Committee for Risk Assessment

RAC

Annex 2

Response to comments document (RCOM)

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

Adopted

28 January 2019

ANNEX 2 - COMMENTS AND RESPONSE TO COMMENTS ON CLH PROPOSAL ON 2-(4-TERT-BUTYLBENZYL)PROPIONALDEHYDE

COMMENTS AND RESPONSE TO COMMENTS ON CLH: PROPOSAL AND JUSTIFICATION

Comments provided during public consultation are made available in the table below as submitted through the web form. Any attachments received are referred to in this table and listed underneath, or have been copied directly into the table.

All comments and attachments including confidential information received during the public consultation have been provided in full to the dossier submitter (Member State Competent Authority), the Committees and to the European Commission. Non-confidential attachments that have not been copied into the table directly are published after the public consultation and are also published together with the opinion (after adoption) on ECHA's website. Dossier submitters who are manufacturers, importers or downstream users, will only receive the comments and non-confidential attachments, and not the confidential information received from other parties.

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Substance name: 2-(4-*tert*-butylbenzyl)propionaldehyde
EC number: 201-289-8
CAS number: 80-54-6
Dossier submitter: Basf SE

GENERAL COMMENTS

Date	Country	Organisation	Type of Organisation	Comment number
21.03.2018	Netherlands		MemberState	1
Comment received				
Toxicokinetics Two in vitro dermal absorption studies used in the SCCS opinion on lysmeral are not included. In these studies, percutaneous absorption and penetration was determined in excised skin of mini pigs and naked rats. The bioavailable portion found was much higher in rats (66.1 and 50.8%) when compared to mini pigs (0.8% and 4.9%), the latter closely resembling human skin. Moreover, the fraction of bioavailable lysmeral was found strongly increased in mini pig skin when moving from dissolved lysmeral (in methylcarbitol or ethanol) to real cream formulations (4.9% vs. 25.7%). Given that the absorption of lysmeral in mini pig skin was much higher when this compound was applied via real cream formulations, it is reasonable to conclude that lysmeral might also better penetrate human skin when it is applied in cream formulations. Since there is no further experimental data on this subject, the SCCS concluded that the maximum fraction of lysmeral being absorbed by human skin might be in the range of 25% rather than at 2,4%. Givaudan, Penetration studies in vitro on the intact skin of naked rats and mini pigs with Lilial 14C, 1982, #56763 Givaudan, Penetration studies on intact skin of naked rat and pig in vitro, 1986, #56764 In figure 3, the curve of the lowest concentration of both lysmeral and TBBA (0,04 µM) lies below the control. Can you explain what could be the reason for this? And could you also present the results in human hepatocytes, as has been done for rat hepatocytes in figure 3? In figure 4 no control values are displayed. Where these included and if so, could you provide these?				

For figure 5b, can you provide the results of the other human hepatocyte measurements, not just the one displayed?

Dossier Submitter's Response

The dossier submitter (DS) acknowledges, that two additional dermal penetrations studies are cited in the SCCS opinion. They represent exploratory screening studies with a limited validity. The studies are not performed according to current guidelines and have limitations in their documentation. In contrast to the information cited by the Netherlands CA, there are further experimental data on this subject (BASF 2016), which was included into the CLH proposal and in submission II to the SCCS.
The SCCS conclusion cited by the NL CA exclusively refers to SCCS´s opinion on submission I. Based on the outcome of the SCCS conclusion on the content of submission I, the DS performed an *in vitro* dermal penetration study in human skin according to current OECD guidelines (OECD TG 428) to clarify the open points on absorbed fractions of Lysmeral in ethanolic and cream formulations on human skin. The type of formulation (i.e. alcoholic vs. 3 different cream formulations) represented a crucial aspect of the study design. In the discussion section of the recent opinion, the SCCS pointed out, that the data from this human in vitro dermal penetration study should be taken into account for a potential MoS calculation. Considering the similar penetration rates of Lysmeral applied in alcoholic and cream formulations, the conclusion, that Lysmeral might better penetrate human skin when applied in cream formulations, cannot been confirmed. Furthermore, the maximum fraction of Lysmeral being absorbed by human skin was found to be in a range clearly below 25%.

The NL CA refers to Figure 3 of the CLH report, indicating TBBA-CoA levels in incubations with the lowest Lysmeral and TBBA concentrations below the control samples. The reason for slightly higher levels of potential TBBA-CoA conjugates stem from some background signals in the non-exposed control samples with the same [M+H+] (928.2113) as TBBA-CoA. Since concentrations for this background ion as calculated with the TBBA-CoA standard curve are very low (0.06-0.14 µM), the signal is too low for MS/MS to identify this background component.
Similar concentrations of this background signal where detected with 0.04 and 0.1 µM Lysmeral or TBBA as in the non-exposed control:

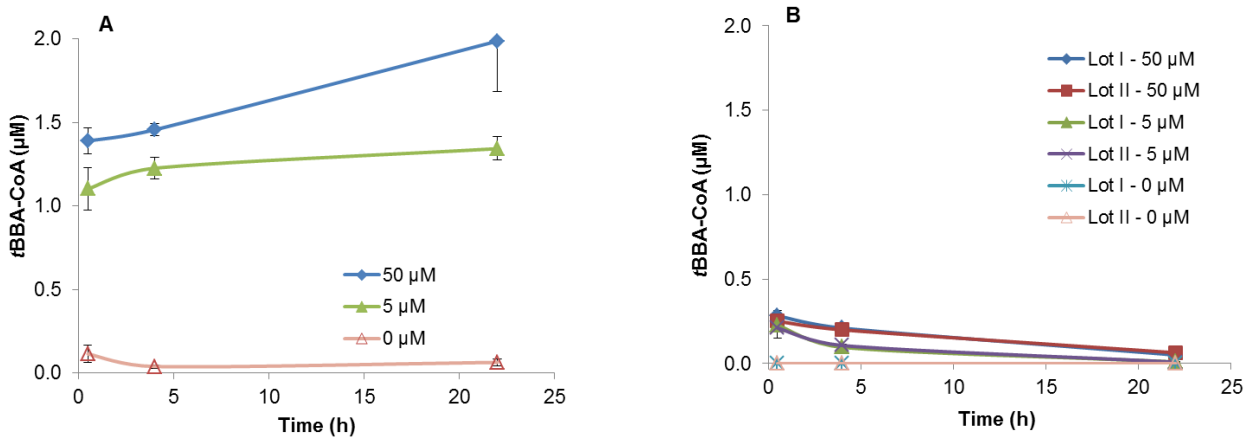
- <LOQ – 0.03 µM (in 0.04 µM Lysmeral incubations)
- 0.09-0.16 µM (in 0.1 µM Lysmeral incubations)
- 0.01 – 0.03 µM (in 0.04 µM TBBA incubations)
- 0.09-0.18 µM (in 0.1 µM TBBA incubations)

Taking into account the broad range of the concentrations used for the TBBA-CoA standard curve (0.005-2 µM), the DS does not consider the background signals in the control and the two lowest test concentrations as significantly different but rather similar.

The NL CA requests to present the results in human hepatocytes, as has been done for rat hepatocytes in Figure 3 of the CLH report. Lysmeral was only tested at two concentrations (5 and 50 µM) in two lots of human hepatocyte cultures. A rapid and almost complete decrease of TBBA-CoA levels was observed within 22 h incubation in human hepatocytes with two independent lots/experiments and two concentrations. The amounts and kinetics of TBBA-CoA formed in the presence of 5 and 50 µM Lysmeral in human hepatocytes resemble the amounts and kinetics of the longer chain Coenzyme A conjugates like Lysmerylic acid CoA in rat hepatocytes. Since significant lower levels of TBBA-CoA were detected with 5 and 50 µM in human hepatocytes compared to rat hepatocytes, the DS did not explore lower test concentrations in human hepatocytes like it has been done in rat hepatocytes as shown in Figure 3 of the CLH report.

The NL CA identified, that no control values are displayed in Figure 4 of the CLH report. Control incubations with plated hepatocytes were always run in parallel. These values were not included in Figure 4, since the concentrations of TBBA-CoA were below the detection level in the controls. In contrast to rat hepatocytes, there was no background signal with the same [M+H+] (928.2113) as TBBA-CoA in the non-exposed control incubations of human hepatocytes. However, the controls were added to a modified Figure 4 for rat and for human hepatocytes as requested, see below.

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 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).



The NL CA asked for results of other human hepatocyte measurements beyond the ones displayed in Figure 5B of the CLH report. So far, TBBA was tested in one lot of human hepatocytes as displayed in Figure 5B. Other human hepatocyte measurements with TBBA as test substance are currently not available. However, there is sufficient evidence which indicate, that levels and kinetics of TBBA-CoA formation from Lysmeral incubation is substantially different in human compared to rat hepatocytes. Lower amounts and no accumulation of TBBA-CoA was observed in human hepatocytes in two independent experiments/Lots and at two concentrations (see Figure 4B of the CLH report). The formation of TBBA-CoA from TBBA incubation is considered as supporting data for Lysmeral, the chemical under scrutiny, for which more data were generated.

RAC's response

Thank you for your comments. Additional information from the Givaudan penetration studies has been taken into account in RAC's assessment.

Date	Country	Organisation	Type of Organisation	Comment number
12.04.2018	Belgium	Procter & Gamble	Company-Downstream user	2

Comment received

The substance under review is used exclusively in fragrances at low concentrations in cosmetic and household care products where dermal exposure is the most relevant route of exposure. An IFRA Industry Standard, in place for many years, restricts the use of this

substance in various consumer products – thus exposure is low and controlled. See attached document ECHA note – An attachment was submitted with the comment above. Refer to public attachment Lysmeral - supporting arguments from PG_CMR2_FINAL_ Apr 2018.docx
Dossier Submitter's Response
agreed
RAC's response
Thank you. Your comment has been noted.

Date	Country	Organisation	Type of Organisation	Comment number
13.04.2018	Belgium		MemberState	3
Comment received				
As a general comment, BE CA would like to remind that the CLH process evaluates the intrinsic danger of a compound for human, and not its potential risk for human. Therefore, all affirmation regarding the relevance of exposure routes of lysmeral for human when used as a fragrance, consumers/workers exposure assessments or any other justification related to risk assessment should never be part of a proper CLH dossier. BE CA is of the opinion that all the affirmations related to risk assessment cannot be taken into consideration during the CLH classification process of lysmeral. - Dermal bioavailability Data's clearly demonstrated that lysmeral becomes systemically available in animals and humans after dermal administration. In an vitro study penetration of [14C]-lysmeral through and into human excised skin was assessed in various formulation (cf. Table 9, p. 16 of the CLH dossier) (OECD 428, GLP, BASF 2016). The DS concluded that the percentage of systematically available lysmeral after skin application was calculated to be between 5 and 7%. BE CA noted that for ethanol (respectively 80,44% and 84,67% for 24h and 72h) and for silicone in water formulation (83,08% for 24h), the total recovery was below the acceptance range of 85%. BE CA would also stress the very low topical doses applied (95 µg/cm² for EtOH 70% formulation vs 5,0 µg/cm² for the three other formulations). According to OECD Guideline 428 (skin absorption: in vitro method), an application of 1-5 mg/cm² should be used in in vitro tests. For this specific study, some other uncertainties also remain. Please provide the thickness of the skin samples, number of samples and number of donors per tested formulation, the measurements for the evaluation of the integrity of the skin (including potential prior storage and electrical resistance of the human skin samples) and the receptor fluid chosen. In rat, in vivo occlusive dermal application of [β-14C]-lysmeral in 70% ethanol for a topical concentration of 0,2 mg/cm² for maximum 6 hours demonstrated a systemic distribution with a main site as liver. Lysmeral was also found in plasma, kidneys, heart and lungs. Radioactivity findings in stomach and intestines might indicate that animals had buccal access to the lysmeral dermal application to some extent. This oral intake might falsely increase the systematic dermal bioavailability results of rat in this study. In an in vivo dermal penetration study on 3 human volunteers, observations include a mean of 1,4% of the applied dose in urine after 6 hours semi-occlusive application (11,37 mg test-substance in 70% ethanol on 10 cm² skin). Radioactivity was below detection limits in all faeces and blood samples. First, we would appreciate further information				

about the timing of blood sampling. BE CA would also be informed if any information is available about the remaining radioactivity in the skin after washing.
Moreover, BE CA would stress that the human in vivo dermal penetration study was realized in a semi-occlusive manner whereas the rats were exposed to [β-14C]-lysmeral via occlusive dermal application. Considering that the vehicle was ethanol and that lysmeral has demonstrated its volatile capacity in the human in vitro dermal absorption study (more than 50% recovered in the charcoal filter), the possibility that a fraction of lysmeral evaporated during semi-occlusive exposure cannot be excluded, therefore underestimating the radioactive fraction found in urine.
DS concluded that "in human only limited percutaneous absorption of lysmeral is observed especially compared to the rat". Taking into consideration the uncertainties, as exposed above, in the human in vitro and in vivo absorption studies, and in the rat study, BE CA is of the opinion that the comparison of dermal absorption rate of human compared to rat is not appropriate and that no conclusion can be made on this basis.
As a general conclusion, BE CA considers that the dermal route should be considered as relevant for human, and that lysmeral demonstrated its capability to be systematically available in human.
- Inhalatory bioavailability
We noticed affirmations in the dossier stating that lysmeral is considered to have a limited bioavaibility via the inhalation route, due to physico-chemical properties, including a reported low vapor pressure (0,25 Pa at 20°C). First, BE CA express their surprise as one of the crucial criteria's for a molecule to be odoriferous is its volatile potential, allowing it to reach the nose receptors. We also noted that the vapour pressure has been determined based on extrapolation of measures at higher temperature and computer calculations. No measure at ambient temperature has been reported.
Finally, the observations in an in vitro percutaneous absorption study using human skin (as presented in the previous section,) showed that 23,92% to 62,88% of the dermally applied lysmeral was recovered in a charcoal filter placed above a Franz cell (BASF 2016), depending on the formulation (water-in-oil, oil-in-water, silicon or ethanol). We would therefore appreciate further elaboration regarding the statement of limited bioavailability via inhalation route.
We also regret that no repeated inhalation toxicity study is available in the CLH report, which might have given some weight to this affirmation.
- Metabolisation
In human, rat, mouse, and rabbit, lysmeral is metabolized in two different primary metabolites, lysmerol and lysmerylic acid. Lysmerylic acid was identified as the main hepatic metabolite which is then metabolized in four secondary metabolites, including p-tert-butyl-benzoid acid (TBBA) and hydroxyl-lysmerylic acid in all tested species.
Human excretion kinetics study showed that the main metabolite found in urine after dermal and oral exposure of lysmeral was TBBA (p. 21-22 of the CLH report).
Interestingly
, the sum of the 4 measured metabolites (lysmerol, lysmerylic acid, hydroxyl-lysmerylic acid and TBBA) and the parent compound (after enzymatic deconjugation) only covered 16,5% of the applied dose 48h after the oral uptake. However, TBHA was not measured during the oral part of the study.
At first glance, this metabolic profile is in contradiction with the findings in an in vitro metabolism study (BASF SE 2010), showing that the main detected metabolite in human hepatocytes was lysmerylic acid after 4 hours exposure (Table 15, p. 25 of the CLH report). Lysmerylic acid being a primary metabolite that is further transformed into TBBA, the difference in metabolic profile results between the two studies might be explained by the short duration of the in vitro study. Therefore, BE CA is of the opinion that TBBA should be considered as a relevant metabolite to human, based on human oral and dermal excretion study.

