Centre on Endocrine Disrupters

Hazard assessment of endocrine disruptors - Assessment of D4.

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DANISH CENTRE ON ENDOCRINE DISRUPTERS

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This report focuses on assessing the endocrine disrupting properties of the substance D4 (Octamethylcyclotetrasiloxane, CAS no.: 556-67-2).

The report contains:

- A description of the performed literature search for D4 (Octamethylcyclotetrasiloxane, CAS no.: 556-67-2), including a preliminary review of ECHA's public dissemination site for D4 (https://echa.europa.eu/registration-dossier/-/registered-dossier/15289)
- For all relevant peer-reviewed studies related to possible reproductive toxicity or endocrine disrupting effects of D4, we have included a summary of methods and results, as well as a quality assessment (Klimisch score), based on the following ECHA / EFSA Guidance Document (2018): https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2018.5311
- All relevant information on endocrine disruption, reproductive- and systemic toxicity, from the published studies, has been tabulated in the following Excel file"D4_EDGD_Appendix-E1_EDdata_final publication", according to the ECHA/EFSA Guidance Document (2018) for ED assessment of pesticides and biocides.
- "Lines of evidence" for in vitro, in vivo mechanistic and in vivo adverse effects, generated based on the tabulated information. The resulting tables are all also included in the Excel file (different sheets), but in this report only "LoE for effects on female reproduction" have been included, as this is the most relevant adverse effect outcome after D4 exposure.
- A discussion of the "human relevance" issue, in relation to the adverse effects on female reproduction. A different interpretation is presented here, compared to the views put forward in review papers by Gentry et al 2017, Franzen et al 2017 & Dekant et al 2017
- Preliminary conclusions on the ED properties of D4

Contents

Abbreviations

AGD – Anogenital distance

Bw – Body weight

CL – Corpora Lutea

ER – Estrogen receptor

GD – Gestational day

PND – Post natal day

PR – Progesterone receptor

S.c. – Subcutaneous

Ss – Statistically significant

E2 - 17β-estradiol

EE - Ethinyl estradiol

KO - Knockout

WT - Wild-type

1. Literature search and review of ECHA dissemination site

We carried out a search in PubMed on April 19th, 2021. The following, relatively broad search string was used: "(Octamethylcyclotetrasiloxane OR Cyclotetrasiloxane OR 556-67-2) AND (rats OR mice OR human OR toxicity or endocrin* OR hormon* OR androgen* OR estrogen* OR thyroid* OR steroid*)

This resulted in 105 hits. We hereafter performed screening of titles and abstracts, and for those studies identified as relevant we did full text reviews. Using this strategy, we identified 16 relevant publications (of these 3 were review articles).

Importantly, all the published studies, except for a single *in vitro* study (Lee et al. 2015), were based on data generated by the registrant.

In addition, we performed a preliminary review of ECHA's public dissemination site for D4 (https://echa.europa.eu/registration-dossier/-/registered-dossier/15289).

Based on the information found in the ECHA dissemination, together with information extracted from review articles on D4 (Gentry et al 2017, Franzen et al 2017, Dekant et al 2017), we extracted a list of all performed *in vivo* studies (included as appendix 1 to this report). It is our assessment that the number of relevant studies is close to 100. Some of the data obtained in these studies have been published in the peer reviewed publications on D4, but there might still be some important toxicity information on D4 present, for which only very limited study summaries are available on the ECHA dissemination site. For a full ED assessment of D4 it would be necessary to include this information in the lines of evidence, mode of action analyses etc.

2. Review of the literature for D4

Overview of the studies

In this section, an overview of the identified *in vitro* and *in vivo* studies is presented. Table 1 shows a study overview, describing model, effect modality and conceptual framework (CF) level (OECD Guidance Document 150). Hereafter study summaries are presented, in the same order. These include descriptions of methods, results and assessment of study quality and reliability. The CF level is in accordance with ECHA/EFSA guidance (ECHA/EFSA 2018).

The quality and reliability of all *in vitro* and *in vivo* studies were assessed, and each study was assigned a reliability score based on the Klimisch categories 1, 2, 3 or 4 (Klimisch et al., 1997) combined with an expert judgement statement. • 1: reliable without restriction • 2: reliable with restriction • 3: not reliable or • 4: not assignable.

Toxcast data is briefly presented below table 1.

Table 1: Study overview, sorted by model (*in vivo, in vitro*) and modalities (EATS).

