of incubation.

Figure 4: TBBA-CoA conjugates detected in plated primary rat (A) or human (B) hepatocytes incubated with two different concentrations of Lysmeral. Plated hepatocytes were exposed to 5 or 50 µM Lysmeral for 0.5, 4 and 22 h and Coenzyme A conjugates analyzed 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, 5 pooled human donors of mixed sex (5 donors).

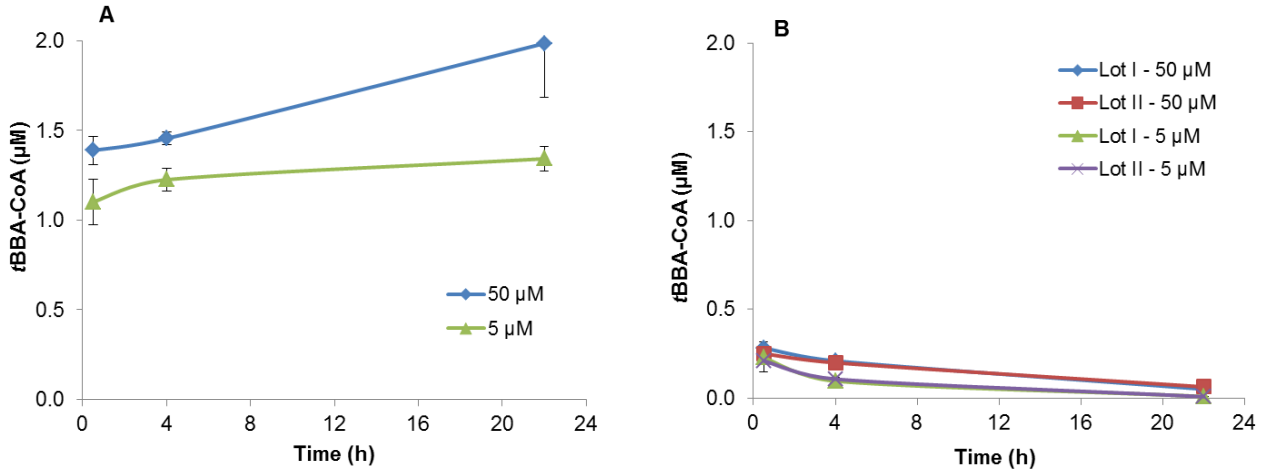
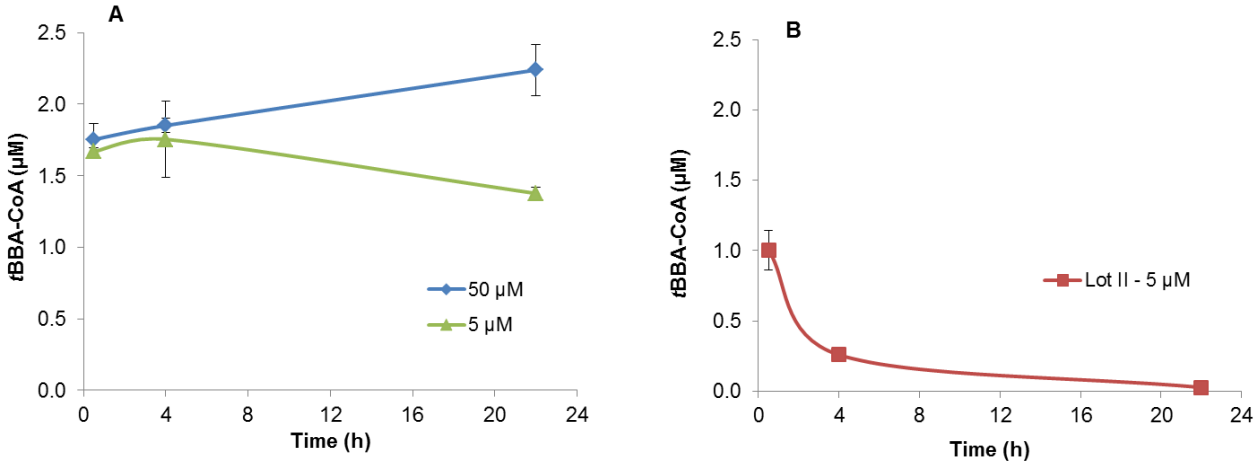


Figure 5: TBBA-CoA conjugates detected in plated primary rat (A) or human (B) hepatocytes incubated with two different concentrations of TBBA. Plated rat hepatocytes were exposed to 5 or 50 µM TBBA for 0.5, 4 and 22 h; data from one representative experiment out of three experiments (with 50 µM test concentration) and two experiments (with 5 µM) are shown. Human hepatocytes from five human donors of mixed sex were exposed to 5 µM TBBA. Coenzyme A conjugates were analyzed by LC-HRMS.



4.1.2 Human information

See Chapter 4.1.1.

4.1.3 Summary and discussion on toxicokinetics

Quantitative data on the toxicokinetics of Lysmeral (2-(4-tert-butylbenzyl)propionaldehyde) are available from rat, mouse, rabbit, guinea pig, dog and rhesus monkey and humans. Based on its physico-chemical properties, Lysmeral is considered to have a high bioavailability via the oral route and a limited bioavailability via the inhalation route. After acute and repeated oral and dermal administration of Lysmeral to experimental animals and humans there is clear evidence of systemic absorption. However, in humans only limited percutaneous absorption of Lysmeral is observed especially when compared to the rat. Distribution predominately to the liver and rapid urinary excretion has been observed in rats after dermal administration and can be assumed for the oral route as well. A detailed in vivo study on the metabolism of Lysmeral is not available.

Comparative assessment of the urinary metabolites in different laboratory animal species reveal species specific differences in the urinary excretion of p-tert-butylbenzoic acid (TBBA) and p-tert-butyl-hippuric acid (TBHA). Furthermore, these data substantiate, that TBBA is formed as common metabolite after administration of Lysmeral, p-tert-butyltoluene (TBT) or p-tert-butylbenzaldehyde (TBB) and their potency for testes toxicity correlates with systemically formed urinary TBBA levels (see chapter 4.11).

On the basis of a qualitative and quantitative evaluation of metabolic profiles for different species in an in vitro metabolism study, a predominant formation of TBBA levels in rat hepatocytes was found when compared to other rodent, non-rodent animal or human hepatocytes. The TBBA levels observed in the model using human hepatocytes were found to be approx. 4 fold lower compared to rat hepatocytes at corresponding incubation concentrations, which reflect plasma levels obtained after oral administration of Lysmeral doses below and above the lowest adverse testicular effect level.

Furthermore, the TBBA levels formed in human hepatocytes after incubation of Lysmeral concentrations related to adverse testicular effect doses were comparable to TBBA levels found in the rabbit, a species not sensitive to testicular toxicity.

In rat hepatocytes, Lysmeral and the metabolite TBBA is rapidly transformed to TBBA-CoA, which leads to an accumulation of stable levels of this conjugate. TBBA - once conjugated to CoA - is not quantitatively transferred to secondary acceptors such as glycine to form TBHA. The observed decrease of physiological CoA conjugate levels in these hepatocytes indicates a competitive inhibition of other CoA dependent cellular processes, leading to cellular toxicity. In human hepatocytes a fundamentally different kinetics was observed in TBBA-CoA formation, since no accumulation of stable conjugate levels were detectable.

Overall, species specific differences in the formation of metabolites have been clearly identified both in vitro and in vivo between responder (e.g. rat) and non-responder species (e.g. mouse, rabbit) with respect to reproductive toxicity. The species specific organ toxicity after repeated oral application of Lysmeral can be attributed to the toxic metabolite TBBA. In vitro studies show significantly lower production of TBBA in humans than in rats, with human TBBA production similar to that observed in rabbits at toxicologically relevant doses. Furthermore, the intracellular formation of stable levels of TBBA coenzyme A complexes is a rat specific effect and does not appear in human cells.

4.2 Acute toxicity

Not evaluated in this report.

4.3 Specific target organ toxicity – single exposure (STOT SE)

Not evaluated in this report.

4.4 Irritation

Not evaluated in this report.

4.5 Corrosivity

Not evaluated in this report.

4.6 Sensitisation

Not evaluated in this report.

4.7 Repeated dose toxicity

4.7.1 Non-human information

For the purpose of this report, evaluation of available data on repeated dose toxicity is mainly focused on adverse effects on the male reproductive organs. However, other adverse effects are discussed in the context of reproductive toxicity. Studies designed for the assessment of fertility and developmental toxicity are discussed in chapter 4.11. Short-term studies in the rat with an application period of 1 to 14 days have been performed for the oral and dermal route. In addition, short-term oral testing for 5 days has been performed in the mouse, guinea pig, and rhesus monkey. Long-term studies are available for the rat and the dog for the oral route.

General adverse effects, such as decreases in body weights and food consumption and/or clinical signs of toxicity were observed after oral administration of Lysmeral (2-(4-tert-butylbenzyl) propionaldehyde). In studies applying more detailed clinical chemistry and histopathological examinations, adverse effects on the liver became evident. At the dose levels showing general and liver toxicity, adverse testicular effects of Lysmeral after oral administration were found as well. Several of the present repeated doses studies mainly focused on testicular toxicity but did not provide full information on liver toxicity after oral administration of Lysmeral. However, when assessed, no testicular toxicity in the absence of other toxicological findings was observed.

Considering the repeated short and long term oral administration studies in rats, a clear effect level for testicular toxicity can be set at 50 mg/kg bw/day. The effect levels observed did not differ between short term (≤ 14 day) and subchronic (90 day) application periods. Adverse testicular findings were observed already after a single oral administration, suggesting acute effects on male reproductive organs. In contrast, dermal administration in rats led to testicular toxicity only at an excessive dose level (above the limit dose), whereas at the limit dose of 1000 mg/kg body weight, no adverse testicular effects were observed. In dogs, general adverse effects, together with liver and testicular toxicity was observed after oral administration at higher dose levels, i.e. 200 mg/kg bw/day. Thus, dogs were found to be less sensitive concerning testicular toxicity after orally administered Lysmeral than rats.

No testicular toxicity was observed in the mouse, guinea pig, rabbit and primates, substantiating species specificity for the testicular toxicity observed after oral administration of Lysmeral. For a summary of available studies with focus on adverse effects of male reproductive organs, see Table 19 in chapter 4.11.

4.7.1.1 Repeated dose toxicity: oral

Rodent studies.

In a 90 day rat oral toxicity study (following OECD 408 with a few deviations), six test groups each consisting of 14 animals per sex were dosed with Lysmeral (analytical purity 97.8%) via gavage with 2, 5, 25, and 50 mg/kg body weight/day (five days a week) (Givaudan 1986A). For the high dose group a satellite group of 14 animals per sex was included for a post-treatment period of 4 weeks. As an adverse clinical sign, alopecia was observed in the females of the high dose group. Organ specific toxicity included the liver, as seen by elevated absolute (24% - 45% and 57% - 69% increase in males and females respectively) and relative (21% - 45% and 59% - 75% increase in males and females respectively) liver weights starting at 25 mg/kg bw/day. A histopathologic correlate (hepatic lipid droplet content) at 50 mg/kg was observed in both genders. Furthermore, a significant decrease in plasma cholinesterase activity ranging from 30% to 70% of respective controls and lower plasma cholesterol levels ranging from 40% to 70% of respective controls at 25 and 50 mg/kg bw/day in both genders was detected.

A slight but significant increase in aspartate aminotransferase activity was observed in males of the high dose group, whereas other liver enzymes such as alanine aminotransferase activities were not influenced. Effects on clinical chemistry were reversible in the recovery group. In addition, in female rats treated with 25 and 50 mg/kg bw/day, elevated absolute (16% - 30% increase) and relative (18% - 36% increase) weights of adrenal glands and hypertrophy of the zona fasciculata were observed. Findings in both, adrenals and liver were shown to be reversible in the recovery group.

In parallel, test substance related testicular toxicity such as spermatoceles in the epididymides and testicular atrophy was observed at 50 mg/kg bw/day (Table 16). Disturbances of spermatogenesis and spermiogenesis, testicular increases in Sertoli cell-only tubules and increased surface density in Leydig cells were described along with a decreased density of spermatozoa, nucleated cells and spermatoceles in the epididymides of the high dose animals. In the 4 week recovery group, the same testicular pathology was observed to a lesser extent. In the lower dose groups (2-25 mg/kg bw/d) either no or low incidences of pathological findings in testes and epididymides comparable to the findings in control group animals were observed. Therefore, a NOAEL for testicular toxicity effects is set at 25 mg/kg bw/day.

Table 16: Histological findings in testes and epididymides in a 90 day rat oral toxicity study (Givaudan 1986A).

Dose group (mg/kg bw/d)	Histological findings (percentage of organs affected)
0	2/14 animals: • Disturbed spermatogenesis (11%) • Sertoli cell-only tubules (11%) • Increased surface density of Leydig cells (11%) • Decreased density of spermatozoa in epididymides* (11%) • Nucleated cells in epididymides* (11%) • Increased number if unusual clear cells in epididymides* (11%)
2	No pathologic findings in the testes and epididymides
5	1/14 animals: • Disturbed spermatogenesis (7%) • Sertoli cell-only tubules (7%) • Increased surface density of Leydig cells (7%)
25	1/14 animals: • Unilateral disturbance of spermatogenesis (4%) • Sertoli cell-only tubules (4%) • Decreased density of spermatozoa in epididymides* (4%) • Increased number if unusual clear cells in epididymides * (4%)
50	14/14 animals: • Disturbed spermiogenesis (21%) • Disturbed spermatogenesis, (29%) • Sertoli cell-only tubules (29%) • Increased surface density of Leydig cells (21%) • Decreased density of spermatozoa in epididymides* (80%) • Nucleated cells in epididymides* (100%) • Spermatoceles in the epididymides* (67%)
50 Recovery group	14/14 animals: • Disturbed spermatogenesis (27%)

(4 weeks)	• Sertoli cell-only tubules (27%) • Increased surface density of Leydig cells (8%) • Decreased density of spermatozoa in epididymides* (33%) • Nucleated cells in epididymides* (67%) • Spermatoceles in the epididymides* (79%)

* Only evaluated in epididymides without spermatocele(s)

Oral administration of Lysmeral (analytical purity 99.1%) and Lysmerylic acid to rats (50 mg/kg bw/day by gavage) for 1, 2, 3, 4 or 14 days was performed in a study with main focus on male reproductive organs (BASF SE 2006A). This study aimed for the comparison of Lysmeral and Lysmerylic acid in terms of potency, time dependency of adverse testicular and spermatotoxic effects and species specificity based on an analogous study performed in mice ((BASF SE 2006B). In rats, slight to severe testicular atrophy with an incidence of 2/5 animals for Lysmeral and 3/5 animals for Lysmerylic acid after a single application, and in all animals after longer application periods was observed (BASF SE 2006A). Generally, testicular effects were described as diffuse tubular testicular degeneration, fine vacuolar change of pachytene spermatocytes up to apoptotic cell death. Furthermore, sperm parameter examined i.e. sperm motility, spermatid count in testes, cauda epididymal sperm count, and sperm morphology were affected solely after an application period of 14 days, as seen for two test substances. Although not statistically significant, body weight gains were found to be decreased by 25% and 20% below controls after application of Lysmeral and Lysmerylic acid for 14 days.

For comparison, oral administration of Lysmeral and Lysmerylic acid via gavage in mice (50 mg/kg bw/day) for 1, 2, 3, 4 or 14 days led to a reduction in the ratio of normal to abnormal sperm in animals exposed only for 3 and 4 days (BASF SE 2006B). However, other treatment periods did not influence this parameter and other sperm parameters, i.e. sperm motility, spermatid count in testes or cauda epididymidis. Single administration (1 day) of Lysmerylic acid led to a significant reduction in the ratio of normal to abnormal sperm and reduced total sperm numbers in cauda epididymidis in the group exposed for 2 days was observed. All other examined sperm parameters were not influenced for all treatment periods. For both substances, macroscopic and microscopic evaluation of the testes revealed no pathological changes in all groups observed. A statistically non-significant decrease in body weight gains (33% below controls) was found after application of Lysmerylic acid after 14 days. Since the changes observed in single sperm parameters were inconsistent, did not follow a kinetic and were not verified histologically in testes, a substance related origin is unlikely. In line, a further study supports the absence of testicular toxicity in mice, as presented further below.

As supportive evidence, several oral gavage studies for 5 consecutive days in rats at doses ranging from 25 to 400 mg/kg bw/day confirmed clinical signs of toxicity, body weight loss and macroscopic changes in the liver starting from 50 mg/kg bw/day Lysmeral. At same dose levels, changes in seminiferous epithelium with degenerated/reduced numbers of germ cells, were found, whereas decreased testes and kidney weights and decreased sizes of prostate & seminal vesicles became evident at higher dose levels (Givaudan 1986B, Givaudan 1991A, Newberne 1990A).

However, oral administration of 100 mg/kg bw/day Lysmeral for five consecutive days in male mice or guinea pigs showed neither any general adverse systemic effects nor adverse effects on the male reproductive organs (Givaudan 1983; Newberne 1990).

Five male albino SPF mice and 5 male Himalayan spotted SPF guinea-pigs (each from Institute of Biological and Medical Research, Füllinsdorf, Switzerland) were orally administered with 100 mg of Lysmeral suspended in rape oil per kg body weight per day. Furthermore, 5 mice and 5 guinea-pigs were dosed in an identical manner with the vehicle. All animals were treated once daily, for 5 consecutive days.

At commencement of treatment, individual body weights were 43 – 61 g for mice and 604 – 676 g for guinea pigs. Animals were acclimatized for 6 days before treatment, were individually caged (temperature 19 – 23° C, mean relative humidity 45 – 65 %, artificial light for 12 hours) and were allowed free access to food and tap water. After the 5th treatment day, all animals were kept in individual metabolism cages for 24 hours to collect urine. Mortality, general symptoms, and body weight development were recorded once daily. After a gross necropsy, testes of all animals were weighed and fixed in mixture of Bouin and embedded in Paraplast Plus.

Testes were sectioned at a nominal thickness of 5 µm and stained with haematoxilin and eosin. Testes and epididymides of all animals were microscopically examined. The condition of the seminiferous tubules of all animals was semiquantitatively evaluated. One hundred cross-sectioned seminiferous tubules were examined per testis cross section. During the inspection, each testis section was meanderingly moved under the microscope and every two (mice) or every three or five (guinea-pigs) cross-sectioned seminiferous tubules were graded. Graduation of the seminiferoµs tubules:

0) normal cellularity of the epithelium

1) normal cellularity of the epithelium, however with some to many degenerated cells or detritus in the lumen of the seminiferous tubule

2) many degenerated cells in the epithelium and disorganization of the epithelial structure

3) severe destruction of the epithelium

All mice and all guinea-pigs survived the test period. Symptoms of incompatibility were not seen. The body weight development was normal. Necropsy findings were observed in the heart, liver, and lung of 1 control mouse as well as in the lung of 1 treated mouse. Changes seen in the guinea-pigs were fine concrements in the milky contents of the urinary bladder (5 control guinea-pigs, 2 treated guinea-pigs) and white spots or regions in the liver (4 control guinea-pigs, 3 treated guinea-pigs). All changes were not considered to be related to treatment because they were incidental and distributed among control and treated animals.

No changes were observed in testes and epididymides of all animals. Absolute and relative testes weights of control and treated animals were comparable. There was no difference between germinal epithelium of control animals and that of treated animals. In mice treated with Lysmeral, 79.6%, 19.6%, 0.7%, 0.1% of the tubuli seminferi were graded 0, 1, 2 and 3 respectively (versus 77.1%, 22.1%, 0.4%, 0.4% for the grades 0, 1, 2 and 3 in control animals, respectively). In line with these results, in guinea pigs treated with Lysmeral, 86.2%, 13.5%, 0.3%, 0.0% of the tubuli seminferi were graded 0, 1, 2 and 3 respectively (versus 87.8%, 12.2%, 0.0%, 0.0% for the grades 0, 1, 2 and 3 in control animals, respectively).

Non-rodent studies.

In a pilot study, Lysmeral (analytical purity 95%) was administered to two male beagle dogs by oral administration via gelatine capsules in subsequently increasing doses (47 -564 mg/kg bw/day) for 9 weeks (Givaudan 1990A). As general adverse effects, occasional vomiting in both animals, diarrhoea in one animal and body weight reduction together with an increase in clinico-chemical parameters (glutamate dehydrogenase, alanine aminotransferase) was found. Histological examinations revealed multifocal inflammation in the liver of the two animals. In parallel, these dogs showed mild atrophy in seminiferous tubules (necrosis of germ cells, multinucleated giant cells in tubular lumen).

Further studies in beagle dogs were performed, i.e. administration of 4.4, 22.3 or 44.6 mg/kg bw/day Lysmeral (analytical purity 97.6%) to each 3 male/female dogs (Givaudan 1990B) or 200 mg/kg bw/day to 3 female dogs for 90 days in gelatine capsules (Givaudan 1990C). In the former study occasional diarrhoea at 22.3 or 44.6 mg/kg bw/day and vomiting at the high dose group was observed but no other alterations and no findings from the latter study were attributable to treatment. In male animals, no alterations on reproductive organs were observed.

Based on the indications of adverse testicular effects observed in the two dogs of the pilot study (Givaudan 1990A), a testicular toxicity screening study in beagle dogs at comparable dose levels was performed for further confirmation. This study intended to clarify, whether testicular toxicity after oral administration of Lysmeral occurs in a non-rodent species. In this study, Lysmeral (analytical purity 99.1%) was administered to groups of 4 purebred male Beagle dogs via gelatine capsules at dose levels of 0, 40, 200 and 1000/500 mg/kg bw/day for 2 weeks (reduction of dose levels in the high dose group due to vomitus and diarrhoea) (BASF SE 2008A). Besides clinical/hematological examinations, urinalyses and a gross-pathological assessment, specific histopathological investigation on reproductive organs and liver was performed.

Systemic effects, such as a retardation in body weight gains and body weight loss in distinct animals together with decreases in food efficiency were observed in combination with vomitus and soft faeces/diarrhea in all animals of the mid and high dose group. Furthermore, significant absolute and relative liver weight increases between 30-40% above control values, and centrilobular hypertrophy of hepatocytes were observed in the mid and high dose groups.

Distinct clinical parameters were altered, i.e. prolongation in activated partial thromboplastin time, increases in serum magnesium, potassium and inorganic phosphate levels in mid/high dose animals and decreased glucose levels in high dose animals. Decreases in aspartate aminotransferase and alanine aminotransferase were found in mid/high dose animals.

A massive diffuse degeneration of seminiferous tubules combined with a hyperplasia of Leydig cells in the testes and an aspermia and epithelial vacuolation in the epididymides was found in one dog of the mid dose, which showed also a decrease in relative testis weights. Furthermore, a reduced size of testes and epididymides was observed in this animal. A second animal in the mid dose group showed a slight, one-sided and focal degeneration of seminiferous tubules, which was observed in historical control data as well and might therefore be considered as spontaneous in nature. In contrast, no such adverse testicular effects were observed in animals of the low and high dose group. Decreases in prostate sizes were observed in low dose and high dose group animals. However, due to the lack of histopathological findings and the absence of a dose response relationship, these effects are not considered to be substance related.

To further clarify the findings of the study above, a follow-up study, involving a higher animal number per dose group and additional andrological/ spermatological examinations prior and during the test substance administration period was performed. Lysmeral was administered to groups of 10 male purebred Beagle dogs via gelatine capsules at concentrations of 0 and 200 mg/kg body weight/day for 2 weeks (BASF SE 2008B).

Mean body weight loss (-0.2 kg compared to 0.1 kg in controls after day 14) due to mainly 2 of 10 animals with a massive body weight loss were observed, together with a slightly reduced food consumption (up to 25% below controls) starting from day 3 onwards. In line, a negative value for food efficiency was found. Vomitus in 7 of 10 animals and diarrhoea in 4 of 10 animals was observed as further parameters for systemic toxicity. Significant increases in absolute and relative liver weights (14% and 17% above controls, respectively) with centrilobular hypertrophy of hepatocytes became evident in the dosed animals.

Furthermore, distinct clinical parameters were statistically significantly altered (values refer to respective mean control levels after day 14), i.e. increases in alanine aminotransferase by 80% and aspartate aminotransferase activities by 310%, prolongation in activated partial thromboplastin time by 10%, decrease in serum triglyceride levels by 35% compared to control. Decreases in red blood cell counts and haemoglobin by 5%, and hematocrit values by 10% together with a decrease in reticulocyte counts by 60% indicate an anemic situation after test substance application.

Increases in serum urea by 45%, creatinine by 25%, calcium by 5% and magnesium levels by 20% indicate adverse effects on the kidneys, however, no kidney weight changes were observed.

Decreases in absolute and relative testes weights by approx. 25% along with a slight to severe degeneration of seminiferous tubules in 9 of 10 animals were observed. Unilateral decrease in testicular length or width was found in 6 of 10 animals.

Furthermore, effects on spermatological parameters, i.e. decrease of progressively motile spermatozoa and/or morphological alterations were found in 9 of 10 dogs after treatment when compared to the values of the respective animals before treatment.

Morphological sperm alterations consisted mainly of mid-piece anomalies (cytoplasmatic droplets) and less frequently in sperm neck anomalies (paraxial tail attachment, cytoplasmatic droplets). Prostate weights were slightly decreased and respective minimal to moderate multifocal atrophies were found in 3 of 10 animals.

A screening study on the male reproductive function in rabbits was performed in order to clarify, whether testicular toxicity or spermatotoxic effcts after oral administration of Lysmeral occurs in a further non-rodent species.

In this screening study, rabbits were treated via gavage for 15 days at doses of 30, 100 and 300 mg/kg bw/day Lysmeral (analytical purity 99.1%; BASF SE 2008C). No test substance related findings on clinical observations, body weights and food consumption were observed in all dosing groups. Neither testes nor cauda epididymis weights were affected. A moderate diffuse degeneration of the seminiferous tubules combined with a moderate oligospermia and a moderate mixed inflammation in the epididymides was observed in 1/5 animals of the low dose group. The inflammation found in the epididymides is assumed to be causative for the degenerative changes in the testis. In the mid dose group, a reduced testes and epididymides size with severe diffuse degeneration of seminiferous tubules in the examined left testis and a severe atrophy plus aspermia in the left epididymides was observed in 1/5 animals. However, sperm evaluation did not reveal any treatment related effect in this or any other treated animal.

Based on the absence of a dose response relationship, the isolated occurrence in one single animal and the absence of adverse effects on spermatological parameters in the respective animal, a treatment related origin of the observed findings seem unlikely.

In a study on primates, using a limited number of animals, oral administration of 100 mg/kg bw/day Lysmeral for 5 days did not lead to any general adverse effects or testicular toxicity (Newberne 1990; Givaudan 1984G). In this study using 2 male rhesus monkeys (Macaca mulatta), clinical signs of toxicity and mortality was monitored and animals were weighed at test day 1 and 6. Animals were sacrificed by perfusion with glutaraldehyde and subjected to a complete necropsy. All organs and tissues were examined grossly and testes and epididymides were examined by histopathology. No endpoints related to liver were investigated. No incompatibility reactions were seen in clinical observations during the in-life period and body weights were not significantly affected. In histological examinations, only small foci in one epididymis of one animal and small hollow spaces in the epithelium of one epididymis of the other animal was observed. The testes of both animals were found to be free of lesions. Seminiferous tubules with orderly arrangement of intact stages of spermatogenesis was predominantly found whereas decreased numbers of spermatozoa per tubule to less than 10 was a minor finding. Unlike effects observed in rats, no tubules with complete loss of cells, sertoli-cell-only-syndrome or with slight/severe reduction and destruction of the germinal epithelium was observed in both testes of the two animals.

The findings in one of the epididymis of each animal does not represent a test substance related effect, since other male reproductive tissues were not affected. Overall, general and testicular toxicity was not observed under the conditions of this study in primates.

4.7.1.2 Repeated dose toxicity: inhalation

No data available

4.7.1.3 Repeated dose toxicity: dermal

Dermal administration of Lysmeral (analytical purity 99.1%) to rats 6 hours per day for 5 days (250, 500 1000, 2000 mg/kg bw/day) caused very slight decrease in body weights by 2% and marked testicular atrophy at the high dose only (Givaudan 1991A). Seminiferous tubules with disorganization of the epithelial structure, decrease of the number of germ cells, increase of the number of degenerating germ cells (inclusive giant cells) were observed in combination with immature/degenerating germ cells in epididymides and the occurrence of spermatocele. No clinical signs and substance related necropsy findings were observed. No further observations were performed in this study to assess adverse effects other than testicular toxicity.

4.7.1.4 Repeated dose toxicity: other routes

No data available

4.7.1.5 Human information

No data available

4.7.1.6 Other relevant information

In vitro data using primary rat hepatocytes indicate inhibition of hepatic lipogenesis and gluconeogenesis by Lysmeral metabolites (McCune et al. 1982). TBBA has been shown to inhibit fatty acid synthesis and glucose synthesis. An inhibition of fatty acid synthesis by 50% required 5-10 µM TBBA. Addition of glycine, a pivotal substrate for hippurate (TBHA) formation, had no effect on TBBA inhibition of lipogenesis. Furthermore TBBA treatment decreased CoA, acetyl-CoA and citrate levels and addition of octanoate protected against the inhibitory effect of TBBA on lipogenesis. In a recent in vitro study in rat hepatocytes, TBBA and Lysmeral were found to rapidly and dose dependently transform to TBBA-CoA conjugates and an accumulation to stable levels occurs (Givaudan 2017, see Chapter 4.1).

As established by these in vitro studies, liver toxicity observed for Lysmeral is likely to be caused by disruption of CoA-dependent metabolic processes triggered by the metabolite TBBA. The formation of TBBA-CoA might either be directly toxic or result in the inhibition of CoA dependent processes. Xenobiotic-acyl-S-CoA thioesters have been described to covalently modify proteins (Darnell et al. 2015; Lassila et al 2015), interfere with endogenous lipid metabolism (Darnell et al. 2013) or deplete the CoA pool. Since the CoA pool is important in a large number of catabolic and anabolic biochemical reactions, but is rather small and cannot be increased quickly, a disruption of a permanent turnover of acyl-CoAs might impair many biochemical pathways (Brass 2002).

The disruption of CoA dependent processes has been confirmed in vivo based on metabolome analyses of Lysmeral and structurally related substances (BASF 2017D). In these studies, Wistar rats were treated for 28 days via gavage with Lysmeral (15 and 45 mg/kg bw/d; two independent experiments). For comparison, the related alcohol Lysmerol (10 and 50 mg/kg bw/d) or meta-Lysmeral (150 and 450 mg/kg bw/d) were administered to Wistar rats accordingly. For each dose group five male and five female animals were used, while the control group consisted of 10 untreated males and 10 untreated females. During the study, the animals were allowed free access to mouse/rat laboratory diet (Provimi Kliba SA, Kaiseraugst, Switzerland) and water (except before blood sampling). For the metabolome analysis of Lysmeral and Lysmerol treated animals, blood samples were taken from retro-orbital venous plexus after 7, 14 and 28 days, respectively. Meta-Lysmeral treated animals were sampled after 28 days only. The blood was centrifuged and plasma metabolome was examined by Metanomics GmbH using GC/MS and LC/MS-MS techniques. Briefly, three types of mass spectrometry analysis were applied to all samples: GC-MS (gas chromatography-mass spectrometry) and LC-MS/MS (liquid chromatography-MS/MS) were used for broad profiling of physiologic metabolites, as described in detail by Roessner et al. (2000) and van Ravenzwaay et al. (2007). Steroid hormones, catecholamines and their metabolites were measured by online SPE-LC-MS/MS (Solid phase extraction-LC-MS/MS; Yamada et al., 2002). For all these metabolites, changes in plasma were calculated as the ratio of the mean of metabolite levels in individual rats in a treatment group relative to mean of metabolite levels in rats in a matched control group (time point, dose level, sex). The ratio of all metabolites assessed in Lysmeral treated animals are listed in Annex 4.

For the identification of biologically relevant changes in the metabolome, common significant changes ($p = 0.05$) for male animals (treated with 45 mg/kg BW/d Lysmeral) have been identified in plasma derived from two independent rat studies. Mean changes of metabolite levels were considered relevant, if statistically significant and changed into the same direction in 4 out of 6 timepoints investigated. The dose chosen is associated with Lysmeral induced liver and testicular toxicity, as seen in other repeated dose and reproductive toxicity studies.

In comparison to these changes, the metabolome ratios of the respective metabolites for Lysmerol and meta-Lysmeral treated male rats have been added (for further information on the methodology refer to Kamp et al., 2012 and van Ravenzwaay et al., 2015). The metabolites showing common significant changes are listed in Table 17.

By comparing the ratios of the 204 different metabolites assessed, a vast majority of metabolites that are commonly changed between the two independent studies represent lipids, fatty acids and fatty acid related metabolites. Plasma levels of all these metabolites are decreased when compared to respective untreated animal levels. These decreases are generally seen after 7, 14 and 28 days exposure. When compared with the metabolite changes induced by Lysmerol treated rats, which is known to lead to comparable testicular toxicity as Lysmeral treatment, a very comparable pattern and a predominant decrease of complex lipids and fatty acid class associated metabolite levels were observed. In contrast, meta-Lysmeral treatment has been shown to lead to no testicular toxicity phenotype and does provide a divergent metabolite pattern especially for complex lipids such as sphingolipids, ceramides and phosphatidylcholines.

Table 17: Common significantly changed plasma metabolites of two independent rat metabolome studies with Lysmeral. Significantly changed metabolites (p = 0.05) found in the high dose group of 2 independent rat studies 7, 14 and 28 days after oral Lysmeral administration are listed as a ratio of the mean of metabolite levels in individual rats in a treatment group relative to mean of metabolite levels in rats in a matched control group (time point, dose level, sex). In comparison, ratios of the respective metabolites for Lysmerol and meta-Lysmeral treated rats have been added. Significant changes to respective controls (p = 0.05) were marked in red (increase) or yellow (decrease).