TBBA can be further transferred to secondary acceptors such as glycine to form p-tert-butyl-hippuric acid (TBHA) or transformed to TBBA-CoA in hepatocytes, such as other chemicals with a para-substituent at the benzyl ring.
We noticed in Figure 1 (p. 24, Metabolic pathway of 14C-Lysmeral) and in the summary on toxicokinetics (p. 30) that TBHA is reported to be only present in rodents. This conclusion is in contradiction with the reporting of the dermal pilot excretion kinetics study with one volunteer (p. 21). The reporting of this study in the CLH dossier indicates that peak levels of lysmerol and lysmerilyc acid were excreted into the urine about 3-6h, whereas TBBA and TBHA peaks appeared in urine about 12h after dermal application. The Givaudan study (1985) found that TBHA was also found in urine samples of dogs, although to a lesser extent that in mouse and guinea pig. Please therefore provide further enlightenment about the relevance of TBHA metabolite to human and other animals.

Dossier Submitter's Response

- Dermal bioavailability

The BE CA noted, that the total recovery was below the acceptance range of 85% for ethanol and silicone in water formulations in the key study for dermal penetration in humans (OECD 428, GLP, BASF 2016). For the two formulations mentioned by the BE CA, a higher proportion of the applied dose has been found in the charcoal filter, demonstrating differences in the volatility of Lysmeral in the experimental in vitro setup used. Importantly, a decrease of the total recovery in mass balance was not correlated with a decrease in the percentage of the Lysmeral dose absorbed. In contrast, an increase in the fraction of evaporated Lysmeral found in the charcoal filter generally resulted in a decrease of the overall recovery. These findings indicate, that a low recovery may be attributable to differences in evaporation with incomplete trapping in the charcoal filter but would not affect the value obtained to determine the dermally absorbed dose of Lysmeral. Furthermore, the OECD Guidance Notes on Dermal Absorption n° 156 (ENV/JM/MONO (2011)36 indicates, that for volatile or unlabeled test substances, a range of 80-120% is acceptable (also quoted in ENV/JM/MONO(2004)2 Guidance notes for the conduct of skin absorption studies n°28). The formulations mentioned by the BE CA met the acceptable range of 80-120%. The DS does not consider, that the recovery values obtained in the study cited substantiate a poor quality on the conduct of the study.

The BE CA stressed the very low topical doses applied in the key in vitro dermal penetration study and cited the OECD TG 428, requesting an application of 1-5 mg/cm² in dermal penetration in vitro tests. The DS wants to point out, that the target dose of 1-5 mg/cm² refers to the test-substance preparation (e.g. cosmetic formulation) and not to the applied dose of the radiolabeled test-substance. In the current experiments, about 5 mg of the test substance preparation / cm² skin surface have been applied. According to OECD TG 428, the test substance preparation (e.g., neat, diluted or formulated material containing the test substance which is applied to the skin) should be the same (or a realistic surrogate) as that to which humans or other potential target species may be exposed. In the dermal penetration study in question, the formulations and the Lysmeral concentrations chosen represent such realistic cosmetic formulations. Therefore, the DS cannot agree with BE CA´s comment, that the tested doses were very low.

The BE CA asked for additional information about the conduct of the dermal penetration study in humans (OECD 428, GLP, BASF 2016):
Skin preparations of 12 donors were used with a thickness of 200 – 400 µm (217 - 389 µm, 261 – 400 µm, 200 – 389 µm, 238 – 386 µm for experiments with the vehicles ethanol, silicone in water, water in oil and oil in water, respectively). Eight Franz-type diffusion cells with a minimum of 4 skin donors were used for each experiment (Lysmeral in a respective formulation and sampling time). The integrity of the skin preparation was determined by

measuring its electrical resistance (TEER). After the resistance measurement, the penetration cells were stored in the refrigerator overnight. The integrity of skin preparations was additionally checked visually immediately before the application of the test substance preparation and immediately before sampling procedures for balancing. The skin preparations used within this study showed a TEER above 1 kΩ (70% tested skin samples were found to be above 10 kΩ) and no physiological damage or leakage of receptor medium to the surface. As receptor fluid, tap water (with 0.01 % NaN_3 for experiments with a sampling time of 72 hours) was chosen, since an aqueous receptor medium was found appropriate to guarantee sufficient solubility of the test substance.

The BE CA asked for further information about the conduct of the dermal penetration study in 3 human volunteers (Huntingdon Research Centre, 1994):
Blood samples (approx. 5 ml) were taken into heparinised tubes immediately before application and at 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 24, 48 and 72 hours afterwards.
After removal of the test substance (6 hours after application), a mean 63.12% (±4.94 SD) of the applied radioactivity was recovered from the gauze dressing used to occlude the site of application. A portion of 3.76% (±1.95 SD) of the dose was removed by washing the skin using an ethanol-moistened swab. Further 3.06% (±2.77 SD) of the applied dose was recovered from gauze dressings used to occlude the treated areas of skin after test substance removal (i.e. 6 - 120 hours after application).
Five successive tape strips (6.25 cm^2 area) were taken after test substance removal (6 hours) and 120 hours after application. It has been observed, that at 6 hours and at 120 hours the proportion of dose removed declined with each successive tape stripping and the mean total amount of dose removed was 0.1127 % (±0.0666 SD) at 6 hours and 0.0012 % (±0.0005 SD) at 120 hours. The values may be multiplied by 16 to account for the area stripped and the total area of application. By applying this multiplication, up to approx. 1.8% of the dose applied could be removed if the total area of treated skin was similarly stripped.

The BE CA noted, that the exposure to the test substance was realized in a semi-occlusive fashion, a fraction of Lysmeral evaporated cannot be excluded underestimating the radioactive fraction found in urine. A certain loss of test substance via evaporation cannot be excluded, considering the mean total recovery of 71.43% (±10.46 SD) ^{14}C-Lysmeral. However, the semiocclusive exposure chosen represents the most likely exposure condition for Lysmeral during e.g. cosmetic product use including fine fragrances.

The BE CA found uncertainties in the human in vitro / in vivo and in the rat absorption studies, and is of the opinion, that a comparison of dermal absorption rates in human vs. rats is not appropriate and that no conclusion can be made on this basis. The DS agrees, that differences in the study designs exist, and that the dermal penetration study with human skin in vitro is the only study according to current guidelines and GLP. Therefore, the human dermal penetration data can be considered as more robust than the rat data. However, higher dermal penetration rates in rat skin compared to human skin has been described elsewhere (see van Ravenzwaay et al., 2004, Human & Experimental Toxicology 23; 421-430) and his aspect has a minor relevance for the CLH proposal on reproductive toxicity of Lysmeral.

The DS agrees with BE CA´s general conclusion, that the dermal route should be considered relevant for humans and that Lysmeral is able to become systematically available in humans.

- Inhalatory bioavailability

The BE CA expressed their surprise as one crucial criteria for a molecule to be odoriferous is its volatile potential, allowing it to reach the nose receptors. The DS would like to give a general overview on properties of fragrance materials and the relevance of their volatile potential:

Fragrances are allocated to three different segments of the so-called fragrance pyramid, based on their evaporation characteristics:

- Base notes, i.e substances of very low volatility (e.g. vanillin: vp = 0.0013 hPa at 20°C)
- Heart notes, i.e. medium volatile fragrance molecules (e.g. Lysmeral: vp = 0.0025 hPa at 25°C);
- Top notes, which are substances with rather high tendency to evaporate (e.g. linalool: vp = 0.3 hPa at 20°C).

As compared to other substances (e.g. ethanol: vp = 78.9 hPa at 25°C; water: vp = 23.4 hPa at 20 °C), most fragrance molecules are generally chemicals with a very low vapour pressure and evaporate only slowly without reaching high concentrations in the surrounding atmosphere.

Lysmeral is a heart note in perfumes with an intensive and impactful olfactory muguet-like profile. Even though being used as a fragrance material, the substance has only a very low vapor pressure (0.0025 hPa at 20 °C), evaporates slowly (substantivity on fibers > 24 hours) and does not reach spontaneously high concentrations in the air. The interaction of Lysmeral with olfactory receptor(s) in the olfactory epithelium is of crucial importance for the use of it as a fragrance material. However, the concentrations needed to trigger the odour sensation are very low. As outlined in the CLH dossier, the human odour threshold for racemic Lysmeral is set at 1-2 ppb (van Gemert, 2003). Therefore, substances even with a low vapour pressure can be sensed and may qualify as fragrance material.

The BE CA commented on the determination of the vapour pressure based on extrapolation and computer calculations. Two studies are available assessing the vapour pressure of Lysmeral (further information can be found in the ECHA disseminated REACh dossier; see https://echa.europa.eu/de/registration-dossier/-/registered-dossier/13572/4/7).

In the first study from 1980, the vapor pressure of Lysmeral with an adequate purity (99.5 %) was measured from 68.1 °C to 277.8 °C. In the more recent study from 1999, the vapour pressure was determined between 90.99 °C and 263.39 °C (purity of the test item: 99.3 %). Despite the long period of 19 years between the conductance of the two tests, the results were highly comparable. Therefore, both studies are considered valid for the determination of the vapour pressure of Lysmeral. The extrapolation from the measured values is generally seen as critical, however, the lowest measured vapour pressure value was 0.17 hPa at 68.1 °C and the vapour pressure at room temperature is expected to be much lower than the measured 0.17 hPa. Since all measured values result in an accurate vapour pressure curve, the DS considers the extrapolated vapor pressure value of 0.0025 hPa at 20 °C as realistic. In addition, the calculated vapor pressure (EPI Suite v. 4.11) is 0.00477 hPa at 25 °C, which is in the same range and further confirms the extrapolated vapour pressure value.

The BE CA referred to the observations in the in vitro human dermal absorption study (BASF 2016), showing that 20-60% of the applied Lysmeral was recovered in a charcoal filter. The DS agrees, that a relevant fraction of evaporated radiolabelled Lysmeral was trapped in the in vitro test setup 14 mm above the skin surface. However, the relevance of these findings for the exposure situation during use is uncertain and the dermal

penetration test system cannot be used to make any prediction concerning the bioavailability of Lysmeral via the inhalation route. When considering general consumer exposure to fragrance materials, 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 (see Cadby et al. 2002; Regulatory Toxicology and Pharmacology 36, 246–252). In contrast, the major route of systemic exposure is almost certainly by deposition on the surface of the skin.
The BE CA noted the absence of a repeated inhalation toxicity study to further elaborate the limited bioavailability via the inhalation route. The DS agrees, that such a study might have delivered further data to add weight to this affirmation, however, it would not have a crucial inpact on the classification decision presented in the CLH proposal submitted.

Overall, BE CA´s comments on the relevance of dermal and inhalative bioavailability are not comprehensible in the light of their initial statement, that all affirmations regarding the relevance of exposure routes of Lysmeral for human when used as a fragrance, consumers/workers exposure assessments or any other justification related to risk assessment should never be part of a proper CLH dossier.

- Metabolisation
The BE CA pointed out, that the sum of the 4 metabolites assessed in the oral pilot study in human volunteers only covered 16,5% of the applied dose (Scherer 2016). The DS would like to emphasize, that this study was intended to develop a human biomonitoring (HBM) method including identification of suitable biomarkers of exposure in human urine. The study protocol did not assess other relevant routes of excretion such as faeces (direct and bilial excretion) or did not determine the amount of metabolites in blood plasma to allow a full balancing. Therefore, the fraction of 16.5% cannot be set into perspective to other routes of excretion. As outlined in the CLH dossier, TBHA was not measured during the oral part of the study due to the high variations concerning inter-day precision at low concentrations and accuracy during method validation. However, it is uncertain, if the addition of the TBHA levels would have had a significant impact on the sum of urinary metabolites measured, considering the 10 fold lower fraction of TBHA compared to TBBA found after dermal application.
The BE CA identified differences in the metabolic profile between the in vitro metabolism study (BASF SE 2010) and the oral pilot study in human volunteers and these might be explained by the short exposure duration of the in vitro study. The DS would like to stress the differences in the scope (determination of the metabolic profile vs. identification of suitable markers for human biomonitoring), the experimental setup (hepatocyte cultures versus human subjects) and the results obtained (metabolite concentrations excreted into the supernatant versus cumulative amount in urine). The detected metabolites in the volunteers urine represent a cumulative amount, to which the metabolite concentrations in the supernatant of the hepatocyte cultures cannot be compared to. Concentrations in hepatocyte supernatants are seen as directly proportional to plasma concentrations in vivo, however, no plasma concentrations have been determined in the oral human volunteer study for an adequate comparison. The present data on human volunteers identified, that Lysmerylic acid is not the main metabolite accumulated in the urine, however, the amount of this metabolite excreted via other routes has not been determined. Overall, we disagree with the direct comparison of the studies made by the BE CA. However, we agree with BE CA´s opinion that TBBA should be considered as a relevant metabolite to humans, although the formation is species dependent and the formation in humans was found to be comparable with non-responder species at testicular and spermatotoxic concentrations.