Reference	Model	EATS or Other	CF level	Klimisch Score	Note
He et al. 2003	*In vitro, In vivo*	E	2,3	2	*Several Uterotrophic & repeated dose (7 days)*
Quinn et al. 2007a	*In vitro, In vivo*	E	2,3	2	*Uterotrophic, Hershberger*
Lee et al. 2015	*In vitro, In vivo*	E	2,3	2	*Uterotrophic*
Farasani and Darbre 2017	*In vitro*	E	2	2	*In vitro proliferation of MCF-10 cells*
McKim et al. 2001a	*In vivo*	E	3	2	*Uterotrophic, 2 rat strains*
Hayden and Barlow 1972	*In vivo*	E	3	3	*Uterotrophic*
McKim et al. 2001b	*In vivo*	T	4	2	*28-day repeated dose tox (1 dose), 6-day study*
Burns-Nass et al. 2002	*In vivo*	E	4	1	*90-day tox 4 doses, all organs*
Jean and Plotzke 2017	*In vivo*	E	4	1	*2-year tox 4 doses, all organs*
Jean et al. 2017	*In vivo*	E	4	2	*2-year tox (only 1 dose) – female repro*
Quinn et al. 2007b	*In vivo*	E	4	2	*Repeated dose tox (2 doses) – female repro*
Meeks et al. 2007	*In vivo*	E	4	2	*2 reprotox. studies reported* *Different phases of reproduction*
Siddiqui et al. 2007	*In vivo*	E	5	1	*2 –gen study* *4 doses*

CompTox dashboard – EDSP21 (18/11-21):

Active in 2/27 assays. 2xERα activation with AC_{50}'s of 19 µM and 37 µM (Toxcast, 2021).

Study summaries; in vitro studies

He et al. 2003

The objective of this study was to investigate the estrogenic mechanism of D4 *in vitro*. Estrogen receptor (ER) binding assays using human ERα and ERβ receptors were performed in triplicates with concentrations of D4 ranging from 1.0×10^{-10} to 1.0×10^{-4} M. At 4 x 10^{-5} M, D4 bound competitively (ss) to ERα (reducing E2 bound to ERα by ~25%) but not to ERβ.

Study assessment: This study is allocated a reliability rating of 2, reliable with restrictions. The study is well-described and appears to be well-performed but does not report chemical purity. The study is assessed to be scientifically acceptable.

Quinn et al. 2007a

The purpose of the study was to determine the estrogenic, progestagenic and androgenic activity of two cyclic siloxanes (D4 and D5). In the *in vitro* part of the study, receptor binding experiments and luciferase reporter gene assays were performed for human estrogen receptor (ER)α, ERβ, progesterone receptor (PR)α or PRβ. Binding experiments were performed in triplicates with 900 ppm D4 exposure for 4 hours. Luciferase reporter experiments were performed in triplicates using MCF-7 cells exposed to D4 concentrations ranging from 0.0001 to 10 µM. Lastly, triplicate "HitHunter (PRα)" assays were performed with concentrations of D4 from 0.0001 to 1 M.

D4 showed statistically significant binding to ERα at 900 ppm, which was the maximal achievable vapor concentration. A 20% inhibition of estradiol binding was seen. The test article was delivered to the test system as a vapor, but the concentration of D4 in the aqueous reaction mixture was determined to be 0.45µM for both receptors by GC/MS. D4 did not appear to bind to ERβ (at 900 ppm), PRα or PRβ as assessed by PR binding assays and the "HitHunter (PRα)" assay (data for PR binding assays not shown). The ERα reporter gene assay showed a 5-fold induction of reporter gene at 10 µM D4 (tested range: 0.1nM-10µM). In this assay, estradiol induced 35-fold reporter gene induction at 10 nM, BPA 10-fold at 20µM. No activation of the PRβ reporter gene assay was observed for any of the tested doses of D4 (0.1-10 µM, data not shown).

Study assessment: This study is allocated a reliability rating of 2, reliable with restrictions. The in vitro studies were well described, the tests were performed in triplicates and although it was not stated whether cytotoxicity was evaluated, the findings do not reflect results from cytotoxicity. Of note, it could very well have been possible to increase the exposure concentration even further to observe any increased response given that no toxicity was observed at 10 µM. The study is scientifically acceptable.