			45 mg/kg bw/d			45 mg/kg bw/d			50 mg/kg bw/d			450 mg/kg bw/d
			Lysmeral			**Lysmeral**			**Lysmerol**			**m-Lysmeral**
Metabolite	**Class**	**Subclass**	day 7	day 14	day 28	day 7	day 14	day 28	day 7	day 14	day 28	day 28
Threonine	**Amino acids and related**	Amino acids. neutral	**1.46**	**1.74**	**2.15**	**1.40**	1.42	**1.79**	**1.92**	**2.63**	**3.06**	0.87
Glutamate		Amino acids. acidic	0.70	0.77	0.76	0.72	0.79	0.61	0.87	0.86	0.77	1.30
Lysine		Amino acids. basic	**6.00**	**6.53**	**8.10**	**4.26**	**6.97**	**5.12**	**8.85**	**4.49**	**5.13**	0.84
Glutamine		Amino acids. basic	0.57	0.80	0.81	0.77	0.72	0.62	0.73	0.68	0.64	0.92
Serine		Amino acids. neutral	**1.37**	**1.54**	**1.69**	**1.25**	**1.32**	**1.61**	**1.92**	**2.06**	**2.57**	1.12
trans-4-Hydroxyproline		Collagen metabolism	0.99	0.85	0.71	0.54	0.58	0.53	0.72	0.67	0.61	0.55
Glycerol. lipid fraction	**Complex lipids. fatty acids and related**	Fatty alcohols	0.33	0.60	0.57	0.29	0.42	0.72	0.37	0.59	0.50	0.86
Palmitic acid (C16:0)		Fatty acids. saturated	0.42	0.56	0.48	0.35	0.43	0.52	0.48	0.52	0.61	1.13
Linoleic acid (C18:cis[9.12]2)		Fatty acids. poly-unsaturated	0.44	0.57	0.38	0.29	0.33	0.49	0.51	0.60	0.62	0.63
Stearic acid (C18:0)		Fatty acids. saturated	0.34	0.51	0.55	0.41	0.67	0.57	0.54	0.58	0.61	0.58
Arachidonic acid (C20:cis[5.8.11.14]4)		Fatty acids. poly-unsaturated	0.21	0.39	0.49	0.26	0.47	0.51	0.44	0.50	0.56	0.77
Glycerol phosphate. lipid fraction		Phospholipid metabolites	0.21	0.54	0.47	0.22	0.46	0.39	0.49	0.54	0.66	0.75
Galactose. lipid fraction		Glycolipids	0.43	0.56	0.66	0.36	0.85	0.74	0.62	0.61	0.68	0.79
Lignoceric acid (C24:0)		Fatty acids. saturated	0.36	0.52	0.60	0.45	0.49	0.61	0.54	0.69	0.76	1.00
Heneicosanoic acid (C21:0)		Fatty acids. saturated	0.93	0.90	0.88	0.71	0.80	0.85	0.99	0.86	0.87	0.94
Heptadecanoic acid (C17:0)		Fatty acids. saturated	0.56	0.59	0.56	0.43	0.60	0.58	0.58	0.52	0.63	0.79
Phosphate. lipid fraction		Phospholipid metabolites	0.49	0.77	0.58	0.66	0.74	0.70	0.65	0.69	0.68	0.81
Myristic acid (C14:0)		Fatty acids. saturated	0.54	0.59	0.53	0.32	0.42	0.75	0.43	0.39	0.63	0.60
myo-Inositol-2-phosphate.		Phospholipid metabolites	0.09	0.49	0.22	0.10	0.44	0.21	0.37	0.27	0.63	0.44

lipid fraction (myo-Inositolphospholipids)												
Behenic acid (C22:0)		Fatty acids. saturated	0.53	0.57	0.80	0.66	0.73	0.70	0.73	0.79	0.83	0.73
Nervonic acid (C24:cis[15]1)		Fatty acids. mono-unsaturated	0.32	0.52	0.51	0.32	0.58	0.69	0.45	0.41	0.52	0.68
Isopalmitic acid (C16:0)		Fatty acids. branched	0.46	0.49	0.34	0.51	0.42	0.35	0.32	0.30	0.32	0.55
16-Methylheptadecanoic acid		Fatty acids. branched	0.37	0.41	0.26	0.32	0.34	0.30	0.41	0.38	0.38	0.37
17-Methyloctadecanoic acid		Fatty acids. branched	0.39	0.42	0.31	0.40	0.49	0.38	0.39	0.42	0.38	0.23
3-O-Methylsphingosine (d18:1)		Sphingolipids	0.22	0.45	0.48	0.27	0.31	0.34	0.49	0.57	0.56	0.91
threo-Sphingosine (d18:1)		Sphingolipids	0.25	0.50	0.51	0.37	0.31	0.51	0.55	0.53	0.64	0.84
Docosapentaenoic acid (C22:cis[7.10.13.16.19]5)		Fatty acids. poly-unsaturated	0.27	0.54	0.37	0.25	0.38	0.47	0.39	0.57	0.54	0.49
5-O-Methylsphingosine (d18:1)		Sphingolipids	0.19	0.48	0.48	0.25	0.33	0.29	0.48	0.56	0.61	0.78
erythro-Sphingosine (d18:1)		Sphingolipids	0.21	0.51	0.51	0.29	0.37	0.37	0.52	0.61	0.63	0.92
myo-Inositol. lipid fraction		Glycolipids	0.41	0.57	0.60	0.41	0.66	0.63	0.54	0.69	0.72	0.88
Sphingomyelin (d18:2.C16:0)		Sphingomyelins	0.58	0.53	0.55	0.87	0.55	0.60	0.63	0.62	0.69	1.13
Phosphatidylcholine (C18:1.C18:2)		Phosphatidylcholines	0.74	0.79	0.83	0.88	0.75	0.76	0.92	0.85	0.78	1.10
Ceramide (d18:1.C24:0)		Ceramides	0.59	0.59	0.44	0.60	0.56	0.48	0.57	0.67	0.66	1.37
TAG (C16:0.C18:2)		Triacylglycerols	0.48	0.55	0.50	0.21	0.29	0.75	0.44	0.67	0.52	0.93
Lysophosphatidylcholine (C20:4)		Lysophosphatidylcholines	0.87	0.95	0.94	0.95	0.93	0.92	0.82	0.95	0.84	1.03
Sphingomyelin (d18:1.C16:0)		Sphingomyelins	0.84	0.88	0.79	0.94	0.88	0.86	0.81	0.83	0.96	1.07
Ethanolamine plasmalogen (C39:4)		Plasmalogens and other ether lipids	0.51	0.66	0.60	0.78	0.61	0.40	0.68	0.49	0.60	0.92
Phosphatidylcholine (C16:0.C20:4)		Phosphatidylcholines	0.83	0.86	0.90	0.96	0.94	0.94	0.98	0.96	0.98	1.00
Sphingomyelin (d18:1.C24:0)		Sphingomyelins	0.52	0.62	0.64	0.65	0.64	0.56	0.65	0.63	0.63	1.11
Unknown lipid (28000473)	**Unknown lipids and others**		0.47	0.46	0.55	0.57	0.58	0.51	0.56	0.66	0.52	0.40
Unknown lipid (68000052)			0.69	0.79	0.91	0.84	0.86	0.80	0.91	0.93	0.92	0.82
Unknown lipid (68000060)			0.59	0.62	0.65	0.57	0.65	0.73	0.66	1.47	2.14	**1.56**
Choline plasmalogen (C36:2) (putative)		Unknown lipid	0.84	0.64	0.63	0.90	0.64	0.60	0.79	0.75	0.83	0.84

PC No 04 (putative)		Unknown lipid	0.41	0.60	0.49	0.64	0.72	0.66	0.48	0.47	0.42	0.68
Lyso PE (C22:0) (putative)		Unknown lipid	0.58	0.65	0.62	0.64	0.54	0.54	0.78	0.92	1.17	0.34
Phosphatidylcholine (C18:0.C20:3)			0.57	0.56	0.68	0.69	0.66	0.60	0.70	0.65	0.67	0.75
Cholesterol. total			0.30	0.47	0.49	0.29	0.42	0.47	0.51	0.45	0.56	0.76
3-Hydroxybutyrate	**Energy metabolism and related**	Ketone bodies	0.24	0.30	0.53	0.25	0.34	0.47	0.31	0.34	0.50	0.78
2-Hydroxybutyrate		Energy metabolism. miscellaneous	**6.75**	**8.07**	**11.12**	**7.80**	**10.13**	**8.72**	**7.10**	**4.28**	**5.94**	0.37
Homovanillic acid (HVA)	**Hormones. signal substances and related**	Catecholamine catabolites	0.29	0.52	0.31	0.59	0.18	0.40	0.27	0.19	0.39	1.25
Metanephrine		Catecholamine catabolites	0.50	0.60	0.42	0.57	0.66	0.68	0.82	0.73	1.11	0.82
beta-Sitosterol	**Miscellaneous**	Diet related	0.43	0.57	0.55	0.54	0.52	0.62	0.49	0.44	0.44	1.01
Campesterol		Diet related	0.24	0.43	0.35	0.33	0.39	0.42	0.41	0.31	0.37	1.13
alpha-Tocopherol	**Vitamins. cofactors and related**	Tocopherols and related	0.52	0.56	0.61	0.51	0.39	0.44	0.43	0.42	0.49	0.97
Coenzyme Q9		Redox-carrier and related	0.21	0.27	0.31	0.30	0.19	0.17	0.32	0.35	0.36	0.84

4.7.1.7 Summary and discussion of repeated dose toxicity

For a detailed discussion of repeated dose studies and studies designed for the assessment of fertility, see Chapter 4.11.

4.8 Specific target organ toxicity (CLP Regulation) – repeated exposure (STOT RE)

Not evaluated in this report.

4.9 Germ cell mutagenicity (Mutagenicity)

Not evaluated in this report.

4.10 Carcinogenicity

Not evaluated in this report.

4.11 Toxicity for reproduction

Table 18: Summary table of relevant reproductive toxicity studies (rangefinding studies and EOGRTS)

Method	Results	Reference
– One-generation range finding study (non-guideline, non-GLP) – rat (Wistar) – oral: via diet – 0, 400, 800, 1700, 3400 ppm in the diet – 0, 14, 28, 62.6, 116.8 mg/kg bw/d (doses Lysmeral males) – 0, 10-15, 18.3-29.4, 62.7, 123.2 mg/kg bw/d (doses / dose range Lysmeral females) – TS purity: 30.7% (a.i. encapsulated)	• **Fertility/reprod. performance - Main effects**: – Testicular toxicity / spermatotoxic effects (for details see Table 19) – Effects on reprod. parameters (for details see Table 20) • **General systemic toxicity - Main effects**: – ↓ Body weights /FC – Changes in liver associated parameters (clinical chemistry, ↑ liver weights) – ↑ Rel. kidney weights • **Developmental toxicity - Main effects (coinciding with maternal toxicity)**: – ↓ pup body weights	BASF SE 2006C
– One-generation range finding study (non-guideline, GLP) – rat (Wistar) – oral: via diet – 0, 230, 750, 2300 ppm in the diet – 0, 2.3-2.8, 7.4-9.1, 25.1-27.5 mg/kg bw/d (dose range Lysmeral males) – 0, 3.3-3.7, 10.6-11.9, 21-34.7 mg/kg bw/d (dose range Lysmeral females) – TS purity: 17.7% (a.i. encapsulated)	• **Fertility/reprod. performance - Main effects**: – Testicular toxicity / spermatotoxic effects (for details see Table 19) – Effects on reprod. parameters (for details see Table 21) • **General systemic toxicity - Main effects**: – ↓ Body weights /FC – Changes in liver associated parameters (clinical chemistry, ↑ liver weights, macroscopic changes), – Hematological changes • **Developmental toxicity - Main effects (coinciding with maternal toxicity)**: – ↓ Pup body weights and early pup survival	BASF SE 2017B
– Modified extended one-generation reproduction toxicity study (OECD Guideline 443, GLP) – rat (Wistar) – oral: via diet – 0, 75, 230, 750 ppm in the diet – 0, 1, 3, 10 mg/kg bw/d (nominal dose Lysmeral) – 0, 1.4, 4.5, 15.1 mg/kg bw/d (overall mean dose Lysmeral) – TS purity: 17.7% (a.i. encapsulated)	• **General systemic toxicity - Main effects**: – ↓ Body weights/FC, – Hematological changes – Changes in liver associated parameters (clinical chemistry, ↑ liver weights, histopathology) • **Developmental toxicity - Main effects (coinciding with maternal toxicity)**: – ↓ Pup body weights. NOAEL (general systemic toxicity): 3 (4.5) mg/kg bw/d NOAEL (developmental toxicity): 3 (4.5) mg/kg bw/d NOAEL (developmental neurotoxicity): 10 (15.1) mg/kg bw/d NOAEL (developmental immunitoxicity): 10 (15.1) mg/kg bw/d NOAEL (fertility/reprod. performance): 10 (15.1) mg/kg bw/d	BASF SE (2017)

Table 19: Summary table of relevant repeated dose and reproductive toxicity studies focused on adverse effects on the male reproductive organs.

Method				Results			Reference
Species	**Study period**	**Dosage** [mg/kg bw/day]	**Protocol**	**LOAEL** testes/sperm [mg/kg bw/day]	**NOAEL** testes/sperm [mg/kg bw/day]	**Adverse male reproductive effects**	
Rat	5 days	Dermal. 250, 500, 1000, 2000; daily for 6 hours TS Purity: 99.1%	Non-guideline; GLP; n=5 males /dose group	2000	1000	**Testicular toxicity:** Marked disorganization of epithelial structure in tubuli seminiferi; ↓ germ cell nr.; ↑ degenerating germ cell nr. inclusive giant cells (5/5); slight -moderate immature/ degenerating germ cells in epididymides (5/5); spermatocele (1/5) **Additional systemic toxicity:** ↓ body weights (slight)	Givaudan 1991A
Rat	1, 2, 3, 4, 14 days	p.o. 50; daily Purity: 99.1%	Non-guideline; non-GLP; n=5 males / time point investigated	n.d.	n.d.	**Testicular toxicity**: slight to severe testicular atrophy (2/5 at day 1; 5/5 at later time points). **Spermatotoxicity:** ↓sperm motility; ↓ testes spermatid count; ↓ cauda epididymal sperm count; affected sperm morphology. **Additional systemic toxicity:** ↓ body weights (day 14)	BASF SE 2006A
Rat	5 days	p.o. 25, 50, 100, 200, 400; daily	Non-guideline; GLP; n=8 males/dose group	50	25	**Testicular toxicity**: degeneration and loss of seminiferous epithelium. **Additional systemic toxicity**: clincial signs; initial body weight loss; macroscopic liver changes; ↓ kidney/ testes weights (at doses above LOAEL).	Givaudan 1986B
Rat	5 days	p.o. 25, 50, 100; daily 5/dose group Purity: 99.1%	Non-guideline; GLP; n=5 males/dose group	50	25	**Testicular toxicity**: minimal and moderate to marked atrophy of testes **Additional systemic toxicity**: initial body weight loss	Givaudan 1991A
Rat	5 days	p.o. 50, 100, 200, 400; daily	Non-guideline; n=8 males/dose group	100	50	**Testicular toxicity:** testicular tubule epithelial degeneration **Additional systemic toxicity**: ↓ body weights; ↓ testis/ kidney weights (at doses above LOAEL).	Newberne 1990A
Rat	90 days	p.o. 2, 5, 25, 50; 5 days/week Purity: 97.8%	OECD TG 408; GLP; n=14/ sex and dose group	50	25	**Testicular toxicity**: (see Table 16) **Additional systemic toxicity**: clinical signs, changes in liver associated parameters (clinical chemistry, ↑ liver weights, histopathology)	Givaudan 1986A

Rat	12 weeks	Feed; 400, 800, 1700, 3400 ppm; daily Purity: 30.7% (a.i. encapsulated)	Non-guideline; non-GLP; n=10 / sex and dose group	62.6 (1700 ppm)	28.0 (800 ppm)	Info given refers to male animals: **Testicular toxicity**: ↓ testis/epididymis weights; moderate diffuse testes degeneration (8/10); moderate to severe focal testes degeneration (2/10); aspermia of epididymides (10/10). **Spermatotoxicity:** 6 mio. testicular spermatid heads (vs. 121 mio. in ctrl.); 2 mio. epididymal sperm heads (vs. 591 mio. in ctrl.); 0% motile sperm; 84.5% morphologically normal sperm. **Effects on reprod. parameters**: see Table 20. **Additional systemic toxicity**: ↓ body weights; changes in liver associated parameters (clinical chemistry, ↑ liver weights); ↑ rel. kidney weights and ↓ seminal vesicle/prostate weights (at doses above LOAEL); minimal to slight hyperplasia of Leydig cells (9/10 males; at doses above LOAEL).	BASF SE 2006C
Rat	10 weeks	Feed; 230, 750, 2300 ppm; daily Purity: 17.7% (a.i. encapsulated)	Non-guideline; GLP; n=10 / sex and dose group	25.1-27.5 (2300 ppm)	7.4 - 9.1 (750 ppm)	Info given refers to male animals: **Testicular toxicity**: ↓ testis/epididymis weights; minimal to moderate tubular degeneration in testis in 3/10 (vs. 1/10 in ctrl.)); minimal to moderate ductal atrophy in epididymis (8 /10); slight to moderate oligospermia (6/10); slight to moderate cellular debris (2/10); not observed in placebo control. **Spermatotoxicity:** ↓ mean fraction of motile sperm (25% vs 85% in ctrl.); ↑ mean fraction of abnormal sperm (72.3% vs 6.2% in ctrl.); ↓ mean sperm head count (469 vs 674 mio/g in ctrl.) in cauda epididymis **Effects on reprod. parameters**: see Table 21 **Additional systemic toxicity**: ↓ body weights; changes in liver associated parameters (clinical chemistry, ↑ liver weights, macroscopic changes); hematological changes.	BASF SE 2017B
Rat	up to 25 weeks	Feed; 75, 230, 750 ppm; daily Purity: 17.7% (a.i. encapsulat	OECD TG 443; GLP; n=10-40 / sex and dose group	--	10.2 - 15.3 (750 ppm)	Info given refers to male animals.	BASF SE 2017

		ed)					
Dog	14 days	Gelatine capsule, 40, 200, 1000/500; daily Purity: 99.1%	Non-guideline; GLP; n=4 males / dose group	200	40	**Testicular toxicity** (1/4): ↓ size testis/epididymis; ↓ weight testis; massive diffuse degeneration of seminiferous tubules; slight hyperplasia of Leydig cells; aspermia and epithelial vacuolation in epididymides; not observed in low/high dose animals. **Additional systemic toxicity**: clinical signs; ↓ body weights; changes in liver associated parameters (clinical chemistry, ↑ liver weights, histopathology).	BASF SE 2008A
Dog	14 days	Gelatine capsule, 200, daily Purity: 99.1%	Non-guideline; GLP; n=10 males / dose group	n.d	n.d.	**Testicular toxicity**: ↓ weight testis/prostate (slight); ↓ testicular length or width of >= 3 mm (6/10); slight to severe degeneration of seminiferous tubules (9/10); minimal to moderate multi focal prostate atrophy (3/10); not observed in ctrls. **Spermatotoxic effects:** ↓ % progressively motile spermatozoa (8/10); ↑ % spermatozoa with damaged plasma membrane (3/10); ↑ % morphological altered spermatozoa (9/10). **Additional systemic toxicity:** body weigh loss; clinical signs, changes in liver associated parameters (clinical chemistry, ↑ liver weights, histopathology); hematologi-cal changes.	BASF SE 2008B
Dog	9 weeks	Gelatine capsule, 47 - 564 Purity: 95%	Non-guideline; n=2 males / dose group	n.d.	n.d.	**Testicular toxicity** (2/2): Mild atrophy of seminiferous tissues (necrosis of germ cells, multinucleated giant cells in lumen of tubules) **Additional systemic toxicity**: clinical signs, body weigh loss; clinical chemistry; liver histopathology.	Givaudan 1990A
Dog	90 Dog days	Gelatine capsule, 4.4, 22.3, 44.6; daily Purity: 97.6%	Similar to OECD TG 409; GLP; n=3 / sex and dose group	> 44.6	44.6		Givaudan 1990B
Mouse	1, 2, 3, 4, 14 days	p.o. 50; daily Purity: 99.1%	Non-guideline; non-GLP; n=5 males / time point investigated	--	50		BASF SE 2006B

Mouse	5 days	p.o. 100; daily	Non-guideline; non-GLP; n=5 males / dose group	--	100		Givaudan 1983
Guinea pig	5 days	p.o. 100; daily 5/dose group	Non-guideline; non-GLP; n=5 males / dose group	--	100		Givaudan 1983
Rhesus monkey	5 days	feed; 100; daily	Non-guideline; non-GLP; n=2 males / dose group	--	100		Givaudan 1984G
Rabbit	15 days	p.o. 30, 100, 300; daily Purity: 99.1%	Non-guideline; non-GLP; n=5 males / dose group	--	300		BASF SE 2008C

4.11.1 Effects on fertility

4.11.1.1 Non-human information

Concerning fertility, a variety of repeated dose studies on male rats (some of them were designed for the assessment of male reproductive organ toxicity), one-generation range finding studies and an extended one-generation reproductive toxicity study (EOGRTS) are available. Furthermore, repeated dose studies on dogs, mice, guinea pigs, rabbits and primates are available for the assessment of reproductive toxicity of Lysmeral (2-(4-tert-butylbenzyl)propionaldehyde; see Chapter 4.7).

Evidence for testicular toxicity of Lysmeral after bolus oral application via gavage is available from existing repeated dose studies in rats, however, data on continuous oral application of Lysmeral was lacking and has been considered in the present one-generation studies. Concerning the properties of Lysmeral as fragrance material, palatability has been assessed in a preliminary feeding test in rats (BASF 2002). Lysmeral (analytical purity 99.1%) has been administered for 14 days to 3 Wistar rats per sex and dose at a dietary concentration of 0, 100 and 1000 ppm. Food consumption and body weights were monitored and animals were examined for signs of toxicity and mortality. Clinical examinations and palpations have been performed.

Mean test substance intake ranged from 10.1-10.6 mg Lysmeral/ kg bw/ day in the low dose group and 97-105.3 mg Lysmeral/ kg bw/ day in the high dose group. Mean food consumption was slightly decreased in males of the high dose group (-6% versus controls at day 7 and 14) and in females of the high dose group (-12% and -3% versus controls after day 7 and day 14 respectively) without gaining statistical significance. Food efficiency has been decreased significantly in high dose males after day 7 and in high dose females at both observation time points.

In male animals, mean body weights were slightly decreased in the high dose group (-5% and -7% compared to controls after day 7 and day 14 respectively) and body weight gains were slightly decreased in the low dose group and more severe in the high dose group (-2% and -20% versus

controls, respectively) at both observation time points. In female animals, slightly decreased body weights were found in the low dose group (-3% versus controls) and a statistically significant decrease has been observed in the high dose group (-10% versus controls) after 14 days. Body weight gains were slightly decreased in the low dose females (-8% and -14%) and significantly decreased in the high dose females (-39% and -42%) after day 7 and day 14 respectively.

No substance related clinical signs of toxicity were observed and no deaths occurred during the observation period.

For further assessment of Lysmeral induced effects after continuous oral application via feed, one-generation range finding studies have been performed using microencapsulated Lysmeral in order to exclude stability issues and palatability induced effects when applied over a longer time period.

This type of test substance administration and the chosen study types allow to:

- verify the relevance of the adverse testicular effects observed after bolus application via gavage compared to a continuous application via feed
- close the gap between the observed adverse changes in testes and sperm and infertility
- to assess the no effect levels concerning fertility for a sound risk assessment

In the older range finding study, the test substance was administered to groups of 10 male and 10 female young Wistar rats (F0 parental generation) via the diet (30.7% Lysmeral, microencapsulated with gelatin based capsules in sunflower oil; BASF SE 2006C). In the 400, 800, 1700 and 3400 ppm group, the uptake of Lysmeral via the diet was accounted to 14.0/15.0, 28.0/29.4, 62.6/62.7 and 116.8/123.2 mg/kg bw/day in males/females respectively. Due to dose adjustment during gestation and lactation, the dams received 200, 400, 850 and 1700 ppm of the test substance in feed, resulting in an uptake of 12.9/10 and 25.8/18.3 mg/kg bw/day Lysmeral for the two low dose groups, respectively (no assessment of the two high dose groups was performed due to the absence of offspring). About 6 weeks after the beginning of treatment, F0 animals were mated to produce a litter (F1).

The female F0 animals were allowed to deliver and rear their F1 pups until weaning (postnatal day 21). The study was terminated with the sacrifice of the F1 weanlings and F0 adult animals.

Male F0 animals showed dose dependent reduced body weights and body weight gains (5-30% and 10-40% below control, respectively) and food consumption was 15% below controls in the high dose group. Increases in relative liver weights (10-20% above control) starting at the 800 ppm dose group and increases in relative kidney weights (15% above control) were found in the high dose group. Significant changes in clinical chemistry such as increased levels of plasma alanine aminotransferase by 20-45%, alkaline phosphatase by 30-55% and a 4 to 5 fold increase of the glutamate dehydrogenase above mean controls were observed starting at 1700 ppm. Mean gamma glutamyltransferase was increased two fold in the high dose group only compared to controls.

Testicular toxicity and spermatotoxicity, i.e. effects on sperm parameters, decreases in relative testes (30-45% below control) and cauda epididymis (30-40% below control) weights, diffuse testes degeneration and aspermia of the epididymis, were observed at the 1700 ppm and 3400 ppm groups. In the high dose group, weights of additional organs were decreased (i.e. seminal vesicle (10%) and prostate (20%) below control) and hyperplasia of Leydig cells was observed.

Maternal toxicity was manifested by decreases in body weights and body weight gain (5-10% and 10-30% below control) during/after premating in the 800 ppm group and at higher doses. During gestation and lactation, mean maternal body weights and body weight gain were approx. 10% below control in the 800 ppm group and food consumption during lactation was 20% below controls. Furthermore, significant changes in clinical chemistry were seen in all dose groups observed, i.e. a 2-8 fold increase in gamma glutamyltransferase and decreases in serum cholinesterase by 50-65% compared to controls. From 800 ppm onward, glutamate dehydrogenase was found to be increased by 5-75% as well. However, no significant changes in mean relative liver or kidney weights were observed.

In the 1700 ppm group only 1 of 8 mated females became pregnant and a relationship to the adverse effects observed in male reproductive organs is indicated.

No viable offspring has been derived from animals treated with 1700 ppm and 3400 ppm microencapsulated Lysmeral. In the 1700 ppm group, the only pregnant female had only 1 implant which was resorbed. In contrast, only a slight and non-significant increase in mean implantation losses have been observed for the two lower dose groups; i.e. 16% and 11% mean losses per litter in dose groups 400 ppm and 800 ppm versus 5% in controls, respectively (see Table **20**). No corpora lutea have been determined in this study

A slight decrease in the mean number of delivered pups per dam (7.9 in dose group 800 ppm versus 9.4 and 8.7 in controls and dose group 400 ppm) was recorded. However, no effects on gestation and the live birth indices became evident, due to the absence of any stillborn in the Lysmeral treated dose groups with offspring.

Pup survival was minimally decreased for postnatal day 0 to 4 (94% in the 800 ppm dose group versus 99% in the 400 ppm dose group and controls), and no pup mortality was observed between postnatal day 4 and 21 in all dose groups with offspring. Overall the respective viability and lactation index was not considered to be affected by treatment.

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) has been recorded for male and female pups. Accordingly the pup body weight gain was decreased in the 400 and 800 ppm dose groups (16% and 21% below controls, respectively).

Table 20: Overview on reproductive parameters from the one-generation range finding study with Wistar rats (BASF SE 2006C).

Dose group [ppm (mg/kg bw/d)]	Fertility index (male)[a]	Fertility index (female)[b]	Mating index (male/female)[c]	Mean implantation sites	Mean Postimplantation loss[d]	Mean pups delivered	Number of litters
0	100 %	100 %	100 %	9.9	5.1±9.27%	9.4±3.95	10
400 (10-15)	100 %	100 %	100 %	8.5	16.2±30.3%	8.7±1.41	9
800 (18.3-29.4)	100 %	100 %	100 %	8.8	11.1±10.16%	7.9±2.23	10
1700 (62.6-62.7)	10 %	13 %	80 %	1*	100 ±0%**	0	0
3400 (116.8-123.2)	0 %	0 %	50 %	0	-	0	0

*p<=0.05, **p<=0.01; mg/kg bw/d (mg/kg body weight/day)
[a]Male fertility index = Number of males proving their fertility / Number of males placed with females * 100
[b]Female fertility index = Number of females pregnant / Number of females mated * 100
[c]Mating index = Number of animals mated or with confirmed matings / number of males placed with females * 100
[d]Postimplantation loss = number of implantations – number of pups delivered / number of implantations

In the recent range finding study, performed to ensure a sound dose selection for the main EOGRTS, groups of 10 male and female Wistar rats were treated with Lysmeral formulated in alginate based microcapsules via diet (17.7% Lysmeral, microencapsulated in sunflower oil; BASF SE 2017B). In the 230, 750, 2300 ppm group, Lysmeral uptake in males was accounted to a mean of 2.8/2.3, 9.1/7.4, 27.5/25.1 mg/kg bw/day (pre-/postmating), respectively. A dose reduction of 50% was performed for females during lactation, and the dams received doses of 3.3-3.6, 10.6-11.9, 30.6-34.7 mg/kg bw/day during premating and gestation and 3.7, 10.7 and 21.0 mg/kg bw/day during lactation in the low, mid and high dose group, respectively. About 2 weeks after the beginning of treatment, F0 animals were mated to produce a litter (F1). The female F0 animals were allowed to deliver and rear their F1 pups until weaning (postnatal day 21), and the study was terminated with the sacrifice of the F1 weanlings and F0 adult animals.

Evidence of systemic toxicity in male F0 animals represented decreases in body weights (starting at the second week of treatment) and body weight gains of 5-11% and 45-84% below control, respectively, in the high dose group. Food consumption was reduced within the first week in mid and high dose males (7% and 9% below control, respectively). Hematological changes in mid and high dose males were observed. Total protein (mid and high dose males), albumin, globulin, cholesterol, triglycerides, sodium and calcium concentrations in serum were significantly decreased and aspartate aminotransferase activity (26% above control) was increased in high dose males. Increases in absolute and relative liver weights (14-30% above control) accompanied by increased incidences of discoloration was observed in high dose males. Liver weight increases were seen to a lower extent in low and mid dose group males (< 10% versus controls).

Indications of testicular and epididymal effects were observed in the high dose males. Weights of the cauda epididymis (-29% / -19%; absolute/relative), epididymides (-16% absolute) and seminal vesicles (-19%; absolute) were reduced compared to the respective placebo control. Histopathology revealed minimal to moderate tubular degeneration in 3 of 10 males and epididymidal changes, i.e. minimal to moderate ductal atrophy (8 /10 males), slight to moderate oligospermia (6 /10 males) and slight to moderate cellular debris (2 /10 males). The mean fraction of motile sperm accounted to 25% in high dose animals (compared to 85%, 88% and 86% in placebo control, low and mid dose males and the mean fraction of abnormal sperm was 72% versus 6-7% in the other test group males. Whereas the mean spermatid head counts in the testis were not significantly affected (i.e. 115 in high dose males versus 124 mio/g in placebo controls), the mean sperm head count in the cauda epididymis was significantly reduced in high dose males (469 mio/g) compared to 674, 760 and 640 mio/g tissue in control, low and mid dose animals. The nature of the capsule material (gelatine or alginate) and the differences in Lysmeral concentrations used (30.7% versus 17.7%) in the two range findings studies might impact the overall bioavailability of the test substance Lysmeral and explain the difference in effective testicular and spermatotoxic dose levels observed.

Maternal toxicity was manifested by an initial body weight loss in the first dosing week and decreases in body weight gain (32-59% below control) during premating and gestation in the high dose females. Accordingly, body weights were significantly decreased starting at day 14 of gestation (10-16% below control) in high dose females. These effects were seen less severe in mid dose females as well. During lactation, mean maternal body weights were approx. 10% below control in the mid and high dose group, respectively, and a recovery of these body weights was observed at the end of lactation.

High dose female food consumption was reduced in the first study week (14% below control) and during lactation (44-48% below controls) and mid dose food consumption was decreased less severe mainly during the first two weeks of lactation as well. Hematological parameters were affected and decreases in serum total protein, albumin, globulin, cholesterol (high dose females), triglycerides, sodium and calcium were seen in mid and high dose females. Furthermore, increase in aspartate aminotransferase (23-47% above control) and gamma glutamyltransferase (9-24 fold above control) in the mid and high dose females and additional increases of creatinine, total bilirubin, chloride and inorganic phosphate in the high dose females were observed.

Whereas the mating index was comparable between test groups, fertility indices were affected in the high dose animals and a reduction of the mean implantations sites were observed (see Table **21**). A slight and non-significant increase in mean implantation losses have been observed in the high dose dams. Consequently, a decrease in the number of litters and a mean number of delivered pups per dam (4.0 in high dose dams versus 11.1 in controls) was observed. An increase in the gestation duration was seen in the high dose group (23 days versus 22.2 days in the respective placebo control), which is considered to be a secondary consequence of the clearly lower number of pups per litter and the affected offspring, leading to a lower stimulus in starting the process of parturition. No effects on live birth indices became evident, since stillborn rates in the Lysmeral treated dose groups were not increased compared to the respective placebo control.

Pup survival was decreased for postnatal day 0 to 4 (86% and 75% in the mid and high dose groups versus 99% and 95% in the low dose group and placebo control group), whereas no pup mortality was observed between postnatal day 4 and 21 in all dose groups.

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) has been recorded for mid and high dose pups. Accordingly, the pup body weight gain was decreased in the mid and high dose groups (13% and 33% below controls, respectively). A higher percentage of live females compared to live males was seen in the high dose group (69% / 31% at day 0 and 67% / 33% at day 21) versus placebo control (53% / 47% at day 0 and day 21). Due to the low number of total pups delivered in this dose group (16 pups versus 111 pups in placebo control group), this finding is considered to be a chance finding, given that changes in the sex ratio has not been observed at any other study.

Table 21: Overview on reproductive parameters from the one-generation range finding study with Wistar rats (BASF SE 2017B).

Dose group [ppm (mg/kg bw/d)]	Fertility index (male)[a]	Fertility index (female)[b]	Mating index (male/female)[c]	Mean implantation sites	Mean Postimplantation loss[d]	Mean pups delivered	Number of litters
0 placebo	100 %	100 %	100 %	11.5	3.8±6.85%	11.1±1.91	10
230 (2.3 – 3.6)	90 %	90 %	100 %	11.8	3.9±6.29%	11.3±1.66	9
750 (7.4 – 11.9)	100 %	100 %	100 %	10.1	3.7±7.77%	9.7±2.36	10
2300 (25.1– 34.7)	40 %	44 %	90 %	4.5**	16.7 ±23.57%	4.0±3.16**	4

**p<=0.01; mg/kg bw/d (mg/kg body weight/day)
[a]Male fertility index = Number of males proving their fertility / Number of males placed with females * 100
[b]Female fertility index = Number of females pregnant / Number of females mated * 100
[c]Mating index = Number of animals mated or with confirmed matings / number of males placed with females * 100
[d]Postimplantation loss = number of implantations – number of pups delivered / number of implantations

In the EOGRTS (according to OECD test guideline No. 443 and OECD Principles of Good Laboratory Practice), encapsulated Lysmeral was administered to groups of 35 male and female Wistar rats for low, mid and control groups and to 40 male and female rats for the high dose group as a homogeneous addition to the food in concentrations of 75, 230 and 750 ppm, based on the outcome of the precedent dose range finding study (BASF 2017). The concentrations of the formulated capsules corresponded to 13, 41 and 133 ppm of the active ingredient Lysmeral and a targeted nominal dose of 1, 3 and 10 mg/kg body weight/day Lysmeral was aimed for. The overall mean dose of Lysmeral administered to the male and female Wistar rats throughout all study phase and across all cohorts was approx. 1.4, 4.5 and 15.1 mg/kg bw/d in the 75, 230 and 750 ppm dose group, respectively. Thus, the targeted Lysmeral dose levels were achieved or exceeded. A negative control group given plain diet and an additional placebo-control group was dosed with capsules without Lysmeral via the diet in parallel.

The F0 animals were treated at least for 13 days prior to mating to produce a litter (F1 generation). Pups of the F1 litter were selected (F1 rearing animals) and assigned to 7 different cohorts (see Table 22) which were continued in the same fashion as their parents and which were subjected to specific post-weaning examinations. F1 Cohort 1B animals selected for breeding were continued in the same dose group as their parents, and the breeding program was repeated to produce a F2 litter. The study was terminated with the terminal sacrifice of the F2 weanlings and F1 Cohort 1B parental animals.

Animals were assessed for their state of health, detailed clinical signs, body weights, food consumption, clinical pathological investigations (including thyroid hormone measurements) and urinalysis. Mating and reproductive performances were investigated in P0 and P1 animals and estrous cycling or sperm parameters were assessed. The offspring was sexed, monitored for their viability, body weights and the presence of nipple/areola anlagen and their sexual maturation was followed via vaginal opening and balanopreputial separation.
Corresponding to their cohorts, F1 animals were assessed by specific (histo-) pathological examinations. Key aspects were general organ and reproductive pathology in cohorts 1 (A/B), specific pathology of central and peripheral nervous system in cohorts 2 (A/B) and specific investigations of immune response in cohort 3. Additional cohort specific investigations included T-cell dependent antibody response to sheep red blood cells or motor activity measurements, auditory startle response and functional observational battery examination. For the add-on cohorts 4 (A/B), blood serum, erythrocytes, brain and muscle tissue were investigated for acetyl cholinesterase activities in F0 parental animals, PND 4 surplus pups, PND 22 and young adult F1-offspring.

Table 22: Cohorts of assigned F1 pups for further post-weaning examinations.