The BE CA stated, that TBBA can be metabolized to TBHA or transformed to TBBA-CoA in

hepatocytes. The DS would like to further specify, that TBBA-CoA formation is an intermediate step between TBBA and TBHA. The activation of benzoates (such as TBBA) via binding to CoA by the action of a mitochondrial ATP dependent Acid:CoA ligase is the initial step for the transfer to the secondary acceptor glycine (see Badenhorst et al. 2004; Drug Metab Rev, Early Online: 1–19). This activation is followed by acylation with glycine via GLYAT to form the respective hippurate (such as TBHA). GLYAT has been isolated in several mammals including rats, rabbits, sheep, cows, rhesus monkeys and humans. Based on this, the occurrence of stable TBBA-CoA levels in rats could be an indication for a disturbance in metabolic glycine conjugation, i.e. an important detoxification mechanism in rodents.

The BE CA noted, that TBHA is reported to be only present in rodents. According to the present data available, TBHA was found after Lysmeral administration in rats, mouse, guinea pigs, dogs, rhesus monkeys (Roche 1985A) and humans (Scherer 2016). However, the comparative urinalysis in the different animals (see Table 14 of the CLH report) showed, that urinary TBHA levels were evidently above TBBA levels in mice and guinea pigs and represented main metabolite. In human urine obtained in the human dermal pilot study, the TBHA level (0.04 %) was found to be below the TBBA level (0.67%). Therefore, these data indicate, that TBHA formation plays a more important role in mice and guinea pigs, than in non-rodent species including humans. Unlike the other rodent species, the rat appears to be different in terms of its low capacity to form the respective hippuric acid.

RAC's response

Thank you for your comment. Your considerations and the clarifications made by the DS have been taken into account in RAC's assessment.

TOXICITY TO REPRODUCTION

Date	Country	Organisation	Type of Organisation	Comment number
11.04.2018	Sweden		MemberState	4

Comment received

FERTILITY
The DS states that there is clear evidence of adverse effects on male fertility, but mechanistic information raises doubt about the relevance for humans. We do not agree with the interpretation of data for human relevance and propose that lysmeral be classified as Repr. 1B H360F based on the following:
• The toxic metabolite TBBA is formed and identified as the most abundant metabolite in humans. The suggested quantitative differences in TBBA-CoA kinetics between rat and human is based on in vitro data. No in vivo data is available to confirm such differences. It is not clarified how these measures extrapolate to in vivo levels and threshold for toxicity.
• Comparable levels of TBBA are excreted in urine after oral administration in rat and human, about 14% of the dose in human and 7-19% in rat.
• The biological plausibility of the proposed MoA is uncertain. Key event(s) leading from "disruption of the lipid synthesis" to "testicular toxicity" are not defined (e.g. not clear if conjugate accumulation occurs in liver or testis).
• Importantly, alternative/multiple MoA(s) have not been considered. In particular, an endocrine disturbing (ED) MoA for lysmeral cannot be ruled out.
• The DS has not considered the effects in females. Nevertheless, there is evidence of toxicity to females, e.g. from the EOGRTS (BASF SE 2017) and studies on analogues (see attachment).
Taken together, the existing information is not sufficient to conclude on a MoA for

lysmeral toxicity or that humans are less sensitive. This is consistent with the recent conclusion by the Scientific Committee on Consumer Safety based on the assessment of lysmeral (SCCS opinion Dec. 2017).

DEVELOPMENT
The DS has not proposed classification for development and argues that developmental toxicity is secondary to maternal toxicity. The SE CA does not agree and suggests classification in category 2 (some evidence of effects) for developmental toxicity based on the following:
Lysmeral causes increased incidence of post-implantation losses, in addition to its effects on pup bw, skeletal variations and neonatal acetylcholine esterase inhibition. An increased incidence of post-implantation losses was seen in the PNDT study and in two generational studies. In the latest study (BASF 2017), the number of delivered foetuses was decreased significantly and was below the historical control range. Mild maternal toxicity was reported at doses where developmental toxicity occurred (See Table 2 in attachment). Thus, a direct effect on development seems possible.

The DS states that a prolonged human uptake of doses inducing systemic toxicity is unlikely. The SE CA notes that human exposure is mainly dermal by use of different types of leave-on and rinse-off cosmetics (SCCS opinion 2017) and washing/cleaning products. There is clear data demonstrating that lysmeral becomes systemically available via this route. Thus, all exposure routes should be considered relevant for hazard assessment.

Please see the submitted attachment for details.

ECHA note – An attachment was submitted with the comment above. Refer to public attachment lysmeral CLH_20180406.docx

Dossier Submitter's Response

FERTILITY
According to comments presented in this document and the attachment, the SE CA does not agree, that existing information is sufficient to exclude human relevance of the testicular toxicity observed in rats. This has been based on the following arguments:

- The SE CA states, that the metabolism of Lysmeral is qualitatively similar in rat and humans, since TBBA is formed as the most abundant metabolite in humans. The DS agrees, that TBBA is found in rats and in humans. However, a direct species comparison in cultured hepatocytes shows differences of the formation of TBBA between rats and humans (BASF SE 2010). Although urinary TBBA levels in humans were found to be higher than other human biomonitoring method (HBM) markers, plasma levels and other compartments (such as faeces) have not been investigated. According to the data in hepatocytes, TBBA is not the most abundant metabolite in humans. Therefore, SE CA´s statement, that TBBA is formed as the most abundant metabolite in humans is not confirmed by the present data.

- The SE CA states, that quantitative differences in TBBA-CoA kinetics are based on in vitro data. The DS would like to clarify, that the SE CA combines two different aspects, that should be assessed separately. The comparative in vitro metabolism study identified toxicokinetic differences (i.e. TBBA formation) between rats and humans (BASF SE 2010). These results demonstrated, that endogenous TBBA levels formed in human cells are lower than in rats and comparable to rabbits (non-responder species). Furthermore, mechanistic studies in hepatocytes identified the

underlying mode of action (stable formation of TBBA-CoA conjugates), which does not occur in human cells (Givaudan 2017; Laue et al. 2017).
The SE CA notes, that no in vivo data are available to confirm such species differences, and it is not clarified, how these measures extrapolate to in vivo levels of the metabolite and threshold for toxicity. The DS agrees, that no full toxicokinetic study in human volunteers with comparable exposure conditions (such as equal doses, routes of exposure, compartments assessed, etc.) is available to compare TK differences/TBBA formation with rats. The differences in the mode of action (stable and sustained TBBA CoA conjugate formation) are difficult to confirm in an experimental in vivo setup in humans. However, studies on human relevance including mechanistic information on the mode of action and comparative metabolization studies strongly depend on the in vitro study setup and data from in vitro tests should not be categorically disregarded.
The extrapolation of the Lysmeral concentrations chosen in the comparative metabolism study (BASF SE 2010) to in vivo levels related to the threshold for toxicity has been provided in the CLH report. The lowest test concentration (10µM) relate to 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. C_{max} 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 C_{max} of 9 µg/ml or approx. 40 µM Lysmerylic acid, i.e. the main metabolite (BASF SE 2006A).

- The SE CA compared rat urinary Lysmeral levels measured after 5 consecutive oral (gavage) application of 50-100 mg/kg bw/d (Roche 1985) with urinary TBBA levels measured in urine after a single oral uptake of approx. 0.1 mg/kg bw (5.26 mg / 60 kg mean body weight assumed; Scherer 2016). Although, the fraction of the doses applied were similar (14% in humans vs. 7-19% in rats), these cannot be considered comparable levels of TBBA due to the 1000 fold difference in the doses applied and the different dosing regimes chosen. As outlined in the CLH report, the human volunteer study was designed as an explorative study to identify suitable Lysmeral exposure markers to develop a HBM. A direct comparison of the in vivo data in the rat and the HBM study is therefore not applicable. The SE CA did not mention the data of the dermal pilot study, in which the TBBA levels represented only 0.67% of the applied dermal dose, although the application route is the most relevant for the general population.

- The SE CA questions the biological plausibility of the proposed MoA and the proposed causality (testicular toxicity caused by disturbing CoA and lipid homeostasis) has not been supported, e.g. by reference to any known testicular toxicants. The key events leading from "disruption of the lipid synthesis" to "testicular toxicity" are not defined (e.g. not clear if conjugate accumulation occurs in testis or liver). The DS disagrees with this comment, since a strong correlation between TBBA-CoA formation, lipid synthesis disruption and testicular toxicity has been established in the CLH report (p. 61 ff.):

  - Complex lipids (VLC-PUFA, ceramides, sphingolipids, phosphatidylcholines) are present in high amounts in mammalian sperm and play an important role for spermatogenesis.
  - Knockout mice with disrupted synthesis pathways for complex lipids (FADS2, ELOVL2, LPAAT3) show disturbances of spermatogenesis and male infertility (Stroud 2009; Zadravec 2011; Iizuka-Hishikawa 2017)

- Complex lipid formation is based on CoA dependent processes.
- Lysmeral treatment results in the disruption of fatty acid and lipid synthesis as observed in vitro and in vivo.
- Lysmeral induced testes toxicity is always observed in the presence of liver toxicity.
- Other chemicals - including known testicular toxicants - potentially transformed to benzoic acid metabolites show strong correlation between sustained formation of benzoyl-CoA in hepatocytes and spermatotoxic/testicular toxicity in rats (see Table 25 of the CLH report).

New toxicological data on position isomers of TBBA in an ex vivo study using a 3D cell culture with primary seminiferous tubules from juvenile Sprague Dawley rats (Bio-Alter®) demonstrated the formation of TBBA-CoA conjugates (see comments submitted by the DS during the public consultation). These data indicate the capacity of male reproductive tissues to form such conjugates. Structural differences between the position isomers (para vs. meta) had an impact on the formation on these conjugates which correlates well with the ability to disturb spermatogenic processes. Accordingly, these new data strengthen our conclusion, that the conjugation of TBBA with CoA represents the mode of action for Lysmeral-induced testes toxicity and spermatotoxicity.

- The SE CA noted, that alternative MoAs have not been considered and an endocrine disturbing MoA cannot be ruled out due to Lysmeral binding to the estrogen receptor, testicular toxicity, effects on several ED-responsive parameters and vitellogenin production in vivo. The DS disagrees with the conclusion made by the SE CA, that an ED MoA cannot be ruled out:

- A hormonal dysregulation as MOA for testes toxicity is unlikely since this adverse effect was observed at doses above 25 mg/kg bw/d independent from treatment duration (even after single oral administration).
- The mechanistic data defining the MOA for testes toxicity (disruption of CoA-dependent metabolic processes) does not represent an ED effect. As presented in Annex 4 of the CLH report, the metabolome data did not show any consistent pattern for steroid hormones (Androstenedione, Testosterone, Progesterone, 11-Deoxycorticosterone, 18-Hydroxy-11-deoxycorticosterone, Corticosterone).
- The in vitro data cited by the SE CA (OECD Level 2) do not serve to prove any ED effect. Only a moderate inhibition of estradiol binding was observed at 3000000 molar excess of Lysmeral. Lysmeral induced estrogen response was lower than with estradiol even at a 10000 fold higher concentration (Charles and Darbre 2009). Furthermore, Lysmeral did not affect basal and gonadotropin induced testosterone formation in primary rat Leydig cells below cytotoxic levels (Roche 1994). The in vitro experiments cited by the SE CA were performed without any adequate metabolic system and are therefore of questionable relevance, since Lysmeral is efficiently metabolized and its systemical presence in vivo is low.
- The findings of the developmental toxicity study (OECD Level 4) in rats do not serve to prove any ED effect (BASF 2004). The decreases in gravid weights of the unopened uteri result from the increased incidence of resorptions and reductions in fetal body weights. No test substance related uterus weight effects were observed in the EOGRTS (see below).
- The EOGRTS in rats (OECD Level 5) does not confirm any ED effect of Lysmeral. No effects on estrous cyclicity (highly sensitive for anti-

/estrogen activities), presence of areolae and nipples (highly sensitive for anti-/androgen activities), anogenital indices, accessory sex glands, thyroid and respective hormones, sexual maturation and puberty (vaginal opening, preputial separation) were observed. The relevance of potential effects on ovary weights, differential ovary follicle counts and uterus weights are discussed further below