Lee et al. 2015

The purpose of the study was to investigate estrogenic activity of D4. In the *in vitro* part of the study, cells from the GH3 cell line (from rat pituitary) were exposed to D4 ($1x10^{-5}$ M) or to 17β-estradiol (E2) ($1x10^{-9}$ M) for 1 day (three replicates/treatment). Gene and protein expression levels of CaBP-9K, ERα, PR and CYP2B1 were measured in the cells. Simultaneous exposure to the ERα inhibitor ICI 182-780 (ICI, $1x10^{-7}$ M) added 30 min prior to treatments was used to investigate if the effects on gene expression level were regulated by the ER-mediated pathway.

CaBP-9K gene expression was increased (ss) in rat GH3 cells exposed to E2 or D4. When cells were exposed to ICI 182-780 in combination with D4 or E2, the gene expression level of CaBP-9K was not affected. Protein expression of CaBP-9K was slightly increased by E2 and D4 but not when ICI was administered simultaneously (though the Western blot shown is hard to assess and does not seem to be a particularly reliable blot). Similarly, PR gene- and protein expression levels were increased by D4 and E2, an effect that was blocked by ICI. Conversely, gene-and protein expressions of ERα were downregulated by E2 and D4 and ICI blocked the effect.

Study assessment: This study is allocated a reliability rating of 2, reliable with restrictions. The study is well described and was scientifically acceptable. However, no CAS-no., lot no. or purity of the chemical is given and only one concentration was tested, for which toxicity was not monitored. Western blots should be interpreted with care.

Farasani and Darbre 2017

The objectives of this study were to investigate any genotoxic effects after short- (1week) or longer-term (30 weeks) exposure to hexamethylcyclotrisiloxane (D3), octamethylcyclotetrasiloxane (D4) or decamethylcyclopentasiloxane (D5) in MCF-10A and MCF-10F immortalized non-transformed human breast epithelial cells. Genotoxic effects were assessed by an ability of cells to grow in suspension culture, from DNA damage measured by comet assays, and from a reduction in levels of DNA repair proteins measured by RT-PCR and western immunoblotting.

MCF-10A and MCF-10F immortalized non-transformed human breast epithelial cells can be grown as monolayer cultures under adherent conditions but under non-adherent conditions do not grow without the addition of 70 nM 17β-oestradiol. Given that this cell proliferation is estrogen-induced, cell proliferation in MCF-10A and MCF-10F cells can be used as an indicator of estrogenic potential of a chemical. 21 days of D4 exposure (0.001-100 μM) increased the total numbers of colonies counted per well (as a measurement of proliferation). Increases were statistically significant from 0.01 μM and were larger than the positive control (70 nM 17β-oestradiol) from around 1 μM D4.

Results from Comet assays and expression of DNA repair proteins is not considered relevant for assessment of the endocrine disrupting potential of D4 and is thus not described here.

Study quality and assessment: This study is allocated a reliability rating of 2, reliable with restrictions. The study is generally well described and includes chemical purity and correct vehicle controls. It is however not described whether cytotoxicity was monitored (though the findings do not reflect results from cytotoxicity) and for the relevant cell proliferation experiments, results from triplicate wells were included but only one independent experiment seems to have been performed.

Study summaries; in vivo studies

Hayden and Barlow (1972)

The purpose of the study was to investigate the effects of a series of organosiloxanes in immature ovariectomized female rats and establish structure-activity relationships. The parameters studied included changes in uterine weight, production of uterine hyperemia, and uterine histology. D4 was only used for studies of uterine weight. The responses were compared to those produced by diethylstilbestrol or estradiol benzoate.

6 Wistar rats/group were dosed by oral gavage once daily for 3 days and autopsied 24 hours after the final treatment. Several doses of siloxanes were used to assess estrogenic activity by measuring uterine weight and establish comparative relative activities to diethylstilbestrol or estradiol benzoate. D4 was rated "+1" meaning a statistically non-significant increase <20%. In conclusion, D4 appeared to have a weak estrogenic activity.

Study quality and assessment: This study is allocated a reliability rating of 3, not reliable. This is based on the fact that the doses used and purity for D4 was not reported, and that any toxicity was either not monitored or not reported. Furthermore, data for the uterine weight increase induced by D4 was not given.