Cohort	Designation	Animals/Cohort	Puberty	Approx. age at necropsy
1A	Reproductive PND90	20 M + 20 F	Yes	13 weeks
1B	Reproductive (=F1 parental animals)	25 M + 25 F	Yes	19-25 weeks
2A	Neurotoxicity PND75-90	10 M + 10 F*	Yes	11 weeks
2B	Neurotoxicity PND22	10 M + 10 F*	No	3 weeks
3	Immunotoxicity	10 M + 10 F*	Yes	8-9 weeks
4A	Cholinesterase PND22	10 M + 10 F*	No	3 weeks
4B	Cholinesterase adult	10 M + 10 F*	Yes	11-12 weeks

*one per litter and representative of 20 litters in total where possible

The high-dose (nominal 10 mg/kg bw/d) produced adverse systemic effects in the F0 parental rats and F1 offspring (see Table 23). In F0 and F1 females of Cohort 1B, food consumption was consistently reduced during lactation (F0 females: 5% and F1 cohort 1B females: 12% below placebo-control), whereas this parameter remained unchanged in males and females of other cohorts. Further, body weights were consistently reduced in high-dose F0 females during gestation and the first two weeks into lactation, which was caused by a reduced body weight gain during different sections of premating and gestation. No such effects were observed in the high-dose F0 males. In line, the high-dose F1 females of Cohort 1B were similarly affected. The decrease of body weight persisted throughout gestation and lactation period for the F2 litters and a reduction of body weight gain was observed during pregnancy. The body weights of the high-dose males in the different F1 cohorts were below the concurrent control throughout the in-life period after weaning (up to 11%).

Effects of the red blood cell parameters were observed in high dose F0 females (higher red blood cell counts, hemoglobin and hematocrit values) and high dose F1 males and females (higher red blood cell counts and hemoglobin values and decreased mean corpuscular volumes). An isolated decrease in mean serum acetylcholinesterase activities was seen in F0 females (-16% in mid dose and -21% in high dose group animals when compared to placebo controls), which was not confirmed in males and other peripheral tissues including erythrocytes. Further, a decrease in the mean acetyl-cholinesterase activities of the Musculus gastrocnemius in the F0 high dose males (-18% compared to placebo controls) was not considered to be an adverse Lysmeral related effect.

Table 23: Overview on selected maternal toxicity and developmental parameters (F1/F2 offspring) from the EOGRTS with Wistar rats (BASF SE 2017).

Finding	Unit	**0** [mg/kg bw/d][a]	**1 (1.4)** [mg/kg bw/d]	**3 (4.5)** [mg/kg bw/d]	**10 (15.1)** [mg/kg bw/d]
F0 - Mean maternal food consumption lactation (d1-21)	grams/ animal/ day	45.1	48.7	46.6	43.0
F1 - Mean maternal food consumption lactation (d1-21)	grams/ animal/ day	49.6	46.4	49.1	43.5
F0 - Mean maternal body weight change (absolute) – premating (d0-13)	grams	19.4	20.6	20.0	16.6
F0 - Mean maternal body weight change (absolute) - gestation (d0-20)	grams	104.7	109.8	106.3	**92.2****
F0 - Mean maternal body weights - gestation (d20)	grams	304.6	311.0	307.2	**290.3***
F0 - Mean maternal body weights - lactation (d14)	grams	268.7	274.3	272.9	**258.4***
F1 - Mean maternal body weight change (absolute) – premating (w0-10)	grams	150.6	152.4	150.6	145.2
F1 - Mean maternal body weight change (absolute) - gestation (d0-20)	grams	108.5	103.9	107.2	**96.8****
F1 - Mean maternal body weights - gestation (d20)	grams	331.9	330.1	326.1	**305.3****
F1 - Mean maternal body weights - lactation (d21)	grams	273.2	269.4	273.8	**262.3***
F1 – Mean pup body weights (males + females; PND1)	grams	6.7	6.9	9.6	**5.7****
F2 – Mean pup body weights (males + females; PND1)	grams	6.8	6.8	6.8	**5.9****
F1 – Mean pup body weights (males + females; PND21)	grams	47.2	49.2	46.5	**42.7****
F2 – Mean pup body weights (males + females; PND21)	grams	49.5	48.1	49.5	**44.5****
F1 - Viability index[b] (Survival PND 0-4)	%	97	100	98	93
F2 - Viability index (Survival PND 0-4)	%	99	94	99	95
F1/F2 - Lactation index [c] (Survival PND 4-21)	%	100	100	100	100
F1 – Mean anogenital distance male / female pups	mm	3.08/1.48	3.1/1.48	3.08/1.47	3.01/1.47
F2 – Mean anogenital distance male / female pups	mm	3.08/1.55	3.01/1.54	3.05/1.54	**2.97*/1.49***
F1 – Mean anogenital index[d] male / female pups		1.62/0.79	1.62/0.79	1.63/0.79	1.67/0.83
F2 – Mean anogenital index male / female pups		1.61/0.83	1.57/0.82	1.60/0.82	1.64/0.83
F1 - Preputial separation	mean days to criterion / body weight at criterion [g]	41.6 / 167.5	41.1 / 169.8	41.5 / 164.9	42.3 / 162.7

*p<=0.05; **p<=0.01
[a]placebo control group, fed with capsules without Lysmeral.
[b]Viability index = number of live pups on PND4 / number of live pups on day of birth * 100
[c]Lactation index = number of live pups on PND21 / number of live pups on PND4 (after culling) * 100
[d]Anogenital index = anogenital distance in mm / cubic root of pup weight in g

Indications for an altered metabolism of liver cells in F0 and F1 high dose females were seen by prolonged prothrombin time (i.e. reduced synthesis of coagulation factors), increased γ-glutamyl transferase (GGT) activity and reduced albumin levels. Significant absolute and relative weight increase of the liver weight (119% and 120%, respectively) were associated with minimal to slight centrilobular hypertrophy accompanied by minimal to slight apoptosis/single cell necrosis of hepatocytes in high-dose F0 females. Furthermore, periportal vacuolation and multinucleated hepatocytes were noted in few animals. In the female F0 mid-dose group (3 mg/kg bw/d) a significant liver weight increase (absolute: 112%, relative: 110%) was within the historical control range values and occurred without a histopathological correlate. Therefore, a treatment related effect is assumed, but was regarded as non-adverse.
A comparable picture was obtained in the F1 high-dose females (i.e. Cohort 1A), showing significant absolute and relative weight increases of the liver (126% and 128%, respectively), that correlated with minimal to slight centrilobular hypertrophy and minimal to slight apoptosis/single cell necrosis of hepatocytes. In the female mid-dose group, the significant absolute and relative liver weight increases (116% and 109% respectively) were above the historical controls. They were assessed as treatment-related and non-adverse, since no histopathological correlate was noted.

There was no evidence from clinical examinations as well as 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 high-dose of 10 mg/kg bw/d (see Table 24). Estrous cycle data, mating behavior, 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 the rats of all groups including control and ranged within the historical control data. A slightly (non-statistically) higher mean percentage of abnormal sperms (9.8%) in the high-dose F0 males above the placebo percentage (6.3%) and the historical control range (6.0-6.6 %) was observed. However, there were no findings in sperm motility (86% versus 88% in placebo control) or sperm head counts in testes (108 mio/g versus 102 mio/g in placebo control) and epididymidis (717 mio/g versus 723 mio/g in placebo control) of these animals. Further, the corresponding F1 offspring males did not show these effects, and there were no indications whatsoever of an impairment of fertility in the F0 and F1 generations. Thus, there is no evidence in the frame of this study that this marginal non-statistical increase is of toxicological relevance.

Table 24: Overview on reproductive parameters from the EOGRTS with Wistar rats (BASF SE 2017).

Finding	Unit	**0** [mg/kg bw/d][e]	**1 (1.4)** [mg/kg bw/d]	**3 (4.5)** [mg/kg bw/d]	**10 (15.1)** [mg/kg bw/d]
F0 - Fertility index (male)[a]	%	94	89	97	98
F1 - Fertility index (male)	%	100	100	96	96
F0 - Fertility index (female)[b]	%	97	91	97	100
F1 - Fertility index (female)	%	100	100	96	100
F0 - Mating index (male/female)[c]	%	97	97	100	98
F1 - Mating index (male/female)	%	100	100	100	96
F0 – Gestation index[f]	%	97	100	100	97
F1 – Gestation index[f]	%	100	96	100	100
F0 - Gestation duration	days	21.9 ± 0.44	22.0 ± 0.37	21.9 ± 0.52	21.9 ± 0.33
F1 - Gestation duration	days	22.2 ± 0.41	22.2 ± 0.50	22.1 ± 0.45	22.1 ± 0.28
F0 – mean estrus cycle duration	days	3.9 ± 0.57	3.8 ± 0.44	3.8 ± 0.43	3.9 ± 0.45
F1 – mean estrus cycle duration	days	4.0 ± 0.23	4.0 ± 0.31	3.9 ± 0.19	3.9 ± 0.10
F1 – Differential ovarian follicle count	Primordial / growing / primordial+growing (mean)	446.3 / 227 / 469	n.a.	n.a.	432.25 / 213.5 / 435.35
F0 - Abnormal sperm in cauda epididymidis	%	6.3 ± 0.6	6.0±0.0	6.0 ± 0.0	9.8 ± 13.2
F1 - Abnormal sperm in cauda epididymidis	%	6.0 ± 0.0	n.a.	n.a.	6.2 ± 0.8
F0 - Mean implantation sites	implants/dam	10.7 ± 3.38	11.4 ± 2.25	11.2 ± 2.23	10.6 ± 2.60
F1 - Mean implantation sites	implants/dam	12.3 ± 1.82	11.6 ± 1.88	12.1 ± 1.83	**10.5 ± 2.13****
F0 - Mean Postimplantation loss[d]	%	4.8 ± 8.6	7.6 ± 9.49	5.4 ± 6.76	7.4 ± 16.23
F1 - Mean Postimplantation loss	%	2.9 ± 6.23	2.5 ± 4.61	5.9 ± 11.09	3.3 ± 4.96
F0 - Mean pups delivered	pups/dam	10.1 ± 3.4	10.6 ± 2.45	10.6 ± 2.33	10.3 ± 1.74
F1 - Mean pups delivered	pups/dam	12.0 ± 2.06	11.3 ± 1.80	11.4 ± 2.02	**10.1 ± 2.19****
F0 - Total number of litters	n	33	31	34	38
F1 - Total number of litters	n	25	24	24	24
F0 – Live birth index[g]	%	99	100	99	99
F1 – Live birth index	%	100	97	100	99

**p<=0.01; n.a.= not assessed; mg/kg bw/d (mg/kg body weight/day).

[a]Male fertility index = Number of males proving their fertility / Number of males placed with females * 100

[b]Female fertility index = Number of females pregnant / Number of females mated * 100

[c]Mating index = Number of animals mated or with confirmed matings / number of males placed with females * 100

[d]Postimplantation loss = number of implantations – number of pups delivered / number of implantations

[e]placebo control group, fed with capsules without Lysmeral.

[f]Gestation index = Number of females with live pups on day of birth / number of females pregnant * 100

[g]Live birth index = Number of liveborn pups at birth / total number of pups born * 100

In F0 animals, implantation was not affected and no postimplantation losses, indicative for Lysmeral induced intrauterine embryo/-/fetolethality, was observed for F0 and F1 animals (Table 24). In high dose F1 females, an observed statistically significant decrease in the mean number of implantation sites (10.5 implants/dam) was within the historical control range (9.4-13.9 implants/dam), was not observed in F0 animals and no other findings supportive of a potential effect on fertility (i.e. follicle numbers, sperm quality) exist, making this finding an incidental event. The finding of decreased mean numbers of delivered F2 pups in these animals is associated with the lower number of implants and not an independent finding. The live birth index was not affected in these and all other treated animals.

The pup body weight development of the high-dose F1 and F2 offspring was affected, as these offspring weighed about 14-16% less than control after birth and did not recover until weaning (weights about 10% below control at PND 21; see Table 23). These body weight effects had no evident influence on postnatal pup survival, neither during early lactation nor later. There was a higher number of cannibalized high-dose F1 pups around PND 1 (20 vs. 1 in placebo control), however, most of these cannibalizations (16) were clustered in 2 litters (total of 5 litters affected with 9, 7, 2, 1 and 1 cannibalized pup(s), respectively), and there were no such effects evident in the high-dose F2 offspring. Thus, these were most likely incidental events. Postnatal survival after PND 4 of the offspring of all test groups until weaning remained unaffected by Lysmeral administration. Furthermore, clinical and/or gross necropsy examinations of the weaned F1 pups revealed no adverse findings.

Lysmeral did not affect blood thyroid hormone levels in F0 parental animals and F1 offspring. The sex ratio of F1 and F2 pups at day of birth and on PND 21 was not affected by Lysmeral administration. In contrast to F1 pups, the anogenital distance of the high-dose F2 pups was statistically significantly below the concurrent placebo-control values (about 4%, respectively) and at the lower limit of the historical control range (2.99-3.15 mm for males, 1.48-1.60 mm for females). In turn, anogenital indices of high-dose male and female F1 and F2 pups were found to be somewhat above the concurrent controls. These effects were considered as a secondary consequence to the lower pup body weights and represent not an independent treatment effect. In addition, further check of sensitive marker of potential endocrine-mediated imbalances (i.e. presence of nipples/areolas) revealed no Lysmeral related effects. For the onset of puberty, no effect of Lysmeral treatment was noted for vaginal opening in the offspring. A statistically significant delay in preputial separation beyond the placebo-control (41.6 days) was observed in the high-dose male F1 offspring (42.3 days), but the delay is well within the historical control range (40.5-45.2) and can in addition be attributed to a general delay in the development of high-dose male F1 offspring. It is thus not considered to be a direct test substance-related effect on male sexual maturation. Overall, the sexual development of the offspring was not impaired by Lysmeral administration.

In male PND 4 pups and PND 76 females of the high dose group, lower peripheral acetylcholinesterase (AChE) activities were seen in serum, erythrocytes and diaphragm tissue (up to 50% of the means in placebo control animals). Such changes were not found in the respective other sex. No corresponding clinical signs of developmental neurotoxicity were evident in male and female F1 offspring at any dose level. There were no compound related effects on motor activity, auditory startle habituation, and in the field observation battery following exposure to the test compound in these animals. The only notable finding in neurobehavioral testing were lower maximum amplitudes in the auditory startle response test of the high-dose F1 males.

However, in comparison to corresponding negative control data (plain diet) and high-dose F1 female data the placebo-control values were rather unusually high, therefore this finding appears to be irrelevant due to high placebo control incidences. Moreover, no such findings were noted in the high-dose F1 females and no corresponding effects were recorded for startle response latency. Thus, this was not regarded as an effect caused by the treatment. In addition, regarding neuropathology, brain weight determination, brain length and width measurements as well as brain morphometry and neuropathological examination by light microscopy did not reveal any neurotoxicological treatment-related findings.

There was no evidence that Lysmeral produced any developmental immunotoxicity. Neither T-cell dependent anti-SRBC IgM antibody response, nor absolute and relative lymphocyte subpopulation cell counts in the spleen tissue (B-, T-lymphocytes, CD4-, CD8-T-lymphocytes and natural killer (NK) cells) displayed any treatment-related changes.

Based on the findings of this extended one-generation reproduction toxicity study the NOAEL for general, systemic toxicity is set at 3 (4.5) mg/kg bw/d for the F0 and F1 parental as well as adolescent animals, based on evidence for distinct liver toxicity, as well as corresponding effects on food consumption, body weights and clinical pathological parameters. The NOAEL for fertility and reproductive performance for the F0 and F1 parental rats is 10 (15.1) mg/kg bw/d, and the NOAEL for developmental toxicity in the F1 and F2 progeny is 3 (4.5) mg/kg bw/d, based on reduced pup body weights in the F1 and F2 offspring, which were observed at the LOAEL of 10 (15.1) mg/kg bw/d. As these weight reductions were only observed in the presence of maternal toxicity, including lower weight gain during pregnancy, they are not regarded as independent effect of the treatment. Although an inhibitory effect of the high dose of Lysmeral on the peripheral AChE activity in pups and adolescent rats cannot entirely be excluded, there were no corresponding effects evident in the neurobehavioral or neuropathological examinations. Accordingly, the NOAEL for developmental neurotoxicity and developmental immunotoxicity for the F1 progeny is 10 (15.1) mg/kg bw/d.

To facilitate comparison with testicular toxicity observed in repeated dose studies a summary of the various studies previously discussed in chapter 4.7 is provided in Table 19 focussing on (no-)effect dose levels for testicular toxicity.

In addition, an in vitro study on chorionic gonadotrophin stimulated testosterone secretion of primary rat Leydig cells after treatment with Lysmeral did not indicate any specific inhibition at the level of testosterone secretion. It was therefore assumed, that the atrophic changes of the testes in vivo is not attributed to a specific and direct action of Lysmeral on the main function of Leydig-cells which is the secretion of testosterone (Roche 1994).

A number of studies with pharmacological agents have demonstrated the pivotal nature of RARα signalling in maintaining functionality in the testis and ablation of the RARα results in testicular toxicity and spermatotoxicityin rodents similar to Lysmeral related findings (Chung et al, 2016, 2013). In order to assess the RAR pathway as a putative mode of action for Lysmeral and TBBA induced testicular toxicity, these substances have been tested in a reporter gene assay to detect potential agonistic or antagonistic activities directed against the human RARα, RARβ and RARγ (BASF 2017C). The CHO cells used, express a receptor hybrid in which the native N-terminal DNA binding domain (DBD) has been replaced with a yeast Gal4 DBD and the reporter vector comprises the firefly luciferase gene functionally linked to the Gal4 upstream activation sequence. Neither incubation with Lysmeral nor TBBA at a concentration range of 0.00128 - 100 µM resulted in a relevant induction of luciferase activity for any of the nuclear receptor investigated. Furthermore, Lysmeral and TBBA did not influence the reporter gene expression in the presence of suitable receptor agonists (i.e. 9-cis and all-trans retinoic acid) at non-cytotoxic concentrations,

demonstrating the absence of a potential antagonistic effect towards human RARα, RARβ and RARγ. Therefore, these results indicate, that Lysmeral and TBBA mediated testicular and spermatotoxicity are not mediated via the RARα, which is highly conserved between species.

Testicular toxicity induced by p-tert-butyl-benzaldehyde (TBB) and p-tert-butyltoluene (TBT) and the common metabolite p-tert-butylbenzoic acid (TBBA).

As described in Chapter 4.1, oral administration of p-tert-butyl-benzaldehyde (TBB, CAS No. 939-97-9; EC No. 213-367-9) and p-tert-butyltoluene (TBT, CAS No. 98-51-1; EC No. 202-675-9) resulted in the formation of systemic p-tert-butyl-benzoic acid (TBBA; CAS No. 98-73-7; EC No. 202-696-3). Lysmeral and both compounds (TBB and TBB) share TBBA as a common metabolite. Both, TBB and TBT administration to rats resulted in adverse testicular effects, which are identical to the testicular findings observed for Lysmeral (for further details see Annex 3). In line with findings for Lysmeral, the rat represents the most sensitive species and evidences for testicular effects in dogs exist, whereas other species, i.e. mouse or guinea pigs, show low susceptibility for testicular toxicity.

Clear evidence of adverse testicular and spermatotoxic effects have been observed for the metabolite TBBA as well. These effects are identical in quality to Lysmeral induced testicular toxicity and have been observed in repeated dose, testicular toxicity screening and fertility studies (for further details see Annex 2).

Based on the lowest adverse effect level for testicular toxicity, TBBA application in rats revealed the highest potency ($LOAEL_{testicular\ toxicity}$ of 8 mg/kg bw/day) and is included in Annex VI of the CLP regulation with a classification as Repr. 1B (H360F; Index No. 607-698-00-1). The lowest potency can be assigned to Lysmeral with a $LOAEL_{testicular\ toxicity}$ of >25 mg/kg bw/day after oral application. TBT and TBB were found to be more potent than Lysmeral but less potent than TBBA with $LOAELs_{testicular\ toxicity}$ of 15 and 25 mg/kg bw/day, respectively. Systemic formation TBBA after oral administration of TBB and TBT was approximately 2-3 fold higher compared to Lysmeral treated animals on the basis of urinary TBBA levels (for details see Chapter 4.1). The magnitude of difference in testicular toxicity potency between Lysmeral and TBT are comparable to the differences observed in the urinary TBBA levels formed. Therefore, testes toxicity potencies correlated with systemically formed urinary TBBA amounts.

The reproductive toxicity data of TBB, TBT and TBBA are used to substantiate the pivotal role of TBBA formation in Lysmeral induced rat testicular toxicity and spermatotoxicity. Based on the accordance in the testicular toxicity profile of Lysmeral, p-tert-benzaldehyde (TBB), p-tert-butyltoluene (TBT) and the shared metabolite para-tert-butylbenzoic acid (TBBA), the formation of the systemic TBBA intermediate represents a metabolic key event for Lysmeral induced testicular toxicity.

Formation of para-alkyl benzoyl-CoA by Lysmeral and Lysmeral-like materials and correlation to rat male reproductive toxicity.

As outlined in Chapter 4.1, Lysmeral and its metabolite TBBA is transformed to TBBA-CoA conjugates in hepatocytes (Givaudan 2017). Additional testing of a wide variety of aldehydes, benzoic acids and other chemicals potentially transformed to benzoic acid metabolites in rat hepatocytes revealed, that chemicals with a para-substituent at the benzyl ring accumulate alkyl-benzoyl-CoA conjugates in a similar fashion as Lysmeral and TBBA (Table 25). All chemicals with

this metabolic outcome (Lysmerylic acid, Lysmerol, BHCA, PMHCA, PHCA, iBMHCA, TBT and p-isopropyl benzoic acid) were reported to cause testicular and spermatotoxic effects in the rat.

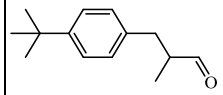
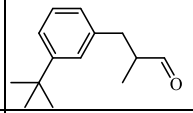
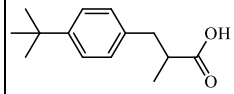
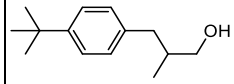
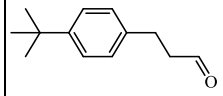
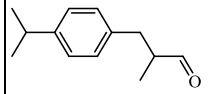
In contrast, meta substituted substances such as meta-Lysmeral and m-iP2MHCA and other structurally related substances (Floralozone, Tropional, Fennaldehyde, Jasmorange, Nympheal™, benzoic acid, p-hydroxy benzoic acid and ethylparaben) showed no accumulation of corresponding alkyl-benzoyl-CoA conjugates especially at the 22 hour time point and did also not affect male reproductive organs such as the testis. Thus, a very strong correlation exists between the metabolic formation of benzoyl-CoA at a sustained elevated level in hepatocytes and spermatoxic and testicular effects in the rat.

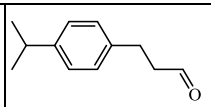
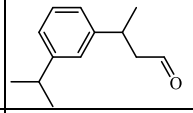
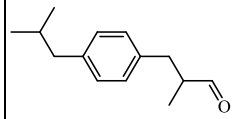
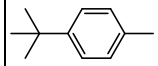
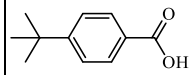
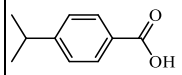
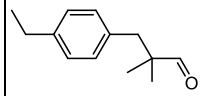
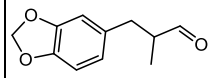
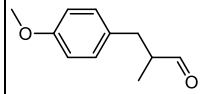
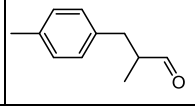
As outlined in Chapter 4.7, Lysmeral and TBBA exposure disrupts CoA dependent intracellular processes which leads to the inhibition of hepatic lipogenesis and gluconeogenesis. Metabolome studies in vivo in rats with Lysmeral and Lysmerol revealed a common decrease of lipids, fatty acids and fatty acid related metabolites, whereas the non-testicular toxicant meta-Lysmeral led to a divergent metabolite pattern especially for complex lipids such as sphingolipids, ceramides and phosphatidylcholines. Complex lipids such as very-long-chain-polyunsaturated fatty acid (VLCPUFA) or ceramides, sphingolipids and phosphatidylcholines containing these PUFAs are present in high amounts in mammalian sperm and play an important role for spermatogenesis. These lipids are considered to stabilize cellular membranes with high curvature such as the rims of the sperm head and provide membrane flexibility needed for efficient sperm formation. Defects in enzymes relevant for synthesis of such lipids are associated with testes toxicity and male infertility. This has been shown in transgenic mice with disrupted enzymes such as FADS2 desaturase, ELOVL2 elongase or LPAAT3 acyltransferase (Stroud 2009; Zadravec 2011; Iizuka-Hishikawa 2017). Disruption of these enzymes are associated with decreases in n-6 and n-3 polyunsaturated fatty acids (PUFAs) such as arachidonic acid, docosapentanoic acid and docosahexanoic acid and result in disturbances of polyunsaturated ceramide, sphingolipid and phosphatidylcholin formation. As seen for the Elovl2 $^{-/-}$ mice, both Sertoli cells and the Leydig cells appeared normal in the interstitium, whereas primary spermatocytes degenerated, formed multinucleated giant cells and a complete arrest of spermatogenesis was observed. The data imply, that ELOVL2 synthetized VLCPUFAs are essential membrane components for normal completion of spermatocyte cytokinesis, accumulating in sphingolipids with these VLCPUFAs. For the other ko. mice, normal spermatozoa formation was disturbed and these animals proved to produce no offspring.

Overall, complex lipids are essential and the disturbance in formation of these lead to disruption in spermatogenesis which results in male infertility. The formation of these lipids is dependent on CoA and disturbances by CoA-TBBA complex formation represent a mode of action of Lysmeral and TBBA induced testicular toxicity. This is further supported by the fact, that Lysmeral induced liver toxicity - which is also caused by disruption of CoA dependent intracellular processes - occurs at doses leading to testicular/spermatotoxic effects.

As outlined in Chapter 4.1, human hepatocytes showed low levels and a rapid and almost complete decrease of TBBA-CoA. The kinetics observed for TBBA-CoA formation in the presence of Lysmeral (Figure 6) or TBBA are similar to those observed for a number of non-reprotoxic chemicals in rat hepatocytes such as meta-Lysmeral, Tropional, Fennaldehyde and Jasmorange (Figure 7 and Table 25). These findings support, that the mode of action for Lysmeral or TBBA induced testes/spermatotoxicity in rats has little relevance in humans.

Table 25: Structures, adverse reprotoxic effects on male rats and accumulation of benzoyl-CoA conjugates in plated rat hepatocytes. All chemicals were tested at 50 µM and selected chemicals were also tested at 5 µM in plated rat hepatocytes. Benzoyl-CoA conjugates refer to the CoA-conjugate formed from the benzoic acid derived from the test chemical and conjugated to CoA. Benzoyl-CoA conjugates were quantified versus a synthetic sample of TBBA-CoA using LC-HRMS analysis. Data for individual repetitions are expressed as % versus the formation of TBBA-CoA from Lysmeral at the given time point and at the corresponding test chemical concentration. Lysmeral was tested in all experiments as reference. bd, below detection level; nd, not determined.*only dose tested.

Name	**CAS Nr.**	**Structure**	**LOAEL for male reprotoxic effects in rat**	**Benzoyl-CoA-conjugate (50 µM)**		**Benzoyl-CoA-conjugate (5 µM)**	
			(mg/kg bw/day)	**4 h**	**22 h**	**4h**	**22h**
3-(4-tert-butylphenyl)-2-methylpropanal (Lysmeral)	80-54-6		50 (Givaudan 1986A)	**100**	**100**	**100**	**100**
3-(3-*tert*-butylphenyl)-2-methylpropanal (*meta*- Lysmeral)	62518-65-4		> 450 (BASF SE 2011A)	18	24	8	13
3-(4-*tert*-butylphenyl)-2-methylpropanoic acid (Lysmerylic acid)	66735-04-4		50* (BASF SE 2006A)	90	94	90	79
3-(*p*-tert-butylphenyl)-2-methylpropanol (Lysmerol)	56107-04-1		50 (BASF SE 2011B)	62	123	119	67
3-(4-*tert*-butylphenyl)-propanal (BHCA)	18127-01-0		25 (100) (RIFM 2016, ECHA 2017F)	115	143	95	102
3-(4-isopropylphenyl)-2-methylpropanal (PMHCA)	103-95-7		75 (ECHA 2017A)	64	66	59	82

3-(4-isopropylphenyl)-propanal (PHCA)	7775-00-0		75 (RIFM 2016)	96	81	90	42
3-(3-isopropylphenyl)-3-methylpropanal (*m*-iP2MHCA)	125109-85-5		> 250 (ECHA 2017B)	2	2	nd	nd
3-(4-isobutylphenyl)-2-methylpropanal (iBMHCA)	6658-48-6		25 (RIFM 2016)	53	44	24	17
p-tert-butyltoluene (TBT)	98-51-1		15 (Furuhashi 2007B)	127	116	nd	nd
p-tert-butylbenzoic acid (TBBA)	98-73-7		6 – 8 (Hunter 1965)	110	98	108	84
p-isopropylbenzoic acid	536-66-3		15 (Givaudan 2011)	77	69	56	35
Floralozone (3-(4-ethylphenyl)-2,2-dimethylpropanal)	67634-15-5		> 250 (RIFM 2016)	1	bd	nd	nd
Tropional (α-methyl-1,3-benzodioxole-5-propanal)	1205-17-0		> 1000* (BASF SE 2010B)	9	10	nd	nd
Fennaldehyde (3-(4-methoxyphenyl)-2-methylpropanal)	5462-06-6		> 1000* (BASF SE 2011B)	12	16	nd	nd
Jasmorange (3-(4-tolyl)propanal)	41496-43-9		> 1000* (BASF SE 2011B)	6	3	nd	nd

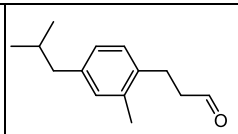
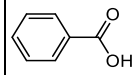
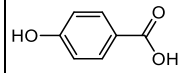
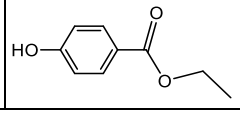
Nympheal™ (3-(4-isobutyl-2-methylphenyl)propanal			> 500 (Laue 2017)	bd	bd	nd	nd
Benzoic acid	65-85-0		> 900 (ECHA 2017C)	1	bd	nd	nd
p-Hydroxy-benzoic acid	99-96-7		> 1000 (ECHA 2017D)	bd	bd	nd	nd
Ethylparaben (ethyl 4-hydroxybenzoate)	120-47-8		> 1043 (ECHA 2017E)	1	bd	nd	nd

Figure 6: Comparison of BMHCA acid-CoA (= Lysmerylic acid-CoA) and TBBA-CoA conjugate formation in plated primary hepatocytes. Hepatocytes from rat (A) and human (B) (Lot I, single donor) were incubated with Lysmeral (50 µM) for 0.5, 4 and 22 h. Coenzyme A conjugates were analyzed by LC-HRMS.

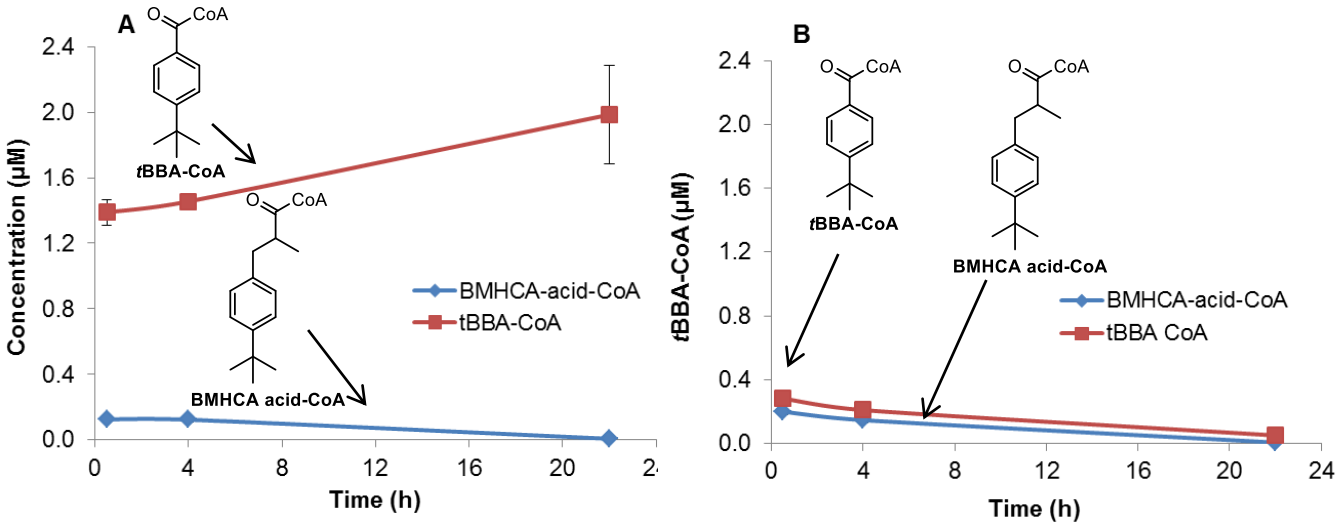
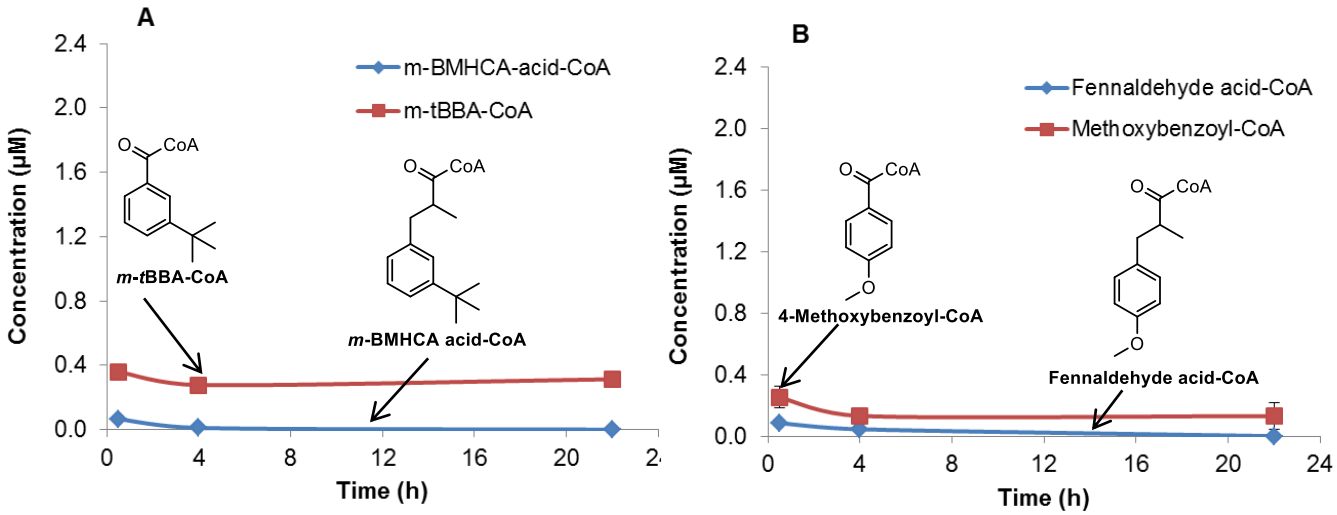


Figure 7: Fate of derivatives of Lysmeral with no male reproductive toxicity in rats in plated rat hepatocytes. Formation of m-BMHCA-acid-CoA (= meta-Lysmerylic acid-CoA) and meta-TBBA-CoA conjugates in the presence of meta-Lysmeral (A) and Fennaldehyde acid-CoA and p-methoxybenzoyl-CoA in the presence of Fennaldehyde (B) in rat hepatocytes. Plated primary rat hepatocytes were incubated with 50 µM meta-Lysmeral (A) or 50 µM Fennaldehyde (B) for 0.5, 4 and 22 h. Coenzyme A conjugates were analyzed by LC-HRMS.



In conclusion, these data provide strong indications, that the formation of para-alkyl-benzoyl-CoA conjugates is an essential step for the observed male reprotoxic rat effects in this close structural chemical group due to the strong correlation of elevated and stable para-alkyl-benzoyl-CoA levels in plated rat hepatocytes and testicular toxicity/spermatotoxicity. Furthermore, liver toxicity in vivo correlates with male reproductive toxicity at comparable doses as well. The liver toxicity is likely to be caused by the disruption of CoA dependent metabolic processes due to conjugation with p-alkyl-benzoic acids. This conjugation further represents a critical hallmark of the reproductive outcome and serves as a mode of action for Lysmeral/TBBA induced testes toxicity and spermatotoxicity.