- In blood plasma of male fish, Vitellogenin (VTG) was statistically elevated compared to the control group in two of three Lysmeral test concentrations. However, the measured VTG values per test group and its statistical significance did not depend on the test concentrations applied. VTG has been measured in test concentrations of 0.0195, 0.0625 and 0.2 mg Lysmeral/L. The corresponding VTG mean values (and standard deviations) were 153.04 (SD 234.95), 977.74 (SD 1733.54) and 38.65 (SD 27.67) µg VTG/ml blood plasma. Mean VTG levels in control and solvent control blood plasma where 2.42 (SD 2.56) and 20.32 (SD 38.58) µg/ml, respectively. Due to the observed lack of dose-response relationship, a plausible link to Lysmeral exposure could not be demonstrated. Moreover, no population-relevant effects on reproduction could be observed. Thus, the observed VTG increases may be due to factors independent from Lysmeral exposure and do not result in adverse effects on the male fish. Furthermore, individual male plasma VTG concentrations varied by as much as 5 order of magnitude in the treatment groups resulting in exceedingly high coefficients of variation. This again rises doubt on a plausible link to Lysmeral exposure. However, a plausible reason for such variation in this study was not identified. Methodological contamination of the blood samples by carryover of blood from previously sampled female fish was excluded since scalpel blades were discarded after each fish. It is known that changes in VTG may also be caused by overt or systemic toxicity and non-endocrine MoAs (e.g. hepatotoxicity), or by confounding factors such as diet or subclinical infection (not specifically assessed in this study). Consequently, a decrease in VTG, while generally considered ED-mediated, needs to be interpreted with caution in combination with other observations, especially since results on VTG levels in male fish are not conclusive: a biologically plausible relationship does not exist between exposure and response. No other evidence of potential endocrine activity (changes in secondary sex characteristics, behavior, or reproduction) were indicated in this study. High VTG values in males were not correlated to low reproduction values. The fertilization rate and the egg production of replicates with males with the high measured vitellogenin values were not markedly decreased in comparison to the control groups range. Thus, population relevant endpoints were not adversely affected by the increased VTG values

- The SE CA stated, that effects reported in female rats have not been considered, respective parameters are not available from most studies or the inclusion of female animals was not clear. The DS agrees, that various short term repeated dose toxicity studies were performed to assess male reproductive effects without including female animals (see column "Protocol" in Table 19 of the CLH report), however, the different reproductive toxicity studies and the developmental toxicity study provided in the CHL report do address female relevant parameters.
  The SE CA identified evidence of female toxicity from the EOGRTS and a study on the structural analogue cyclamal. The DS disagrees, that the information provided by the SE CA represents any evidence for female reproductive toxicity. The SE CA

provided data in a summary table in the submitted attachment, showing that mean ovary weights were reduced below the historical control range in F0 females. However, the SE CA did not include the related data of the F1 generation for a full comparison of the whole dataset. No reduction in mean ovary weights were observed in F1A/B animals, representing animals treated with Lysmeral during all sensitive windows of sexual development (see updated table below). Furthermore, the reduction of ovary weights in the F0 females (absolute: 97.067 mg; relative: 0.045%) were minimally below historical control range (absolute: 109.542 – 130.320 mg; relative: 0.046 – 0.056%). No histopathological correlate has been observed, and the variances in mean ovary weights are rather physiological changes due to sexual cycling than treatment related changes.
The SE CA also mentioned decreases in uterus weights, however, these findings were not statistically significantly different from controls, and could also not be confirmed in F1 animals (see table below). The SE CA referred to a decrease in DOFC in F1 females, which often becomes apparent prior to a change in organ weights. However, the reduction in DOFC was not statistically significant (mean values for primordial/growing follicles 432.25/213.5 in high dose F1 females vs. 446.3/227 in controls) and no changes in estrous cycle duration was observed (mean days from estrous to estrous: 3.9-4 days in high dose F0, F1A and F1B females vs. 3.9-4 days in respective controls). Therefore, the data cited by the SE CA do not represent all data, that can be extracted from an EOGRTS, and their interpretation cannot be confirmed by the DS.

Table 1. Updated Table 1 of the SE CA on selected organ weights (%) relative to the placebo controls obtained from F0, F1A and F1B cohorts of an EOGRTS (BASF 2017):, **p<=0.01**

Dose (mg/kg bw/d)	Cohort	0	1	3	10
Abs. ovary weight	F0	100	99	94	**88**
	F1A	100	99	105	108
	F1B	100	94	98	92
Rel. ovary weight	F0	100	96	93	**89**
	F1A	100	96	99	111
	F1B	100	95	100	97
Abs. uterus weight	F0	100	94	90	84
	F1A	100	97	109	117
	F1B	100	112	104	102
Rel. uterus weight	F0	100	92	88	85
	F1A	100	94	102	118
	F1B	100	113	106	107

Adverse effects on male reproductive organs were observed in the rat one-generation reproduction toxicity study with cyclamal. The SE CA cited adverse effects on weight and histopathology of male and female reproductive organs and fertility, and further specified a statistically significant reduction in ovary and non-gravid uterus weights in treated F0 females. According to the information provided in the EChA disseminated REACh dossier for cyclamal*, the absolute and relative weights of the non-gravid uterus (with the cervix) was significantly decreased and the weights of the left and right ovary were significantly decreased at the high dose level of cyclamal (150 mg/kg bw/d) as compared to controls (no further details given). However, no microscopic changes in the uterus or ovaries were reported, that could be correlated with the differences in these organ weights. Furthermore, no apparent treatment related effects on estrous cyclicity, mating and fertility

parameters were observed in females treated with the high dose. Pregnancies occurred in all of the 25 treated female rats and all pregnant dams delivered litters, when mated with untreated male rats. This is in contrast to treated males at this dose level, producing only one pregnancy (1/24) after matings with untreated female rats. These data clearly demonstrate a predominant adverse effect on testes/sperm formation and male fertility by cyclamal, which is in line with the data provided in Table 25 of the CLH report.

*https://echa.europa.eu/de/registration-dossier/-/registered-dossier/5681/7/9/2

- The SE CA stated, that the EOGRTS was performed at too low doses to fully conclude on the toxicity, since the high dose group of the EOGRTS (10 mg/kg/d) was below the fertility LOAEL (40 mg/kg/d) and only minimal systemic toxicity was observed (reduced mean bw <6% and increased liver weights <20% with minimal histopathology). Based on the data available for Lysmeral, the DS considers the LOAEL for male reproductive effects above 25 mg/kg bw/d but clearly below 40 mg/kg bw/d, considering the effect level of 2300 ppm (25.1-27.5 mg/kg bw/d) for testes and sperm effects observed in the recent one-generation range-finding study (BASF 2017B). Furthermore, the SE CA cited the nominal high dose (10 mg/kg bw/d) used in the EOGRTS, although the test substance was applied via feed and the actual dose ingested in the high dose group (750 ppm) was above 10 mg/kg bw/d. The overall mean dose of analytically verified Lysmeral administered to the male and female Wistar rats throughout all study phases and across all cohorts was 15.1 mg/kg bw/d and the range of the mean Lysmeral intake accounted to 11.2-18.8 mg/kg bw/d (females) and 10.2-19.2 mg/kg bw/d (males) during different phases of the study and cohorts used. Given the variability of the actual dose ingested (mean doses up to 19 mg/kg bw/d) and the effect levels observed in the associated one-generation range finding study (25.1-27.5 mg/kg bw/d), the use of 750 ppm as a high dose for the EOGRTS is considered adequate to ensure sufficient numbers of offspring to comply with the requirements of the OECD TG 443 and the final decision of the SE CA on the substance evaluation under CoRAP. The final decision clearly stated, that:

  "the fact that the substance is toxic to testis and gives maternal toxicity requires special attention in the selection of doses (number of and intervals between), which should ensure that a high enough number of pups are produced while still allowing reproductive effects to be identified"

  With respect to this request made by SE CA, particular attention should be drawn to the fact that the high dose (25.1-27.5 mg/kg bw/d) in the preceding one-generation range-finding study (BASF 2017B) produced an appreciable impairment of the male ability to reproduce. Only 4 out of 10 mating pairs were able to produce living offspring and the litter size in those fertile pairs was only 36% of the control (4.0 pups vs. 11.1 in control). The application of dose levels in the EOGRTS, closer to the 25.1-27.5 mg/kg bw/d dose than the effective doses of 10.2-19.2 mg/kg bw/d in males would have significantly increased the risk that the goal set by SE CA to "*ensure that a high enough number of pups are produced"* was not met. In addition, the request of SE CA for certain non-standard additions to the OECD 443 protocol (such as the measurement of AChE in red blood cells, serum and in a variety of nervous tissues) generated the additional demand for a distinctly higher number of offspring available for these non-standard cohorts. This went well beyond the 5 male and 5 female offspring per litter necessary for all fertility, neurotoxicity and immunotoxicity cohorts in a (full) standard study design.

The additional demand was only met by increasing the number of F0 mating pairs set into the study. These study additions further increased the pressure to make absolutely sure, that enough offspring for all cohorts were produced. Dose levels close to 25.1-27.5 mg/kg bw/d would have created a high uncertainty in the study design and would have required to set in at least a triplicate number of F0 animals into the EOGRTS, if a similarly impaired fertility as in the range-finding study was considered, which is neither applicable nor desirable from an animal welfare perspective.

As outlined above, by setting the doses as they effectively were, the requirements of the SE CA have been met adequately.

Furthermore, the SE CA asserts, that only minimal systemic toxicity was observed in the high dose group of the EOGRTS. Besides the effects mentioned by the SE CA the following adverse and test substance related effects were observed in the high dose F0 parental animals:

- Decreased food consumption in the females during lactation (5% below placebo-control)
- Decreased body weights in the females towards the end of gestation (up to 5% below placebo-control) and during the first two weeks of lactation (up to 6% below placebo control),
- decreased body weight change in the females during several sections of premating and gestation
- Prolonged prothrombin time (HQT) in both sexes
- Increased red blood cell (RBC) counts, hemoglobin and hematocrit values in females
- Increased γ-glutamyl transferase (GGT) activities in females
- Decreased albumin levels in females
- Increased absolute (119%) and relative (120%) liver weight in females
- Slight histopathological changes in the liver: Hypertrophy, hepatocellular, centrilobular (19 females); Apoptosis/single cell necrosis (12 females); periportal vacuolation (4 females); multinucleated hepatocytes (3 females)

The DS disagrees with the position of SE CA to disregard these effects and consider them as evidence of "only minimal systemic toxicity". Particularly the liver apoptosis/single cell necrosis in 12 F0 females as one crucial step towards liver atrophy, in combination with first signs of liver cell degeneration, is considered to be a serious adverse event.
Based on these findings, systemic toxicity has been observed. Effect doses of an adequate one-generation toxicity range finding study (BASF 2017B) have been used for a sound dose setting of the main EOGRTS.

The DS does not agree with the SE CA, that the existing information is not sufficient to conclude on a MoA for toxicity or that humans are less sensitive. Furthermore, the statement of the SE CA, that this is consistent with the cited SCCS opinion is not correct, since the data on the TBBA-CoA conjugate formation as the MoA have been derived very recently, were not considered in the evaluation of the SCCS and cannot be found in the SCCS opinion.
Overall, the arguments provided by the SE CA are not considered sufficient to change the conclusions made in the CLH report on the MoA for Lysmeral induced testes toxicity in rats and the doubts about the relevance of these effects for humans. The DS agrees with

the SE CA´s comment, that human exposure is mainly dermal and that Lysmeral becomes systemically available in animals and humans via this route. Since Lysmeral is neither used in flavour applications nor in lipstick, toothpaste and mouthwash products, oral exposure is unlikely and SE CA´s conclusion, that hazard data from oral exposure routes should be considered relevant, cannot be followed.

DEVELOPMENT
The SE CA suggests a classification of Lysmeral for developmental toxicity in category 2 based on an increased incidence of post-implantation losses in addition to effects on pup body weights, skeletal variations, anogenital distance (AGD) and neonatal acetylcholine esterase inhibition.

Postimplantation loss:
A statistically significant increase in the incidence of postimplantation loss has been observed in the key study for developmental toxicity (PNDT study; BASF 2004) only, whereas this finding was either not dose dependent and/or not statistically significant in the two range finder studies (see Table 3)). Furthermore, Lysmeral did not have a consistent impact on the number of postimplantation losses in the EOGRTS, although tested in the range of the LOAEL of the PNDT.

Anogenital distance (see Table 2):
A reduced AGD in F2 (mean 2.97 mm males; 1.49 mm females) was mentioned. However, no reduction in the respective anogenital indices were observed (ratio AGD to body weight), which indicates a dependency of the AGD reduction with the reduced pup weights. The AGD reduction was at the lower limit of the historical control range (2.99-3.15 mm males; 1.48-1.60 mm females) and was reduced for both males and females, which is unlikely for a specific sex hormonal effect. AGD reduction was not observed in F1 pups, therefore, a relation to Lysmeral treatment is uncertain.

Table 2. Mean AGD and AG index data of the EOGRTS (BASF 2007).

	Dose [mg/kg bw/d]	0	1.4	4.5	15.1
Anogenital Distance	F1 males; mean [mm]	3.08	3.10	3.08	3.01
	F1 females; mean [mm]	1.48	1.48	1.47	1.47
	F2 males; mean [mm]	3.08	3.01	3.05	2.97*
	F2 females; mean [mm]	1.55	1.55	1.54	1.49*
Anogenital Index	F1 males; mean	1.62	1.62	1.63	1.67
	F1 females; mean	0.79	0.79	0.79	0.83
	F2 males; mean	1.61	1.57	1.60	1.64
	F2 females; mean	0.83	0.82	0.82	0.83

Neonatal acetylcholinesterase inhibition:
A decrease in AChE activities was observed in serum erythrocytes and diaphragm tissue in male pups at PND 4 and in females at PND 76. No changes were found in the other sex at PND 4 and PND 76. Although these results were not fully conclusive an inhibitory effect of the compound on the peripheral AChE activity in pups and adolescent rats cannot be excluded. However, no corresponding developmental neurotoxicity was observed in the respective EOGRTS cohorts. Therefore, this finding is not considered sufficient to justify SE CA´s proposal for a classification of Lysmeral for developmental toxicity.

The post-implantation loss and skeletal variations observed in the key PNDT study (BASF 2004) were always observed together with evident maternal toxicity (see Table 3) such as e.g. strongly impaired body weight gains or even losses during a certain phase of gestation (6-8 days p.c.). Reductions in pup body weights seen in the one-generation

range-finding studies and the EOGRTS also occurred in the presence of maternal systemic toxicity.