McKim et al. (2001a)

The objective was to investigate the estrogenic and anti-estrogenic activity of the cyclic D4 and the linear siloxane hexamethyldisiloxane in the Uterotrophic assay. Immature female rats (12 pups per group) from two different strains were used in the study. Sprague-Dawley rats (18 days old) and Fisher-344 rats (21 days old) were dosed by oral gavage with 0, 10, 50, 100, 250, 500, or 1000 mg/kg/day of D4 for 4 days. For evaluation of anti-estrogenic properties of D4, EE was co-administered with either D4 (500 mg/kg/day) or ICI 182-780 (positive control). The uterine weight was measured and the epithelial cell height in uterus was measured histologically (n=6 or 12 per group, methods section indicates 6, figure text 12). Relative potency of the different chemicals tested (EE, diethylstilbestrol dipropionate and Coumestrol) was evaluated based on the effects on uterine weight.

Female body weight was decreased in the highest D4 dose-group on day 21 (SD rats) or on days 23, 24 and 25 (Fisher rats). Compared to the other compounds tested, D4 was the least potent in increasing uterine weight (absolute and relative). Higher doses of D4 were necessary to get the same increase in uterine weight compared with the weak estrogenic compound Coumestrol. Uterus weight was significantly increased by 250, 500 and 1000 mg/kg/day of D4. Co-administration of D4 with EE attenuated the effect of EE on uterine weight suggesting an anti-estrogenic effect of D4. D4 was less effective in inhibiting the effect of EE on uterine weight compared to ICI. Uterine epithelial cell height was increased by EE and D4 in a dose-dependent manner. In conclusion, D4 appeared to have weak estrogenic and anti-estrogenic properties.

Study quality and assessment: This study is allocated a reliability rating of 2, reliable with restrictions. CAS-number D4 was not provided and there was a discrepancy between numbers of uteri investigated for epithelial cell height mentioned in the methods section compared to the figure text. The study is assessed to be of medium quality.

He et al. 2003

Several *in vivo* studies were performed, and other siloxanes were also tested in the Uterotrophic assay.

Female B6C3F1 mice (6-7 weeks old, n=6/group) were dosed orally with 1, 10, 50, 100, 250, 500, and 1000 mg/kg D4 for 7 days and serum estradiol was measured. Serum estradiol was reduced by approximately 50 % by 1000 mg/kg bw/day D4 exposure and from 10 to 1000 mg/kg bw/day D4, estradiol levels were reduced in a dose-dependent manner (which however did not seem to have been statistically assessed).

Subsequently intact, sham-operated and adrenalectomised (ADX) B6C3F1 mice (n=8/group) were dosed for 7 days with 1000 mg/kg bw/day of D4, and serum estradiol and corticosterone were measured. Serum estradiol was reduced (ss) in intact, sham-operated and ADX mice. Serum corticosterone was increased (ss) in intact and sham-operated mice, whereas corticosterone was not increased in ADX mice. This suggested that the D4-induced decrease in estradiol was independent of corticosterone levels and an association between stress-like elevated corticosterone levels and suppression of the hypothalamic-pituitary-gonadal axis could be excluded.

D4 was tested in a Uterotrophic assay in ovariectomized female B6C3F1 mice dosed orally to 1000 mg/kg/day D4 for 3 days (n=5/group). A uterine peroxidase assay was performed on uteri from the ovariectomized mice. A dose-response study was additionally performed for the Uterotrophic assay using 0, 1, 10, 50, 100, 250, 500 and 1000 mg/kg D4 for a 3-days exposure period (n=5/group) in ovariectomized mice. To investigate if the effects on uterine weight were mediated through ER, two more studies were performed. The Uterotrophic assay was repeated with 1000 mg/kg D4 in ovariectomized mice with pre-treatment with the ER antagonist ICI 182,780. Finally, ERα knockout mice (αERKO) and wild-type (WT) (129/J/C57BL/6J) controls

were ovariectomized (n=5) and used in an Uterotrophic assay with oral dosing of 1000mg/kg D4 (WT) or 250, 500, 1000 mg/kg (αERKO) for 3 days.

1000 mg/kg D4 induced increased (ss) uterine weight (approximately 3-fold compared to control assessed from graph) in the Uterotrophic assay and increased (ss) uterine peroxidase activity (approximately 5-fold compared to control assessed from graph) in ovariectomized rats. The dose-response study in the Uterotrophic assay showed statistically significant increases of uterine weight at doses of 250mg/kg D4 and above (approximately 140%, 150%, and 450% of control at respectively 250, 500, and 1000 mg/kg D4 assessed from graph). Pre-treatment with ICI 182,780 blocked the D4-induced increase in uterine weight, indicating that the effects on uterus weight are ER-mediated. Additionally, the D4-induced increase in uterus weight was absent in exposed αERKO mice, suggesting that the effects on uterus weight were more specifically mediated through ERα.