Besides the differences in the formation of TBBA levels, the kinetics of TBBA-CoA conjugation fundamentally differs in human hepatocyts in terms of lower concentrations and a transient and rapid decrease compared to the rat, which strongly indicates, that the observed metabolic fate of Lysmeral is rat specific and the testicular toxicity/spermatotoxicity is a species-specific effect with little relevance for humans.

4.11.1.2 Human information

No data available.

4.11.2 Developmental toxicity

Table 26: Summary table of relevant developmental toxicity studies

Method	Results	Reference
– Prenatal Developmental Toxicity Study (OECD Guideline 414, GLP) – rat (Wistar) – oral: gavage – 0, 5, 15, 45 mg/kg bw/d (nominal dose) – 0, 4.1, 12.7, 40.7 mg/kg bw/d (actually ingested) – TS purity: 98.1%	• **General maternal toxicity - Main effects**: Clinical signs, ↓ body weights (incl. transient body weight loss), changes in liver associated parameters (clinical chemistry, ↑ liver weights). • **Prenatal developmental toxicity - Main effects (coinciding with maternal toxicity):** ↑ total resorptions/postimplantation loss, ↓ mean gravid uterus weights, ↓ in fetal body weights and associated ↑ in skeletal variations. NOAEL (maternal toxicity): 5 (4.1) mg/kg bw/d NOAEL (prenatal developmental toxicity): 5 (4.1) mg/kg bw/d	BASF SE (2004)

4.11.2.1 Non-human information

For the assessment of developmental toxicity of Lysmeral (2-(4-tert-butylbenzyl)propionaldehyde), a study in Wistar rats was performed in accordance with the OECD test guideline No. 414 in line with the OECD Principles of Good Laboratory Practice (BASF SE 2004). Lysmeral (analytical purity 98.1%) was administered via gavage at nominal doses of 5, 15 and 45 mg/kg bw/day from day 6 through day 20 post coitum (p .c.). The effective dose levels amounted to 4.1; 12.7 and 40.7 mg/kg body weight/day.

Clear signs of maternal toxicity were observed starting at the mid dose level. The high dose animals showed transient salivation. Slight but statistically significant reduction of mean food consumption (18% below controls) was observed in the high dose group on day 6-8 p.c. By study termination, food consumption was comparable to control animals (see Table 28). Although no evident decrease in food consumption was detectable in mid dose animals, mean maternal weight gains significantly decreased on day 6-8 p.c of about 56 % below control which 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, the corrected body weight gain was statistically significantly lower (about 32% below control), representing a direct, substance-related sign of maternal toxicity.

Concerning clinical chemistry, 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 respective clinical parameters, only the liver weight changes in the mid and high dose group were considered as adverse. In high dose animals, reduced mean gravid uterus weights (20% below controls) were observed.

Gestational parameters such as number of corpora lutea, implantation sites and preimplantation loss were not influenced by the test substance at any dose level (see Table **27**). However, mean postimplantation losses (mainly early resorptions) were found to be increased significantly in the high dose group. In animals receiving 45 mg/kg bw per day, mean resorptions accounted to 15.1% per dam compared to 4.4%; 4.7% or 4.9% resorptions at 0, 5 or 15 mg/kg body weight/day, being outside the historical control range. Subsequently, a decrease in the mean number of fetuses and live fetuses per dam became evident in the high dose group, i.e. 7.4, when compared to controls or lower dose groups (8.1; 8.2 and 8.8 at 0; 5 and 15 mg/kg body weight/day). These high dose findings were slightly below the historical controls of the mean number of fetuses per dam. Sex distribution and placental weights were not influenced by the test substance. No dead fetuses, abortions or premature births have been observed in control and all dose groups of this study.

Table 27: Overview on reproductive parameters from the developmental toxicity study in Wistar rats (BASF SE 2004).

Dose group [mg/kg bw/d]	Conception rate[a]	Pregnant at terminal sacrifice	Mean Corpora lutea	Mean implantation sites	Mean Preimplantation loss[b]	Mean Postimplantation loss[c]	mean number of fetuses / life fetuses
0	92%	92%	9.2±1.41	8.5±1.5	7.6±12.33%	4.4±7.35%	8.1±1.5
5 (4.1)	88%	88%	9.0±1.25	8.6±1.4	4.6±7.42%	4.7±7.59%	8.2±1.18
15 (12.7)	92%	92%	9.9±1.29	9.3±1.22	5.5±7.17%	4.9±10.56%	8.8±1.37
45 (40.7)	92%	92%	9.3±1.36	8.8±1.68	5.9±9.36%	**15.1±20.25%***	7.4±2.15
HCD	92%	92%	9.2-11.3 (range per study)	8.1-10.2 (range per study)	3.5-12.2% (range per study)	3.4-11.3% (range per study)	7.6-9.8 (range per study)

*$p \leq 0.05$, mg/kg bw/d (mg/kg body weight/day); HCD (Historical control data)
[a]Conception rate = number of pregnant animals / number of fertilized animals * 100
[b]Preimplantation loss = number of corpora lutea - number of implantations / number of corpora lutea * 100
[c]Postimplantation loss = number of implantations - number of live fetuses / number of implantations * 100

Sporadic malformations were observed in 3 out of 170 or 1.8% of all high dose group fetuses. Three out of 23 or 13% of the litters were affected in this dose group. Findings were reported as anasarca with a small spleen, polydactyly due to a supernumerary phalanx and cervical hemivertebra. The mean percentages of affected fetuses per litter with total malformations amounted to 0; 0; 0 and 2.4% at 0; 5; 15; or 45 mg/kg bw/day. These findings are not regarded as sufficient evidence for a selective teratogenic effect of Lysmeral, since the observed malformations lacked a consistent pattern and were not found in any other dose group. Furthermore, they occurred in very few of the large number of examined fetuses and its low incidence is to be found within the respective control range of the given testing laboratory; i.e. affected fetuses (0-2.7%), affected litters (0-25%) and affected fetuses/litter (0-2.79%).

External variations were not observed and soft tissue variations (dilated renal pelvis, ureters and/or cerebral ventricles) occurred in a dose independent manner in all test groups including control animals. The mean percentages of affected fetuses per litter with total soft tissue variations amounted to 7.8%, 7.7%, 3.6% and 5.3%, in controls, low, mid and high dose animals, respectively.

Skeletal variations were seen in all tested dose groups and litters including controls. Every litter was affected, resulting in 100% litter incidence for all groups assessed. Although within the historical control range and lacking a relation to dosing, a statistically significant increase in mean percentages of affected fetuses per litter were found in mid and high dose animals (99.1% and 98.3% in mid and high dose groups versus 89.1%, 92.%, in controls and the low dose group, respectively). The fetal incidence of skeletal variations in total was increased accordingly.

As shown in Table 28, these skeletal variations mainly represent delays and minor disturbances in ossification of the skull, sternebrae and pubic girdle. Non or incompletely ossified structures were statistically significantly increased in the mid and/or high dose group compared to the concurrent control, and incidences of single findings were above the study related and/or current historical control range, i.e. incomplete ossification of supraoccipital, sternebra, pubis or unossified sternebra. As described in detail for skeletal structural variations further down, these findings coincide with decreased mean fetal body weights. These were dose dependently lowered in the mid (10% below controls) and high dose groups (20% below controls). Approximately 85 % of the fetuses in the high dose group and 50 % in the mid dose group showed body weights below one standard deviation of the control group mean body weight (< 3.3 g). Decreases in mean maternal body weight gains or even body weight losses in combination with decreased food consumption occurred at these dose levels as well.

Although significantly increased, structural skeletal variations such as supernumerary (14^{th}) ribs were found in control and dosed animals at high incidences within historical control ranges, whereas incidences for a supernumerary thoracic vertebra (14^{th}) or a misshapen sacral vertebra (1^{st} sacral arch; right or left side) were increased in the high dose group fetuses above historical control ranges.

One fetus of one litter in the control group showed a supernumerary thoracic vertebra which places the study specific control group parameters into the lower part of the updated current historical control range. High dose group incidences are well above the historical control range whereas mid dose group values are within the updated current historical data range. Both dose groups contained single litters with multiple fetuses showing a supernumerary thoracic vertebra. Findings from these litters are the main driver for the increased fetal incidences and affected fetuses/ litter given in Table 28. Out of 23 litters, a single mid dose litter with 4 affected fetuses and 2 mid dose litters with 2 affected fetuses each were observed. Similarly, a single litter contained 4 affected fetuses and 3 litters contained 2 affected fetuses each in the high dose group. For each of these litters, a decreased mean litter weight has been observed, i.e. ≤3.3 g in the mid dose group and ≤3.0 g in the high dose group. The respective dams showed a decreased body weight gain in the mid dose and a body weight loss in the high dose group on day 6-8 p.c. Furthermore, increases in absolute and/or relative liver weights and changes in clinical chemistry were noted. Three additional litters contained 1 fetus with such a variation in the mid and high dose group, being within the range of incidence per litter also observed in the control group. One additional vertebra in the thoracolumbar region is generally considered to be a variation in the rat and occurs quite frequently in the rabbit (Solecki 2001).

A misshapen sacral vertebra was found in 1 fetus of 2 control group litters each (fetal incidence of 2.1% and litter incidence of 8.7%). Incidences in the high dose group exceeded the historical control data. Two of 23 litters contained 2 fetuses with the named variation. In addition, 7 litters contained 1 affected fetus each, leading to an increase in the litter incidence. For all these litters, mean fetal body weights were decreased (≤3.0 g/litter) and respective dams experienced a body weight loss and decreased food consumption on d6-8 p.c., changes in clinicochemical parameters and increases in absolute and/or relative liver weights. Furthermore, the observed structural changes in the morphology of the sacral vertebra were minor and provide, together with the high control animal incidence, the rationale for classifying them as variations.

As described above, the observed skeletal variations are well correlated to the statistically significantly decreased mean fetal body weights and maternal adverse effects in the respective dose groups. Such a delay in fetal body weight development and subsequent increase in skeletal variations is considered to be caused by the evident maternal toxicity.

Significantly increased mean percentages of affected fetuses per litter with incomplete ossification of parietal (29.8% versus 11.7% in controls) or interparietal (36.0% versus 20.7% in controls) with unchanged cartilage was observed only in the low dose group and considered to be spontaneous in nature, since no dose dependency was observed. Discoloration of fetal livers was evident in some mid and high dose animals with a mean percentage of affected fetuses per litter of 1.7% and 15.5%, respectively, being in line with the liver changes of the respective dams.

Based on these findings, the NOAEL is set at 5 (4.1) mg/kg bw/day for maternal and prenatal developmental toxicity.

Table 28: Maternal systemic toxicity based on body weight/ changes and food consumption. Effects on mean fetal body weights on a litter basis and occurrence of statistically significantly increased fetal skeletal variations (expressed as mean percentage of affected fetuses/litter). All statistically significant differences, which showed a dose-response relationship and/or were outside historical control ranges (at date of study) were marked in bold types. ; *p ≤ 0.05; **p ≤ 0.01; mg/kg bw/d (mg/kg body weight/day).

Finding		**0** [mg/kg bw/d]	**5 (4.1)** [mg/kg bw/d]	**15 (12.7)** [mg/kg bw/d]	**45 (40.7)** [mg/kg bw/d]	**HCD report[1]** Mean % (range)	**HCD 2012[2]** Mean % (range)
Mean maternal food consumption (d6-8 p.c.)	grams/ animal/ day (± SD)	15.4 ±1.87	15.0 ±1.73	14.7 ±1.46	12.7 ±1.76**	-	-
Mean maternal body weight change (d6-8 p.c.)	grams (± SD)	5 ±3.52	4.2 ±2.16	2.2 ±2.84**	-5.5 ±3.65**	-	-
Mean maternal body weight change (d6-20 p.c.)	grams (± SD)	75.3 ±8.4	75.7 ±12.51	77.0 ±7.94	56.8 ±21.55**	-	-
Mean maternal body weights (d20 p.c.)	grams (± SD)	264.4 ±16.75	265.2 ±23.12	268.1 ±16.12	247.2 ±24.11*	-	-
Mean net body weight change from day 6[3]	grams (± SD)	30.0 ±6.67	30.6 ±8.43	30.6 ±7.41	20.5 ±14.92**	-	-
Mean Gravid Uterine weight	grams (± SD)	45.2 ±7.65	45.1 ±6.59	46.4 ±6.81	36.3 ±9.64	-	-
Mean fetal weights	grams (± SD) (on a litter basis)	3.6 ±0.28	3.5 ±0.20	3.3 ±0.17**	2.9 ±0.29**	3.5 (2.8-4.2)	3.6 (2.6-5.5)
Misshapen sacral vertebra	Fetal incidence %	2.1	0	2.7	**12**	0.7 (0-3.5)	1.8 (0-7.1)
	Litter incidence %	8.7	0	13	**39***	3.4 (0-16.7)	7.6 (0-28)
	Affected Fetuses/litter	1.7	0	3	**11.9****	0.8 (0-3.8)	1.9 (0-7.5)
Supernumerary thoracic vertebra	Fetal incidence %	1	2.1	**10**	**14**	1.9 (0-4.7)	2.9 (0-10.1)
	Litter incidence %	4.3	9.1	**26***	**30***	7.3 (0-16.7)	10.7 (0-30)
	Affected Fetuses/litter	1.1	2.7	**9.8***	**13.6****	1.9 (0-5.3)	3.0 (0-11.5)
Supernumerary rib (14th), cartilage present	Fetal incidence %	1	2.1	**10**	8.7	4 (0-8.7)	6.2 (0-19.2)
	Litter	4.3	9.1	26*	22	13.4	20

	incidence %					(0-29.2)	(0-62.5)
	Affected Fetuses/litter	1.1	2.3	9.8*	8.4*	4.1 (0-10.2)	6.2 (0-18.3)
Supernumerary rib (14th), cartilage not present	Fetal incidence %	41	31	51	39	41.9 (31.3-58.1)	48.8 (31.2-72)
	Litter incidence %	78	64	91	65	81.2 (65.2-95)	85.7 (65.2-100)
	Affected Fetuses/litter	38.5	30.7	51.1*	36.7	41.7 (32.4-58.8)	48.6 (32.4-73.1)
Incomplete ossification of supraoccipital, unchanged cartilage	Fetal incidence %	13	19	17	**22**	14 (5.8-21.4)	17.9 (5.8-46.2)
	Litter incidence %	39	55	43	**61**	41.5 (22.2-60)	48.1 (22.2-92)
	Affected Fetuses/litter	12.8	20.1	17.2	**24.6***	14 (6.2-21.2)	17.8 (6.2-45.1)
Supraoccipital hole(s)	Fetal incidence %	23	17	28	42	31.3 (8-59.3)	29.2 (4-59.3)
	Litter incidence %	52	50	70	87*	70.7 (33.3-100)	67.4 (19-100)
	Affected Fetuses/litter	23.4	17.3	28.4	39.8*	31.7 (8.6-60.5)	29.4 (3.6-60.5)
Incomplete ossification of skull, unchanged cartilage	Fetal incidence %	0	4.3	2.7	7.6	5.4 (0-10.8)	5.5 (0-15.2)
	Litter incidence %	0	14	13	26*	17.3 (0-30.4)	18.1 (0-44.0)
	Affected Fetuses/litter	0	5.1*	2.8*	6.7**	5.4 (0-11)	5.4 (0-15.8)
Unossified sternebra, unchanged cartilage	Fetal incidence %	3.1	8.5	13	**46**	10.6 (3.4-35.7)	7.9 (0.9-35.7)
	Litter incidence %	13	32	26	**83****	32.2 (13-70.8)	24.9 (4.2-70.8)
	Affected Fetuses/litter	3.3	9.4	12.8	**49.9****	11.1 (3.4-39)	8.0 (0.8-39)
Incomplete ossification of sternebra, unchanged cartilage	Fetal incidence %	47	61	**76**	**77**	56 (38.5-73.7)	64.3 (38.5-87.3)
	Litter incidence %	78	91	100*	96	88.5 (70-100)	91.6 (70-100)
	Affected Fetuses/litter	46.1	60.1	**76.7****	**76.6****	55.9 (38.4-74.6)	63.6 (38.4-87.2)
Bipartite ossification of sternebra, unchanged cartilage	Fetal incidence %	0	0	2.7	0	0.8 (0-4.9)	0.4 (0-4.9)
	Litter incidence %	0	0	13	0	2.9 (0-13)	1.6 (0-13)
	Affected Fetuses/litter	0	0	2.8*	0	0.8 (0-4.6)	0.4 (0-4.6)
Incomplete ossification of sacral arch, cartilage present	Fetal incidence %	1	2.1	0	12	12.5 (0-46.2)	6.9 (0-46.2)
	Litter incidence %	4.3	9.1	0	39**	31 (0-87)	18.8 (0-87)
	Affected Fetuses/litter	1.1	2.4	0	16.2**	12.2 (0-46.1)	6.8 (0-46.1)
Incomplete ossification of pubis, cartilage present	Fetal incidence %	0	0	**1.8**	**5.4**	0.4 (0-1.2)	0.3 (0-1.9)
	Litter incidence %	0	0	**8.7**	**22***	2 (0-5.6)	1.6 (0-8.3)
	Affected Fetuses/litter	0	0	**2**	**8****	0.4 (0-1.2)	0.4 (0-2.1)
Incomplete ossification of interparietal,	Fetal incidence %	21	**36**	21	11	21.6 (13.6-33.3)	23.4 (12.7-36.1)

unchanged cartilage	Litter incidence %	52	**82***	65	35	58.5 (37.5-81.8)	60.4 (37.5-82.6)
	Affected Fetuses/litter	20.7	**36***	20.9	9.8	21.5 (12.5-33.3)	23.3 (12.4-35.3)
Incomplete ossification of parietal, unchanged cartilage	Fetal incidence %	11	**30**	14	14	15.2 (3.2-29.9)	15.0 (3.2-29.9)
	Litter incidence %	39	68*	52	48	43.4 (12.5-68.2)	43.7 (12.5-68.2)
	Affected Fetuses/litter	11.7	**29.8****	13.6	13.4	15.4 (3.1-27.6)	15.3 (3.1-27.6)
Total fetal skeletal variations	Fetal incidence %	91	91	99	98	94.6 (88-99.2)	96.6 (88-100)
	Litter incidence %	100	100	100	100	100 (100-100)	100 (100-100)
	Affected Fetuses/litter	89.1	92	99.1**	98.3*	94.7 (87-99.2)	96.6 (87-100)

*$p \leq 0.05$; **$p \leq 0.01$; mg/kg bw/d (mg/kg body weight/day).
[1]HCD report: historical control data available at the study finalisation date.
[2]HCD 2012: updated historical control databasis, i.e. including data available up to 2012.
[3]Net Weight Change From Day 6 = Terminal Body Weight minus Uterine Weight minus Day 6 Body Weight

Further information on developmental toxicity can be deduced from the respective endpoints of the one-generation range-finding studies and the EOGRTS. In the older range-finding study, no viable offspring has been derived from animals treated with 1700 ppm (63 mg/kg bw/d) and 3400 ppm (120 mg/kg bw/d) microencapsulated Lysmeral (BASF SE 2006C; for further details see Chapter 4.11.1.1). In the 1700 ppm group, the only pregnant female had only 1 implant which was resorbed. In contrast, only a slight and non-significant increase in mean implantation losses have been observed for the two lower dose groups; i.e. 16% and 11% mean losses per litter in dose groups 400 ppm (10-15 mg/kg bw/d) and 800 ppm (18-29 mg/kg bw/d) versus 5% in controls, respectively (see Table **20**). A slight decrease in the mean number of delivered pups per dam (7.9 in dose group 800 ppm versus 9.4 and 8.7 in controls and dose group 400 ppm) was recorded. However, no effects on gestation and the live birth indices became evident, due to the absence of any stillborn in the Lysmeral treated dose groups with offspring. Pup survival was minimally decreased for postnatal day 0 to 4 (94% in the 800 ppm dose group versus 99% in the 400 ppm dose group and controls), and no pup mortality was observed between postnatal day 4 and 21 in all dose groups with offspring. Overall the respective viability and lactation index was not considered to be affected by treatment.

No effects in sex ratios have been observed and pup necropsy revealed only sporadic and non-dose related findings, including post mortem autolysis, situs inversus, hemorrhagic thymus, dilated renal pelvis and a small kidney. The overall pup incidence for these observations was 6.4%, 2.6%, and 1.3% in controls and dose groups 400 ppm and 800 ppm respectively.

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) has been recorded for male and female pups. Accordingly the pup body weight gain was decreased in the 400 and 800 ppm dose groups (16% and 21% below controls, respectively).

In the recent range finding study (BASF SE 2017B), a slight and non-significant increase in mean postimplantation loss have been observed in the high dose dams (2300 ppm, 25-35 mg/kg bw/d; (see Table 21). No effects on live birth indices were observed, since stillborn rates in the Lysmeral treated dose groups were not increased compared to the respective placebo control. An observed decrease in the number of delivered pups per dam (4.0 in high dose dams versus 11.1 in controls) can predominantly be attributed to lower numbers in implantation sites and affected fertility indices.

Pup survival was decreased for postnatal day 0 to 4 (86% and 75% in the mid and high dose groups versus 99% and 95% in the low dose group and placebo control group), whereas no pup mortality was observed between postnatal day 4 and 21 in all dose groups.

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) has been recorded for mid (750 ppm, 7-12 mg/kg bw/d) and high dose pups. Accordingly the pup body weight gain was decreased in the mid and high dose groups (13% and 33% below controls, respectively).

Taken together, significant Lysmeral related developmental toxicity in the one-generation rangefinding studies have been observed predominantly in terms of decreased pup weights and indications for effects on early pup survival exist. 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 hematological parameters (for further details see Chapter 4.11.1.1.).

In the EOGRTS, nominal doses of 1, 3 and 10 mg/kg body weight/d Lysmeral (approx. 1.4, 4.5 and 15.1 mean mg/kg bw/d throughout all study phase and across all cohorts) were administered to groups of 35-40 male and female Wistar rats as a homogeneous addition to the food (BASF 2017).

No postimplantation losses, indicative for Lysmeral induced intrauterine embryo/-/fetolethality, was observed for F0 and F1 generation animals up to the highest dose level tested (Table 24). A decrease in the mean number of implantation sites (10.5 implants/dam) in high dose F1 females was within the historical control range, was not observed in respective F0 animals and no other findings supportive of a potential effect on fertility (i.e. follicle numbers, sperm quality) exist, making this finding an incidental event. The finding of decreased mean numbers of delivered F2 pups in these animals is associated with the lower number of implants and not an independent finding. The live birth index was not affected in these and all other treated animals.

The pup body weight development was affected in high-dose F1 and F2 offspring (about 14-16% less than control after birth and no recovery until weaning; see Table 23). 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. A higher number of cannibalized high-dose F1 pups around PND 1 were mostly clustered in 2 litters and no such effects evident in the high-dose F2 offspring. Thus, these were most likely incidental events.

Blood thyroid hormone levels in parental animals 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 (not observed in 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 and represent not an independent treatment effect.

Further marker of potential endocrine-mediated imbalances (i.e. presence of nipples/areolas) revealed no Lysmeral related effects. A delay in preputial separation in the high-dose male F1 offspring, was within the historical control range and can be attributed to a general delay in the development of high-dose male F1 offspring. No effect of Lysmeral treatment for vaginal opening was noted. Overall, the sexual development of the offspring was not impaired by Lysmeral administration.

During the course of development, i.e. in high dose PND 4 male pups and PND 76 females, lower peripheral acetylcholinesterase (AChE) activities were observed in serum, erythrocytes and diaphragm tissue. However, no corresponding signs of developmental neurotoxicity were observed. There was no evidence that Lysmeral produced any developmental immunotoxicity.

Overall, developmental toxicity observed in the EOGRTS represent only reductions in pup body weights in the high dose F1 and F2 offspring (10 mg/ kg bw/d nominal; approx. 15 mg/kg bw/d ingested). This dose level resulted in adverse maternal liver effects, effects on food consumption, body weights and clinical pathological parameters (for further details see Chapter 4.11.1.1).

4.11.2.2 Human information

No data available.

4.11.3 Other relevant information

No data available.

4.11.4 Summary and discussion of reproductive toxicity

Summary of Fertility

Repeated dose studies in male rats, partly with focus on male reproductive organs, provide evidence for adverse effects on male reproductive organs in rats after oral Lysmeral (2-(4-tert-butylbenzyl) propionaldehyde) administration. These effects were observed concomitantly with signs of general toxicity and adverse effects on the liver. The subchronic repeated dose toxicity study provides a NOAEL for testicular toxicity effects after oral administration at 25 mg/kg bw/day (Givaudan 1986A), and according to the findings from further repeated dose and reproductive toxicity studies, these effects can be expected to occur at doses above this NOAEL. This effect level was found to be independent from treatment duration. Adverse testicular findings were observed even after a single oral administration. These data support the conclusion for a clear dose threshold for the induction of testicular toxicity in rats independent of dose duration.

Accordingly, impairment of male fertility combined with signs of general toxicity and changes in clinical parameters of the liver was observed in the one-generation range finding studies in the rat after oral administration of Lysmeral. Due to the obvious testicular and spermatotoxic effects of Lysmeral, the relation between the observed lack of pregnancies, lack of delivered offspring and impairment of male fertility is clearly indicated. These findings were obtained at comparable dose levels also used in repeated dose studies. In contrast, dermal administration on rats led to no testicular toxicity except for dose levels above the limit dose. In the EOGRTS, oral administration of Lysmeral via feed did not affect male or female fertility and reproductive performance of parents and offspring at doses up to 10 mg/ kg bw/d nominal (approx. 15 mg/kg bw/d ingested). In dogs, general adverse effects together with liver and testicular toxicity were observed after oral administration, however, adverse testes effects occurred at higher dose levels than in the rat. Considering the findings from the available studies in dogs, a NOAEL for testicular toxicity is set at 44.6 mg/kg bw/day. No testicular toxicity was observed in the mouse, guinea pig, rabbit and primates.

Identical adverse testicular effects and species specificity has been observed after oral administration of p-tert-benzaldehyde (TBB) and p-tert-butyltoluene (TBT). The rat has been found to be the most sensitive species for TBB and TBT induced testicular toxicity. In analogy to Lysmeral, systemic formation of p-tert-butylbenzoic acid (TBBA) has been observed after oral administration of TBB and TBT. Clear evidence of adverse testicular and spermatotoxic effects - identical in quality to Lysmeral - have been observed for the metabolite TBBA as well. Based on the lowest adverse effect level for testicular toxicity, TBBA application in rats revealed the highest potency and is included in Annex VI of the CLP regulation with a classification as Repr. 1B (H360F; Index No. 607-698-00-1). TBB and TBT showed lower potencies in exerting comparable testes effects. Lysmeral showed the lowest potency in testes toxicity when compared to TBB, TBT and especially to TBBA. Testes toxicity potencies correlated well with systemically formed urinary TBBA amounts. Therefore TBB, TBT and Lysmeral all share TBBA as common metabolite and the formation of the systemic TBBA intermediate represents a metabolic key event for Lysmeral induced testicular toxicity.

A strong correlation between the formation of TBBA-CoA conjugates in rat hepatocytes, disruption of lipid synthesis and testicular toxicity has been found. Complex lipids are present in high amounts in mammalian sperm and play an important role for spermatigenesis. Their synthesis depends on intracellular process, that requires a sufficient pool of available CoA. Lysmeral treatment was found to disrupt fatty acid/ lipid synthesis and induced testes toxicity is always observed in the presence of liver toxicity. Other chemicals potentially transformed to benzoic acid metabolites show a strong correlation between sustained formation of benzoyl-CoA complexes in hepatocytes and spermatoxic/testicular toxicity in rats.

Taken together, the comparable pattern of testicular effects, the species dependencies and the observed differences in potencies substantiate, that the formation of systemic TBBA is a metabolic key event for Lysmeral and TBB/TBT induced testicular toxicity. Furthermore, the conjugation of TBBA with CoA represents the mode of action for Lysmeral induced testes toxicity and spermatotoxicity.

Summary of Developmental Toxicity

Developmental toxicity of Lysmeral has been assessed by oral (gavage) administration of Lysmeral to pregnant rats in a developmental toxicity study according to OECD test guideline No. 414.

High dose dams (41 mg/kg bw/d) showed clinical signs (transient salivation), transient reduction of mean food consumption and body weight loss on day 6-8 p.c. Mean body weight gain was decreased over the entire treatment phase resulting in lower mean body weights on day 13 - 20 p.c. and net body weight gain compared to controls. Increased levels of alanine aminotransferase and glutamate dehydrogenase, decreases serum cholinesterase levels and organ weight changes (increased liver weights, reduced uterus weights) were noted.

In mid dose dams (13 mg/kg bw/d) body weight gains were transiently decreased on day 6-8 p.c. Furthermore, alanine aminotransferase levels were increased, serum cholinesterase levels were decreased and increased liver weights were found.

These findings reflect a Lysmeral induced general systemic and liver toxicity for high dose and less pronounced for mid dose dams.

The number of mainly early resorptions was increased due to postimplantation losses in the high dose group whereas gestational parameters were not significantly influenced in lower dose groups (5, 15 mg/kg bw/d). Subsequently, the number of fetuses and live fetuses per dam was found to be slightly below the respective historical control range in the high dose group.
Concomitantly, prenatal developmental toxicity in terms of reduced fetal body weights was observed in the mid and high dose groups. These findings coincided with significant maternal toxicity at the same dose levels.

Sporadic malformations were observed, which lacked a consistent pattern, occurred in very few of the large number of examined fetuses and there incidences were found within the respective historical control ranges. External variations were not observed and soft tissue variations occurred in a dose independent manner in all test groups including control animals.

In contrast, an overall incidence of skeletal variations was statistically significantly increased in mid and high dose animals. These variations represented mainly delays and minor disturbances in ossification processes of the skull, sternebrae and pubic girdle. Supernumerary (14^{th}) ribs were found in control and dosed animals at high incidences, and structural variations like a supernumerary thoracic vertebra (14^{th}) or a misshapen sacral vertebra (1^{st} sacral arch) were found to be increased evidently in the high dose group fetuses. The observed skeletal variations are well correlated to statistically significantly decreases in mean fetal body weights and evident maternal toxicity in the respective dose groups. Clustering of incidences for a supernumerary or misshapen vertebra in single litters was observed, and a maternal predisposition which affects the respective offspring in situations of maternal stress conditions could be hypothesized here.

Supernumary ribs and delays of ossification in rodent offspring are among the common endpoints related to chemical exposure stress (ECETOC, 2004). Delays in ossification are by definition transitory, occur in conjunction with decreased fetal weights and represent an indicator for adverse effects on fetal maturation rather than a teratogenic potential (Daston, 2007).

Overall, the increased numbers of fetuses with common skeletal variations are considered an embryo-/fetotoxic effect due to fetal growth retardations, representing a manifestation of a non-specific stress on the dams and not a teratogenic effect of Lysmeral. Increased early resorptions and the subsequent decrease in number of fetuses are further manifestations of the non–specific maternal stress induced by Lysmeral administration.

The findings of the one-generation rangefinder studies are largely consistent with the effects observed in the present key teratogenicity study. Slight, non-significant and dose independent increases in postimplantation losses were found in dose groups having offspring. A slight reduction in the number of delivered pups has been observed at doses not affecting fertility indices. Furthermore, a significant reduction in birth weights, pup weights at weaning and pup weight gain has been seen when compared to controls. These findings coincided with adverse systemic effects to the dams. No effects on the gestation and live birth indices were observed due to the absence of any stillborn in the dosed animals. Whereas effects on early pup survival occurred, lactation indices were not significantly affected and no test substance related findings in pup necropsy have been found.

Furthermore, the highest Lysmeral dose tested in the EOGRTS (in the range of the LOAEL of the developmental toxicity study) resulted in pup body weight reductions of the F1 and F2 offspring and was associated with adverse maternal liver and general systemic effects. Lysmeral did not have a consistent impact on the number of postimplantation losses, delivered pups and pup survival up to this dose. Further developmental toxicity endpoints including developmental neurotoxicity and immunotoxicity were not affected by treatment with Lysmeral.

Taken together, developmental toxicity has been observed at doses leading to evident maternal toxicity and is considered to be a secondary non-specific consequence of general systemic toxicity in the dams. Therefore, these findings do not warrant a classification with respect to developmental toxicity.

Discussion

Adverse effects of Lysmeral on the male reproductive system have been observed in various oral repeated dose toxicity studies in rats and were confirmed in feeding one-generation range-finding studies, whereas no evidence for testicular toxicity was observed in the mouse and guinea pig. Considering non-rodent species, the dog has been shown to be susceptible towards testicular toxicity as well, however at higher dose levels than the rat. Testicular toxicity in rats and dogs after oral Lysmeral application was only observed at dose levels showing also general signs of toxicity in these animals. In studies with more detailed observations, the liver was found to be the main affected organ upon treatment with doses also inducing testicular toxicity. In contrast, short-term oral exposure to rabbits did not indicate a potential of Lysmeral to induce testicular toxicity. Furthermore in rhesus monkeys, no indication of testicular toxicity, at doses causing testicular toxicity in the rats, was observed. Therefore, the rat represents the most susceptible species, and it appears, that a single oral exposure to Lysmeral above a clearly defined threshold dose seems sufficient to cause testicular toxicity.

Testicular effects and species dependencies identical to Lysmeral have been observed after application of p-tert-benzaldehyde (TBB) and p-tert-butyltoluene (TBT). Para-tert-butylbenzoic acid (TBBA) is formed as metabolite after administration of TBT, TBB or Lysmeral. Therefore TBB, TBT and Lysmeral all share the same metabolite, namely TBBA. TBBA application in rats revealed the highest testicular toxicity potency based on effect levels and is included in Annex VI of the CLP regulation with a classification as Repr. 1B (H360F). In line, TBB and TBT showed lower potencies in exerting comparable testes effects. Lysmeral showed the lowest potency in testes toxicity when compared to TBB, TBT and especially to TBBA. Testes toxicity potencies correlated well with systemically formed urinary TBBA amounts. Overall, these findings substantiate, that the formation of systemic TBBA represents a metabolic key event for Lysmeral and TBB/TBT induced testicular toxicity.

Based on the clear evidences from animal studies, it is considered appropriate to classify Lysmeral for reproductive toxicity, i.e. adverse effects on fertility. In determining the respective hazard category, the assessment of the relevance of the hazard to humans is to be considered. The adverse effects on male reproductive organs are considered to underlie species specific mechanisms and the present data indicate, that primates are considerably less or even not susceptible towards the testicular toxicity observed in the dog and more effectively in the rat. In accordance, quantitative differences in the formation of metabolites such as TBBA exist and the urinary excretion of glycine conjugated TBBA differs between the rat and the other rodent species investigated, i.e. mouse and guinea pig.

On the basis of a qualitative and quantitative evaluation of metabolic profiles for different species in an in vitro metabolism study, a predominant formation of TBBA concentrations in rat hepatocytes was found when compared to other rodent, non-rodent animal or human hepatocytes. The TBBA concentrations found in the model using human hepatocytes were approx. 4 fold lower compared to rat hepatocytes at corresponding incubation concentrations of Lysmeral (10, 50 100 µM). The Lysmeral concentrations used reflect plasma levels obtained after oral administration of Lysmeral doses below and above the lowest adverse testicular effect level. Furthermore, the TBBA levels formed in human hepatocytes after incubation of Lysmeral concentrations related to adverse testicular effect doses (50 and 100 µM) were comparable to TBBA levels found in the rabbit, a species not sensitive to testicular toxicity. Although, the metabolite TBBA is classified as Repr. 1B (H360F), its endogenous formation after Lysmeral exposure is species dependent and the formation in humans is found to be comparable to species, showing no Lysmeral induced testicular toxicity.

A strong correlation has been established between the formation of TBBA-CoA conjugates in rat hepatocytes, disruption of lipid synthesis and testicular toxicity. It is concluded, that the conjugation of TBBA (classified as Repr. 1B (H360F)) with CoA represents the mode of action for Lysmeral induced testes toxicity and spermatotoxicity. The kinetics of TBBA-CoA conjugation fundamentally differs in human hepatocytes when compared to the rat. Lower and transient concentrations due to a rapid decrease of TBBA-CoA conjugates have been observed in human hepatocytes, which strongly indicates, that the observed metabolic fate of Lysmeral is rat specific and the testicular toxicity/spermatotoxicity is a species-specific effect with little relevance for humans.