The SE CA mentioned a significant decrease in the number of delivered pups below the historical control range in the EOGRTS (BASF 2017). As presented in Table 24 of the CLH report, this finding was only found in pup numbers of the F1 generation but not confirmed in pup numbers of the F0 generation. The finding of decreased mean numbers of delivered pups in F1 animals is subsequent to the lower number of implants and not an independent finding. The 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 SE CA referred to a mild maternal toxicity consisting of decreased mean body weights and increased liver weights at doses where developmental toxicity occurred and provided a table for illustration. However, the table provided is incomplete in terms of adverse maternal effects observed. All these findings were described in Chapter 4.11.2.1 of the CLH report and the DS has updated and commented it (see Table 3).

The SE CA concludes, that a direct effect on development seems possible. However, based on the SE CA´s comments provided, the DS disagrees with this conclusion and does not consider these arguments robust enough to conclude on a classification of Lysmeral for developmental toxicity in category 2.

Table 3. Updated Table 2 of SE CA comments - Overview: maternal and developmental toxicity in the reproduction toxicity studies.

Study	mg/kg/d	Maternal Toxicity	Developmental toxicity
PNDT (BASF SE 2004)	13	DS Comment: not all maternal effects sufficiently presented in this dose group: • statistically significantly impaired mean body weight gain on days 6 - 8 p .c. (56% below ctrls.) • statistically significantly increased alanine aminotransferase (19% above ctrl.) • statistically significantly decreased serum and erythrocyte cholinesterase (17% and 9% below ctrl.) • statistically significantly increased absolute and relative liver weights (13% and 11 % above ctrls.)	DS Comment: not all developmental effects sufficiently presented in this dose group: • statistically significantly lower mean fetal body weights (8% below ctrl.) • statistically significantly increased rate of fetuses/litter with skeletal variations (delays/ minor disturbances in ossification, predominantly of vertebrae and sternebrae; supernumerary 14th ribs)
	41	***Mean bw decrease 7%*** ***Rel liver w increase 19%*** ***Gravid uterus w decrease 20%*** DS Comment: not all maternal effects sufficiently presented: • Transient salivation in 5 of 25 dams • Reduced food consumption on days 6 - 8 p .c. (18% below ctrl.) • Reduced mean body weights on days 13 - 20 p.c. (7% below ctrl. at termination) • Statistically significantly impaired mean body weight gains with **body weight loss** on days 6 - 8 p.c . and statistically significantly impaired mean body weight gain if calculated for the entire treatment phase (25% below ctrl.) • Reduced net body weight gain (32% below ctrl.) and mean gravid uterus weights (20% below ctrl.) • Increased alanine aminotransferase (29% above ctrl.) and glutamate dehydrogenase (79% above ctrl.) • Decreased serum and erythrocyte cholinesterase (43% and 16% below ctrl.) • Increased absolute and relative liver weights (11 % and 19% above ctrl.)	***Post-imp loss 15% (4% in cont.)*** ***Pups decreased mean bw 19%*** DS Comment: not all developmental effects sufficiently presented: • statistically significantly increased resorption rate (postimplantation loss 15.1%) and a lower number of live fetuses/dam (7.4 versus 8.1 in ctrls.) • statistically significantly lower mean fetal body weights (19% below ctrls. • statistically significantly increased rate of fetuses/litter with skeletal variations (delays/minor disturbances in ossification, predominantly of skull, vertebrae and sternebrae; supernumerary 14th ribs)
One-gen range finding (BASF SE 2006)	10-14	***AChE inhibition*** DS comment: not all maternal effects sufficiently presented: • Decreased serum cholinesterase (49% below ctrl.) • Increased gamma-glutamyltransferase (107% above ctrl.)	***Post-imp loss 16% (5% in cont.)*** DS comment: Increase in post-implantantion loss was not significant (16.2±30.3% vs. 5.1±9.27% in ctrls) and not dose dependent. Not all developmental effects sufficiently presented: • Decreased birth weight (19% below ctrls.) • Decreased weight at weaning (17% below ctrl.) • Decreased weight gain (16% below ctrl.)
	18-29	***Mean bw decrease 11%*** ***Liver w increase <20%*** ***AChE inhibition***	***Post-imp loss 11% (5% in cont.)*** ***Pups decreased mean bw 22%***

			DS comment: not all maternal effects sufficiently presented: • Decreased food consumption during lactation (20% below ctrls.) • Decrease of body weights (5% below ctrls.), weight gain (11 % below ctrls.) during premating • Decrease of body weights (11 % below ctrls.), weight gain (12% below ctrls.) during gestation • Decrease of body weights (10% below ctrls.), weight gain (9% below ctrls.) during lactation • Increase in gamma-glutamyltransferase (287% above ctrl.) and glutamate dehydrogenase (6% above ctrl.) • Decreased serum cholinesterase (57% below ctrl.)	Increase in post-implantantion loss was not statistically significant (11.1±10.16% vs. 5.1±9.27% in ctrls.) and not dose dependent. Further details on pup body weights: • Decreased birth weight (22% below ctrls.) • Decreased weight at weaning (21% below ctrl.) • Decreased weight gain (21% below ctrl.)	
	One-gen range finding (BASF SE 2017)	7-12	DS Comment: not all maternal effects sufficiently presented in this dose group: • Decreased food consumption during lactation (8-20% below ctrls.) • Decrease of body weight gain (12-36% below ctrls.) during premating • Decrease of body weight gain (8-30% below ctrls.) during gestation • Decrease of body weights (6-9% below ctrls.) during lactation • Decrease in hematocrit, platelets, • Increase in prothrombine time • Increase in gamma-glutamyltransferase (9 fold above ctrl.) and aspartate aminotransferase (23% above ctrl.) • Decrease in total protein, albumin, globulin, triglycerides, Calcium • Increased absolute and relative liver weights (23 % and 25% above ctrl.) + Discoloration (2/10 animals)	DS Comment: not all developmental effects sufficiently presented in this dose group: • Decreased pup birth weights (17% below ctrls.) • Decreased pup body weights (13-21% below ctrls.) at weaning • Decreased pup weight gain (13% below ctrls.) PND 1-21 • Decreased pup survival (86% versus 95% in ctrls.) PND 0-4 (not observed for PND 4-21).	
		25-35	***Mean bw decrease 6%*** ***Haematology,*** ***Rel. liver w increase 25%*** DS comment: not all maternal effects sufficiently presented: • Decreased food consumption during premating (8-14% below ctrls.) and lactation (44-48% below ctrls.) • Body weight loss during first premating week • Decrease of body weights (10-16 % below ctrls.), weight gain (32-43% below ctrls.) during gestation • Decrease of body weights (6-11% below ctrls.), weight gain (26% below ctrls.) during lactation • Decrease in hemoglobin, hematocrit,MCV, platelets, eosinophils • Increase in prothrombine time, reticulocytes • Increase in gamma-glutamyltransferase (24 fold above ctrl.) and aspartate aminotransferase (47% above ctrl.) • Increase in creatinine, total bilirubin, Cloride, Phosphate	***Post-imp loss 17% (4% in cont.)*** ***Pups delivered/dam 4 (11 in cont.)*** ***Pups decreased mean bw 21%*** DS comment: Increase in post-implantantion loss was not statistically significant (16.7 ±23.57% vs. 3.8±6.85% in ctrls). The decrease in the number of delivered pups / dam is to be attributed to lower numbers in implantation sites and affected fertility indices. Further details on developmental effects: • Decreased pup birth weights (18% below ctrls.) • Decreased pup body weights (30-32% below ctrls.) at weaning • Decreased pup weight gain (33% below ctrls.) PND 1-21 • Decreased pup survival (75% versus 95% in ctrls.) PND 0-4 (not observed for PND 4-21).	

		• Decrease in total protein, albumin, globulin, cholesterol, triglycerides, Calcium • Increased absolute and relative liver weights (19 % and 28% above ctrl.) + Discoloration (9/10 animals)	
EOGRTS (BASF SE 2017)	10	***Mean bw decrease <6%*** ***Liver w increase <20%*** ***AChE inhibition*** DS comment: AChE inhibition in serum (mean -21%, median -31%) was an isolated finding with no AChE changes in other peripheral tissues including erythrocytes and in brain sections. Not all maternal effects sufficiently presented: • Decreased food consumption during lactation (5% below ctrls.) • Decreased body weights towards end of gestation (up to 5% below below ctrls.) and during the first two weeks of lactation (up to 6% below ctrls.) • Decreased body weight change during several sections of premating and gestation • Prolonged prothrombin time • Increased red blood cell counts, hemoglobin and hematocrit • Increased γ-glutamyl transferase (4 fold above ctrl.) • Decreased albumin • Increased absolute (119%) and relative (120%) liver weight • Liver histopath (minimal to slight): hepatocellular, centrilobular hypertrophy (19/21); apoptosis/single cell necrosis (12/21); periportal vacuolation (4/21); multinucleated hepatocytes (3/21)	***F2 male+female decreased AGD (4%)*** ***AChE inhibition (up to 50%)*** ***Pups decreased mean bw 10%*** DS comment: A relation between the reduced AGD in F2 to Lysmeral treatment is uncertain. Information on AChE inhibition needs to be further specified: A decrease in AChE activities in serum erythrocytes and diaphragm tissue in male pups at PND 4 and in females at PND 76. No changes were found in the other sex at PND 4 and PND 76. No corresponding clinical signs of developmental neurotoxicity observed. Further information on pup body weights are provided: • Decreased F1 pup body weights during lactation (up to 16% below ctrls.), pup body weight gain during lactation (8% below ctrls.) • Decreased F2 pup body weights during lactation (up to 14% below ctrls.), pup body weight gain during lactation (10% below ctrls.)

RAC's response

Thank you for your comment. Your considerations and the clarifications made by the DS have been taken into account in RAC's assessment.

Date	Country	Organisation	Type of Organisation	Comment number
10.04.2018	Germany		Individual	5

Comment received

e the applicant's suggestion to classify 2-(4-tert-butylbenzyl)propionaldehyde (Lysmeral) as Repr. 2, H361f. A detailed rationale for this will be given below and in the attached file.

1. Introduction

Lysmeral has been shown to induce testicular toxicity and spermatotoxicity when administered orally to rats and at higher dose levels to dogs. Infertility in rats due to 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 Lys-meral for reproductive toxicity, with special regards to adverse effects on fertility ap-plying a weight of evidence approach analyzing the available data.

2. Summary of fertility studies

Numerous repeated dose studies on male rats, one-generation range finding stud-ies and an extended one-generation reproductive toxicity study (EOGRTS) on the compound are available. Furthermore, repeated dose studies on dogs, mice, guinea pigs, rabbits and primates were performed for the assessment of reproductive toxicity of Lysmeral.
The present report correctly concludes that these studies "provide evidence for ad-verse effects on male reproductive organs in rats after oral Lysmeral (2-(4-tert-butylbenzyl) propionaldehyde) administration. These effects were observed concom-itantly 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, and according to the findings from further re-peated 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 ob-vious testicular and spermatotoxic effects of Lysmeral, the relation between the ob-served lack of pregnancies, lack of delivered offspring and impairment of male fertili-ty 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 repro-ductive 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 tes-tes 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 po-tency 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 poten-cies 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 hepato-cytes, 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 spermatogenesis. Their synthesis depends on an intracellular process, that re-quires 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 pres-ence of liver toxicity. Other chemicals potentially transformed to benzoic acid metab-olites show a strong correlation between sustained formation of benzoyl-CoA com-plexes in hepatocytes and spermatoxic/testicular toxicity in rats.
Taken together, the comparable pattern of testicular effects, the species dependen-cies and the observed differences in potencies substantiate, that the formation of systemic TBBA is a metabolic key event for Lysmeral and TBB/TBT induced testicu-lar toxicity. Furthermore, the conjugation of TBBA with CoA represents the mode of action for Lysmeral induced testes toxicity and spermatotoxicity."
I support the authors' conclusions about the equivalence of effects of Lysmeral and the assumed active metabolite, TBBA. I am also in line with the authors that there is a clear species specificity in the observed testicular toxicity.

3. Species specificity of fertility effects

As outlined in the previous chapter, there is clear evidence about a species speci-ficity of the observed testicular toxicity. The differences are so striking to even allow to classify different investigated species in responder and non-responder species. Rats and dogs were found to be responder species, while rabbits, guinea pigs and primates turned out to be non-responders. The most relevant question now was to extrapolate these data to the human situation in order to conclude whether humans can be expected to be responders or non-responders. In order to do so, it was im-portant to evaluate toxicokinetics in different animal species and compare them to the available data in humans.

4. Toxicokinetics

Based on the available data, the provided report correctly summarizes that "quantita-tive data on the toxicokinetics of Lysmeral 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 percutane-ous absorption of Lysmeral is observed especially when compared to the rat. Distri-bution 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 de-tailed 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-tertbutyl-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 po-tency for testes toxicity correlates with systemically formed urinary TBBA levels.
On the basis of a qualitative and quantitative evaluation of metabolic profiles for dif-ferent species in an in vitro metabolism study, a predominant formation of TBBA lev-els 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 conju-gate levels in these hepatocytes indicates a competitive inhibition of other CoA de-pendent cellular processes, leading to cellular toxicity. In human hepatocytes a fun-damentally different kinetics was observed in TBBA-CoA formation, since no accu-mulation of stable conjugate levels were detectable.
Overall, species specific differences in the formation of metabolites have been clear-ly 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."
I fully endorse the conclusions of the authors of the report. The observed species specificity of testicular (and other forms of) toxicity can be fully explained by species specific toxicokinetics. There is also enough data available to conclude that human toxicokinetics resemble those of non-responder species, clearly showing that hu-mans are expected to be non-responders too.
5. Proposed mechanism of action

Taking together the above outlined data, the following mechanism of action can be derived and is highly plausible:
The observed testicular toxicity is not a direct effect of the compound under consid-eration, Lysmeral. It could be shown that formation of TBBA is responsible for induc-ing the observed testicular toxicity, since TBBA administered itself leads to the same effects on testes. It could also be shown that, if TBBA conjugates to CoA, this com-plex will be more stable and less subject to metabolism/excretion, thus increasing systemically bioavailable concentrations of TBBA. These processes could be con-firmed to take place in studies in rats, the most sensitive species for testicular (and other forms of) toxicity. In contrast to this, non-responder species (mice, guinea pigs, primates) show a significantly lower production of TBBA, thus preventing the for-mation of high enough systemic levels to induce testicular toxicity, for which a clear threshold was shown. All available human data clearly show that human belongs to non-responder species. Not only does this proposed mechanism of action plausibly underline the sequence of events needed to exert the observed testicular effects, it also shows that rat data cannot be simply extrapolated to the human situation due to fundamental species differences and that in this particular case only non-responder species data are relevant for the human situation.