Study assessment: This study is allocated a reliability rating of 2, reliable with restrictions. The study is well-described although it is a complex study using several mouse lines. However, information of the CAS-number and purity of the compounds are not provided. The study is assessed to be of high quality.

Quinn et al. 2007a

A Uterotrophic and a Hershberger assay was performed. In both assays exposure to D4 (700 ppm) was through whole-body inhalation, 16 hours/day. In the Uterotrophic assay, exposure in ovariectomized adult Wistar and Fisher 344 rats (n=10/group for D4 experiments and n=6 for ethinyl estradiol experiments) lasted 3 days and in the Hershberger assay exposure of Fisher 344 rats (n=10/group) lasted 10 days.

For comparison of estrogenic activity, rats were given subcutaneous doses of EE (0.3, 1.0, and 3.0 mg/kg/day) and genistein (10, 25, and 50 mg/kg/day) followed by a control inhalation exposure of filtered air to mimic exposure conditions.

In the Uterotrophic assay, D4 increased uterine wet (maximal effect: 2.7-fold in Sprague Dawley rats) and blotted (maximal 2.6-fold in Sprague Dawley rats) weight. As did uterine weights after exposure to positive controls EE (max 6.5-fold) and genistein (data not shown). D4 exposed uteri were fluid filled and had increased luminal (maximal increase: 2.6-fold compared to control) and epithelial cell height (maximal increase: 1.6-fold compared to control) in both strains tested. For comparison, highest EE dose increased luminal cell height with maximum 2.8-fold.

The anti-estrogenic effect was also investigated: the highest dose of EE (3 mg/kg/day) was given in combination with the ER antagonist, ICI 182,789, or D4. However, the results were unclear. D4 mildly (80% of EE control) suppressed the ethinyl estradiol-induced increase in uterine weight in Fischer 344 rats, indicating a weak anti-estrogenic activity of D4. This effect, however, was not

observed in the measurement of the uterine epithelial cell height and Sprague Dawley rats did not exhibit any anti-estrogenic effects of D4 in any of the measured endpoints.

The Hershberger assay showed no androgenic or anti-androgenic activity of D4.

Study assessment: This study is allocated a reliability rating of 2, reliable with restrictions. The study is well described and is assessed to be of high quality; however, the housing conditions and body weight of the animals was not reported.

Lee et al. 2015

A Uterotrophic assay in immature female Sprague Dawley rats (18 days old) dosed s.c. for 4 days (5 animals/group) with ethinyl estradiol (EE) (3 µg/kg) or D4 (500 or 1000 mg/kg) was performed. 4 more groups of rats were co-treated with the ERα inhibitor ICI (3mg/kg) 30 min. before EE or D4 administration. Animals were killed 1 day after the last dosage. Livers and uteri were weighed and gene- and protein expression of CYP2B1/2 was measured in the livers. Gene and protein expression was measured in uteri from the Uterotrophic assay for evaluation of the expression levels of the estrogenic biomarkers CaBP-9K, ERα and PR.

In the Uterotrophic assay, the positive control EE increased (ss) the relative uterus weight markedly. No effects on uterus weight were seen with administration of D4.

Gene-and protein expression of CaBP-9K, ERα and PR was measured in rat immature uterus. The gene expression levels of CaBP-9K were increased (ss) by EE and D4 (from 500 mg) and the effect was blocked by ICI. Protein expression of CaBP-9K was similarly induced by EE and D4 and the effect was inhibited by ICI, protein expression was, however, not quantified. Both protein and gene expression levels were more elevated after EE exposure than with D4. E.g. EE increased CaBP-9K gene expression by 170 fold and D4 only by 3 fold.

Expression of PR mRNA was suppressed (ss) by EE and by the high dose of D4 to a similar level. Co-treatment of ICI further decreased PR gene expression levels in controls, in EE treated animals and in the low-dose D4 group. In the high-dose D4 exposure group ICI treatment increased PR levels compared to D4 alone. At protein level, the opposite effects were observed with EE and D4 increasing PR protein expression (assessed from Western blot, not quantified).