Based on differences in the endogenous formation and the mode of action between rat and humans, the harmonized classification of the metabolite TBBA as Repr. 1B (H360F) has no implication for the classification proposal provided for Lysmeral.

The adverse testicular effects in the rat and dog were observed after administration of Lysmeral via the oral route. Because of its properties as fragrance material, Lysmeral administration needed to be performed via gavage or encapsulation. Besides test substance stability issues, palatability was a major obstacle due to the unpleasant smell of concentrated Lysmeral in order to attain study relevant doses. Dermal application of Lysmeral represents the most appropriate route of administration, having regard to the likely route of human exposure as a fragrance material (for further details see Annex 1). In contrast, the administration via gavage or encapsulation represents an unrealistic and non-relevant form of application. Compared to oral studies, dermal administration of Lysmeral in rats led to testicular toxicity only at an excessive dose level, clearly above the limit dose, whereas at 1000 mg/kg body weight, no adverse testicular effects were observed. When compared to doses leading to rat testicular toxicity, a prolonged human uptake of Lysmeral doses inducing systemic toxicity (testes toxicity or spermatotoxic effects) is highly unlikely (for further details see Annex 1).

Overall, studies on species variability provide clear evidence, that higher order mammalian species including humans are less or not susceptible than rats. This is due to the observed lack of testicular toxicity in these studies, differences in metabolic profiles (including TBBA formation) and fundamental differences concerning the mode of action identified (sustained TBBA-CoA conjugation). In support, testicular toxicity in susceptible species has a clear threshold and it is highly unlikely that Lysmeral levels taken up by humans would lead to the formation of relevant systemic levels of TBBA. Taken together, the relevance of the species specific testes toxicity observed in test animals for humans is doubtful.

4.11.5 Comparison with criteria

Lysmeral (2-(4-tert-butylbenzyl)propionaldehyde) has been found to induce testicular toxicity and spermatotoxicity when administered orally to rats and at higher dose levels to dogs. Infertility in rats due adverse effects of orally administered Lysmeral on the male reproductive system has been confirmed in feeding one-generation range-finding studies. Based on clear evidences from experimental animals, it is considered appropriate to classify Lysmeral for reproductive toxicity, i.e. adverse effects on fertility.

The CLP regulation criteria for classification as reproductive toxicants are as follows:

The classification in Category 1A (Known human reproductive toxicant) “is largely based on evidence from humans”.

The classification of a substance in Category 1B (Presumed human reproductive toxicant) “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.”

Besides the reference to clear evidences from experimental animals, the CLP regulation further states in its criteria for classification in Category 1B:

“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.”

Further, substances are classified in Category 2 (Suspected human reproductive toxicant), "when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1".

In determining the appropriate hazard category for the adverse effects on fertility, the assessment of the relevance of the given hazard to humans needs to be taken into account.

- Species specificity for Lysmeral induced testicular toxicity has been observed. Adverse effects of Lysmeral on the male reproductive system at a clearly defined threshold dose have been found in rats whereas no evidence for testicular toxicity was observed in the mouse and guinea pig. Considering non-rodent species, the dog has been shown to be susceptible towards Lysmeral induced testicular toxicity. In contrast, short-term oral exposure to rabbits did not indicate a potential of Lysmeral to induce testicular toxicity. Furthermore in rhesus monkeys, no indication of testicular toxicity, at doses causing testicular toxicity in the rats, was observed.

- Based on the accordance in the testicular toxicity profile of Lysmeral, p-tert-benzaldehyde (TBB), p-tert-butyltoluene (TBT) and the shared metabolite para-tert-butylbenzoic acid (TBBA), the formation of the systemic TBBA intermediate (classified as Repr. 1B (H360F)) represents a metabolic key event for Lysmeral induced testicular toxicity.

- Species specificity for Lysmeral induced testicular toxicity is reflected by species dependent differences in the conversion of Lysmeral to TBBA in hepatocytes. TBBA formation in human hepatocytes is of low magnitude compared to rats and is comparable to concentrations found in the rabbit at toxicologically relevant doses, a species not sensitive to Lysmeral induced testicular toxicity.

- A strong correlation has been established between the formation of TBBA-CoA conjugates in rat hepatocytes, disruption of lipid synthesis and testicular toxicity. The conjugation of TBBA with CoA represents the mode of action for Lysmeral induced testes toxicity and spermatotoxicity. In human hepatocytes, lower and transient concentrations due to a rapid decreases of TBBA-CoA conjugates strongly indicate, that testicular toxicity/spermatotoxicity is a species-specific effect with little relevance for humans.

- Because of the properties of Lysmeral as fragrance material leading to palatability issues, substance administration needed to be performed via gavage or encapsulation. However, dermal application of Lysmeral represents the most appropriate route of administration, having regard to the likely route of human exposure as a fragrance material. Dermal studies in rats showed no testicular toxicity up to the limit dose.

Altough the human exposure considerations provided in Annex 1 do not represent a main argument, for the the classification for Lysmeral, they are included to support the classification proposal made in this CLH report. Based on these considerations, a prolonged human uptake of Lysmeral doses inducing systemic toxicity (testes toxicity or spermatotoxic effects) is highly unlikely.

A clear evidence for a species specificity and, if at all, a low human susceptibility concerning Lysmeral induced testicular toxicity raises doubt about the relevance of the effect for humans. Furthermore, evident reproductive toxicity has been observed after substance administration via gavage or encapsulation, representing a non-relevant form of application. In support, a prolonged human uptake of Lysmeral doses inducing systemic toxicity (testes toxicity or spermatotoxic effects) is highly unlikely, when compared to doses leading to rat testicular toxicity.

As outlined in the CLP criteria listed above, a classification in Category 2 (Suspected human reproductive toxicant; Repr. 2 - H361f; CLP regulation EC/1272/2008) is appropriate, whereas classification in Category 1A or 1B (Known or presumed human reproductive toxicant) is not justified.

Concerning developmental toxicity, the CLP regulation states as a basis of classification:

"…Classification as a reproductive toxicant is intended to be used for substances which have an intrinsic, specific property to produce an adverse effect on reproduction and substances shall not be so classified if such an effect is produced solely as a non-specific secondary consequence of other toxic effects."

As outlined in Chapter 4.11.4, developmental toxicity has been observed at doses leading to evident maternal toxicity and is considered a secondary non-specific consequence of general systemic toxicity in the dams. Therefore, based on the present data, no classification concerning developmental toxicity is warranted.

Classification for effects on or via lactation is intended to indicate when a substance may cause harm due to its effects on or via lactation, and it is independent of consideration of the reproductive toxicity of the substance. According to Table 3.7.1 (b) of the CLP-regulation, classification for effects on or via lactation can be assigned on the:

a) human evidence indicating a hazard to babies during the lactation period; and/or

b) results of one or two generation studies in animals which provide clear evidence of adverse effect in the offspring due to transfer in the milk or adverse effect on the quality of the milk; and/or

c) absorption, metabolism, distribution and excretion studies that indicate the likelihood that the substance is present in potentially toxic levels in breast milk.

The present data do not allow to specifically assess the effects of Lysmeral on or via lactation. No human evidence indicating a hazard to babies during the lactation and no 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, classification for effects on or via lactation is therefore not warranted.

4.11.6 Conclusions on classification and labelling

Lysmeral (2-(4-tert-butylbenzyl)propionaldehyde) has been identified to induce testicular toxicity when administered orally to rats and at higher dose levels to dogs. Infertility in rats due adverse effects of orally administered Lysmeral on the male reproductive system has been confirmed in feeding one-generation range-finding studies. In an EOGRTS, Lysmeral doses below the identified LOAEL for testicular toxicity did not affect male or female fertility and reproductive performance of parents and offspring. Based on clear evidences from animal studies, it is considered appropriate to classify Lysmeral for reproductive toxicity, i.e. adverse effects on fertility.

Based on the accordance in the testicular toxicity profile of Lysmeral, p-tert-benzaldehyde (TBB), p-tert-butyltoluene (TBT) and the shared metabolite para-tert-butylbenzoic acid (TBBA), the formation of the systemic TBBA intermediate represents a metabolic key event for Lysmeral induced testicular toxicity. On the basis of the effective doses determined, Lysmeral possesses evidently a lower potency for testicular toxicity than TBBA.

Furthermore, a strong correlation has been established between the formation of TBBA-CoA conjugates in rat hepatocytes, disruption of lipid synthesis and testicular toxicity. The conjugation of TBBA with CoA was found to be the mode of action for Lysmeral induced testes toxicity and spermatotoxicity.

In determining the respective hazard category, the assessment of the relevance of the given hazard to humans needs to be taken into account. Species specificity for Lysmeral induced testicular toxicity has been observed. Adverse effects of Lysmeral on the male reproductive system at a clearly defined threshold dose have been found in rats but not in the mouse and guinea pig. Considering non-rodent species, the dog has been shown to be susceptible towards Lysmeral induced testicular toxicity. In contrast, short-term oral exposure to rabbits did not indicate a potential of Lysmeral to induce testicular toxicity. Furthermore in rhesus monkeys, no indication of testicular toxicity, at doses causing testicular toxicity in the rats, was observed

Species specificity for Lysmeral induced testicular toxicity is reflected by species dependent differences in the conversion of Lysmeral to TBBA in hepatocytes. TBBA formation in human hepatocytes is of low magnitude compared to rats. In fact, TBBA formation in humans is comparable to levels produced in species that did not demonstrate testicular toxicity (i.e. rabbits) at biologically relevant doses. In addition, the kinetics of TBBA-CoA conjugation, i.e. the identified mode of action, fundamentally differs in human hepatocyts when compared to the rat. Lower and transient concentrations due to a rapid decrease of TBBA-CoA conjugates have been observed in human hepatocytes, which strongly indicates, that the observed metabolic fate of Lysmeral is rat specific. Overall, clear evidences for a low susceptibility of humans regarding Lysmeral induced testicular toxicity further support the absence of its human relevance.

Because of the properties of Lysmeral as fragrance material leading to palatability issues, substance administration needed to be performed via gavage or encapsulation. This represents a non-relevant form of application. Studies via the relevant route to humans (i.e. dermal) in rats showed no testicular toxicity up to the limit dose (1000mg/kg bw/day). As a supportiv argument, a prolonged human uptake of Lysmeral doses inducing systemic toxicity (testes toxicity or spermatotoxic effects) is highly unlikely and the relevance of the observed testicular effects for humans is doubtful.

Overall, clear evidence for a species specificity and, if at all, a low human susceptibility concerning Lysmeral induced testicular toxicity question a relevance for humans.

Therefore, a classification as substance to be suspected of damaging fertility, i.e. Repr. 2 (H361f; regulation 1272/2008), is warranted**.**

Developmental toxicity has been observed at doses leading to evident maternal toxicity and is considered to be a secondary non-specific consequence of general systemic toxicity in the dams. Therefore, based on the present data, no classification concerning developmental toxicity is warranted.

Based on currently available data, classification for effects on or via lactation is not warranted.

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.

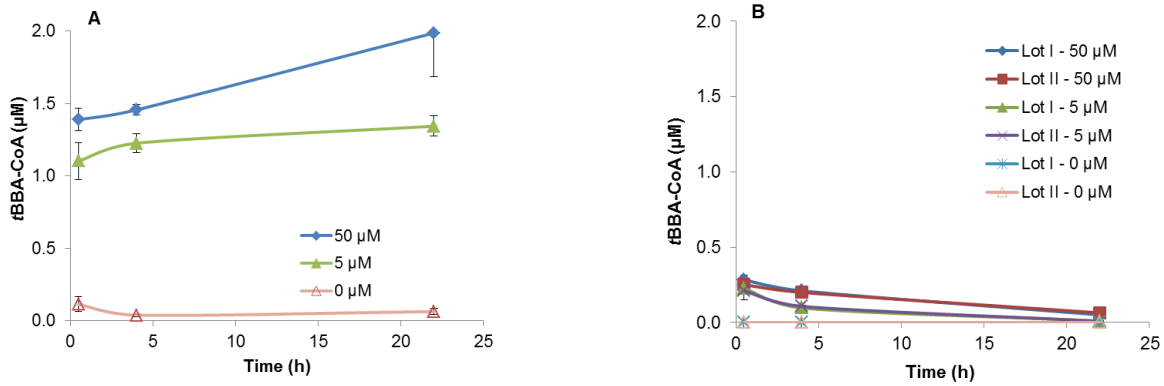
To address the proposed mode of action (MoA), the DS summarised published *in vitro* data indicating an inhibitory capacity of TBBA on lipogenesis and gluconeogenesis in rat hepatocytes. Addition of glycine, which represents a relevant substrate to form the respective hippurate (TBHA), did not affect 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).

Additional key elements

During public consultation the DS provided a summary of a recent study investigating the correlation between the formation of TBBA-CoA conjugates and testicular/sperm toxicity, as well as details of the human *in vitro* and *in vivo* penetration studies.

A 3D-culture of primary rat seminiferous tubules was treated with 2, 10, and 50 µmol/L of para- or meta-TBBA. Cytotoxicity, blood-testis barrier functionality via trans-epithelial electrical resistance (TEER) measurements and cell numbers of different somatic and germ cell populations were quantified. In addition, the content of TBBA conjugated with CoA were assessed in cell culture lysates.

Results indicate a direct spermatotoxic effect of p-TBBA, but not of m-TBBA. It was shown that p-TBBA-CoA-conjugates can also be formed in testicular tissue. Although concentrations measured in this study were approximately 100-fold lower than in hepatocytes *in vitro*, the different potential of p-TBBA vs m-TBBA to form stable CoA-conjugates was confirmed.

The DS also provided the details on the *in vitro* human skin penetration study and the *in vivo* dermal penetration study in human volunteers, both of which were not considered of major relevance for the hazard assessment and classification of Lysmeral.

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 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*: 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	LOAEL (general toxicity, males): 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**.

4.12 Other effects

Not evaluated in this dossier.

5 ENVIRONMENTAL HAZARD ASSESSMENT

Not evaluated in this dossier.

6 OTHER INFORMATION

This substance has been registered according to the requirements of the REACH legislation. In addition, the substance is under evaluation in the framework of the Community Rolling Action Plan (CoRAP). The listing was based on concerns regarding human health due to CMR properties wide dispersive use and consumer use. The evaluation started in the year 2012 and further information was required to clarify concerns regarding endocrine disrupting properties and developmental toxicity. This information requirement included to conduct an Extended one-generation reproductive toxicity study (EOGRTS) in rats, oral route (test method: OECD 443) with the extension of Cohort 1B to mate the F1 animals to produce a F2 generation, the 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. This CLH report provides an update including the required information on the EOGRTS and further relevant information on the proposed classification.

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8 ANNEXES

ANNEX 1: HUMAN LYSMERAL UPTAKE AND REPRODUCTIVE TOXICITY HAZARD ASSESSMENT.

Summary

Lysmeral has been identified to induce testicular toxicity when administered orally to rats and dogs, but not to mice, guinea-pigs, rabbits and monkeys. Orally administered Lysmeral in rats resulted in the formation of systemic p-tert-butyl benzoic acid (TBBA). TBBA has been found to induce identical testicular toxicity in rats at significantly lower doses compared to Lysmeral. It is therefore considered appropriate to classify Lysmeral for reproductive toxicity, i.e. adverse effects on fertility.

The adverse testicular effects in the rat and dog were observed after administration of Lysmeral via the oral route. Besides test substance stability issues, palatability was a major obstacle due to the unpleasant smell of concentrated Lysmeral in order to attain study relevant doses. Therefore, administration needed to be performed via gavage or encapsulation, representing an unrealistic and non-relevant form of application with respect of realistic use patterns.

The dermal route is the most relevant route to humans in both an occupational and consumer setting (exposure via inhalation may occur but to a much lower extent). Compared to oral studies, dermal administration of Lysmeral in rats led to testicular toxicity only at an excessive dose level, clearly above the limit dose, whereas at 1000 mg/kg body weight, no adverse testicular effects were observed. Furthermore, compared to rats, limited percutaneous absorption of the test substance in humans was observed.

The exposure assessment performed here is meant to support the identification of human relevance and includes the estimation of Lysmeral doses taken up by workers and the general population in comparison to the effective level of the most sensitive species via the oral route. Taking conservative assumptions into account, the estimated Lysmeral doses in an occupational setting are of low magnitude. Due to the much lower Lysmeral concentrations handled in formulated consumer products, potential Lysmeral doses taken up by the consumer would be significantly lower than for the worker in manufacture, compounding, formulation processes and when using products for industrial and professional cleaning.

The potential for effects from the estimated worker Lysmeral uptake under normal working conditions or following an accidental acute event would result in systemic doses of Lysmeral or respective metabolites such as TBBA, far below levels that have been shown to induce testicular toxicity in the rat.

In conclusion, an assessment of the human relevance of the hazard has been made in order to determine the appropriate hazard category for Lysmeral. Altough the human exposure considerations do not represent the main argument, they support the classification proposal for Lysmeral, which mainly relies on the the observed species differences in metabolism and the fundamental differences concerning the mode of action identified. The human exposure considerations demonstrate, that a human relevance is questionable when considering occupational exposure and the significantly lower exposure of the general population. Furthermore, a low odour threshold and unpleasant perception at toxicologically relevant but unrealistic concentrations (1000 fold above the respective odour threshold) are intrinsic properties of Lysmeral, which make a prolonged human uptake of effective internal doses as in animals highly unlikely.

Based on the fact that testicular toxicity in susceptible species has a clear threshold, it is extremely unlikely that Lysmeral levels taken up by humans would lead to the formation of relevant systemic levels of TBBA, resulting in testicular toxicity. This leads to the conclusion, that a hazardous situation for humans would be unrealistic and the relevance of the observed testicular effects of Lysmeral for humans is doubtful.

Introduction and approach

Lysmeral is used as fragrance in a wide number of industries. It shows an intensive, radiant, floral odor with a typical lily-of-the-valley note. As a component of fragrance mixtures the main uses include cosmetic/personal care products and washing/cleaning products. In this annex, an assessment of human doses, that result from current uses of Lysmeral is compared with doses from respective animal studies, showing testicular toxicity of Lysmeral in order to support the determination of the appropriate hazard classification.

The human odour threshold for racemic Lysmeral is set at 1-2 ppb (0.01 mg/m^3 for R-Lysmeral, >2.5 mg/m^3 for L-Lysmeral; van Gemert, 2003). Due to its properties, a concentration 1000 fold above the human odour threshold would usually be perceived as very unpleasant. Therefore a prolonged human uptake of Lysmeral doses inducing systemic toxicity, i.e. testes toxicity or spermatotoxic effects, is highly unlikely. As a general approach, perceivable Lysmeral concentrations would result in a calculated internal dose of 0.007 mg/kg Lysmeral (considering a daily respiration volume of 20 m^3 and an average body weight of 60 kg for the general population). Given an allometric scaling factor of 4, the perceivable odour threshold would result in human doses approximately 3 orders of magnitude below non-effective rat dose levels concerning testicular toxicity.

Exposures of Lysmeral to the general public are minimal, since the substance is only used in trace amounts in final products, is poorly absorbed dermally in humans and has a low volatility (vapor pressure = 0.25 Pa). Thus, Lysmeral doses taken up by a consumer would be expected to be very low and significantly lower than by workers involved in manufacturing, compounding, formulating and in industrial/professional uses in final products, i.e. cleaning. Specific occupational exposure via potential relevant routes of exposure (i.e. dermal and inhalation), have been assessed using conservative assumptions in first tier or higher tier exposure estimation tools (ECETOC TRA, Stoffenmanager, RiskofDerm). It includes the potential occupational exposure to Lysmeral during manufacturing, compounding, formulating and industrial/professional uses in final products.

Exposure assessment

For the assessment of the workplace, models, i.e. ECETOC TRA, Stoffenmanager 5.1 or RISKOFDERM 2.1 have been used. This assessment is supported by workplace measurements covering several process categories.

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 2-(4-*TERT*-BUTYLBENZYL)PROPIONALDEHYDE

Exposures in the workplace

It is generally assumed that oral exposure to industrial chemicals in the workplace can be discounted and therefore, it is considered unlikely that any oral uptake with liquid Lysmeral will occur during manufacture, compounding, formulation and industrial/professional uses in final products. Ingestion is usually controlled by straightforward good hygiene practices such as segregating working and eating facilities and adequate washing prior to eating. Overall, a putative oral uptake has not been included in a combined worker exposure estimation.

For the assessment of the uptake via the dermal route, a low dermal absorption of max. 7% of the applied dose is assumed based on in vitro dermal penetration data as a worst case, given that a value of 2% was derived in an in vivo study with human subjects (BASF 2016; Huntingdon Research Centre Ltd 1994; for further details see Chapter 4.1.). External dermal exposure has been calculated by the first tier exposure estimation tool ECETOC TRA or the higher tier exposure estimation tool RISKOFDERM 2.1 (see Table 30), and an internal dermal exposure has been calculated assuming a 7% dermal absorption of the external dermal dose. For process categories involving handling of higher Lysmeral concentrations during manufacture, compounding and formulation, the use of suitable gloves as personal and product protective equipment has been included in the exposure assessment resulting in an additional reduction by a factor of 10 of the external dermal dose.
Inhalation of Lysmeral is considered to be of low relevance, since Lysmeral has a low vapor pressure (0.25 Pa) and the major industrial scenarios do not include the formation of aerosols. Inhalation has been estimated using the first tier estimation tool ECETOC TRA or the higher tier estimation tool Stoffenmanager 5.1 (see Table 30). The given external inhalation exposure estimate has been calculated into an internal inhalation estimate assuming a 100% absorption via inhalation, a mean worker respiratory volume of 10 m^3 and a mean body weight of 70 kg. For confirmation of the calculated estimates, occupational measurements by personal air sampling are available, covering the relevant process categories in the manufacture exposure scenario (Table 29). These data support, that the calculations made are based on conservative assumptions, leading to an overestimation compared to the realistic levels not exceeding 1 µg/m^3. Furthermore in a model setup, a stationary measurement of the air concentration 5 cm above the liquid surface of Lysmeral resulted in a concentration of 0.18 mg/m^3 at room temperature. Sampling has been performed for 150 minutes above a dish containing 20 ml Lysmeral.
This setup represents a worst case scenario compared to realistic scenarios and further confirms the conservatism in the calculation for certain process categories, where relevant uptake via inhalation might occur (e.g. mixing or industrial/professional spray applications of final products). Such model setup can also be used for the assessment of Lysmeral uptake following an accident in the workplace such as spilling. The concentrations of Lysmeral in air would be considered not to exceed 0.18 mg/m^3 as determined in the setup, i.e. air concentration 5 cm above the liquid surface. Considering a respiration volume of 10 m^3 during a shift and a mean body weight of 70 kg, a daily internal dose would result in 0.026 mg/kg bw for workers not wearing respiratory protection. When comparing with the no adverse effect level for testicular toxicity in the rat as most sensitive species including an allometric scaling factor of 4, the margin of safety would be approx. 250 for such an accidental situation.

Table 29: BASF SE Workplace measurements. Personal air sampling according to EN 481 und 482 have been performed. For the determination of Lysmeral concentrations in air, defined air volume have been collected into a cartridge containing 2,4-dinitrophenylhydrazine. Adsorbed Lysmeral has been eluted with acetonitrile and quantified by liquid chromatography via comparison with a calibrator solution.

Detection limit (mg/m^3)*	Measurement duration (minutes)	Description of Task
< 0.001	250	Activities in laboratories
< 0.00098	240	Activities in laboratories
< 0.00098	240	Transfer of substance into drums
< 0.00098	240	Transfer of substance into drums
< 0.00098	240	Transfer of substance into drums
< 0.001	500	Drum filling and control activities in production facility
< 0.001	500	Sampling and control activities in production facility
< 0.001	500	Sampling and control activities in production facility
< 0.00098	240	Activities in production facility - not further specified
< 0.00098	240	Activities in production facility - not further specified
< 0.001	250	Activities in production facility - not further specified
< 0.001	250	Activities in production facility - not further specified
< 0.001	250	Activities in production facility - not further specified

*All measurement results have been below the detection limit given in the table

As presented in Table 29, the external dermal dose in a worker specific exposure scenario does not exceed 0.4 mg/kg bw day on the basis of the conservative assumptions of ECETOC TRA, leading to a potential internal dose of 0.03 mg/kg bw/day. Furthermore, the calculated mean inhalation exposure estimates during an 8 hour shift do not exceed 0.43 mg/m^3 resulting in an internal dose of 0.06 mg/kg bw/day.

Overall, the comparison of the combined internal exposure estimate (dermal and inhalation) of all relevant process categories for Lysmeral and the no adverse effect level for testicular toxicity in the most sensitive species, i.e. rats, revealed a margin above 100. This margin of safety has been derived by comparing conservative exposure estimations and a NOAEL from the rat as most sensitive species for Lysmeral induced testicular toxicity. Therefore, the calculated margin of safety demonstrate, that the uptake of Lysmeral in the workplace will not have an impact on the fertility of male workers.

Table 30: Combined exposure assessment and risk characterization of all relevant process categories in the workplace.

Exposure scenario	Process category PROC[1]	Exposure estimate long-term inhalation external [mg/m³]	Exposure estimate long-term inhalation internal [mg/kg bw/d] [2]	Exposure estimate long-term systemic dermal external [mg/kg bw/d]	Exposure estimate long-term systemic dermal internal [mg/kg bw/d] [3]	Exposure estimate long-term systemic combined internal [mg/kg bw/d]	NOAEL [mg/kg bw/d] [4]	Margin of safety[5]
Manufacture	2	0.0097	0.0014	0.1371	0.0096	0.0110	25	2276
	8b (vessel)	0.0201	0.0029	0.2114	0.0148	0.0177	25	1416
	8b (drums)	0.0091	0.0013	0.0000[7]	0.0000[7]	0.0013	25	19248
	15	0.0201	0.0029	0.0343	0.0024	0.0053	25	4736
Compounding	1	0.0170	0.0024	0.0034	0.0002	0.0027	25	9356
	3	0.0021	0.0003	0.0686	0.0048	0.0051	25	4896
	5 (automated)	0.0097	0.0014	0.0691	0.0048	0.0062	25	4012
	5 (manual)	0.0446	0.0064	0.0124	0.0009	0.0072	25	3452
	8a	0.0393	0.0056	0.3429	0.0240	0.0296	25	844
	8b	0.0101	0.0014	0.4157	0.0291	0.0305	25	820
	9	0.2128	0.0304	0.1714	0.0120	0.0424	25	588
	15	0.0115	0.0016	0.0343	0.0024	0.0040	25	6180
Formulation	1	0.0043	0.0006	0.0009	0.0001	0.0007	25	37424
	3	0.0039	0.0006	0.0171	0.0012	0.0018	25	14228
	5	0.0395	0.0056	0.3429	0.0240	0.0296	25	844
	8a	0.0047	0.0007	0.1371	0.0096	0.0103	25	2436
	8b	0.0408	0.0058	0.3429	0.0240	0.0298	25	836
	9	0.0851	0.0122	0.0686	0.0048	0.0170	25	1472
	14	0.4256	0.0608	0.0343	0.0024	0.0632	25	396
	15	0.0102	0.0015	0.0086	0.0006	0.0021	25	12144
Cleaning industrial	1	0.0009	0.0001	0.0003	0.00002	0.0001	25	171724
	2	0.0851	0.0122	0.0137	0.0010	0.0131	25	1904
	4	0.2979	0.0426	0.0686	0.0048	0.0474	25	528
	7[6]	0.1061	0.0152	0.4286	0.0300	0.0452	25	552
	8b	0.0851	0.0122	0.1371	0.0096	0.0218	25	1148
	10	0.0950	0.0136	0.2743	0.0192	0.0328	25	764

	13	0.0851	0.0122	0.1371	0.0096	0.0218	25	1148
Cleaning professional	1	0.0009	0.0001	0.0003	0.0000	0.0001	25	171724
	2	0.2979	0.0426	0.0137	0.0010	0.0435	25	576
	4[6]	0..2913	0.0416	0.0686	0.0048	0.0464	25	540
	8a	0.2979	0.0426	0.1371	0.0096	0.0522	25	480
	8b	0.1703	0.0243	0.1371	0.0096	0.0339	25	736
	10[6]	0.2913	0.0416	0.2743	0.0192	0.0608	25	412
	11[6]	0.2581	0.0369	0.1071	0.0075	0.0444	25	564
	13	0.1703	0.0243	0.1371	0.0096	0.0339	25	736

[1]process categories (PROCs) are defined according to REACH Guidance on information requirements and chemical safety assessment Chapter R.12: Use descriptor system.
[2]calculated as follows: Exposure estimate inhalation external x respiration volume (10 m3) / body weight (70 kg)
[3]calculated as follows: Exposure estimate dermal external x dermal penetration human (7% of applied dose)
[4]NOAEL for testicular toxicity in rats
[5] NOAEL / systemic combined internal exposure estimate
[6] Example scenario leading to the highest calculated exposure values of this PROC
[7] Filling of drums is performed in a fully automated fashion. No dermal worker exposure expected.

Exposures via consumer products

Lysmeral is found in a broad array of consumer products as a component of fragrance mixtures. However, Lysmeral has not been used as flavor ingredient. The main sources of Lysmeral stem from cosmetic/personal care products and washing/cleaning products. Lysmeral is further included in air care products and biocidal products. As described above, estimated worker doses of Lysmeral are of low magnitude. Due to the much lower levels of Lysmeral in formulated consumer products, an uptake of Lysmeral by a consumer would be in general significantly lower than for the worker. Lysmeral is used in trace amounts in final product up to 0.75 % in washing/cleaning products and up to 1.42 % in personal care products. Highest use levels for Lysmeral were determined for air care products (up to 10%), which contain high concentrations of fragrance mixes. Despite the obvious intention of incorporating fragrances into consumer products for their olfactory properties, inhalation appears to represent a minor route of systemic exposure to fragrances, even when highly exaggerated airborne levels and rather unlikely exposure scenarios are used (Cadby 2002). Furthermore Lysmeral has a low volatility (vapor pressure = 0.25 Pa), making exposure via the inhalation route less likely. Since Lysmeral is not used as flavor agent nor is included in oral care products, only indirect oral exposure (e.g. via dishwashing residues) is to be expected. Therefore, the major route of systemic exposure is almost certainly by deposition on the surface of the skin.
A broad variety of consumer products contain Lysmeral as an integral part of fragrance mixtures included in these products. However, the final concentrations in these products are usually in the range or below the concentrations found in the main product/article categories described above. Based on the back-calculation from urinary metabolite levels of spot urines samples of 40 adult volunteers, median daily exposures with Lysmeral were determined to be approx. 200 µg/d or 3 µg/kg bw/d (based on a mean body weight of 60 kg), indicating a significantly lower exposure of Lysmeral by a consumer than the estimations made for worker exposure (Scherer 2016). Overall, the exposure of the general population to products containing Lysmeral will not have an impact on male fertility.

Conclusion

In conclusion, based on the high margins of safety in relation to oral dosing studies in the rat as most sensitive for Lysmeral induced testicular toxicity, it is unlikely that the uptake of Lysmeral would lead to the formation of relevant systemic levels of metabolites, such as p-tert-butylbenzoic acid, in the human, meaning the likelihood of a concern would be very low.

ANNEX 2: OVERVIEW ON STUDIES ADRESSING TESTICULAR TOXICITY INDUCED BY P-TERT-BUTYLBENZOIC ACID (TBBA).

Table 31. Summary of relevant studies for p-tert-butylbenzoic acid (TBBA) with focus on adverse effects of the male reproductive organs.

Species	Test substance	Study period	Dosage	LOAEL/$C_{testes/sperm}$	NOAEL/$C_{testes/sperm}$	Reference
Rat	TBBA	Single treatment	p.o. 500, 630, 800, 1000, 2000 mg/kg bw n = 4 / sex and dose	500 mg/kg bw Testicular toxicity: macroscopic changes, ↓ testes weights testicular atrophy (4/5)	<500 mg/kg bw	Hunter 1965
Rat	TBBA	Single treatment	p.o. 700, 720 mg/kg bw n = 10 / sex and dose	700 mg/kg bw Testicular toxicity: ↓ testes weights hypospermatogenesis (10/10)	<700 mg/kg bw	Hazleton 1986
Rat	TBBA	Single treatment	inhalation 495, 668, 958, 1802 mg/m^3; 4 hour whole body n = 6 / sex and dose	495 mg/m^3 Testicular toxicity: ↓ testes weights ↓ in spermatid counts, tubular degeneration, reduction of spermatogonia, multinucleated giant cells in tubuli seminiferi, vacuolization in Sertoli cell plasma	<495 mg/m^3	Lu 1987
Rat	TBBA	5 days	p.o. 12.5, 25, 50, 100 mg/kg bw/d; n = 8 males / dose	25 mg/kg bw/d Testicular toxicity: ↓ testes weights; degeneration of germ cells (spermatids and spermatocytes); ↓ number of spermatozoa; sporadic giant cells Additional systemic toxicity: Mortality, body weight loss, macroscopic changes in liver/kidney, ↑ liver weights	12.5 mg/kg bw/d	Givaudan 1982D

Rat	TBBA	90 days	p.o. 100, 316, 1000, 3160, 10000 ppm in feed n = 10 / sex and dose	100 ppm (6-8 mg/kg bw/d) Testicular toxicity: ↓ testes weights; testes atrophy (degenerated epithelium of seminiferous tubules) Additional systemic toxicity: mortality, clinical signs, ↓ body weights; changes in clinical chemistry; liver and kidney toxicity (↑ organ weights, macroscopic changes, histopathology)	<100 ppm (6-8 mg/kg bw/d)	Hunter 1965
Rat	TBBA	70 days	p.o. 20, 100, 500 ppm in feed n = 10 males / dose	100 ppm (7.9 mg/kg bw/d) Testicular toxicity: ↓ testes weights; minor lesions of the germinative epithelium; impaired fertility	20 ppm (1.6 mg/kg bw/d)	Hoechst 1987
Rat	TBBA	28 days	dermal 7.5, 15, 30, 60 mg/kg bw/d n = 8 / sex and dose	60 mg/kg bw/d Testicular toxicity: ↓ testes weights; ↓ mitotic activity in spermatogonia; germinal epithelium degeneration, ↓ spermatozoa; presence of multinucleated spermatocytes. Additional systemic toxicity: ↓ body weights; ↑ liver/kidney weights.	30 mg/kg bw/d	Shell 1975

Rat	TBBA	90 days	dermal 17.5, 35, 70, 140 mg/kg bw/d n = 13 / sex and dose	70 mg/kg bw/d Testicular toxicity: ↓ testes weights; moderate to severe diffuse seminiferous tubular degeneration. Spermatotoxic effects: ↓ sperm head count and LDH-X enzyme activities. Additional systemic toxicity: ↓ body weights, liver and kidney toxicity (↑ organ weights, changes in clinical chemistry, histopathology)	35 mg/kg bw/d	Cagen 1989, Lu 1987
Rat	TBBA	7 days	inhalation 12.5, 106, 525 mg/m^3; 6 hours/day, whole body n = 8 / sex and dose	12.5 mg/m^3 Spermatotoxic effects: ↓ testicular sperm counts Testicular toxicity (>12.5 mg/m^3): ↓ testes weights; multifocal to diffuse degeneration of the germinal epithelium. Additional systemic toxicity: Mortality, ↓ body weights, clinical signs, hematological/clinical changes; liver and kidney toxicity (↑ organ weights, histopathology); CNS lesions.	<12.5 mg/m^3	Shell, 1982

Rat	TBBA	28 days	inhalation 1.5, 4.7, 15.7 mg/m^3; 6 hours/day, snout-only n = 5 / sex and dose (organ assessment)	No testicular toxicity observed.	15.7 mg/m^3	HRC 1995

Evidences for testicular toxicity after oral administration of p-tert-butylbenzoic acid were found in an acute oral toxicity study in albino Carworth Farm rats (Hunter 1965). Groups of 4 animals/sex were administered with doses of 500, 630, 800, 1000 and 2000 mg/kg bw. When male survivors were examined 18 or 24 days after treatment, testicular atrophy was observed in 4/5 males exposed to a single dose of 500 mg/kg bw. Testes were shrunken, their parenchyma was pinkish and felt like "bags of jelly". These organs weighted 50- 60% of the normal weight. This bilateral atrophy was due to a degeneration of the generative cells in the seminiferous tubules. In contrast, ovaries of the surviving females were of normal appearance and presented no evidence of abnormal oogenesis at microscopy. Cohabitation of surviving male animals with untreated female animals did not result in pregnancies, indicating disturbed male fertility. An oral LD50 of 735 mg/kg bw was determined in this study.