6. Exposure routes

Though CLP is exclusively based on the intrinsic hazard of a compound under con-sideration, the exposure hazard needs to be taken into account. In the human situa-tion skin absorption will be the main route of exposure. In animal studies it could be shown that Lysmeral is highly bioavailable via the oral route, while bioavailability after dermal exposure is much lower. In the most responsible responder species, rat, a clear threshold level of 50 mg/kg for inducing testicular (and other) toxicity was found with a NOAEL of 25 mg/kg after oral treatment. 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 ad-verse testicular effects were observed. When compared to doses leading to rat tes-ticular toxicity, a prolonged human uptake of Lysmeral doses inducing systemic tox-icity (testes toxicity or spermatotoxic effects) is highly unlikely. In humans, dermal penetration is even lower than in rats: while in rats a dermal absorption rate of ap-prox. 19 % was found, the maximum absorption in humans was determined at 7 %.
Taking this altogether, assuming the most relevant, dermal, route of exposure in humans, the formation of systemically available levels above the threshold for in-duction of testicular effects can be excluded.

7. Developmental toxicity

An OECD 414 study was performed in rats. The present report correctly summarizes the findings of this study: "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 aminotransfer-ase and glutamate dehydrogenase, decreases serum cholinesterase levels and or-gan 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 loss-es 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 his-torical 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 de-lays and minor disturbances in ossification processes of the skull, sternebrae and pubic girdle. Supernumerary (14th) ribs were found in control and dosed animals at high incidences, and structural variations like a supernumerary thoracic vertebra (14th) or a misshapen sacral vertebra (1st 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 evi-dent 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. 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 poten-tial.

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 nonspecific 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 range finder 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 coin-cided 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 ani-mals. 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 gen-eral 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 immu-notoxicity were not affected by treatment with Lysmeral.
Taken together, developmental toxicity has been observed at doses leading to evi-dent maternal toxicity and is considered to be a secondary non-specific conse-quence of general systemic toxicity in the dams. Therefore, these findings do not warrant a classification with respect to developmental toxicity."
I fully agree with the assessment of these data by the authors of the report and en-dorse their conclusion that the available data do not warrant a classification with respect to developmental toxicity.

8. Summary and conclusions

Based on these data and applying weight of evidence in interpreting them, I con-clude that the observed testicular toxicity is a species-specific phenomenon with very limited relevance for humans, as shown by the following facts:
1. Testicular toxicity in animal studies is only observed at dose levels causing other forms of toxicity, e.g. hepatotoxicity.
2. There are striking inter-species differences in terms of testicular toxicity, with the rat being the most sensitive species.
3. Mechanistic studies clearly indicate that testicular toxicity is highly correlated with the formation of para-tert-butylbenzoic acid (TBBA), representing a key me-tabolite for Lysmeral induced testicular toxicity.
4. It could be shown that direct TBBA administration leads to the same testicular ef-fects as observed for Lysmeral, thus underlining that TBBA is the relevant metabo-lite.
5. There is good evidence to show that specifically formation of TBBA-CoA is critical in terms of testicular toxicity.
6. Both in vivo and in vitro studies on toxicokinetics show that the rat is the species producing the highest levels of both TBBA and TBBA-CoA.
7. This strongly supports the hypothesis that the formation of these two metabolites is a key event for the induction of testicular toxicity and can explain the species specificity of

the observed effect, allowing to distinguish between responder and non-responder species.
8. Studies on testicular toxicity showed that rats, and to a lesser degree, dogs were responder species, while mice, guinea pigs, Rhesus monkeys and rabbits belonged to the non-responder species.
9. Even in the most responsible responder species, rat, a clear threshold level of 50 mg/kg for inducing testicular (and other) toxicity was found with a NOAEL of 25 mg/kg.
10. Based on toxicokinetics, it could be shown that the human metabolism is clos-est to the rabbit situation and, consequently, the formation of TBBA or TBBA-CoA levels above the threshold to induce testicular toxicity in humans is most unlike-ly.
11. Therefore, the assumption is well justified that humans belong to non-responder species.
12. The most likely human route of exposure is skin absorption, a route that only induced testicular toxicity even in the most sensitive species, rat, in doses higher than the limit dose of 1000 mg/kg even in the most sensitive species, rat.
13. Dermal absorption in humans is significantly lower than in rats. Consequent-ly, the formation of systemic levels capable to induce testicular toxicity in humans is most unlikely.
14. In summary, the available data form an excellent pattern which, based on the mode of action and underlying toxicokinetics, explains the observed species-specific effects regarding testicular toxicity and plausibly shows that humans belong to the non-responder species.
15. The available data strongly support the conclusion that Lysmeral does not have "an intrinsic property to produce an adverse effect on reproduction" in humans.
16. Consequently, the proposal to classify Lysmeral in Category 2 for fertility ef-fects is scientifically well justified and no higher classification is warranted.
17. Sporadic effects in fetuses and pups are considered variations and develop-mental retardations observed only in the presence of evident maternal toxicity and do not warrant any classification for developmental toxicity.

ECHA note – An attachment was submitted with the comment above. Refer to public attachment LysmeralJB.pdf

Dossier Submitter's Response
Agreed.
RAC's response
Thank you. Your comment has been noted.

Date	Country	Organisation	Type of Organisation	Comment number
13.04.2018	Denmark		MemberState	6
Comment received				
Please see comments attached ECHA note – An attachment was submitted with the comment above. Refer to public attachment CLH Lysmeral DK comments.docx				
Dossier Submitter's Response				
The DS agrees with the DK CA, that serious effects on testes and sperm were consistently reported in repeated dose studies by oral gavage of varying duration in rats. However, studies in other rodents and the dermal application to rats (at/below the limit dose) did not lead to these effects.				

The DK CA mentioned a low purity of the micro-encapsulated test material in the EOGRTS (BASF 2017), raising doubts concerning the results, as the doses chosen appear to be very low. This argument cannot be confirmed by the DS. The analytically verified fraction of the active ingredient Lysmeral in the test material was 17.7%, which is due to the encapsulation material used to ensure full palatability and stability of the test substance in feed. The alginate/glycerin capsules contained a nucleus with Lysmeral (30% in refined sunflower oil). The nominal and actually ingested doses presented in the CLH report always refer to the active ingredient Lysmeral. The dose chosen for the EOGRTS were defined by a suitable range finding study (BASF 2017B), and does not raise doubts concerning the results, as indicated by the DK CA.

It appears, that the submitted document of the DK CA is truncated since an incomplete sentence has been found in the second paragraph:
"Studies in other species lack information on"
Therefore, the DS is unable to respond to the mentioned lack of information in studies with other species.

The DK CA mentioned species differences (quantitative rather than qualitative) in toxicokinetics to refute human relevance. According to DK CA, an *in vitro* study in different species is used to support this line of argument, whereas the exclusivity of the proposed mode of action is not proved. As outlined in Comment Nr. 4, two different aspects should be assessed separately to fully address the human relevance:

- The comparative in vitro metabolism study identified toxicokinetic differences in TBBA formation between rats and humans (BASF SE 2010). Based on these results, the endogenous TBBA levels formed in human cells are lower than in rats and comparable to rabbits (non-responder species).
- Mechanistic studies in hepatocytes identified the underlying mode of action (stable formation of TBBA-CoA conjugates), which does not occur in human cells (Givaudan 2017; Laue et al. 2017).

These two lines of argumentation depend on several in vitro studies and the postulated MoA was supported by evidences from in vivo studies as well. Since a formation of stable and sustained levels of TBBA-CoA conjugates were found in rat but not in human hepatocytes, the lack of a qualitative differences between these two species cannot be confirmed.

Overall the DS disagrees with the DK CA, that Lysmeral should be classified as reproductive toxicant Cat 1B, H360, since the arguments provided do not support this proposal.

RAC's response

Thank you for your comment. Your considerations and the clarifications made by the DS have been taken into account in RAC's assessment.

Date	Country	Organisation	Type of Organisation	Comment number
04.04.2018	France		MemberState	7

Comment received

Fertility

Section 4.11

Could you please explain the very low purity of tested substance reported for the one

generation studies and the EOGRTS? Is it rather corresponding to the concentration of the tested substance in the capsule? It should be noted that microencapsulation is often used to limit the toxicity of a substance. Therefore, it is not surprising that studies performed with microencapsulated lysmeral did not show the same level of effects in testis.

Consistent testicular effects with spermatotoxicity have been reported in repeated-dose toxicity studies in rats. Among the reprotoxicity studies, similar effects were observed in the one-generation study (BASF, 2006C). In this study, the effects on testis and sperm parameters occurred from 62 mg/kg bw/day, dose not associated with significant general systemic effects (clinical changes and increased liver and kidney weights that may be considered as adaptive effects). Effects on male reproduction lead to unfertility with clear decrease of mating and fertility index from 62 mg/kg bw/day. Effects on sperm, testis and fertility indices were also observed in the recent one generation range finding study at about 25 mg/kg bw/day (BASF, 2017C).

In contrast, the EOGRTS was performed with very low doses (up to about 10-15 mg/kg bw/day). FR considers that the lower doses chosen compared to the one-generation range finding study are not sufficiently justified. Considering the LOAEL for testicular toxicity from repeated-dose toxicity studies (about 50 mg/kg bw/day), from the older one-generation range finding study (about 62 mg/kg bw/day) and from the recent one-generation range finding study (about 25 mg/kg bw/day), the lack of reproductive effects in the EOGRTS should not be used to discard the reproductive toxicity of lysmeral in rats.

Page 61:
It is stated that the LOAEL for testicular toxicity is > 25 mg/kg bw/day for lysmeral. However, effects on sperm, testis and fertility indices were already observed at this dose in the recent one generation range finding study.

Discussions on fertility:
All the argumentation to decrease the relevance of the effects for humans is based on the formation of TBBA. Even if it is noted that TBBA has a similar toxicity on testis and sperm as lysmeral, it is difficult to conclude firmly that the testicular effects of lysmeral are solely attributed to TBBA. Based on the overall dataset, it cannot be excluded a direct toxicity linked to the parent molecule or to another metabolite. For example, Lysmeric acid (representing about 74.4-88.1% of lysmeral in humans [page 25]) also induces similar testicular effects as reported in page 33.

Moreover, it is stated that TBBA levels formed in human hepatocytes were comparable to those in rabbits. However, when we look at Table 15, TBBA levels in hepatocytes are higher in humans (up to 7.5%) than in rabbits (up to 2%). In addition, low levels of TBBA, as urinary metabolite, are reported for dogs (Table 14; 3% versus 11% in rats) which showed testicular effects at 200 mg/kg bw/day.

Therefore, FR considers that a classification for fertility is required. FR thinks that there are still uncertainties regarding the mode of action proposed since no other modes of action have been investigated. In addition, it should be noted that there is no data presented to confirm that TBBA is formed in humans at concentrations that do not lead to testicular toxicity. Thus, FR considers that the argumentations set in the CLH report, solely based on one specific mode of action linked to the formation of one particular metabolite, should be more deeply discussed at the RAC level to conclude on the most appropriate category for classification as toxic for fertility.

Developmental toxicity

Developmental effects such as foetal lethality (as characterized by a decrease in the mean number of delivered pups per dams in the fertility studies and post-implantation losses in the prenatal developmental toxicity studies) and significant reduction in birth weight, not reversible at weaning, were consistently found. These effects occurred at doses leading to maternal toxicity. Considering the severity and the non-reversibility of the developmental effects, FR considers that comparison to CLP criteria for developmental toxicity should be more described in order to conclude if a classification is justified or not.

Dossier Submitter's Response

Fertility

The FR CA asked for further explanations concerning the low purity of the test substance. As outlined in Comment Nr. 6, the low purity/content of the test material is due to the encapsulation material used. The alginate/glycerin capsules contained a nucleus with Lysmeral (30% in refined sunflower oil). The concentration of Lysmeral in the test substance and in feed was analytically verified and the nominal and actually ingested doses presented in the CLH report always refer to the active ingredient Lysmeral.

The intention to encapsulate Lysmeral was to ensure full palatability and stability of the test substance in feed but not to limit its toxicity, as mentioned by the FR CA. When comparing the effect levels for testes and sperm toxicity (LOAEL = 25.1-27.5 mg/kg bw/d) in the one-generation range-finding study with encapsulated Lysmeral (BASF 2017B) with the subchronic repeated dose toxicity study in rats with gavaged Lysmeral (LOAEL = 50 mg/kg bw/d; Givaudan 1986), the assumption made by the FR CA cannot be confirmed at least for Lysmeral.