Single oral dose by gavage with 700 mg/kg bw of p-tert-butylbenzoic acid to 10 male Sprague-Dawley albino rats resulted in a high mortality rate (7/10 rats) within 24 hours (Hazleton 1986). After administration of 720 mg/kg bw 2/10 rats died within 2 hours. In both groups the only treatment-related macroscopic and microscopic observation was small testes observed in one animal and hypospermatogenesis of the testes in all treated animals. Even though the cells appeared normal, these animals had fewer spermatogenic cells in the seminiferous tubules than controls. Mean absolute and relative testes weights were significantly lower in treated animals.

Single whole body exposure for 4 hours to respirable dust of p-tert-butylbenzoic acid (0, 495, 668, 958 or 1802 mg/m^3) in 6 Fischer 344 rats/sex resulted in reduced testes weights (50% compared to controls) in all p-tert-butylbenzoic acid exposed animals (Lu 1987). Reduction in spermatid counts, tubular degeneration, reduction of spermatogonia, multinucleated giant cells in the tubuli seminiferi and vacuolization in the plasma of Sertoli cells was observed. The LC50 determined in this study was >1802 mg/m^3.

In a testicular toxicity screening test using Fü albino SPF rats (8 males/ dose group) were orally treated with 12.5, 25, 50 and 100 mg p-tert-butylbenzoic acid /kg bw/day for 5 consecutive days, whereas 4 control males received the vehicle only (Givaudan 1982D). Mortality, general symptoms, body weights were recorded and all rats were autopsied. The testes of all rats were examined microscopically. One animal in the 100 mg/kg bw/ day dose group died by unknown causes. No signs of intoxication were observed, but a dose-related loss of body weights were observed from 25 mg/kg bw/ day onward. As necropsy findings, marmoration of the liver, delineation of hepatic lobules, pale livers and kidneys were observed in some animals of all dose groups. Increases in mean liver weights by 9% compared to the control group were found in the high dose group. Mean testes weights decreased by 15% compared to controls at the top dose.

Treatment related histological changes in the seminiferous epithelium were found. Cross-sectioned seminiferous tubules of controls and rats treated with 12.5 mg/kg bw/day showed normal patterns with orderly germ cell arrangement. At higher dose levels, degeneration of germ cells especially of spermatids and spermatocytes were observed and number of spermatozoa were reduced. Sporadic giant cells were found. These changes showed a dose related increase in incidence and severity.

A $NOAEL_{general\ toxicity}$ and a $NOAEL_{testicular\ toxicity}$ of 12.5 mg/kg bw/ day can be set for this study.

In an oral 90 day study, p-tert-butylbenzoic acid was administered to albino Carworth Farm rats (10 animals/sex/group) via the diet containing doses of 0, 100, 316, 1000, 3160 and 10000 ppm (0, 6, 21, and 75 mg/kg bw/d for males and 0, 8, 27, 89 mg/kg bw/d for females for doses up to 1000 ppm; two top doses not calculated) (Hunter 1965). In this study food consumption and body weights were recorded and urinalysis, hematology, clinical chemistry, gross and microscopic examinations were performed. Dietary levels of 3160 and 10000 ppm resulted in high percentages of premature deaths or animals to be killed in extremis, whereas no deaths were observed in the three lower exposure groups. Hind limb paralysis was reported for one male and one female exposed to diet concentration of 3160 ppm and one female at 1000 ppm. Final body weights were significantly decreased in males at diet concentrations from 316 ppm onward and in female rats from 1000 ppm onward. The feed consumption was reduced by 50-70% compared to controls in the 3160 and 10000 ppm dose group. In hematology, only changes with minor pathological relevance, i.e. decreased erythrocyte counts and changes in the differential blood counts were observed in the two high dose groups. Reduced total protein levels in male rats receiving 100 to 1000 ppm and dose-dependent increases in urea concentrations in males and female rats from 1000 ppm onward were found. In the urinalysis, increased urine volume and reduced urine osmolarity was found in treated rats from 3160 ppm onwards and protein concentrations were elevated in animals of the 10000 ppm dose group.

Increased relative liver and kidneys weights were observed in all dose groups and animals of the two top doses revealed congested and speckled livers and hydronephrosis, hydroureter, ureteral obstructions, hematuria in the urinary tract at necropsy. Findings in liver and kidney were confirmed microscopically. Sinusoidal congestion and fatty degeneration of centrilobular hepatocytes were found and intra-luminal cell debris, necrosis of the tubular epithelium, papillary necrosis were reported as the causes of the obstructive urinary tract lesions. Renal tubular necrosis and papillary necrosis was evident in all treated animals, showing dose dependent increases in incidences.

Evident testicular toxicity was observed by decreased testes to body ratios in all treated males. Testes weights were statistically significantly ($p<0.05$) reduced in the 1000 ppm (1.21 g) and 316 ppm (2.67 g) exposure groups compared to controls (3.45 g). Bilateral atrophy of the testes was found in all treated animals. The testes atrophy was related to degenerated epithelium of seminiferous tubules.

The authors indicate that atrophy of the testis was found even in the lowest dosage group of 100 ppm. Overall, a NOAEL on male reproductive organ toxicity and general toxicity could not be determined.

A $LOAEL_{general\ toxicity}$ and a $LOAEL_{testicular\ toxicity}$ of 100 ppm has been derived, referring to 6 or 8 mg p-tert-butylbenzoic acid/kg bw/day.

In a fertility study with male Wistar rats, ten animals per dose group were fed diets containing 0, 20, 100, or 500 ppm p-tert-butylbenzoic acid continuously for a period of 70 days before starting with mating trials (Hoechst, 1987). On the basis of the recorded food consumption a mean daily intake of 1.6 (20 ppm), 7.9 (100 ppm) and 41 (500 ppm) mg p-tert-butylbenzoic acid/kg bw/d was calculated. During exposure, the animals were checked regularly for general condition, behavior, and body weight and food consumption. Each male was then mated to two non-exposed virgin females for a period of one week (first mating trial) and the females were checked daily for cyclicity and sperm. Proof of fertility was taken from successful impregnation of at least one of the two females. Males that had not been fertile during the first trial were kept for another 70 days without dietary test substance exposure and then were again mated to virgin females for a period of one week (second mating trial - recovery group). Length of gestation, numbers of live and dead born, sex, weight and any externally visible anomalies of the newborns, which were finally sacrificed, have been assessed. Males were terminated at delivery of their impregnated dams or at the end of the mating trials and macroscopically investigated. Organ weights were taken of brain, heart, liver, spleen, kidney, testes and epididymides. Testes, epididymides, prostate and seminal vesicles were subjected to histopathological investigation. Females were terminated either one day after delivery or 25 days after the last mating trial and macroscopically investigated and numbers of implantation sites counted.

Reversible reduction in body weight gains was observed in treated animals at the 500 ppm dose level only, resulting in 14% lower body weights than controls at the end of the treatment period. Parental organ weights for brain, heart, liver, spleen and kidneys of the treated groups did not differ from those of the controls.

No treatment-related effects were observed for duration of the gestational period and on parturition and no differences in the numbers of live born per litter, sex ratio and mean body weights of the newborns between the controls and the treatment groups were found. No externally visible anomalies in newborns were recorded.

No pregnancies were produced during the first mating interval from males exposed to dietary levels of 500 ppm. Three males inseminated one female each; however, no pregnancies resulted, whereas from the other 7 males no sperm was detected in vaginal smears of their female partners. In contrast, all males of former 500 ppm group impregnated one or both females during the second mating trial 70 days after the end of the treatment period, i.e. the recovery group. One male of the 100 ppm group was not successful to impregnate but one of its two females was sperm positive in the first mating interval (one of the two females was impregnated by this male animal in the second mating period). All other males in this dose group, at lower dose or in the control group proved to be fertile by impregnating one or two females.

In males of the 500 ppm group mean absolute testes weights were reduced (2.76 g) in comparison to that of the controls (3.14g) after the recovery period, whereas testes weights at lower dose groups did not differ from controls. Exposure to 500 ppm p-tert-butylbenzoic acid resulted in minor lesions of the germinative epithelium which were confined to few tubules only, but no histopathological changes were found in prostate, seminal vesicles, epididymides and its sperm. No histopathological differences were observed in lower dose groups when compared to controls.

Overall, a $NOAEL_{testicular\ toxicity}$ of 20 ppm (1.6 mg/kg bw/d) and a $NOAEL_{general\ toxicity}$ of 100 ppm (7.9 mg/kg bw/d) can be derived from the study based on the finding of infertility/inability to impregnate at dietary dosages of 100 ppm and reduced body weight gain at 500 ppm.

Evidences for testicular toxicity via dermal application of p-tert-butylbenzoic acid have been found in a subacute and subchronic dermal toxicity study.

In a 28 day dermal study, Carworth Farm E strain rats (8 animals/sex and dose) received 0, 7.5, 15, 30 and 60 mg/kg bw/d 4-tert-butylbenzoic acid (0.2 mg/kg of 3.75, 7.5, 15 or 30% w/v solutions of p-tert-butylbenzoic acid in DMSO) topically on shaved skin (Shell 1975). Sixteen animals per sex served as control being exposed to solvent only. Body weights were recorded daily. Four animals/group were necropsied at the end of the study and the liver, kidneys and the gonads were examined histologically.

No mortality or clinical signs were observed, but body weight gains were reduced in animals exposed to 30 and 60 mg/kg bw/d. Significantly lower final body weights of males of these dose groups were observed compared to controls. Dose-related significant increases in absolute and relative liver weights were seen in female rats of all dose groups and in male rats exposed to 15 mg/kg bw/d and above. Increased relative kidney weights were observed in two top doses of female rats. No other relevant and test substance related adverse effect was observed in the liver and the kidneys of all treated animals.

Testicular toxicity has been observed by a decrease in relative and absolute testes weights in rats receiving 60 mg/kg bw/d. Histopathology of the testes revealed a degeneration of germinal epithelium in these animals. Approximately half of the tubuli seminiferi were affected. Reduced mitotic activity in spermatogonia led to germinal epithelium degeneration and reduction of spermatozoa. Multinucleated spermatocytes were found in the affected tubuli.

A $NOAEL_{testicular\ toxicity}$ of 30 mg/kg bw/d can be derived from this dermal study based on the findings of testes weight reduction and effects on the germinative epithelium. A $NOAEL_{general\ toxicity}$ can be set at 7.5 mg/kg bw/d for males and A $LOAEL_{general\ toxicity}$ can be set at 7.5 mg/kg bw/d for females based on the liver effects.

Fischer 344 rats (20 animals/sex and dose) were treated topically (once a day /five days a week) on skin clipped free of hair with 1.0 ml/kg of p-tert-butylbenzoic acid and diethanolamine salt prepared in deionized water (simulating cutting fluid) for either 7 weeks (7 animals/sex/group) or 13 weeks (13 animals/sex/group) (Cagen 1989; Lu 1987). Treatment resulted in daily exposures of 0, 17.5, 35, 70 and 140 mg/ kg bw/ d p-tert-butylbenzoic acid. Study examinations included observation of clinical signs, body weights, food consumption, clinical chemistry, hematology and urinalysis. Furthermore macroscopic findings, organ weights of lungs with trachea, larynx, liver, kidneys, brain, heart, uterus, and spleen have been investigated. Histopathology of various organs/tissues (including those weighed and sciatic nerve and spinal cord) has been performed. Absolute and relative testis weights, sperm head count and LDH-X enzyme assays as a measure for surviving spermatocytes and spermatids were performed.

No exposure related deaths or any clinical signs of toxicity were observed up to the top dose. Significantly lower body weights and body weight gains were observed for males and females exposed to 140 mg/kg bw and for females exposed to 70 mg/kg bw.

A significant increase in urine volume occurred during week 13 at 70 and 140 mg/kg bw/d in males and at 140 mg/kg bw/d in females. Cholesterol concentrations were reduced in all dose groups, and the levels of BUN and phosphorus were increased in males/females at 70 and 140 mg/kg bw/d.

Dose-related significant increases in relative and absolute hepatic and renal weights were seen in all dose groups. In line, in the 70 and 140 mg/kg bw/d dose groups cytoplasmic vacuolation in the liver; pallor, dilatation, degeneration and regeneration of distal convoluted tubular epithelium, tubular casts, interstitial nephritis and papillary necrosis of the kidneys has been observed microscopically. Liver cell vacuolation was also evident in 17.5 and 35 mg/kg bw/d dose groups (females). This lesion was characterized as multifocal to diffuse perilobular to panlobular, lipidpositive vacuolation of hepatocytes. Accompanying aberrations in clinical chemistry values suggested altered hepatic and renal function. In general, a contribution of DEA to the observed effects in this study, especially on the liver metabolism cannot be ruled out.

Concerning testicular toxicity and spermatotoxicity, absolute and relative testes weights, sperm head count and LDH-X enzyme activities were significantly reduced compared to controls after dermal exposures of 70 and 140 mg/kg bw for 7 and 13 weeks.

Furthermore, exposure related microscopic lesions occurred in rat of the 70 and 140 mg/kg bw exposure groups. Lesions were characterized by moderate to severe diffuse seminiferous tubular degeneration. Most affected tubules were reported to contain spermatogonia, primary and secondary spermatocytes, early spermatids and Sertoli cells but were devoid of late spermatids. Occasionally, few seminiferous tubules in the same testis contained only Sertoli cells and a few spermatogonia. Testicular giant cells were reported to be quite numerous in the degenerative tubules and occurred in even greater numbers within epididymal tubular lumina.

Overall, a $NOAEL_{testicular\ toxicity}$ of 35 mg/kg bw/d can be derived from the study based on the findings of testes weight reduction, hypospermia and degeneration of the germinative epithelium, whereas a NOAEL for other adverse systemic effects, i.e. on the liver, could not be established, resulting in a dermal LOAEL of 17.5 mg/kg bw/day.

In an subacute inhalation toxicity study, groups of Fischer 344 rats (8 animals per sex and concentration) were exposed (whole body) to concentrations of 12.5, 106, or 525 mg/m^3 for 6 hours/day on 4 consecutive days, followed by 3-4 days without test substance treatment and subsequent 3 additional exposure days (Shell, 1982). Mean particle diameters ranged from 3.6 to 4.3 µm (MMAD). Mortality and clinical signs, body weights, clinical chemistry and hematology were assessed. Rats were terminated on the day after the last exposure. Organ weights for liver, kidneys, lungs, heart, spleen and brain, gross necropsy were assessed and histopathology on spinal cord, nasal passages, trachea, larynx, lungs, liver, kidneys and all lesions was performed. Furthermore, testes weights, sperm counts, and testicular histology were investigated.

Unscheduled deaths occurred at concentrations of 106 and 525 mg/m^3. Animals of the mid and high concentration groups showed concentration-dependent significant loss of body weights during the study period. Abnormal neurobehaviour has been observed, i.e. fore and hind limb neuropathy, hunched posture, tremors, convulsions, gait abnormalities, prolapsed penis, hypoactivity and abnormal respiration) in the mid and high dose groups. High concentration females had a mild but significantly decreased haemoglobin concentrations and hematocrit and increases in mean white blood cell counts were found in mid and high concentration groups. Clinical chemistry parameters were affected such as a reduced activity of alkaline phosphatase in all treated females.

Macroscopic findings (perineal and abdominal urine staining, dehydration, white powder on the haircoat, small red thymus, bright red lungs, pinpoint red gastric foci, enlarged tan livers, reduced digesta and body fat stores) were primarily noted in mid and high concentration groups.

Absolute and relative organ weights of the liver and kidneys were increased in all animals of the 106 and 525 mg/m³ groups, and females of the 12.5 mg/m³ group showed also increased absolute and relative liver weights.

In histopathology, treatment-related lesions were seen in the kidneys (bilateral multifocal cytoplasmic eosinophilia of cortical tubular cells, intracytoplasmatic vacuolation) of all treated animals. In mid and high concentration group animals, livers showed vacuolar degeneration in peripherilobular/periportal or panlobular hepatocytes (negative for lipid staining) and increased rate of mitotic cells. The thymus in animals of the mid and high concentration groups showed lymphocytic necrosis and atrophy in the cortical region plus medullary congestion and haemorrhage. In the 106 and 525 mg/m³ concentration groups, spinal cords showed severe focal or regional poliomyelopathy. Lesions of the central nervous system were reported for animals, which demonstrated clinical signs of paraplegia.

Testicular toxicity and spermatotoxicity was observed in terms of small soft testes and focal epididymal lesions in animals of the mid and high concentration groups. Absolute and relative weights of the testis were reduced for males of the mid and high concentration groups, which revealed to be lethal. Testicular sperm counts (left testis of all surviving rats) decreased concentration dependent and a reduction by 21% was still observed in animals of the low concentration group (12.5 mg/m³). From histopathological analysis no apparent effects on spermatogenesis were observed in the low concentration group, however, multifocal to diffuse degeneration of the germinal epithelium has been found in the testes of higher concentration groups. Severe tubular changes consisting of absence of late spermatids, reduction in spermatogenic cell types, giant cell bodies, cellular debris, and atrophy of epididymides were observed.

Overall, a $LOAEC_{testicular\ toxicity}$ and a $LOAEC_{general\ toxicity}$ of 12.5 mg/m³ can be derived from this study based on the findings of a reduction of testes sperm numbers and effects on liver and kidney.

Testicular toxicity could not be confirmed in a 28-day inhalation study with a specific design to evaluate neurotoxicity (HRC 1995). Sprague Dawley rats (8 animals per sex and concentration) were exposed to a particulate atmosphere containing 1.5, 4.7 or 15.7 mg/m³ p-tert-butyl benzoic acid (MMAD values of 3.2 - 3.9 µm) by snout-only exposure for 6 hours a day, 5 days a week (Monday to Friday) for 4 weeks.

Standard clinical observations/body weights and neurobehaviour was examined within a functional observational battery (FOB), organ weight analysis, macroscopic and microscopic examination of the adrenals, heart, kidneys, liver, lungs, spleen, testes with epididymides, and gross abnormalities was performed and specific neurohistopathological examinations have been conducted in this study.

No test substance related clinical signs, bodyweight changes, effects on food consumption, macroscopic or microscopic changes were observed. Minor differences in female absolute liver weights, i.e. an increase by 9% compared to controls were observed.

Behavioral observations revealed a slight increase in the incidence of body tremor, that further increased during the observation time of 4 weeks at 15.7 mg/m3 and a significant decrease in activity counts with tendency towards decreased rearing counts was found.

The number of males with decreased arousal and urinating/defecating while in the arena was increased. Also, facial staining and hair loss occurred with slightly increased frequency in high concentration males. No similar findings were noted among treated females.

Microscopic examinations of the organs examined including the testis and epididymides revealed no lesions, which were attributable to treatment with p-tert-butylbenzoic acid.

Putative spermatotoxic effects associated with occupational exposure to p-tert- butylbenzoic acid were investigated in a cohort of 90 male volunteers working in a p-tert-butylbenzoic acid producing facility (Whorton 1981). The control group consisted of 103 volunteers who did not work in the facility and who had not been exposed to any known testicular toxin. Exposures were indexed (with a weighted relative exposure point system) and an exposure index for each person was calculated based on the relative exposure point values and the amount of time in a given job. Medical evaluations were based on self-administered questionnaire for marital and reproductive history and smoking history, administered questionnaire for work history and genitourinary medical history, a brief physical examination of the male genitalia, venous blood sample for haematology and clinical chemistry and two semen samples that were analyzed for volume, sperm count and sperm morphology. Because of the large range of sperm counts found in healthy men who have not been exposed to chemicals that could influence sperm production, the distribution of sperm counts in a group of subjects was taken as a criterion of possible testicular damage in order to improve the evaluation.

Of the 90 participants 33 had undergone a vasectomy and did not contribute to semen analysis. A total of only 51 of the 90 participants provided at least one semen sample. Thirty-nine men provided a total of two semen samples. Exposure indices were similar between both groups, the semen sample providing men as well as the non semen providing participants. Analysis of the sperm count data of the 51 individuals of the study group yielded a median sperm count of 72 million sperm/ml semen, while that of the control group was 78 million sperm/ml. Eight individuals in the study group (15.7 %) had sperm counts of less than 20 million sperm/ml (e.g. in the sub-fertile range), compared to 7 subjects in the control group (6.8%). The authors calculated that this difference was not significant and concluded that p-tert-butylbenzoic acid, at the exposures experienced at that plant, had no clinically detectable effect on testicular function of the workers. Also, there were no indications that p-tert-butylbenzoic acid caused infertility in men who took part in this study. No adverse effects on liver and kidney function or on blood composition were observed. The levels of the hormones studied were in the normal range in the semen providing and the other participants.

To obtain a better statistical analysis, the control group was increased in size by including 232 men who had served as controls in other similar studies. Of the group of non-exposed men (n= 335), 25 (7.5%) had sperm counts less than 20 million/ml. It is reported, that depending on the process used for statistical analysis, the slight difference between the study subjects and the non-exposed group might or might not have been significant. Closer analysis of the urological-clinical data for the men with oligospermia in the study group of the plant revealed that a multitude of other potential factors, such as orchitis after mumps, testicular hernias and sclerosis of the penis could have been responsible for the reduced sperm density. The urological-clinical data for the control group could not be evaluated to further improve the statistical analysis.

The small size of the study group together with the manifold urological findings makes the toxicological significance of the difference from the control group questionable.

ANNEX 3: OVERVIEW ON STUDIES ADRESSING TESTICULAR TOXICITY INDUCED BY P-TERT-BUTYL-BENZALDEHYDE (TBB) AND P-TERT-BUTYLTOLUENE (TBT).

Table 32: Summary of relevant studies for p-tert-butylbenzaldehyde (TBB) and p-tert-butyltoluene (TBT) with focus on adverse effects of the male reproductive organs.

Species	Test substance	Study period	Dosage	LOAEL/$C_{testes/sperm}$	NOAEL/$C_{testes/sperm}$	Reference
Rat	TBB	5 days	p.o. 6.5, 12.5, 25, 50 mg/kg bw/d; n = 8 males / dose	25 mg/kg bw/d Testicular toxicity: disorganisation/ severe destruction germinal epithelium; ↑ degenerated cells; ↓ spermatozoa; ↓ testes weights (>25 mg/kg bw/d); Additional systemic toxicity: body weight loss, clinical signs, macroscopic changes in liver.	12.5 mg/kg bw/d	Givaudan 1981
Rat	TBB	5 days	p.o. 100 mg/kg bw/d n = 7 males / dose	100 mg/kg bw/d Testicular toxicity ↓ testes weights, minimal to moderate degeneration of spermatids and spermatocytes (5/7); minimal reduction of spermatozoa (1/7); minimal to moderate appearance of multinucleate giant cells (7/7) Additional systemic toxicity: body weight loss.	n.d.	EPA (TSCAT) 1982
Mouse	TBB	5 days	p.o. 100 mg/kg bw/d n = 6 males / dose	No evident testicular toxicity observed	100 mg/kg bw/d	Givaudan 1984A

Guinea pig	TBB	5 days	p.o. 100 mg/kg bw/d n = 5 males / dose	No evident testicular toxicity observed	100 mg/kg bw/d	Givaudan 1984B
Dog	TBB	5 days	p.o in gelatine capsule. 100 mg/kg bw/d n = 2 males / dose	100 mg/kg bw/d Testicular toxicity: seminif. tubules only with early stages of spermatogenesis+ Sertoli cells (1/2) (bilateral - graded as minimal change)	n.d.	Givaudan 1984C
Rat	TBT	5 days	p.o. 12.5, 25, 50, 100 mg/kg bw/d; n = 8 males / dose	50 mg/kg bw/d Testicular toxicity: disorganisation/ severe destruction germinal epithelium; ↑ degenerated cells; ↓ spermatozoa; ↓ testes weights (>50 mg/kg bw/d); Additional systemic toxicity: body weight loss, clinical signs, macroscopic changes in liver.	25 mg/kg bw/d	Givaudan 1982C
Rat	TBT	5 days	p.o. 200 mg/kg bw/d n = 7 males / dose	200 mg/kg bw/d Testicular toxicity: ↓ testes weights degeneration of spermatocytes /spermatids, ↓ spermatozoa, appearance of giant cells Additional systemic toxicity: body weight loss, clinical signs	n.d.	EPA (TSCAT) 1982
Mouse	TBT	5 days	p.o. 100 mg/kg bw/d n = 6 males / dose	No evident testicular toxicity observed (see Table 33)	100 mg/kg bw/d	Givaudan 1984D

Guinea pig	TBT	5 days	p.o. 100 mg/kg bw/d n = 5 males / dose	Slight testicular toxicity observed in single animals (see Table 33)	n.d.	Givaudan 1984E
Dog	TBT	5 days	p.o in gelatine capsule. 100 mg/kg bw/d n = 2 males / dose	100 mg/kg bw/d Testicular toxicity: seminif. tubules only with early stages of spermatogenesis+ Sertoli cells (1/2) (bilateral - graded as minimal change)	n.d.	Givaudan 1984F
Rat	TBT	28 days	p.o. 1.5, 5, 15, 50 mg/kg bw/d n = 12 / sex and dose	50 mg/kg bw/d Testicular toxicity ↓ testes/epididymis weights, atrophy of seminiferous tubules, hyperplasia of Leydig cells, ↓ sperm count. Additional systemic toxicity: Changes in clinical chemistry and hematology, ↑ liver weights (incl. histopathology)	15 mg/kg bw/d	Furuhashi 2007A
Rat	TBT	50-52 days (males) 41-45 days (females)	p.o. 1.5, 5, 15, 50 mg/kg bw/d n = 12 / sex and dose	15 mg/kg bw/d Testicular toxicity: ↓ testes/epididymis weights, atrophy of seminiferous tubules, hyperplasia of Leydig cells, Spermatotoxic effects: ↓ sperm motility/velocity/viability, ↓ sperm count, ↑ abnormal sperm, impaired fertility Additional systemic toxicity: Mortality, clinical signs, ↓ body weights.	5 mg/kg bw/d	Furuhashi 2007B

In SFP albino rats, a 5-day oral exposure to p-tert-butylbenzaldehyde (TBB) caused marked testicular damage. Eight male rats per dose group received 0, 6.5, 12.5, 25 or 50 mg/kg bw/d of the test material via gavage once daily for 5 consecutive days; 4 additional control animals received the respective vehicle in the same manner (Givaudan 1981). Body weights, clinical signs were assessed and gross necropsy was performed. Liver, kidneys and testes were weighed. The testes of all rats were microscopically examined.

No mortality was observed. Three rats treated with 12.5 mg/kg bw/d showed slight aggressiveness on test days 3 and 4. From days 3 to 6, a slight loss of hair was seen in one animal of the 50 mg/kg bw/d group. The test material did not affect body weights of animals treated with 6.5 and 12.5 mg/kg bw/d. Rats treated with 25 mg/kg bw/d initially showed a slight weight loss and returned to normal at the end of the treatment. The animals of the highest dose group showed a severe weight loss throughout the study. During the dissection, a marbled liver was recorded in 2 rats treated with 50 mg/kg bw/d. In one rat treated with 25 mg/kg bw/d a small dell was seen in the right kidney.

Testes weights of rats treated with 50 mg/kg bw/d were significantly lower than those recorded for the controls. Histopathological changes of the testes were circumscribed in the seminiferous epithelium. Interstitial cells and Sertoli cells were unaffected. Disorganization of the epithelial structure, degeneration of cells and reduction of the spermatozoa were observed. One testis of a control rat showed about 80 % convoluted tubules with a normal epithelium, and about 20 % convoluted tubules with a normal epithelium, but with degenerated cells or detritus in the lumen. The same ratio occurred in the animals of the 6.5 and in the 12.5 mg/kg bw/d dose group. An alteration of this ratio was seen from the 25 mg/kg bw/d group on. In addition, severe injuries were observed in the seminiferous epithelia of the testes of one animal treated with 25 mg/kg bw/d. Moderate to severe injuries were discovered in the seminiferous epithelia of all rats treated with 50 mg/kg bw/d.
The NOAEL derived for testicular toxicity in rat was 12.5 mg/kg bw/d TBB, based on the histological changes and a slight decrease of the testicular weights. Systemic toxicity in terms of body weight changes were found at doses also exerting testicular toxicity.

A similar test protocol has been applied in a subacute testicular toxicity screening study using p-tert-butyltoluene (TBT). Eight male SFP albino rats per dose group received 12.5, 25, 50, 100 mg/kg bw/d via gavage once daily for 5 consecutive days; 4 additional control animals received the respective vehicle in the same manner (Givaudan 1982C).
No mortality was observed and clinical signs (loss of hair, shaggy fur, hunched posture, lethargy and diarrhea) occurred in the dose group 50 and 100 mg/kg bw/d. A slight and transient body weight loss was observed at 25 mg/kg bw/d, and a more severe body weight loss (reaching the initial weight by the end of the study period) was observed at 50 mg/kg bw. A marked progressive body weight loss was found in rats treated with 100 mg/kg bw/d. Necropsy revealed a delineation of hepatic lobules and pale livers at the 50 and 100 mg/kg bw/d dose groups.
Testes weights were decreased by 23% in rats of the 100 mg/kg bw/d dose group compared to controls. In line, histopathological examinations revealed severe cell-deformations in the germinal epithelium in rats treated with 50 and 100 mg/kg bw/d.

Controls, 12.5 and 25 mg/kg bw/d dose groups showed approximately 85 % convoluted tubules with a normal epithelium, and about 15 % convoluted tubules with a normal epithelium, but with degenerated cells or detritus in the lumen. An alteration of this ratio was seen at 50 mg/kg bw/d group and above. Furthermore the incidence of tubuli with severe destruction of the epithelium which mainly consisted of spermatogonial and Sertoli cells dose dependently increased at 50 and 100 mg/kg bw/d, i.e. 20% and 86% respectively.
The NOAEL derived for testicular toxicity in rat was 25 mg/kg bw/d TBT, based on the histological changes and a slight decrease of the testicular weights. Systemic toxicity in terms of body weight changes were found at doses also exerting testicular toxicity.

In further testicular toxicity screening studies with male SFP albino rats (7 males) and mice (6 males), Himalayan guinea pigs (5 males) and Beagle dogs (2 males), 100 mg/kg bw/d TBB was administered orally (gavage or via gelatin capsules) once daily for five consecutive days (EPA (TSCAT) 1982, Givaudan 1984A; Givaudan 1984B; Givaudan 1984C). In these experiments, body weights and clinical signs were monitored. Rats, mice, guinea pigs were inspected by gross necropsy. Testes were weighed in rats, mice, guinea pigs and histopathological examination of the testes was performed in all species examined.

In rats, no mortality occurred throughout the study and all animals appeared normal. During the first two days an initial body weight loss was observed, but these rats showed subsequent weight gain at the end of the treatment period. However, final mean body weights in treated animals were still below controls. One of the 7 tested animals had an agenesia of the left kidney and testis. Testes weights of the treated animals were decreased compared to controls whereas weight of liver and kidney showed no relevant difference. Histological examination revealed signs for acute hepatitis and acute interstitial nephritis occurred in animals of treated and control groups. Since these histological findings are commonly seen according to the authors, a parasitic infestation is considered to be the cause rather than compound related effects.
In the testes the treated animals showed injuries in the seminiferous epithelium. Five treated animals showed minimal to moderate degeneration of spermatids and spermatocytes. One treated animal showed a minimal reduction of spermatozoa and all treated animals showed minimal to moderate appearance of multinucleate giant cells. Sertoli cells and Leydig cells were unaffected. In contrast, these findings were not observed in control animals.

In mice, no treatment-related findings on body weights and clinical signs of toxicity were observed. At necropsy, a turbid pericardiopleural region with deposits was seen in 2 treated mice, but has been considered to be unrelated to treatment with the test substance. Testes weights of the treated animals showed no effect when compared to control animals. Histological examination revealed a slight damage of the germinal epithelium in testes of 1 control and 4 treated animals. A marginally higher incidence of seminiferous tubules showing, many degenerated cells in the epithelium and disorganization of the epithelial structure (0% versus 0.2%) or severe destruction of the epithelium (0.2% versus 1.2%) were observed for control and test substance treated animals respectively.

In guinea pigs, no treatment-related clinical findings, changes on body weights or necropsy findings were observed. The mean testes weights of the control group and of the treated group were not significantly altered. In histology, a slight damage of the germinal epithelium was seen in 2 control animals and in 1 treated animal.
Furthermore, marginal incidences of seminiferous tubules showing severe destruction of the epithelium (0.2% and 0.1%) were observed for control and test substance treated animals respectively.

In dogs, application of TBB resulted in a slight body weight loss up to day 6 (Dog 1, treated: 12.2 kg on day 1, 11.6 kg on day 6; dog 2, treated: 11.1 kg on day 1, 10.0 kg on day 6). There were about 60 cross sectioned seminiferous tubules with nearly total depopulation of germinal epithelium in both testes of one dog. In these seminiferous tubules, early stages of spermatogenesis and Sertoli cells were preserved only. With the exception of the occurrence of multinucleated giant cells - a background finding seen also in the control animal- , no abnormalities were discovered in the testes of the other treated dog. No changes were seen in epididymides of both dogs. The slight damage of germinal epithelium of one dog was considered to be related to treatment.

Analogous testicular toxicity screening studies have been performed for TBT using male SFP albino rats (7 males) and mice (6 males), Himalayan guinea pigs (5 males) and Beagle dogs (2 males). A single dose (200 mg/kg bw/d in rats and 100 mg/kg bw/d in other animals) TBT was administered orally (gavage or via gelatin capsules) once daily for five consecutive days (EPA (TSCAT) 1982, Givaudan 1984D; Givaudan 1984E; Givaudan 1984F;).

In rats, no mortalities but lethargy and shaggy fur was recorded. Slight body weight loss during the first 3 days was observed with a tendency to return to normal body weights at the end of treatment. Necropsy findings revealed inflammation in the liver in one treated animal. Testes weights decreased compared to controls. Histopathology revealed changes of the seminiferous epithelium in terms of degeneration of spermatocytes and spermatids, reduction of spermatozoa and appearance of giant cells, whereas Sertoli and Leydig cells were unaffected.

In mice, no treatment-related clinical signs and findings on body weights and in necropsy were observed. Testes weights of the treated animals were slightly increased compared to control animals. Histological examination revealed a slight damage of germinal epithelium in testes of 1 control and 3 treated animals. A marginally higher incidence of seminiferous tubules showing, many degenerated cells in the epithelium and disorganization of the epithelial structure (0% versus 0.2%) or severe destruction of the epithelium (0.2% versus 0.7%) were observed for control and test substance treated animals respectively.

In guinea pigs, no mortality, treatment-related clinical findings, changes in body weights or necropsy findings were observed. The mean testes weights of the control group and of the treated group were not significantly altered. In histology, a slight damage of germinal epithelium was seen in 2 control animals and in 1 treated animal and a moderate damage was seen in one TBT treated animal. Slightly increased incidences of seminiferous tubules showing many degenerated cells in the epithelium and disorganization of the epithelial structure (1.8% vs. 0%) or severe destruction of the epithelium (2.7% vs. 0.2%) were observed for TBT treated versus control animals respectively. However, the severity of these findings were much lower when compared the testicular effects observed in rats under the same testing conditions.

The results presented in Table 33 refer to the percentage of tubuli seminiferi of TBT treated rats, mice and guinea pigs, showing a certain histopathological grade. Administration of TBT to mice and guinea pigs did not evidently increase the occurrence of degenerated cells, disorganization of the epithelial structure or severe destruction of the epithelium, when compared to the respective controls. These findings need to be seen in context of a comparable study performed in rats, showing a high percentage of tubuli seminiferi with severe destruction of the epithelium. Accordingly, rats were identified to be a responder to TBT induced testicular toxicity in contrast to mice and guinea pigs.

Table 33: Percentage of affected tubuli seminiferi per histological grade of severity observed in studies with rats, mice and guinea pigs receiving 100 mg/kg bw/d TBT versus controls (Givaudan 1982C, Givaudan 1984D; Givaudan 1984E). Each testis section was meanderingly moved under the microscope and cross-sectioned seminiferous tubules were graded. Grade 0 = normal cellularity of the epithelium; Grade 1 = normal cellularity of the epithelium, however with some to many degenerated cells or detritus in the lumen of the seminiferous tubule; Grade 2 = many degenerated cells in the epithelium and disorganization of the epithelial structure; Grade 3 = severe destruction of the epithelium.

	Dose (mg TBT/kg bw/d)	**Histologic Grade (severity)**			
		0	**1**	**2**	**3**
Rat	**0**	**85.63 %**	**14.37 %**	**0**	**0**
	100	**0**	**1.25 %**	**12.44 %**	**86.31 %**
Mouse	**0**	**95.8 %**	**4.1 %**	**0**	**0.2 %**
	100	**94.8 %**	**4.3 %**	**0.2 %**	**0.7 %**
Guinea pig	**0**	**97.5 %**	**2.3 %**	**0**	**0.2 %**
	100	**91.0 %**	**4.5 %**	**1.8 %**	**2.7 %**

In dogs, no mortality, symptoms of incompatibility or effects on body weights were observed after application of TBT. A small quantity of seminiferous tubules with nearly total depopulation of germinal epithelium in both testes of one dog was observed, i.e. approx 20 in testis 1 and 10 in testis 2. These tubules showed early stages of spermatogenesis and Sertoli cells only. With the exception of the occurrence of multinucleated giant cells - a background finding seen also in the control animal- , no abnormalities were discovered in the testes of the other treated dog. No changes were seen in epididymides of both dogs. The observed slight damage of germinal epithelium of one dog cannot be clearly attributed to treatment.

TBT induced testicular toxicity in rats was confirmed in a subacute toxicity study equivalent to OECD TG 407. TBT was administered to 12 Sprague-Dawley rats/sex/dose by gavage at dose levels of 0, 1.5, 5, 15, 50 mg/kg bw/day for 28 days (Furuhashi 2007A). Satellite groups were allowed a 14-day recovery.

No deaths occurred and no treatment-related clinical signs of toxicity or changes in body weights were observed. Lower food consumption was noted in the 15 mg/kg males and in both sexes given 50 mg/kg. Higher water intake was noted in the males at 15 mg/kg and in both sexes at 50 mg/kg. Clinical chemistry parameters such as decreases in total protein, albumin, cholesterol, triglycerides and ions and increases in AST, A/G ratio, gamma-GTP, bilirubin, urea nitrogen and creatinine were affected starting from dose group 5 mg/kg bw/d for males and 15 mg/kg bw/d for females. Hematology parameters, i.e. shortened APTT, prolonged PT and decreased fibrinogen concentration were affected starting from dose group 5 mg/kg bw/d for males and 50 mg/kg bw/d for females.

Urinalysis revealed a higher urinary volume, decreased urinary specific gravity, and decreased protein levels from dose group 15 mg/kg bw/d for males and 50 mg/kg bw/d for females. Organ weight measurement showed increased absolute/ relative liver weights starting from dose group 15 mg/kg bw/d onward. Increased relative kidney weights, increased relative adrenals weights and decreased absolute ovary weights were found in the high dose females. No gross pathological findings were reported. In histopathology a hypertrophy of periportal hepatocytes in high dose males and females has been observed.

Testicular toxicity was evident in terms of decreased absolute and relative testis and absolute epididymis weights in males of the 50 mg/kg bw/d dose group. In histopathology, atrophy of seminiferous tubules, hyperplasia of Leydig cells and a decrease in sperm count in the lumen of the ductus epididymis was found in the high dose males. At the termination of the recovery period, atrophy of seminiferous tubules in the testes and decrease in sperm in the epididymides were still evident in the males which had received 50 mg/kg bw/d.

Taken together a $NOAEL_{general\ toxicity}$ can be set at 1.5 mg/kg bw/d (males) and 5 mg/kg bw/d (females) and a $NOAEL_{testicular\ toxicity}$ can be set at 15 mg/kg bw/ day for this study.

In a reproduction / developmental toxicity screening study by the same authors in accordance with OECD TG 421, TBT was administered to 12 Sprague-Dawley rats/sex/dose by gavage at dose levels of 0, 1.5, 5, 15, 50 mg/kg bw/day.

Mortality was observed at 50 mg/kg bw/d (1 male and 6 females) and at 15 mg/kg bw/d (1 female). Hypothermia, decrease in locomotor activity, soiled fur, reddish urine, hypothermia, adoption of a prone position, a staggering gait, piloerection, lacrimation, bradypnea, diarrhea, and muscle relaxation were noted starting from 15 mg/kg bw/d. Body weights were decreased in males from 15 mg/kg bw/d onward and in females from 5 mg/kg bw onward. Transient decreases in food consumption was noted in males at 50 mg/kg bw/d and in females at 5 mg/kg bw/d during the lactation period.

No changes attributable to administration of the test substance were noted in the estrous frequency, copulation index, or number of days before copulation. No substance related changes were noted at 1.5, 5, or 15 mg/kg bw/d in terms of numbers of corpora lutea, implantation sites, implantation rate or gestation index.

Concerning the offspring, number of pups on day 0 of lactation was zero at 50 mg/kg bw/d, since no females conceived. In the 15 mg/kg bw/d dose group, lower numbers of pups, number of pups on day 0 of lactation, the delivery index, birth index, live birth index, number of pups on day 4 of lactation, and the viability index on day 4 of lactation was observed. The number of stillbirths tended to be higher in this dose group. No external abnormalities attributable to the compound administration were noted. Lowered pup body weights in both sexes on Days 0 and 4 of lactation were found at 5 and 15 mg/kg bw/d.

Concerning testicular toxicity and spermatotoxicity, decreased absolute testis and epididymis weights at 15 mg/kg bw/d were observed. The relative testis and epididymis weights were lowered at 50 mg/kg bw/d. Sperm examinations revealed a decreased motility ratio, path velocity, straight line velocity, curvilinear velocity, viability, survivability, number of sperm, number of sperm/g of the left caudae epididymidis and an elevation of the proportion of abnormal sperm at 15 and 50 mg/kg bw/d. A higher value for beat cross frequency was noted at 15 mg/kg bw/d. At necropsy, atrophy of the testes and epididymides was noted in males at 15 and 50 mg/kg bw/d. Histopathological examinations confirmed an atrophy of seminiferous tubules, hyperplasia of Leydig cells and decrease in sperm in the epididymides at 15 and 50 mg/kg. Furthermore, the fertility index was significantly decreased at 15 and 50 mg/kg bw, and no animal became pregnant in the high dose group, substantiating the adverse effects of TBT on male fertility.

Taken together a $NOAEL_{general\ toxicity}$ can be set at 5 mg/kg bw/d and a female specific $NOEL_{general\ toxicity}$ can be set at 1.5 mg/kg bw/d. A $NOAEL_{testicular\ toxicity}$ can be set at 5 mg/kg bw/ day and a $NOAEL_{developmetal\ toxicity}$ can be set at 1.5 mg/kg bw/ day for this study.

ANNEX 4: METABOLOME ANALYSIS

Table 34: Ratio of plasma metabolites of two independent rat metabolome studies with Lysmeral. Plasma metabolites were investigated in 2 independent rat studies 7, 14 and 28 days after oral Lysmeral administration of 2 doses (15 and 45 mg/kg bw/d, respectively). Metabolites are listed as a ratio of the mean of metabolite levels in individual rats in a treatment group relative to mean of metabolite levels in rats in a matched control group (time point, dose level, sex). Data are provided for females (f) and males (m) separately. Significant changes to respective controls (p = 0.05) were marked in red (increase) or yellow (decrease)

			15 mg/kg bw/d						45 mg/kg bw/d						15 mg/kg bw/d						45 mg/kg bw/d					
			Lysmeral												Lysmeral											
Metabolite	**Class**	**Subclass**	**f7**	**f14**	**f28**	**m7**	**m14**	**m28**	**f7**	**f14**	**f28**	**m7**	**m14**	**m28**	**f7**	**f14**	**f28**	**m7**	**m14**	**m28**	**f7**	**f14**	**f28**	**m7**	**m14**	**m28**
Threonine	Amino acids	Amino acids, neutral	**1.42**	**1.89**	**1.50**	1.27	**1.39**	1.23	1.28	**1.99**	**1.82**	**1.46**	**1.74**	**2.15**	**1.42**	**1.70**	**1.51**	0.99	1.20	1.14	1.16	**1.75**	**2.37**	**1.40**	1.42	**1.79**
Tryptophan	Amino acids	Amino acids, aromatic	1.09	1.20	1.14	0.93	0.90	0.90	0.66	0.87	0.75	0.88	1.07	**1.12**	0.87	0.86	1.08	0.86	0.92	0.95	0.85	0.84	1.37	1.07	**1.12**	1.01
Arginine	Amino acids	Amino acids, basic	1.05	**1.22**	1.16	**1.10**	1.15	1.01	0.92	1.08	1.10	**1.37**	**1.59**	1.76	1.00	1.35	**1.40**	1.25	1.08	1.24	1.17	1.26	**1.46**	1.19	1.20	1.15
Citrulline	Amino acids related	Urea cycle and related	0.91	0.85	0.91	1.04	1.04	0.90	0.91	0.87	0.76	0.93	0.99	1.10	0.97	0.84	1.00	1.04	0.96	1.13	1.01	0.92	1.15	0.96	0.80	0.79

Glutamate	Amino acids	Amino acids, acidic	0.73	0.84	0.70	1.09	0.85	0.89	0.67	0.91	0.79	0.70	0.77	0.76	0.89	**1.16**	0.84	0.90	1.12	0.88	0.80	1.08	0.90	0.72	0.79	0.61
Phenylalanine	Amino acids	Amino acids, aromatic	0.89	1.06	1.05	0.84	0.76	1.04	1.00	1.10	0.96	0.93	1.04	1.06	1.20	0.92	1.13	0.95	0.91	0.98	1.05	0.99	0.95	1.03	0.93	1.04
alpha-Tocopherol	Vitamins, cofactors and related	Tocopherols and related	0.48	0.51	0.73	0.84	0.89	0.99	0.40	0.44	0.61	0.52	0.56	0.61	0.41	0.53	0.42	0.54	0.53	0.65	0.31	0.43	0.36	0.51	0.39	0.44
Pantothenic acid	Vitamins, cofactors and related	Acyl-carriers and related	0.78	0.86	1.05	1.04	1.04	1.86	0.84	1.28	0.75	**1.23**	**1.94**	**1.99**	1.32	1.27	1.11	**1.59**	**1.36**	1.33	**1.46**	**1.71**	1.13	1.38	0.96	1.08
Taurine	Amino acids	Amino acids, S-containing	NA	1.54	NA	0.78	1.20	1.10	**1.49**	1.65	1.22	1.13	1.15	NA	1.22	**1.40**	1.04	1.17	1.00	1.18	**1.61**	**1.90**	1.08	**1.60**	**1.31**	1.35
Malate	Energy metabolism and related	Citrate cycle	0.76	0.92	0.80	0.71	0.90	0.88	0.81	0.79	0.86	0.73	0.74	0.88	0.74	1.05	0.76	0.97	0.83	1.39	0.73	1.01	0.62	1.00	1.00	0.94
Fructose-6-phosphate	Energy metabolism and related	Glycolysis/ Gluconeogenesis	1.23	0.99	0.74	0.88	1.02	1.25	1.31	1.05	**1.42**	0.93	1.03	1.07	1.30	2.76	1.48	1.29	1.12	1.81	1.76	**2.47**	1.28	1.30	1.73	1.39

Salicylic acid	Miscellaneous	Miscellaneous	1.12	0.77	1.07	0.56	0.69	0.81	1.10	0.87	1.16	0.65		0.67	0.61	0.99	0.88	1.14	0.54	1.06	1.07	1.04	1.06	1.22	0.66	1.06	1.15
Coenzyme Q9	Vitamins, cofactors and related	Redox-carrier and related	0.33	0.34	0.44	0.50	0.46	0.58	0.35	0.49	0.42	0.21		0.27	0.31	0.36	0.44	0.49	0.36	0.33	0.58	0.39	0.69	0.46	0.30	0.19	0.17
Cholic acid	Complex lipids, fatty acids and related	Bile acids	0.04	0.47	0.02	0.26	**3.01**	0.03	0.22	0.01	0.10	0.44		1.15	0.76	0.18	0.14	0.01	3.83	2.54	11.55	0.36	1.27	0.59	2.08	0.15	0.24
Creatinine	Amino acids related	Creatine metabolism	0.92	1.13	1.32	1.08	0.90	1.07	1.08	1.03	0.96	1.33		1.24	1.14	1.05	1.00	0.77	1.11	0.90	0.96	1.02	1.25	1.02	**1.35**	1.04	0.91
Creatine	Amino acids related	Creatine metabolism	NA	0.77	NA	1.23	1.41	0.96	1.33	0.82	0.83	1.15		1.26	NA	1.37	**1.27**	1.23	**1.35**	1.23	1.37	1.89	**1.45**	1.21	**1.52**	**1.37**	1.21
Kynurenic acid	Amino acids related	Tryptophan metabolism	NA	1.32	NA	0.46	1.01	1.04	0.88	1.61	0.07	0.67		1.62	NA	1.07	0.56	0.32	0.80	0.41	0.95	0.82	0.89	0.78	0.65	0.75	0.65
Taurocholic acid	Complex lipids, fatty acids and related	Bile acids	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		NA	NA	2.07	1.01	2.45	0.80	0.71	1.48	2.08	1.28	1.71	1.48	0.94	1.50

Glycochenodeoxycholic acid	Complex lipids, fatty acids and related	Bile acids	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		NA	NA	1.03	0.26	0.73	0.66	0.65	0.98	1.13	0.97	1.47	0.16	0.12	0.13
Glycerol, lipid fraction	Complex lipids, fatty acids and related	Fatty alcohols	0.76	0.65	0.71	0.70	0.73	0.61	0.98	1.20	1.06	0.33		0.60	0.57	0.64	0.51	0.62	0.38	0.36	0.38	0.85	0.65	0.91	0.29	0.42	0.72
Palmitic acid (C16:0)	Complex lipids, fatty acids and related	Fatty acids, saturated	0.56	0.59	0.66	0.67	0.69	0.63	0.54	0.82	0.76	0.42		0.56	0.48	0.67	0.49	0.55	0.53	0.50	0.44	0.78	0.53	0.72	0.35	0.43	0.52
Linoleic acid (C18:cis[9,12]2)	Complex lipids, fatty acids and related	Fatty acids, poly-unsaturated	0.73	0.60	0.54	0.59	0.71	0.58	0.79	0.87	0.76	0.44		0.57	0.38	0.69	0.45	0.48	0.41	0.42	0.37	0.91	0.49	0.53	0.29	0.33	0.49
Oleic acid (C18:cis[9]1)	Complex lipids, fatty acids and related	Fatty acids, mono-unsaturated	1.15	**1.20**	**1.30**	1.04	1.42	1.29	1.26	**1.58**	**1.70**	0.87		1.01	0.81	1.22	0.94	1.00	0.74	0.99	0.73	**1.55**	1.15	**1.43**	0.73	0.74	1.07

Stearic acid (C18:0)	Complex lipids, fatty acids and related	Fatty acids, saturated	0.46	0.53	0.53	0.66	0.75	0.66	0.35	0.52	0.51	0.34	0.51	0.55	0.50	0.43	0.45	0.65	0.69	0.86	0.43	0.37	0.40	0.41	0.67	0.57
Arachidonic acid (C20:cis [5,8,11, 14]4)	Complex lipids, fatty acids and related	Fatty acids, poly-unsaturated	0.31	0.47	0.45	0.60	0.64	0.62	0.19	0.45	0.43	0.21	0.39	0.49	0.33	0.45	0.42	0.54	0.58	0.81	0.25	0.29	0.38	0.26	0.47	0.51
Docosahexaenoic acid (C22:cis [4,7,10, 13,16,19]6)	Complex lipids, fatty acids and related	Fatty acids, poly-unsaturated	0.25	0.48	0.53	0.76	0.77	0.81	0.15	0.40	0.48	0.23	0.54	0.71	0.33	0.61	0.52	0.51	0.73	0.81	0.29	0.30	0.56	0.20	0.50	0.61
Cholesterol, total			0.34	0.52	0.59	0.64	0.86	0.68	0.21	0.47	0.44	0.30	0.47	0.49	0.40	0.45	0.52	0.63	0.74	0.92	0.28	0.24	0.42	0.29	0.42	0.47
beta-Sitosterol	Miscellaneous	Diet related	0.41	0.61	0.67	0.63	0.78	0.73	0.29	0.54	0.45	0.43	0.57	0.55	0.45	0.49	0.63	0.63	0.53	0.68	0.33	0.35	0.43	0.54	0.52	0.62
Glycerol phosphate, lipid fraction	Complex lipids, fatty acids and related	Phospholipid metabolites	0.35	0.53	0.48	0.65	0.53	0.59	0.17	0.43	0.44	0.21	0.54	0.47	0.33	0.45	0.29	0.69	0.50	0.69	0.19	0.25	0.25	0.22	0.46	0.39

Galactose, lipid fraction	Complex lipids, fatty acids and related	Glycolipids	0.35	0.64	0.52	0.59	0.82	0.81	0.29	0.50	0.41	0.43	0.56	0.66	0.41	0.38	0.32	0.70	0.87	0.80	0.26	0.19	0.37	0.36	0.85	0.74
Lignoceric acid (C24:0)	Complex lipids, fatty acids and related	Fatty acids, saturated	0.40	0.60	0.66	0.54	0.75	0.65	0.21	0.43	0.49	0.36	0.52	0.60	0.48	0.48	0.48	0.59	0.70	0.76	0.27	0.24	0.41	0.45	0.49	0.61
Campesterol	Miscellaneous	Diet related	0.33	0.43	0.45	0.57	0.69	0.58	0.12	0.41	0.33	0.24	0.43	0.35	0.29	0.37	0.33	0.46	0.44	0.49	0.21	0.25	0.30	0.33	0.39	0.42
Hexadecanol	Complex lipids, fatty acids and related	Fatty alcohols	0.87	0.80	0.80	1.07	1.12	1.06	0.91	0.80	0.93	0.96	0.93	0.94	0.60	0.81	0.79	0.90	1.05	0.91	0.65	0.89	0.74	1.01	0.96	0.66
Heneicosanoic acid (C21:0)	Complex lipids, fatty acids and related	Fatty acids, saturated	0.82	0.87	0.93	0.90	0.97	0.90	0.88	0.91	0.98	0.93	0.90	0.88	0.92	0.80	0.93	0.81	0.78	1.02	0.87	0.91	0.98	0.71	0.80	0.85
Dodecanol	Complex lipids, fatty acids and related	Fatty alcohols	0.52	0.63	0.67	0.79	0.83	0.76	0.46	0.75	0.71	0.45	0.75	0.82	0.61	0.73	0.59	0.73	0.83	1.01	0.70	0.85	0.64	0.68	0.81	1.02

Heptadecanoic acid (C17:0)	Complex lipids, fatty acids and related	Fatty acids, saturated	0.70	0.60	0.69	0.77	0.77	0.80	0.59	0.70	0.55	0.56	0.59	0.56	0.70	0.65	0.66	0.52	0.63	0.81	0.62	0.70	0.59	0.43	0.60	0.58
Eicosanoic acid (C20:0)	Complex lipids, fatty acids and related	Fatty acids, saturated	0.71	0.62	0.80	1.06	1.21	0.84	NA	0.86	0.70	0.76	0.88	0.69	0.80	0.65	0.96	0.77	0.90	1.38	0.59	0.72	0.98	0.62	0.86	0.74
Tricosanoic acid (C23:0)	Complex lipids, fatty acids and related	Fatty acids, saturated	0.38	0.70	0.64	0.68	0.88	0.84	0.33	0.49	0.61	0.48	0.75	0.82	0.59	0.64	0.53	0.72	0.76	0.85	0.32	0.37	0.49	0.37	0.61	0.54
Phosphate, lipid fraction	Complex lipids, fatty acids and related	Phospholipid metabolites	0.52	0.64	0.65	0.83	0.82	0.67	0.44	0.61	0.61	0.49	0.77	0.58	0.52	0.59	0.55	0.67	0.78	0.88	0.44	0.54	0.56	0.66	0.74	0.70
Myristic acid (C14:0)	Complex lipids, fatty acids and related	Fatty acids, saturated	0.72	0.76	0.88	0.75	0.69	0.53	0.66	1.03	1.08	0.54	0.59	0.53	0.67	0.61	0.40	0.39	0.35	0.27	0.56	0.78	1.00	0.32	0.42	0.75

myo-Inositol-2-phosphate, lipid fraction (myo-Inositolphospholipids)	Complex lipids, fatty acids and related	Phospholipid metabolites	0.16	0.36	0.22	0.55	0.41	0.56	0.08	0.13	0.18	0.09	0.49	0.22	0.16	0.29	0.13	0.53	0.50	0.59	0.09	0.09	0.08	0.10	0.44	0.21
Behenic acid (C22:0)	Complex lipids, fatty acids and related	Fatty acids, saturated	0.51	0.73	0.69	0.79	0.77	0.83	0.39	0.54	0.63	0.53	0.57	0.80	0.55	0.72	0.69	0.72	0.85	1.05	0.43	0.42	0.43	0.66	0.73	0.70
4-Hydroxysphinganine (t18:0, Phytosphingosine), total	Complex lipids, fatty acids and related	Sphingolipids	0.60	0.82	0.90	1.05	1.05	0.96	0.55	0.79	0.64	0.46	0.74	0.67	0.46	0.75	0.80	0.61	0.92	0.80	0.49	0.66	0.54	0.42	0.53	0.42
Nervonic acid (C24:cis[15]1)	Complex lipids, fatty acids and related	Fatty acids, mono-unsaturated	0.22	0.56	0.59	0.63	0.78	0.81	0.26	0.38	0.46	0.32	0.52	0.51	0.33	0.58	0.56	0.75	0.68	1.18	0.22	0.19	0.37	0.32	0.58	0.69
Urea	Amino acids related	Urea cycle and related	0.87	1.18	1.26	0.84	0.83	1.10	1.17	**1.36**	**1.23**	1.33	**1.60**	**1.58**	0.86	0.95	0.85	0.79	1.02	1.16	1.02	1.23	1.04	1.20	**1.23**	1.20
Isopalmitic acid (C16:0)	Complex lipids, fatty acids and related	Fatty acids, branched	0.75	0.62	0.70	0.74	0.71	0.59	0.54	0.62	0.55	0.46	0.49	0.34	0.63	0.35	0.50	0.54	0.54	0.53	0.55	0.55	0.60	0.51	0.42	0.35

Palmitoleic acid (C16:cis[9]1)	Complex lipids, fatty acids and related	Fatty acids, mono-unsaturated	0.85	0.76	0.82	0.56	0.45	0.50	0.82	0.87	1.22	0.66	0.55	0.37	1.08	0.71	0.67	0.49	0.18	0.18	1.38	1.64	1.03	0.72	0.62	0.65
Unknown lipid (28000473)			0.49	0.80	0.96	0.53	0.55	0.58	0.56	1.02	1.10	0.47	0.46	0.55	0.50	0.62	0.68	0.41	0.74	0.70	0.58	0.49	0.83	0.57	0.58	0.51
14-Methylhexadecanoic acid	Complex lipids, fatty acids and related	Fatty acids, branched	1.02	0.87	1.01	0.91	1.00	0.92	0.88	0.91	0.95	0.84	0.96	0.73	0.95	0.70	0.89	0.73	0.98	0.74	0.86	0.80	0.93	0.64	0.85	0.73
gamma-Linolenic acid (C18:cis[6,9,12]3)	Complex lipids, fatty acids and related	Fatty acids, poly-unsaturated	1.27	0.65	1.30	0.85	1.37	0.96	1.63	**2.55**	1.82	0.87	1.24	0.94	0.81	0.83	0.75	0.72	0.56	0.56	**2.06**	1.39	1.58	0.62	0.76	1.39
16-Methylheptadecanoic acid	Complex lipids, fatty acids and related	Fatty acids, branched	0.53	0.54	0.69	0.65	0.54	0.51	0.50	0.48	0.70	0.37	0.41	0.26	0.58	0.29	0.44	0.37	0.46	0.53	0.65	0.32	0.36	0.32	0.34	0.30
17-Methyloctadecanoic acid	Complex lipids, fatty acids and related	Fatty acids, branched	0.45	0.65	0.44	0.91	0.78	0.56	0.55	0.65	0.49	0.39	0.42	0.31	0.50	0.51	0.46	0.41	0.50	0.59	0.43	0.44	0.36	0.40	0.49	0.38
dihomo-gamma-Linolenic acid (C20:cis[8,11,14]3)	Complex lipids, fatty acids and related	Fatty acids, poly-unsaturated	0.39	0.50	0.42	0.84	0.75	0.91	0.39	0.52	0.53	0.51	0.51	0.55	0.49	0.28	0.42	0.55	0.55	0.44	0.62	0.44	0.58	0.35	0.43	0.52
Eicosaenoic acid (C20:1) No 02	Complex lipids, fatty acids and related	Fatty acids, mono-unsaturated	1.58	0.79	0.67	0.92	1.02	0.63	0.63	0.84	1.10	0.24	0.51	0.36	0.48	0.49	0.59	0.31	0.77	0.96	0.54	0.60	0.59	0.32	0.42	0.62

3-O-Methylsphingosine (d18:1)	Complex lipids, fatty acids and related	Sphingolipids	0.27	0.50	0.59	0.44	0.66	0.72	0.20	0.39	0.33	0.22	0.45	0.48	0.21	0.25	0.41	0.44	0.48	0.72	0.19	0.19	0.20	0.27	0.31	0.34
threo-Sphingosine (d18:1)	Complex lipids, fatty acids and related	Sphingolipids	0.33	0.50	0.65	0.53	0.63	0.67	0.25	0.43	0.44	0.25	0.50	0.51	0.28	0.47	0.45	0.47	0.51	0.78	0.37	0.23	0.25	0.37	0.31	0.51
Docosapentaenoic acid (C22:cis[7,10,13,16,19]5)	Complex lipids, fatty acids and related	Fatty acids, poly-unsaturated	0.42	0.49	0.50	0.69	0.55	0.49	0.24	0.57	0.57	0.27	0.54	0.37	0.42	0.52	0.47	0.46	0.25	0.32	0.72	0.49	0.97	0.25	0.38	0.47
5-O-Methylsphingosine (d18:1)	Complex lipids, fatty acids and related	Sphingolipids	0.25	0.52	0.55	0.49	0.66	0.69	0.21	0.35	0.29	0.19	0.48	0.48	0.13	0.36	0.38	0.49	0.52	0.72	0.20	0.18	0.22	0.25	0.33	0.29
erythro-Sphingosine (d18:1)	Complex lipids, fatty acids and related	Sphingolipids	0.28	0.56	0.65	0.52	0.73	0.66	0.21	0.38	0.38	0.21	0.51	0.51	0.12	0.37	0.45	0.51	0.55	0.87	0.17	0.14	0.24	0.29	0.37	0.37
Elaidic acid (C18:trans[9]1)	Complex lipids, fatty acids and related	Fatty acids, mono-unsaturated	0.78	0.71	0.79	0.84	0.76	0.69	0.54	0.70	0.86	0.52	0.69	0.55	0.84	0.54	0.67	0.56	0.58	0.75	1.13	0.45	0.85	0.53	0.70	0.50
myo-Inositol, lipid fraction	Complex lipids, fatty acids and related	Glycolipids	0.43	0.57	0.51	0.68	0.76	0.75	0.28	0.45	0.54	0.41	0.57	0.60	0.37	0.47	0.42	0.70	0.76	0.80	0.27	0.30	0.39	0.41	0.66	0.63
Pyruvate	Energy metabolism and related	Glycolysis/Gluconeogenesis	0.52	0.78	0.54	0.68	0.70	0.67	0.70	0.85	1.21	0.86	1.30	**1.23**	0.53	0.96	0.70	0.82	0.99	1.20	0.66	1.22	1.27	1.53	**2.04**	1.31

Alanine	Amino acids	Amino acids, neutral	0.68	0.67	0.83	0.90	1.02	1.03	0.64	0.89	1.21	0.91	1.10	1.45	0.59	0.93	0.89	0.99	1.19	1.32	0.65	0.93	1.01	0.94	1.00	0.78
Glycine	Amino acids	Amino acids, neutral	**1.29**	1.20	1.00	1.17	1.21	1.18	1.15	0.94	0.92	**1.46**	1.29	1.14	1.03	0.97	0.90	1.11	1.34	1.20	1.07	0.83	0.75	0.69	0.89	0.69
Methionine	Amino acids	Amino acids, S-containing	0.97	0.94	1.01	1.06	0.98	1.09	1.08	0.94	1.00	1.19	1.47	1.38	1.05	1.02	**1.21**	1.00	1.12	1.08	0.88	0.97	1.18	1.06	1.09	1.02
5-Oxoproline	Amino acids related	Amino acid metabolites	0.80	0.82	0.83	1.03	0.91	0.89	0.77	0.74	0.75	0.68	0.79	0.74	1.08	1.04	0.88	1.05	0.92	0.75	1.03	0.81	0.98	0.92	0.80	0.62
Cysteine	Amino acids	Amino acids, S-containing	0.66	0.93	0.99	0.85	1.25	1.03	0.65	0.96	0.98	0.88	1.36	1.33	0.77	0.77	1.11	1.13	1.25	0.90	0.72	0.74	1.09	0.99	**1.66**	1.26
Citrate	Energy metabolism and related	Citrate cycle	0.92	0.85	0.82	0.98	0.82	0.88	0.85	0.82	0.80	0.67	0.82	0.87	0.90	0.81	0.69	0.81	0.99	0.82	0.74	0.96	0.77	0.91	0.89	0.89
Lysine	Amino acids	Amino acids, basic	**2.35**	**2.45**	**4.03**	**1.90**	**2.74**	**2.80**	**4.17**	**4.07**	**4.89**	**6.00**	**6.53**	**8.10**	**3.61**	**2.69**	**3.99**	**2.84**	**3.17**	**2.04**	**4.80**	**3.45**	**3.73**	**4.26**	**6.97**	**5.12**
myo-Inositol	Carbohydrates and related	Polyols	1.09	1.09	1.20	1.01	0.99	0.97	1.35	**1.32**	**1.46**	0.98	1.30	1.28	**1.44**	**1.90**	1.14	1.06	1.44	1.06	**1.45**	**2.55**	1.26	1.34	1.75	1.23
Sucrose	Carbohydrates and related	Disaccharides	2.29	0.75	1.34	1.03	0.53	2.91	0.52	2.23	0.86	1.93	0.93	1.01	1.25	1.51	0.75	0.55	1.02	1.33	1.21	1.43	0.46	0.64	0.99	1.46
Mannose	Carbohydrates and related	Monosaccharides	0.94	0.81	0.75	0.96	0.87	0.85	0.87	0.72	0.66	0.72	0.70	0.73	0.94	0.89	0.90	0.95	0.93	0.88	0.90	0.70	0.91	0.88	0.86	0.79
Glucose	Carbohydrates and related	Monosaccharides	0.95	0.95	0.93	0.81	0.83	0.83	**1.21**	1.06	1.00	1.10	1.11	0.90	1.16	1.30	**1.15**	1.38	**1.49**	**1.61**	1.10	1.30	**1.36**	**1.64**	**1.42**	1.33
Glycerol, polar fraction	Complex lipids, fatty acids and related	Lipid precursors	1.17	0.95	0.97	0.96	0.93	0.77	1.21	1.17	0.99	0.81	0.79	0.60	1.02	0.90	0.86	0.87	0.88	0.73	1.02	**1.21**	1.04	0.94	0.81	0.70

Spermidine	Miscellaneous	Polyamines	0.80	0.88	0.75	0.99	0.88	0.81	0.60	0.58	0.56	0.62	0.81	0.91	1.22	1.26	0.59	0.59	0.83	1.24	1.01	0.82	0.87	0.93	0.80	1.04
Glutamine	Amino acids	Amino acids, basic	0.86	0.96	0.77	0.90	0.92	1.02	0.76	0.66	0.71	0.57	0.80	0.81	0.83	0.89	0.83	1.03	1.00	0.80	0.81	0.74	0.89	0.77	0.72	0.62
Ornithine	Amino acids related	Urea cycle and related	0.91	0.93	0.91	1.07	1.20	1.25	0.86	0.86	0.94	0.98	1.26	1.30	0.99	1.05	1.25	1.18	1.26	0.99	0.88	1.06	1.18	0.84	0.78	0.75
Phosphate (inorganic and from organic phosphates)	Miscellaneous	Miscellaneous	1.01	0.95	1.13	0.95	0.87	1.02	**1.21**	1.02	1.23	0.98	1.06	0.99	1.17	0.99	0.95	0.91	0.93	0.84	1.15	1.03	1.25	1.14	1.08	0.87
Serine	Amino acids	Amino acids, neutral	**1.26**	**1.43**	**1.37**	1.15	1.28	**1.28**	1.22	**1.72**	**1.59**	**1.37**	**1.54**	**1.69**	**1.56**	**1.42**	**1.61**	**1.29**	**1.58**	1.15	**1.44**	**1.87**	**1.82**	**1.25**	**1.32**	**1.61**
Glycerol-3-phosphate, polar fraction	Complex lipids, fatty acids and related	Lipid precursors	1.17	0.95	1.11	0.83	1.11	**1.25**	1.32	1.09	**1.53**	0.84	1.12	1.18	1.06	2.13	1.10	0.93	1.24	0.94	1.01	1.48	0.84	0.70	0.95	1.41
Valine	Amino acids	Amino acids, branched chain	0.85	0.91	1.03	0.91	0.91	1.02	0.98	1.07	1.02	0.97	1.16	**1.18**	0.87	0.83	1.24	0.84	0.99	1.07	1.15	1.08	1.34	1.11	0.90	0.95
Aspartate	Amino acids	Amino acids, acidic	1.07	1.12	0.90	1.06	0.91	0.96	1.01	1.26	1.02	0.78	0.93	0.77	1.08	**1.95**	0.93	0.79	1.05	0.89	1.14	1.26	0.90	1.10	0.88	0.63
Isoleucine	Amino acids	Amino acids, branched chain	0.73	0.77	0.95	0.91	0.87	1.05	0.88	0.85	1.01	0.81	1.02	1.02	0.80	0.75	1.12	0.81	0.96	0.98	1.02	0.97	1.02	0.93	0.86	0.84
Leucine	Amino acids	Amino acids, branched chain	0.73	0.85	0.99	0.97	0.85	1.04	0.98	0.98	1.07	0.95	1.13	**1.19**	0.91	0.84	1.18	0.81	0.99	0.97	1.27	1.13	1.09	1.05	0.96	0.88
Proline	Amino acids	Amino acids, neutral	0.91	0.87	0.92	1.01	1.06	1.12	0.84	0.76	0.86	0.90	1.11	1.20	0.89	0.93	1.01	1.12	1.15	1.16	0.79	0.90	1.13	0.83	0.86	0.85
Threonic acid	Vitamins, cofactors and related	Ascorbic acid and related	0.90	0.78	0.70	1.03	0.79	1.06	1.31	0.78	0.86	1.08	1.00	1.12	1.04	0.97	0.95	0.63	0.80	1.02	0.97	1.05	0.86	0.98	0.92	1.03