The FR CA mentioned testes and sperm effects at a dose, not associated with significant general systemic effects (clinical changes and increased liver and kidney weights that may be considered as adaptive effects). The DS does not consider the findings observed to be adaptive effects, since next to impaired body weight development, clinical chemistry parameters (i.e. increased glutamate-dehydrogenase and gamma-glutamyltransferase levels) indicated adverse liver effects, however, histopathological examinations of this organ have not been performed to further confirm adversity (see Table 3 of Comment Nr.4).

The FR CA considers, that the doses chosen in the EOGRTS are not sufficiently justified when compared to the one-generation range finding study. As outlined in Comment Nr.4, the LOAEL for male reproductive effects in the relevant one-generation range-finding study was set at 2300 ppm (25.1-27.5 mg Lysmeral/kg bw/d in males) for testes and sperm effects observed (BASF 2017B). Female animals of this dose group received 21-34.7 mg Lysmeral/kg bw/d and also showed clear adverse effects on food consumption, body weight development, liver associated parameters (organ weight, discoloration and clinicochemical parameters) and hematological effects (see Table 3 of Comment Nr.4). In the EOGRTS, the overall mean dose of analytically verified Lysmeral administered to male and female rats throughout all study phases and across all cohorts was 15.1 mg/kg bw/d and the range of the mean Lysmeral intake accounted to 11.2-18.8 mg/kg bw/d (females) and 10.2-19.2 mg/kg bw/d (males) during different phases of the study and cohorts used. Given the variability of the actual dose ingested (mean doses up to 19 mg/kg bw/d) and the male testes effect levels observed in the associated one-generation range finding study (25.1-27.5 mg/kg bw/d), the use of 750 ppm as a high dose for the EOGRTS is considered adequate to ensure sufficient numbers of offspring to comply with the requirements of the OECD TG 443 and the final decision of the SE CA on the substance evaluation under CoRAP. Furthermore,

systemic toxicity has been observed in the high dose group of the EOGRTS. Particularly the liver apoptosis/single cell necrosis in 12 F0 females as one crucial step towards liver atrophy, in combination with first signs of liver cell degeneration, is considered to be a serious adverse event.

The FR CA noted, that the lack of reproductive effects in the EOGRTS should not be used to discard the reproductive toxicity of Lysmeral in rats. The DS did not intend to discard any reproductive toxicity of Lysmeral in rats via the EOGRTS. There is evidence for animal species specific adverse reproductive toxicity (i.e. testes and sperm effects) to classify Lysmeral for reproductive toxicity, i.e. adverse effects on fertility (Cat. 2), as outlined in the CLH report. The required EOGRTS was an outcome of the substance evaluation under CoRAP.

The FR CA mentioned effects on sperm, testis and fertility indices observed in the 2300 ppm dose group of the recent one-generation range-finding study (BASF 2017B). The mean test substance intake in the premating phase for males of this dose group was 27.5 mg Lysmeral/kg bw/d. Furthermore, several oral (gavage) subacute and a subchronic 90 day repeated dose toxicity study in rats as well as the one-generation range finding study using Lysmeral in gelatine capsules identified the NOAEL for male reproductive effects at 25 mg Lysmeral/kg bw/d (see Table 19 in the CLH report). Therefore the male reproductive LOAEL is set above the NOAEL of 25 mg Lysmeral/kg bw/d, although the present data indicate an effect level already at 27.5 mg Lysmeral/kg bw/d.

Discussions on fertility:
The FR CA noted, that all arguments provided to lower the human relevance of male reproductive effects of Lysmeral is based on the formation of TBBA. As outlined in Comment Nr.4, the lack of human relevance was not only based on the species dependent toxicokinetic differences in the formation of the metabolite TBBA, but also on the underlying mode of action, i.e. stable and sustained formation of TBBA-CoA conjugates, which does not occur in human cells.

The pivotal role of TBBA for Lysmeral induced testes toxicity and sperm effects has been demonstrated by the findings from studies with Lysmeral, TBBA, TBB and TBT (see Table 19, 31, 32 and Chapter 4.1.1 of the CLH report):

- All these substances show a very comparable testes toxicity profile when administered to rats.
- Lysmeral, TBB, TBT share TBBA as a common metabolite.
- TBBA shows highest potency of testes toxicity, i.e. lowest LOAEL, for testes toxicity
- The potency of testes toxicity correlates with the amount of TBBA endogenously formed.

The FR CA further stated, that a direct toxicity linked to the parent molecule or to another metabolite such as Lysmerylic acid. A direct testicular toxicity of the parent compound seems unlikely, since significant systemic occurrence of Lysmeral is not to be expected. Lysmeral plasma levels could not be detected after oral application (BASF SE 2006A and 2006B) and was nearly completely metabolized in the comparative in vitro metabolism study (BASF SE 2010). It is evident, that Lysmerylic acid induces testicular toxicity, as this metabolite represents an intermediate step between the parent compound and TBBA. As presented on page 27 of the CLH report, the direct CoA conjugate of Lysmerylic acid was only transiently formed at low levels within 0.5-4 hours and not detectable after 22 hours incubation with Lysmeral in the mechanistic studies in rat hepatocytes (Givaudan 2017). Given the mode of action identified, i.e. stable TBBA-CoA conjugate formation, the direct action of the metabolite Lysmerylic acid is unlikely.

The FR CA referred to Table 15 of the CLH report and found, that TBBA levels in hepatocytes are higher in humans (up to 7.5%) than in rabbits (up to 2%). The TBBA fraction of 7.5% in human hepatocytes was only found after incubation of 10 µM, which was identify to relate to plasma levels observed after oral administration of no adverse testicular effect levels of Lysmeral. In contrast, incubations with 50-100 µM Lysmeral covered plasma levels obtained after doses exerting testicular toxicity (see page 23 of the CLH report). At these concentrations, TBBA levels were comparable between the human and rabbit setup (2%/1.3% in rabbits versus 3.1%/1.9% in humans after incubation with 50 µM/100 µM, respectively). Concerning the differences in urinary TBBA levels in rats and dogs, no comparative in vitro study with dog hepatocytes is available to set the differences in urinary TBBA concentrations in vivo into perspective. Such a study would allow to better compare concentrations in dog hepatocyte supernatants with other species, since these are seen as directly proportional to given plasma concentrations in vivo.

Developmental toxicity.
The FR CA identified developmental effects such as foetal lethality (decrease in the mean number of delivered pups per dams in fertility studies and postimplantation losses in the key developmental toxicity study), non-reversible reduction in birth weights, and asked for further description of the comparison of the developmental effects to CLP criteria due to the severity and the non-reversibility of the developmental effects.

The DS agrees, that a significant increase in postimplantation loss was identified in the developmental toxicity study (BASF 2004), but would like to add further information on cited findings concerning the decreased numbers of delivered pups / dam in the one-generation range finding studies:

- Only a slight decrease in the mean number of delivered pups per dam (7.9±2.23 in dose group 800 ppm versus 9.4±3.95 in ctrls.) was found in the older range finding study without effects on gestation and live birth indices in dose groups with any offspring (BASF 2006).
- A decrease in the mean number of delivered pups per dam (4.0±3.16 in high dose dams versus 11.1±1.91 in ctrls.) was observed in the recent range finding study (BASF 2017B). This is a consequence of a reduction of the mean implantation sites and the affected fertility indices, and a relation to the affected male fertility/spermatotoxicity is very likely. The increase in mean implantation losses observed was slight and not statistically significant.
- In the EOGRTS (BASF 2017), a decrease in the number of delivered pups per dam was only found for the F1 generation (10.1 ± 2.19 in high dose dams versus 12.0 ± 2.06 in ctrls.) but not confirmed in the F0 generation (10.3 ± 1.74 in high dose dams versus 10.1 ± 3.4 in ctrls.). The finding of decreased mean numbers of delivered pups in F1 animals is associated with the lower number of implants and not an independent finding. The decrease in the mean number of implantation sites (10.5 implants/dam) was within the historical control range (9.4-13.9 implants/dam) and was not observed in F0 animals.

The CLP criteria define, that:

"Substances are classified in Category 2 for reproductive toxicity 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. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification. Such effects shall have been observed 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 the other toxic effects."

In addition, the CLP further defines, that:

"…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."

"Based on pragmatic observation, maternal toxicity may, depending on severity, influence development via non-specific secondary mechanisms, producing effects such as depressed foetal weight, retarded ossification, and possibly resorptions and certain malformations in some strains of certain species. However, the limited number of studies which have investigated the relationship between developmental effects and general maternal toxicity have failed to demonstrate a consistent, reproducible relationship across species. 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. Moreover, classification shall be considered where there is a significant toxic effect in the offspring, e.g. irreversible effects such as structural malformations, embryo/foetal lethality, significant post-natal functional deficiencies."

The present key developmental toxicity study for Lysmeral identified increased numbers of fetuses with common skeletal variations, fetal growth retardations and increased early resorption rates, but not Lysmeral related teratogenicity/ structural malformations were observed. The findings of the one-generation rangefinder studies showed slight, non-significant and/or dose independent increases in postimplantation losses, a slight reduction in the number of delivered pups at doses not affecting fertility indices and a reduction in birth/pup weights. Data of the EOGRTS also showed pup body weight reductions associated with adverse maternal liver and general systemic effects. In this study, Lysmeral did not have a consistent impact on the number of postimplantation losses, delivered pups and pup survival. Further developmental toxicity endpoints including developmental neurotoxicity and immunotoxicity were not affected by treatment with Lysmeral excluding significant postnatal functional deficiencies.

Developmental effects observed in the key developmental toxicity study coincided with significant maternal toxicity at the same dose level, and are considered manifestations of the non–specific maternal stress induced by Lysmeral administration (see Table 3 of Comment Nr. 4). The reduction in the number of delivered pups at doses not affecting fertility indices are slight and always coincided with adverse systemic effects to the dams. In line, the reduction in birth weights and pup weights at weaning occurred and relate to adverse systemic effects to the dams.

The DS concludes, that 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 a classification with respect to developmental toxicity is not justified.

RAC's response

Thank you for your comment. Your considerations and the clarifications made by the DS have been taken into account in RAC's assessment.

Date	Country	Organisation	Type of Organisation	Comment number
13.04.2018	Belgium		MemberState	8

Comment received

Fertility
Lysmeral has been identified to induce undoubtedly testicular toxicity when administrated orally to rats from 50 mg/kg bw/day and at higher dose levels to dogs.
The observations in rat after repeated exposure include atrophy of testes, degeneration and loss of seminiferous epithelium and spermatotoxicity (decrease in sperm motility, in testes spermatid count and in cauda epididymal sperm count ; affected sperm morphology) from 50 mg/kg bw/day (cf Table 19, p. 45 of the CLH dossier). Effects are observed after short-term and long-term exposure. Infertility in rats due adverse effects of orally administrated lysmeral on the male reproductive system has been confirmed in feeding one-generation range-finding studies, resulting in lack of pregnancies, lack of delivered offsprings and impairment of male fertility
Findings in dog are reported at higher doses (200 mg/kg bw/day) include decrease in size and weight of testis, massive diffuse degeneration of seminiferous tubules, slight hyperplasia of Leydig cells. Spermatotoxicity findings in dog include aspermia, decrease in sperm motility and alteration in and morphological altered spermatozoa.
Repeated toxicity studies are also available in mouse, guinea pig, rhesus monkey and rabbit but demonstrated no observed effects after short-term exposure. However, considering the low applied dose, BE CA is of the opinion that these species should not be concluded as not sensitive to lysmeral induced testicular toxicity and that potential testicular effects at higher dose cannot be excluded.
- Mode of action
The formation of TBBA metabolite has been clearly demonstrated to be correlated with testicular toxicity. Moreover, TBBA is already classified as Repr. 1B (H360F). However, no justification is given on the absence of testicular toxicity of the parent compound or the other metabolites in the CLH report.
A correlation has been further established between the formation of TBBA-CoA conjugates in rat hepatocytes, disruption of lipid synthesis and testicular toxicity. Nevertheless, other chemicals with a para-substituent at the benzyl ring accumulate alkyl-benzoyl-CoA conjugates in a similar pattern as TBBA. These chemicals include among others lysmeral, lysmerylic acid, lysmerol. All chemicals with this metabolic outcome were reported to cause testicular and spermatotoxic effects in the rat, as stated on p. 62 of the CLH report. Therefore, BE CA believes that TBBA should not be considered as the only cause of testicular toxicity.
In conclusion, although species species differences in the metabolic rates of lysmeral have been identified, BE CA is of the opinion that the findings in rat should be considered as relevant to human. BE CA is of the opinion that a Repr 1B (H360F) classification for fertility effects of lysmeral is warranted.

Dossier Submitter's Response

Fertility
The BE CA identified, that Lysmeral induces testicular toxicity when administrated orally to rats at doses starting at 50 mg/kg bw/day. However, based on the administration strategy used (gavage vs encapsulation and choice of capsule material), effect levels for testicular toxicity in rats are below 50 mg/kg bw/day and only slightly above the NOAEL of 25 mg/kg bw/d (see effect levels found in the recent one-generation range-finding study (BASF 2017B) and Comment Nr.7).
The BE CA mentioned a slight hyperplasia of Leydig cells after Lysmeral treatment in dogs. The DS would like to add, that this finding was only observed in 1 of 4 dogs in the mid dose (200 mg/kg bw/d) of one study (BASF 2008A) and was not confirmed in the

follow-up study with 10 male animals at the same dose (BASF 2008B). Therefore, the relation of this finding to Lysmeral treatment is questionable.