Lactate	Energy metabolism and related	Glycolysis/Gluconeogenesis	0.51	0.71	0.57	0.73	0.59	0.81	0.54	0.69	1.42	0.69	0.77	1.19	0.73	1.46	0.64	1.24	0.91	1.29	0.38	1.04	0.57	1.37	1.00	1.01
Glycolate	Miscellaneous	Miscellaneous	0.86	1.00	0.97	0.98	0.97	1.07	1.03	0.99	0.96	1.02	0.90	0.94	0.93	0.90	0.93	0.98	1.03	1.06	0.81	1.15	0.91	1.02	0.89	1.02
Asparagine	Amino acids	Amino acids, basic	0.74	0.77	0.92	0.95	0.99	0.93	0.71	0.85	0.72	0.81	1.05	0.97	0.98	1.08	0.96	0.96	0.85	0.78	0.94	0.97	0.96	0.79	0.79	0.72
Ascorbic acid	Vitamins, cofactors and related	Ascorbic acid and related	1.01	1.13	1.07	0.97	1.00	1.02	1.27	1.04	1.04	1.19	**1.16**	1.14	1.18	0.92	1.22	1.15	**1.30**	**1.43**	1.25	1.22	1.19	**1.35**	**1.27**	1.25
Tyrosine	Amino acids	Amino acids, aromatic	1.06	1.06	1.02	1.01	0.98	1.05	1.03	1.11	1.04	1.02	**1.27**	**1.55**	1.14	0.95	1.06	1.01	1.07	0.83	1.24	1.13	1.06	0.92	0.86	0.79
Unknown polar (38000389)			0.50	0.49	0.98	0.69	0.98	0.71	0.46	0.55	0.41	1.08	1.02	0.86	0.29	0.46	0.41	0.82	0.98	1.18	0.22	0.48	0.54	0.54	**3.33**	0.88
3-Hydroxybutyrate	Energy metabolism and related	Ketone bodies	0.66	0.61	0.86	1.03	0.93	1.07	0.30	0.33	0.48	0.24	0.30	0.53	0.55	0.66	0.51	1.20	1.37	0.77	0.29	0.37	0.45	0.25	0.34	0.47
Deoxyribonucleic acids, total	Carbohydrates and related	Nucleobase related saccharides	0.93	**1.26**	0.80	1.02	0.95	**1.20**	1.15	**1.31**	1.24	0.98	**1.15**	1.05	**1.29**	1.11	1.01	0.85	1.07	1.04	**1.21**	**1.54**	1.41	1.10	**1.36**	1.43
Unknown polar (38000433)			1.15	0.82	0.95	0.89	0.85	1.00	1.20	**1.12**	0.78	1.05	0.90	0.98	0.99	0.98	**1.43**	1.18	1.10	1.22	0.89	0.62	1.39	**1.27**	0.96	1.18
trans-4-Hydroxyproline	Amino acids related	Collagen metabolism	**1.19**	0.89	0.80	**1.36**	1.15	**1.21**	0.93	0.81	0.72	0.99	0.85	0.71	0.83	0.83	0.71	0.93	0.80	0.93	0.86	0.71	0.70	0.54	0.58	0.53
Cytosine	Nucleobases and related	Pyrimidine metabolism	0.95	1.16	0.90	0.97	0.99	1.06	**1.23**	**1.23**	**1.32**	1.11	1.04	**1.12**	**1.29**	1.11	1.13	0.96	1.14	1.12	**1.32**	**1.37**	1.28	1.19	**1.30**	1.35
Unknown polar (38000449)			0.55	0.44	0.90	0.64	0.84	0.73	0.43	0.52	0.59	0.84	0.98	0.74	0.24	0.45	0.43	0.67	0.87	1.04	0.16	0.44	0.61	0.44	**4.03**	0.84

1,5-Anhydrosorbitol	Carbohydrates and related	Polyols	0.96	0.88	0.95	1.05	1.05	1.13	1.07	1.00	0.92	1.09	1.05	**1.14**	1.05	0.99	1.09	0.91	0.69	0.57	1.03	**1.25**	1.08	1.22	0.75	0.55
Histidine	Amino acids	Amino acids, basic	1.01	1.12	**1.42**	**1.10**	0.99	**1.10**	1.19	**1.18**	**1.61**	**1.18**	**1.37**	**1.55**	1.11	1.24	**1.47**	0.99	**1.23**	1.16	**1.55**	**1.65**	**1.97**	1.03	1.25	**1.24**
Uracil	Nucleobases and related	Pyrimidine metabolism	0.89	1.00	0.90	1.02	0.75	0.84	1.07	1.05	**1.38**	0.96	0.98	1.13	1.20	1.39	0.72	0.86	0.88	0.94	**1.42**	**1.62**	0.98	1.07	1.04	0.88
Xylitol	Carbohydrates and related	Polyols	1.01	1.23	**1.33**	1.39	1.20	1.60	1.03	1.15	1.35	1.18	0.93	**2.12**	1.66	1.49	1.13	**1.53**	**1.70**	**1.84**	1.27	1.12	0.98	0.99	1.16	0.79
3-Hydroxyindole	Amino acids related	Tryptophan metabolism	0.88	1.06	1.08	1.07	1.20	1.06	0.56	0.94	0.95	0.90	**1.56**	**1.76**	0.87	0.89	0.92	1.04	0.85	0.90	0.80	1.06	1.12	0.66	1.09	0.77
Glucuronic acid	Carbohydrates and related	Sugar acids	1.01	1.06	0.91	1.07	1.23	1.42	1.04	0.79	1.04	1.36	**1.45**	1.21	1.18	0.90	0.74	1.00	1.62	1.13	1.22	1.11	0.93	**1.26**	1.37	0.79
Indole-3-acetic acid	Amino acids related	Tryptophan metabolism	**1.98**	**2.05**	**1.51**	0.71	0.78	0.92	**2.71**	**3.86**	**4.90**	**2.02**	**2.39**	**3.21**	**2.47**	1.55	**1.68**	1.03	0.94	0.86	**1.91**	**3.24**	**2.18**	1.55	1.34	**1.60**
Pseudouridine	Nucleobases and related	Pyrimidine metabolism	1.02	0.93	**1.21**	1.04	0.98	1.03	1.23	**1.27**	**1.44**	1.10	**1.29**	**1.30**	1.10	0.86	0.86	0.95	1.03	1.10	1.10	1.20	1.05	1.06	1.11	1.23
Uric acid	Nucleobases and related	Purine metabolism	0.82	0.91	0.77	1.11	0.89	0.97	1.07	0.84	0.94	0.93	0.93	0.91	1.40	1.64	0.64	1.02	0.72	1.08	1.15	1.91	0.98	1.19	0.85	1.06
scyllo-Inositol	Carbohydrates and related	Polyols	0.98	0.95	1.11	0.78	0.89	0.96	0.97	1.05	1.27	1.00	**1.61**	1.24	1.15	1.32	0.84	1.04	1.41	1.11	1.11	**1.68**	1.02	1.40	1.60	1.26
Erythronic acid	Carbohydrates and related	Sugar acids	**1.32**	0.92	1.17	0.95	0.90	1.07	1.11	1.12	1.21	0.83	1.26	0.90	1.33	1.12	1.06	0.91	1.02	0.94	1.18	1.19	1.06	1.25	1.01	1.14
Allantoin	Nucleobases and related	Purine metabolism	1.01	1.09	0.96	1.12	1.16	1.17	1.12	1.21	0.98	1.19	**1.44**	**1.26**	**1.21**	1.12	1.02	1.25	1.24	1.25	1.14	1.21	1.21	1.14	1.21	1.03

3-Hydroxyisobutyrate	Amino acids related	Amino acid metabolites	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Cystine	Amino acids	Amino acids, S-containing	0.97	1.13	1.34	0.95	**1.42**	1.11	0.87	**1.24**	**1.39**	1.13	1.25	**2.00**	**2.12**	0.96	1.38	1.16	1.13	1.00	1.14	0.95	**1.49**	1.15	**1.62**	**1.50**
3-Phosphoglycerate (3-PGA)	Energy metabolism and related	Glycolysis/Gluconeogenesis	1.34	1.32	**1.57**	1.22	0.90	1.30	2.43	**2.85**	**2.94**	1.20	1.05	**1.66**	**2.49**	1.46	**1.64**	0.92	0.70	0.90	**3.18**	**1.69**	1.24	1.40	0.93	1.21
2-Hydroxybutyrate	Energy metabolism and related	Energy metabolism, miscellaneous	1.14	1.16	1.36	1.12	1.22	1.31	**2.43**	**2.63**	**3.71**	**6.75**	**8.07**	**11.12**	1.44	0.90	0.92	**1.91**	**1.45**	**1.68**	**3.08**	**3.13**	1.40	**7.80**	**10.13**	**8.72**
Ketoleucine	Amino acids related	Amino acid metabolites	0.64	0.70	0.70	0.73	0.70	0.73	0.78	0.77	0.93	1.02	1.20	0.96	0.75	0.98	0.67	1.17	0.87	1.13	1.14	1.08	0.84	1.72	0.99	0.75
Mannosamine	Carbohydrates and related	Amino sugars	0.99	**1.22**	1.31	0.94	1.01	0.99	**1.54**	**1.33**	1.42	1.29	**1.29**	1.53	0.56	1.42	1.18	1.41	1.09	**1.28**	0.62	1.38	1.35	**2.26**	1.02	1.35
Glucosamine	Carbohydrates and related	Amino sugars	0.87	1.24	0.72	0.42	0.77	0.66	**1.10**	1.11	0.94	1.43	1.15	1.14	0.96	1.15	1.01	1.19	1.08	**1.74**	0.50	0.50	1.53	1.49	1.00	0.74
Indole-3-lactic acid	Amino acids related	Tryptophan metabolism	0.95	1.24	1.11	0.92	0.96	0.86	0.81	0.83	0.75	1.06	1.27	**1.11**	0.84	0.93	1.08	0.87	1.05	0.89	1.04	0.89	1.26	1.00	1.10	0.91
Unknown polar (58000010)			1.10	0.88	1.19	0.99	0.82	0.93	0.77	1.10	0.83	1.13	0.71	1.29	0.98	0.71	0.88	0.53	0.78	1.19	1.20	1.32	0.92	1.65	0.97	0.92
Unknown polar (58000014)			0.62	0.74	**1.57**	0.91	1.03	1.96	0.95	1.04	0.63	0.65	0.73	0.70	0.98	3.82	NA	0.86	0.76	0.84	NA	1.73	0.63	0.81	1.16	0.95
Unknown polar (58000017)			0.80	1.27	0.76	1.11	0.94	0.71	0.62	1.00	0.85	1.03	0.74	0.80	1.17	1.20	1.13	1.14	1.71	1.17	NA	0.61	1.33	0.65	0.83	0.88
Hippuric acid	Miscellaneous	Miscellaneous	0.80	1.22	0.78	0.72	0.90	0.70	0.39	0.44	0.28	0.56	0.50	0.52	0.44	0.22	0.77	0.30	0.55	0.92	0.58	0.37	1.05	0.40	0.46	0.47

3-Indoxylsulfate	Amino acids related	Tryptophan metabolism	1.15	0.42	0.91	0.68	3.25	1.96	1.15	0.84	1.14	1.31	1.69	1.26	0.79	0.56	0.93	0.68	0.80	1.08	0.97	0.65	1.23	0.68	0.99	0.78
Succinate	Energy metabolism and related	Citrate cycle	1.02	0.96	0.89	1.04	0.95	0.91	0.94	0.99	0.88	0.96	1.06	1.14	0.97	0.99	0.99	1.14	0.99	0.99	1.01	0.99	0.98	1.05	0.96	1.01
Unknown polar (58000132)			0.88	0.76	1.02	1.11	0.97	1.06	0.87	0.90	0.82	0.93	0.97	0.90	1.11	1.02	0.80	0.88	0.86	1.30	1.01	0.94	0.95	0.99	0.83	0.99
(NaAc)2 (NaFormate)2 Cl(35) Artefact	Unknown	Unknown polar	0.93	**1.37**	1.02	0.96	0.94	0.59	0.89	1.01	0.95	0.96	1.06	0.96	1.35	0.78	1.13	0.85	1.03	0.85	1.15	0.94	1.01	1.23	0.81	0.83
Unknown polar (58000141)			0.82	0.96	0.87	1.09	1.09	1.08	1.04	0.85	0.93	0.78	0.87	0.80	0.86	0.97	1.02	1.11	1.03	1.00	0.78	0.93	1.06	0.86	1.05	0.99
Unknown polar (58000142)			0.92	0.93	1.09	1.01	0.95	1.07	1.00	1.02	0.91	0.95	1.22	0.83	0.89	0.73	1.06	0.85	0.91	1.01	0.87	0.76	1.05	0.80	1.02	0.90
Unknown polar (58000143)			1.00	1.01	0.91	0.84	0.85	0.95	0.83	1.09	0.76	0.85	0.92	0.98	1.11	1.24	1.05	1.17	1.08	1.01	1.00	1.07	1.24	1.36	1.03	1.02
Unknown polar (58000148)			1.11	1.03	1.07	1.04	0.73	0.64	1.15	1.05	0.89	1.01	0.96	1.01	1.01	0.88	0.96	0.84	0.88	1.08	1.07	0.90	0.89	1.05	1.10	0.88
Unknown polar (58000149)			1.22	0.86	1.07	1.11	0.92	1.01	0.71	0.92	1.02	0.87	0.99	0.87	0.88	0.98	0.93	1.07	0.97	**1.58**	0.90	0.85	0.80	0.86	1.14	1.08
Unknown polar (58000158)			0.92	0.69	0.86	1.08	1.09	0.69	0.77	0.95	0.80	0.82	**2.48**	0.45	1.17	0.99	0.85	0.91	0.76	0.86	1.22	0.97	0.90	0.93	1.01	1.08
Unknown polar (58000162)			1.06	0.92	0.86	1.19	0.90	0.74	1.01	0.81	0.79	0.93	0.84	1.02	0.97	0.73	1.28	0.85	0.83	0.79	0.92	0.65	1.09	0.81	0.85	0.87
Unknown polar (58000166)			0.91	0.99	0.70	1.10	1.09	1.26	0.84	0.95	1.01	0.78	0.79	1.09	0.84	0.90	0.89	1.13	0.79	0.81	1.05	0.93	1.08	1.06	0.79	1.07
Unknown polar (58000167)			0.95	0.68	0.76	0.85	0.84	0.56	0.90	0.78	0.73	0.84	0.80	0.63	0.82	0.64	0.69	0.67	0.92	0.75	1.03	0.63	0.85	0.87	1.15	0.71
(NaFormate)1 (NaAc)6 Cl(37) Artefact	Unknown	Unknown polar	0.87	0.99	0.70	0.85	0.81	1.05	1.04	1.05	0.99	0.93	0.98	1.00	0.89	0.96	0.98	1.09	0.96	0.62	1.24	1.32	1.06	0.96	1.13	0.72

Lysophosphatidylcholine (C18:2)	Complex lipids, fatty acids and related	Lysophosphatidylcholines	0.94	0.98	0.89	0.89	1.00	0.82	1.04	0.96	0.81	1.03	0.98	0.83	0.94	0.83	0.87	1.01	0.92	0.88	1.00	0.93	0.82	1.01	0.89	0.83
Lysophosphatidylethanolamine (C22:5)			1.03	1.19	**1.23**	0.80	1.31	**1.28**	0.90	1.41	1.06	0.80	**1.23**	1.07	0.92	0.90	0.93	1.16	1.03	1.21	0.91	0.90	1.09	1.18	1.10	1.12
Lysophosphatidylcholine (C16:0)	Complex lipids, fatty acids and related	Lysophosphatidylcholines	0.86	1.00	0.94	1.11	1.01	1.05	0.82	0.88	0.78	1.04	0.99	0.98	0.99	0.97	0.87	0.99	1.03	0.89	0.93	0.80	0.85	1.00	0.88	0.99
Lysophosphatidylcholine (C18:1)	Complex lipids, fatty acids and related	Lysophosphatidylcholines	**1.12**	**1.18**	**1.31**	1.17	**1.20**	**1.14**	1.01	**1.16**	**1.27**	0.99	1.14	1.09	1.22	1.08	**1.17**	1.01	1.15	**1.35**	1.17	**1.25**	**1.27**	1.08	1.05	1.10
Lysophosphatidylcholine (C18:0)	Complex lipids, fatty acids and related	Lysophosphatidylcholines	0.93	0.90	0.97	0.99	0.95	1.01	0.88	0.87	0.92	0.94	0.90	0.94	0.92	0.98	0.96	0.92	0.95	0.99	0.91	0.88	0.96	0.97	0.95	0.92
Sphingomyelin (d18:2,C16:0)	Complex lipids, fatty acids and related	Sphingomyelins	0.54	0.51	0.63	0.83	0.79	0.83	0.22	0.44	0.45	0.58	0.53	0.55	0.64	0.71	0.59	0.84	0.80	0.72	0.64	0.54	0.54	0.87	0.55	0.60
Sphingomyelin (d18:2,C18:0)	Complex lipids, fatty acids and related	Sphingomyelins	0.78	0.61	0.83	0.81	0.92	1.11	0.56	0.55	0.48	0.83	0.87	0.82	1.10	0.74	0.80	0.88	0.99	0.83	0.94	0.59	0.77	1.09	0.79	0.76
Phosphatidylcholine (C16:1,C18:2)	Complex lipids, fatty acids and related	Phosphatidylcholines	0.74	0.70	0.87	0.71	0.84	0.66	0.74	1.27	1.25	0.97	0.91	0.81	0.93	0.78	0.91	0.88	0.62	0.58	1.17	1.50	1.51	0.87	0.92	1.11

Phosphatidylcholine (C16:0,C22:6)	Complex lipids, fatty acids and related	Phosphatidylcholines	0.50	0.76	0.88	0.97	0.97	1.04	0.49	0.73	0.82	0.70	0.95	1.01	0.57	0.76	0.84	0.93	1.08	1.04	0.58	0.64	0.83	0.79	0.91	0.96
Phosphatidylcholine No 02	Complex lipids, fatty acids and related	Phosphatidylcholines	0.69	0.98	0.89	0.94	0.98	0.88	0.58	0.93	0.58	0.69	0.89	0.83	0.68	0.76	0.95	0.84	0.88	1.04	0.60	0.59	0.91	0.68	0.93	0.99
Phosphatidylcholine (C16:0,C18:2)	Complex lipids, fatty acids and related	Phosphatidylcholines	0.56	0.84	0.82	0.99	0.98	0.93	0.72	0.85	0.93	0.89	0.93	0.92	0.83	0.82	0.85	0.99	0.96	0.99	0.76	0.75	0.88	0.94	0.94	0.91
Phosphatidylcholine (C18:1,C18:2)	Complex lipids, fatty acids and related	Phosphatidylcholines	0.75	0.77	0.90	0.91	0.90	0.84	0.67	0.87	0.98	0.74	0.79	0.83	0.81	0.74	0.67	0.88	0.79	0.92	0.63	0.65	0.69	0.88	0.75	0.76
Ethanolamine plasmalogen (C39:5)	Complex lipids, fatty acids and related	Plasmalogens and other ether lipids	0.62	0.57	0.76	0.77	0.91	0.76	0.41	0.49	0.60	0.70	0.78	0.70	0.40	0.76	0.77	1.09	1.26	0.79	0.85	0.48	0.48	1.03	0.98	0.49
Phosphatidylcholine (C16:0,C16:0)	Complex lipids, fatty acids and related	Phosphatidylcholines	0.80	0.92	0.85	0.92	0.99	0.93	0.81	1.13	1.15	0.95	0.95	1.00	0.82	0.93	0.85	0.87	0.96	0.95	0.98	1.25	0.83	0.87	0.97	0.67
Choline plasmalogen (C36:2) (putative)	Unknown	Unknown lipid	0.69	0.81	0.65	0.64	0.90	0.66	0.53	0.63	0.61	0.84	0.64	0.63	0.46	0.53	0.48	0.69	0.79	1.04	0.48	0.46	0.51	0.90	0.64	0.60
Choline plasmalogen (C18-vinyl,C20:4)	Complex lipids, fatty acids and related	Plasmalogens and other ether lipids	0.78	0.86	0.96	1.06	**1.46**	1.23	0.41	0.86	0.95	0.92	1.12	**1.11**	0.58	1.08	1.17	1.25	**1.90**	**1.44**	0.71	0.77	0.81	1.58	1.27	0.91

Phosphatidylcholine (C18:0,C20:4)	Complex lipids, fatty acids and related	Phosphatidylcholines	0.57	0.97	0.83	1.01	0.99	**1.01**	0.85	0.85	0.86	0.88	0.96	1.02	0.85	0.94	0.97	0.96	0.98	1.00	0.81	0.94	0.91	0.99	0.98	0.95
PC No 04 (putative)	Unknown	Unknown lipid	0.48	0.50	0.58	0.81	0.84	0.70	0.37	0.45	0.43	0.41	0.60	0.49	0.44	0.59	0.54	0.56	0.70	0.86	0.42	0.51	0.47	0.64	0.72	0.66
Sphingomyelin (d18:1,C23:0)	Complex lipids, fatty acids and related	Sphingomyelins	0.66	0.80	0.80	0.85	0.97	0.78	0.45	0.58	0.47	0.68	0.84	0.84	0.55	0.83	0.74	0.31	0.89	0.89	0.44	0.51	0.77	0.82	0.72	0.71
DAG (C18:1,C18:2)	Complex lipids, fatty acids and related	Diacylglycerols	0.69	0.42	0.48	0.59	0.71	0.75	0.84	1.41	1.40	0.46	0.42	0.38	0.55	0.47	0.59	0.50	0.48	0.58	1.31	1.38	1.22	0.36	0.51	0.90
Ceramide (d18:1,C24:0)	Complex lipids, fatty acids and related	Ceramides	0.46	0.43	0.39	0.62	0.76	0.57	0.47	0.64	0.55	0.59	0.59	0.44	0.70	0.41	0.51	0.46	0.51	0.63	0.81	1.11	0.54	0.60	0.56	0.48
TAG (C16:1,C16,1) and TAG (C14:0,C18:2) (putative)	Unknown	Unknown lipid	1.40	0.70	0.72	0.51	0.57	0.60	**3.81**	**2.54**	**5.71**	0.72	0.74	0.77	0.50	0.33	0.64	0.37	0.75	0.51	2.53	2.96	2.08	0.16	0.55	1.30
TAG (C18:2,C18:2)	Complex lipids, fatty acids and related	Triacylglycerols	0.77	0.38	0.76	0.48	0.45	0.63	2.48	**1.96**	2.54	0.64	0.58	0.46	0.24	0.21	0.30	0.21	0.22	0.19	1.62	1.12	1.04	0.14	0.33	0.36
TAG (C16:0,C18:2)	Complex lipids, fatty acids and related	Triacylglycerols	0.68	0.30	0.61	0.49	0.66	0.67	1.41	**1.99**	2.37	0.48	0.55	0.50	0.21	0.19	0.25	0.23	0.26	0.52	1.10	0.90	0.98	0.21	0.29	0.75

TAG (C18:1,C18:2)	Complex lipids, fatty acids and related	Triacylglycerols	**1.31**	0.67	1.01	0.57	0.96	0.81	**3.93**	**3.26**	**3.66**	0.76	0.94	0.69	0.29	0.28	0.44	0.31	0.43	0.46	1.84	2.07	1.64	0.42	0.38	0.49
Unknown lipid (68000033)			0.95	0.84	0.87	0.87	0.85	0.89	0.82	0.92	0.97	0.90	0.87	0.89	0.96	1.01	0.88	1.05	0.92	1.05	0.96	0.94	0.93	0.76	1.02	0.91
Unknown lipid (68000034)			0.99	0.90	0.99	0.93	0.80	0.90	1.03	0.98	1.13	0.75	0.79	0.70	0.86	1.10	0.90	0.73	0.79	0.82	0.94	1.21	1.09	0.59	0.76	1.07
Cholesterylester, total	Complex lipids, fatty acids and related	Cholesteryleslers	0.84	0.87	0.91	0.89	0.86	0.90	1.04	1.04	1.21	0.82	0.73	0.73	0.76	0.80	0.74	0.50	0.51	0.82	1.11	0.99	0.80	0.42	0.59	0.99
Lysophosphatidylcholine (C17:0)	Complex lipids, fatty acids and related	Lysophosphatidylcholines	0.82	0.78	0.75	0.85	0.92	0.95	0.52	0.60	0.62	0.72	0.67	0.65	0.71	0.75	0.70	0.82	0.77	1.14	0.60	0.58	0.64	0.74	0.82	0.68
Lyso PE (C22:0) (putative)	Unknown	Unknown lipid	0.67	0.70	0.67	0.84	0.90	0.75	0.42	0.45	0.50	0.58	0.65	0.62	0.55	0.56	0.72	0.71	0.70	0.88	0.43	0.42	0.55	0.64	0.54	0.54
Lysophosphatidylcholine (C20:4)	Complex lipids, fatty acids and related	Lysophosphatidylcholines	0.92	1.00	0.98	0.95	1.01	1.02	0.90	0.97	0.94	0.87	0.95	0.94	0.96	0.92	0.98	0.90	0.96	1.01	0.94	1.01	0.96	0.95	0.93	0.92
TAG (C16:0,C16:1)	Complex lipids, fatty acids and related	Triacylglycerols	1.09	0.85	0.73	0.58	0.61	0.56	**2.99**	**2.50**	**4.67**	0.47	0.67	0.66	0.50	0.46	0.70	0.66	0.24	0.31	2.92	3.03	2.61	0.49	0.57	1.41
TAG (C18:2,C18:3)	Complex lipids, fatty acids and related	Triacylglycerols	0.92	0.42	0.61	0.40	0.51	0.45	2.82	**1.78**	4.03	0.72	0.57	0.49	0.36	0.34	0.25	0.24	0.21	0.17	2.25	2.39	1.49	0.15	0.26	0.51

Cholesterylester C20:4	Complex lipids, fatty acids and related	Cholesterylesters	0.76	1.11	1.00	1.10	1.06	0.97	0.30	0.45	0.26	1.09	1.17	**1.27**	0.79	0.76	0.77	1.29	1.19	1.19	0.31	0.26	0.45	1.31	0.80	0.82
Sphingomyelin (d18:1,C16:0)	Complex lipids, fatty acids and related	Sphingomyelins	0.74	0.71	0.92	0.96	0.92	1.00	0.58	0.70	0.60	0.84	0.88	0.79	0.96	0.93	0.93	0.93	0.98	1.00	0.95	0.83	0.94	0.94	0.88	0.86
Ethanolamine plasmalogen (C39:4)	Complex lipids, fatty acids and related	Plasmalogens and other ether lipids	0.43	0.45	0.57	0.70	0.71	0.57	0.30	0.56	0.63	0.51	0.66	0.60	0.42	0.58	0.61	0.63	0.86	0.96	0.32	0.49	0.48	0.78	0.61	0.40
Phosphatidylcholine (C16:0,C20:5)	Complex lipids, fatty acids and related	Phosphatidylcholines	0.60	0.77	0.82	0.94	0.95	0.84	0.71	0.87	0.91	0.88	0.94	0.84	0.75	0.78	0.73	0.94	0.74	0.69	0.65	0.67	0.74	0.86	0.81	0.76
Phosphatidylcholine (C16:0,C20:4)	Complex lipids, fatty acids and related	Phosphatidylcholines	0.36	0.74	0.70	0.99	0.98	0.97	0.61	0.74	0.78	0.83	0.86	0.90	0.79	0.80	0.82	0.95	0.96	0.98	0.68	0.83	0.82	0.96	0.94	0.94
Phosphatidylcholine (C18:0,C18:2)	Complex lipids, fatty acids and related	Phosphatidylcholines	0.74	0.83	0.84	0.95	0.97	0.90	0.71	0.75	0.88	0.90	0.93	0.86	0.79	0.87	0.76	0.91	0.94	0.92	0.68	0.72	0.71	1.02	0.91	0.77
Phosphatidylcholine (C18:0,C18:1)	Complex lipids, fatty acids and related	Phosphatidylcholines	0.74	1.02	1.09	0.94	1.20	**1.09**	0.68	0.85	1.10	1.04	**1.19**	**1.14**	0.74	0.87	0.85	0.98	1.09	**1.29**	0.87	0.92	0.77	1.17	0.93	1.00
Unknown lipid (68000052)			0.41	0.76	0.75	0.94	0.96	0.91	0.52	0.64	0.77	0.69	0.79	0.91	0.66	0.76	0.78	0.83	0.89	0.92	0.52	0.70	0.68	0.84	0.86	0.80

Phosphatidylcholine (C18:0,C20:3)			0.40	0.61	0.61	0.68	0.85	0.77	0.36	0.54	0.54	0.57	0.56	0.68	0.49	0.59	0.56	0.69	0.75	0.80	0.39	0.50	0.43	0.69	0.66	0.60
Sphingomyelin (d18:1,C24:0)	Complex lipids, fatty acids and related	Sphingomyelins	0.45	0.49	0.61	0.67	0.78	0.68	0.52	0.48	0.49	0.52	0.62	0.64	0.49	0.59	0.58	0.53	0.78	0.72	0.48	0.67	0.40	0.65	0.64	0.56
Phosphatidylcholine (C18:0,C22:6)	Complex lipids, fatty acids and related	Phosphatidylcholines	0.57	0.77	0.84	1.03	0.99	1.07	0.42	0.61	0.65	0.70	1.01	1.08	0.51	0.89	0.82	0.80	1.12	1.04	0.51	0.60	0.68	0.68	0.95	0.98
TAG (C52:5 (H) or C50:2 (Na)) (putative)	Unknown	Unknown lipid	1.25	0.60	0.78	0.42	0.62	0.53	**3.42**	**2.93**	4.83	0.69	0.52	0.55	0.33	0.19	0.29	0.11	0.10	0.26	2.13	1.65	1.62	0.29	0.37	0.62
TAG (C16:0,C18:1, C18:3)	Complex lipids, fatty acids and related	Triacylglycerols	1.20	0.28	0.45	0.36	0.41	0.44	**3.05**	**2.30**	3.30	0.47	0.43	0.36	0.27	0.16	0.27	0.17	0.17	0.27	1.48	1.61	1.21	0.21	0.39	0.36
TAG (C18:1,C18:2, C18:3)			0.92	0.47	0.73	0.43	0.45	0.53	2.91	**2.28**	2.85	0.57	0.48	0.32	0.48	0.48	0.57	0.19	0.15	0.39	1.28	1.45	1.14	0.19	0.31	0.37
TAG (putative)			0.73	0.45	0.64	0.47	0.45	0.45	1.66	1.46	1.91	0.44	0.42	0.28	0.39	0.46	0.35	0.18	0.15	0.40	0.89	1.25	0.90	0.12	0.19	0.42
Unknown lipid (68000060)			0.52	0.50	0.55	1.14	0.97	0.93	0.32	0.42	0.66	0.59	0.62	0.65	0.62	0.79	0.60	0.91	0.87	0.84	0.41	0.42	0.84	0.57	0.65	0.73
Homovanillic acid (HVA)	Hormones, signal substances and related	Catecholamine catabolites	1.07	0.36	0.90	0.64	0.80	0.71	0.73	0.48	0.28	0.29	0.52	0.31	0.60	0.57	0.69	0.65	0.34	0.58	0.62	1.28	0.59	0.59	0.18	0.40
3,4-Dihydroxyphenylacetic acid (DOPAC)	Hormones, signal substances and related	Catecholamine catabolites	**1.33**	1.21	1.04	1.21	1.06	1.02	1.17	**1.19**	1.11	1.03	**1.19**	1.05	0.78	0.75	0.87	0.86	0.31	0.79	0.89	0.98	0.78	0.74	0.31	0.66

3,4-Dihydroxyphenylglycol (DOPEG)	Hormones, signal substances and related	Catecholamine catabolites	**1.21**	1.15	0.91	**1.14**	1.14	0.87	1.17	1.08	1.12	**1.17**	**1.25**	**1.18**	1.20	1.17	0.94	1.15	1.17	0.77	1.09	**1.33**	0.91	0.95	0.90	0.59
Serotonin (5-HT)	Hormones, signal substances and related	Serotonin and serotonin metabolism	0.63	0.53	0.16	1.41	0.71	0.49	0.11	0.09	0.13	0.87	0.26	0.37	0.54	1.86	1.38	3.00	0.35	0.77	1.07	1.19	1.09	1.63	0.73	1.12
Normetanephrine	Hormones, signal substances and related	Catecholamine catabolites	0.91	0.94	0.98	0.95	0.71	0.86	0.83	0.79	0.99	0.72	0.87	0.68	0.79	0.99	1.00	1.02	1.20	1.04	0.91	1.18	0.80	0.89	0.79	0.54
Metanephrine	Hormones, signal substances and related	Catecholamine catabolites	0.86	1.01	1.09	0.50	0.67	0.78	0.81	0.68	0.71	0.50	0.60	0.42	0.80	0.72	0.62	1.03	1.30	0.70	0.98	0.85	0.77	0.57	0.66	0.68
Dopamine	Hormones, signal substances and related	Catecholamines	0.86	0.84	1.04	0.75	1.07	**1.14**	0.86	0.91	0.95	1.09	1.07	0.62	1.10	1.12	0.91	0.91	1.14	1.04	1.12	1.15	1.07	0.85	0.91	0.95
Noradrenaline (Norepinephrine)	Hormones, signal substances and related	Catecholamines	1.07	1.15	1.14	1.11	1.10	0.98	1.11	0.85	1.09	1.03	0.95	1.12	1.08	1.31	1.03	1.11	0.94	0.81	0.96	1.09	1.02	0.88	0.78	0.76
Adrenaline (Epinephrine)	Hormones, signal substances and related	Catecholamines	0.98	1.36	1.87	0.25	0.31	0.90	1.10	0.65	0.74	0.84	0.62	0.69	0.73	1.44	1.17	0.66	1.12	0.50	1.47	1.04	0.83	0.44	0.85	0.94

3,4-Dihydroxyphenylalanine (DOPA)	Hormones, signal substances and related	Catecholamine precursors	0.68	0.87	0.61	**1.46**	1.08	1.08	0.64	0.90	0.87	**1.35**	**1.40**	**1.82**	1.01	**1.16**	1.51	1.15	1.03	1.00	1.00	**1.20**	0.93	1.16	0.92	0.96
3-Methoxytyrosine	Hormones, signal substances and related	Catecholamine catabolites	1.07	**1.28**	**1.18**	1.14	**1.22**	1.17	**1.20**	1.32	1.15	**1.16**	**1.33**	**1.42**	0.92	0.92	**1.18**	1.22	1.20	1.18	1.15	1.02	0.89	1.06	1.13	1.34
4-Hydroxy-3-methoxyphenylglycol (HMPG)	Hormones, signal substances and related	Catecholamine catabolites	1.21	0.96	0.89	1.00	0.84	0.87	0.76	0.97	1.06	0.88	1.05	1.07	1.19	1.08	1.01	**1.21**	**1.22**	0.85	0.98	1.19	0.78	1.13	0.88	0.81
Histamine	Hormones, signal substances and related	Other hormones, signal substances and related	1.18	1.23	1.06	1.13	1.06	1.28	1.00	1.00	2.06	1.19	1.03	**3.26**	1.35	1.34	0.75	0.93	1.14	2.17	1.25	**1.66**	1.15	1.03	1.20	2.00
Androstenedione	Hormones, signal substances and related	Steroids and related	1.84	NA	3.23	3.26	1.39	0.62	1.17	NA	1.08	1.21	**2.25**	1.19	0.49	1.51	0.39	1.90	1.50	0.82	1.23	0.54	0.79	0.98	0.36	1.93
Testosterone	Hormones, signal substances and related	Steroids and related	0.49	0.40	0.57	2.96	1.16	0.80	0.50	0.31	0.47	0.88	**2.81**	1.16	0.44	0.24	0.48	**1.21**	1.11	0.71	0.31	0.47	0.42	0.65	0.39	1.31

Progesterone	Hormones, signal substances and related	Steroids and related	1.44	0.55	0.35	0.40	0.86	0.32	1.22	0.52	0.75	0.43	0.60	0.48	0.74	0.29	1.30	1.92	1.14	1.35	1.01	0.42	0.35	**2.83**	1.79	0.88
21-Hydroxyprogesterone (11-Deoxycorticosterone)	Hormones, signal substances and related	Steroids and related	0.58	0.17	0.43	0.47	0.54	0.21	0.70	0.87	0.37	0.83	0.49	0.28	2.78	1.52	0.97	1.00	1.52	1.06	3.99	0.76	1.78	**2.12**	2.84	1.01
18-Hydroxy-11-deoxycorticosterone	Hormones, signal substances and related	Steroids and related	0.61	0.27	0.59	0.24	0.24	0.12	0.73	0.80	0.49	0.35	0.30	0.14	1.09	1.11	0.77	1.17	1.22	1.10	1.89	0.65	0.70	1.61	1.44	1.19
Corticosterone	Hormones, signal substances and related	Steroids and related	0.90	0.42	0.77	0.37	0.39	0.19	0.76	0.84	0.69	0.33	0.62	0.31	1.24	1.08	0.90	**1.84**	1.90	1.04	1.68	0.66	0.77	**1.82**	2.45	1.27