The BE CA considered the doses used in the studies with the mouse, guinea pig, rhesus monkey and rabbit as low and these species should not be concluded as not sensitive to Lysmeral induced testicular toxicity, since potential testicular effects at higher doses cannot be excluded. The DS acknowledges, that the studies in mice, guinea-pigs and rhesus monkeys were performed at single doses only. These studies have been designed to allow a comparison with the subacute rat studies, aiming for the assessment of male reproductive organ toxicity. Since the rat testes toxicity LOAEL has been identified to be above but close to the NOAEL of 25 mg/kg bw/d, the dose chosen (100 mg/kg bw/d) is considered adequate to relate it to the responder-species rat. Based on the data in dogs, the testicular toxicity effect level has been confirmed at 200 mg/kg bw/d but could also be in the range >44.6 – 200 mg/kg bw/d (see Table 19 of the CLH report). However, the study performed in the rabbit, covered 3 doses up to 300 mg/kg bw/d and did not show testicular toxicity at doses, that were confirmed in dogs. Overall, a species dependent sensitivity for Lysmeral induced testicular toxicity can be postulated, the rat is the most susceptible species and the present data do not give any indication of testicular toxicity in other rodents (mice, guinea pigs). For non-rodent species, data in the rabbit do not indicate Lysmeral induced testicular toxicity at doses effective in dogs and the limited study in rhesus monkeys did not show testicular toxicity, at doses causing testicular toxicity in the rats.

Mode of action
The BE CA asked for a justification on the absence of testicular toxicity of the parent compound or other metabolites in the CLH report. As outlined in Comment Nr.7, a direct testicular toxicity of the parent compound seems unlikely, since significant systemic occurrence of Lysmeral is not to be expected. Lysmeral plasma levels could not be detected after oral application (BASF SE 2006A and 2006B) and was nearly completely metabolized in the comparative in vitro metabolism study (BASF SE 2010). The quantitatively main metabolite Lysmerylic acid represents an intermediate step between the parent compound and TBBA. The direct CoA conjugate of Lysmerylic acid was only transiently formed at low levels within 0.5-4 hours and not detectable after 22 hours incubation with Lysmeral in the mechanistic studies in rat hepatocytes (Givaudan 2017). Given the mode of action identified, i.e. stable TBBA-CoA conjugate formation, the direct action of the metabolite Lysmerylic acid is therefore unlikely.
However, the pivotal role of TBBA for Lysmeral induced testes toxicity and sperm effects has been demonstrated by the findings from studies with Lysmeral, TBBA, TBB and TBT:

- All these substances show a very comparable testes toxicity profile when administered to rats.
- Lysmeral, TBB, TBT share TBBA as a common metabolite.
- TBBA shows highest potency of testes toxicity, i.e. lowest LOAEL, for testes toxicity
- The potency of testes toxicity correlates with the amount of TBBA endogenously formed.

The BE CA mentioned, that TBBA should not be considered as the only cause of testicular toxicity. It is argued, that chemicals with a para-substituent at the benzyl ring accumulate alkyl-benzoyl-CoA conjugates in a similar pattern as TBBA and cause testicular and spermatotoxic effects in the rat. Lysmerylic acid and Lysmerol were explicitly mentioned. The DS would like to clarify, that Lysmerylic acid and Lysmerol are identified metabolites of Lysmeral. Both substances have the ability to be further metabolized to TBBA. As outlined in Table 25 of the CLH report, the benzoyl-CoA conjugates (defined in Figure 2 of the CLH report) measured refer to the conjugate formed from the test chemical specific benzoic acid metabolite and CoA. In the case of Lysmerylic acid and Lysmerol, the

respective benzoic acid metabolite is TBBA. This also applies to TBT and BHCA, whereas PMHCA, PHCA, iBMHCA, and p-isopropyl benzoic acid would be metabolized to benzoic acid metabolites, which do not represent Lysmeral specific metabolites. Therefore, BE CA´s argument, that TBBA should not be considered as the only cause of testicular toxicity based on the other chemicals with a para-substituent at the benzyl ring, cannot be followed.
Due to the strong correlation between the formation of TBBA-CoA conjugates, disruption of lipid synthesis and testicular toxicity in the rat and the argumentation given above, the involvement of other metabolites in Lysmeral induced testes and sperm toxicity is unlikely.

The BE CA acknowledged species differences in the metabolic rates of Lysmeral but considers findings in rats as relevant to humans, justifying a Repr 1B (H360F) classification. The DS disagrees with this consideration, since next to the metabolic rate differences (i.e. low formation of endogenous TBBA levels), the absence of the underlying mode of action (stable formation of TBBA CoA conjugates) in humans need to be considered.

RAC's response

Thank you for your comment. Your considerations and the clarifications made by the DS have been taken into account in RAC's assessment.

Date	Country	Organisation	Type of Organisation	Comment number
12.04.2018	Belgium	Procter & Gamble	Company-Downstream user	9

Comment received

A thorough review of all the available data related to reproductive toxicity of LYSMERAL is more than sufficient for a robust hazard assessment of this substance. Under current hazard classification guidelines and although the data do not indicate a human health risk under the current conditions of use, we consider it appropriate to classify Lysmeral as CMR2 (Repro Cat 2, H361f: Suspected of damaging fertility). See attached document for detailed comments.

ECHA note – An attachment was submitted with the comment above. Refer to public attachment Lysmeral - supporting arguments from PG_CMR2_FINAL_ Apr 2018.docx

Dossier Submitter's Response

agreed

RAC's response

Thank you. Your comment has been noted.

Date	Country	Organisation	Type of Organisation	Comment number
13.04.2018	Germany	COTY	Company-Downstream user	10

Comment received

COTY's views on the harmonized classification and labelling of 2-(4-tert-butylbenzyl) propionaldehyde (EC Nr. 201-289-8; CAS Nr. 80-54-6, " BMHCA")

BMHCA is used as fragrance ingredient in cosmetic products, including but not limited to fine fragrances, shampoos and toilet soaps. It does not occur in nature and is not used as flavoring agent. The main route of human exposure is dermal absorption.

The newly submitted data to ECHA support the safety of BMHCA as used in cosmetic products, and additional studies are in progress to reaffirm some of the findings.

Our expert toxicologists have reviewed the updated CLH dossier and support the hazard classification of Repro. Cat. 2 of BMHCA.

Dossier Submitter's Response
agreed
RAC's response
Thank you. Your comment has been noted.

Date	Country	Organisation	Type of Organisation	Comment number
21.03.2018	Netherlands		MemberState	11

Comment received

Fertility
It is proposed to classify in Cat. 2 rather than Cat. 1B, mainly because rats form higher levels of TBBA and TBBA-CoA conjugate than other species, including humans. However, the evidence of the absence of effects on the testes in mice, guinea pigs, rhesus monkeys and rabbits is based on tests with limited duration, dose level and possibly limited general toxicity (not always stated). Please provide information on the general toxicity in these studies and whether these studies were performed at the maximum tolerable dose level.
The 90-day studies with dogs did report testis effects, albeit at higher doses than in rats, which indicates that the difference between species is quantitative rather than qualitative. Moreover, the in vitro metabolism study (BASF SE 2010) showed that human hepatocytes formed TBBA at higher levels than mice or rabbit hepatocytes and about a factor four below the levels in rat hepatocytes. An in vivo study even showed formation of TBBA in a rhesus monkey on the same level as in rats.
Considering these factors and the severe effects on fertility observed in both one-generation range finding studies and the repeated dose studies, Cat. 1B may be warranted.

Development
In both one generation range finding studies and in the PNDT, an increase was found in post-implantation loss at doses in the range of ~15-40 mg/kg bw/d, which is a developmental effect. Please clarify why this effect is considered secondary to the maternal toxicity.
Could you provide the individual data of both maternal and developmental effects in the PNDT study? This may clarify whether the foetal toxicity was related to maternal toxicity or not.

Please include a calculation of the ED10 showing whether a SCL is required or not.

Dossier Submitter's Response

The NL CA asked for information on the general toxicity on the studies in mice, guinea pigs, rhesus monkeys and rabbits, and if these were performed at the maximum tolerable dose level. Furthermore, these studies were identified to have a limited duration, dose level and possibly limited general toxicity. The DS would like to clarify, that these studies were not dosed up to the maximum tolerable dose levels and they lack parameters to get a full overview on further systemic/organ toxicity according to current OECD guidelines for repeated dose toxicity studies. However, these studies have been designed to

compare with the subacute rat studies, aiming for the assessment of the male reproductive system as the relevant target organ (see also Comment Nr.8). According to the data derived from rats, the exposure duration and doses chosen were adequate to potentially affect sperm and testes. The rat testes toxicity LOAEL has been identified to be above but close to the NOAEL of 25 mg/kg bw/d, and single oral exposure was sufficient to initiate testicular toxicity. The dosing regimen used in the other rodent species mouse and guinea pig (daily oral gavage application of 100 mg/kg bw/d for 5 consecutive days) is considered adequate to relate it to the responder-species rat. Rabbits were treated for 15 consecutive days with oral gavage doses up to 300 mg/kg bw/d and did not show testicular toxicity at doses, that were confirmed to be effective in dogs. The limited study in rhesus monkeys (dosed at 100 mg/kg bw/d for 5 consecutive days) did not show testicular toxicity, at doses causing clear testicular toxicity in rats. All test substance related adverse systemic effects observed were reported in Chapter 4.7.1.1. of the CLH report and further details on the conduct of the studies can be found in the disseminated REACh dossier (https://echa.europa.eu/de/registration-dossier/-/registered-dossier/13572/7/9/2).

The NL CA referred to studies with dogs, which indicate, that the difference between species is quantitative rather than qualitative. The DS agrees, that quantitative differences in species exist in terms of the capacities for endogenous TBBA formation. However, the absence of the underlying mode of action (stable formation of TBBA-CoA conjugates) in humans appears to be a qualitative difference to rats. Unfortunately, the absence of this mode of action in other non-responder species has not been assessed in the studies currently available.

The NL CA mentioned, that human hepatocytes formed TBBA at higher levels than mice or rabbit hepatocytes and that formation of urinary TBBA levels in 1 of 2 rhesus monkeys was on the same level as in rats. The DS agrees, that the TBBA levels formed in human hepatocytes was higher than in murine cells. However, TBBA levels were comparable between the human and rabbit setup at Lysmeral concentrations, that relate to plasma levels affecting the testis in vivo (2%/1.3% in rabbits versus 3.1%/1.9% in humans after incubation with 50 µM/100 µM, respectively). As discussed in Comment Nr.7, a higher TBBA fraction in human hepatocytes was only found after incubation of 10 µM Lysmeral, which relate to plasma levels with no adverse testicular effects (1.3% in rabbits versus 7.5% in humans). Concerning the comparable urinary TBBA levels in 1 rhesus monkey, the DS would like to emphasize, that the 24 hour urine TBBA represents a cumulative amount of the excreted metabolite. The concentrations in hepatocyte supernatants are seen as directly proportional to given plasma concentrations in vivo. 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 (see also page 23 of the CLH report).

According to the NL CA, the factors considered and the severe effects on fertility observed may warrant a classification Cat. 1B. The DS disagrees with this conclusion, since the arguments provided are not considered sufficient to change the conclusions made in the CLH dossier. Furthermore, the severity of effects on fertility observed in rats after oral application of Lysmeral is not considered to justify a classification Cat. 1B, given the doubts about the relevance of these effects for humans.

A calculation of the ED10 showing whether a SCL is required for male fertility effects were requested by the NL CA. In general the NOAEL for testicular toxicity is found to be 25 mg/kg bw/d and the LOAEL is above this dose in the most sensitive species independent

of treatment duration. Therefore, a robust ED10 would be considered not to be below the given NOAEL, independent from the parameter taken into account. Given the boundaries for the medium potency group, i.e. > 4 mg/kg ≤ 400 mg/kg, no justification for a SCL is given below the generic concentration limits (3%). Concerning a SCL above the generic concentration limits, the dataset does not provide evidence, that ED10 values with parameters for testes toxicity and spermatotoxicity could be placed in the low toxicity group. Development The NL CA asked for a clarification, why the post-implantation losses observed in the one-generation range finding studies and in the PNDT are considered secondary to the maternal toxicity. The DS would like to further specify, that a statistically significant increase in postimplantation losses were only observed in the PNDT study (BASF 2004). In the range finding studies, this finding was either not dose dependent and/or not statistically significant (BASF 2006, 2017B). Furthermore, Lysmeral did not have a consistent impact on the number of postimplantation loss in the EOGRTS, although tested the range of the LOAEL of the PNDT. Developmental effects such as postimpantation losses coincided with significant maternal toxicity at the same dose level, and are considered manifestations of the non–specific maternal stress induced by Lysmeral administration (for details see Table 3 of Comment Nr. 4). Individual data for both maternal and developmental effects for the PNDT study will be submitted to RAC representatives via the RAC secretariat.
RAC's response
Thank you for your comment. Your considerations and the clarifications made by the DS have been taken into account in RAC's assessment.

Date	Country	Organisation	Type of Organisation	Comment number
11.04.2018	Germany	BASF SE	Company-Manufacturer	12
Comment received				
additional information to the previously submitted CLH proposal SPS-013920-17 ECHA note – An attachment was submitted with the comment above. Refer to public attachment Summary for RAC final.pdf ECHA note – An attachment was submitted with the comment above. Refer to confidential attachment 80-54-6.zip				
Dossier Submitter's Response				
Submitted by the DS.				
RAC's response				
Thank you for the additional information. It has been taken into account in RAC's assessment.				

PUBLIC ATTACHMENTS
1. CLH Lysmeral DK comments.docx [Please refer to comment No. 6]
2. Lysmeral - supporting arguments from PG_CMR2_FINAL_ Apr 2018.docx [Please refer to comment No. 2, 9]
3. Summary for RAC final.pdf [Please refer to comment No. 12]
4. lysmeral CLH_20180406.docx [Please refer to comment No. 4]
5. LysmeralJB.pdf [Please refer to comment No. 5]

ANNEX 2 - COMMENTS AND RESPONSE TO COMMENTS ON CLH PROPOSAL ON 2-(4-TERT-BUTYLBENZYL)PROPIONALDEHYDE

CONFIDENTIAL ATTACHMENTS
1. 80-54-6.zip [Please refer to comment No. 12]