For ERα, gene expression was reduced (ss) by EE and D4 to a similar level, and simultaneous exposure to ICI reduced the expression level further for EE exposure and increased expression in the high D4-group compared to D4 alone. No effect on ERα protein expression was observed after D4 treatment, whereas EE alone and in combination with ICI and D4 with ICI decreased the protein expression (again assessed from blot, no quantification).

Relative liver weights were increased (ss) by the high dose of D4 (around 10%) but not by EE.

Gene expression of CYP2B1/2 was increased (ss) markedly (around 30-fold for 1000 mg/kg dose group) compared to controls in the D4 dose-group in a dose-dependent manner and treatment with ICI did not alter the effect of D4. In contrast, CYP2B1/2 gene expression was decreased (ss) by EE and co-administration of ICI and EE decreased the gene expression additionally compared to EE alone.

Study assessment: This study is allocated a reliability rating of 2, reliable with restrictions. The study was well described. However, no CAS-no., lot no. or purity of the chemicals were given, and protein expression was not quantified. Furthermore, it was not mentioned whether statistical model presumptions were met (normal distribution, homogeneity of variances etc.) and the uterus weights were only shown as relative to body weight, but it was not mentioned whether body weights were affected by the exposures.

McKim et al. 2001b

The purpose of the study was to evaluate the effects of D4 exposure on hepatic and thyroid cell proliferation and hypertrophy with respect to time and concentration. A 4-week repeated dose toxicity study was conducted using female Fischer 344 rats (10/group). Rats were exposed via whole body inhalation to 0 ppm or 700 ppm D4 vapors (6 h/day; 5 days/week). Liver and thyroid weight and histopathology was assessed at day 6, 13, and 27. In an additional short study, rats (10/group) were exposed to 0, 7, 30, 70, 150, 300, or 700 ppm D4 for 6 days (animals were exposed to D4 vapors for 5 days and euthanized the morning of day 6) to assess dose-response effects on liver weight and proliferation.

Liver-to-body weight ratios in animals exposed to 700 ppm D4 were increased 18, 20, and 22% over controls on days 6, 13, and 27 respectively. In the 6-day study, D4 increased liver-to-body weight ratios in a dose-dependent manner.

Hepatic cell proliferation/hepatic hyperplasia (assessed by BrdU incorporation and PCNA expression) following exposure to D4 was increased compared to controls and was highest on day 6 (15–22%) but was at or below control values by day 27. This pattern of transient hyperplasia was observed in all hepatic lobes examined. Results were compared to effects observed after phenobarbital exposure and this pattern of transient hyperplasia was similar to the pattern of effects observed following treatment with phenobarbital. Similarly, D4 reduced the number of nuclei (e.g., cells) relative to controls by 9% on day 6, 16% by day 13, and 11% by day 27 indicating hepatocellular hypertrophy. Examination of H & E-stained sections from D4-treated and control liver supported the presence of hypertrophy; however, there were no other lesions that could be attributed to exposure.

In the 6-day study, hepatic hyperplasia increased in a dose-dependent manner. The lowest exposure concentration of D4 that resulted in a statistically significant increase in BrdU incorporation was

70 ppm. For PCNA expression, the values at the low end of the exposure curve were variable and it is unclear from these data at what point dose-related increases begin.

Exposure to 700 ppm D4 produced a small but statistically significant increase in thyroid-to-body weight ratios on day 6 (17%), 13 (18%) and 27 (23%).

Thyroid cell proliferation (BrdU incorporation) was increased after 6 (8%) and 13 (7%) days. On day 27, thyroid cell proliferation in D4-treated animals had returned to control values. In a concentration-response experiment with only 6 days of exposure, no effect of D4 was observed on thyroid BrdU incorporation.

The authors compare the observed effects to phenobarbital (PB). A common property of PB and PB-like compounds is their ability to increase thyroid cell proliferation, which in rodents is associated with an increased incidence of thyroid tumors. The effects on thyroid cell proliferation may be initiated by an induction of hepatic thyroxine metabolizing enzymes, which decreases the concentration of circulating thyroxine. This reduction in thyroxine triggers a compensatory increase in circulating TSH, which causes hyperactivity in thyroid tissues leading to hyperplasia. In conclusion, D4 increased liver weight and induced dose-dependent but transient hepatic hyperplasia in a manner like phenobarbital. Furthermore, D4 exposure increased thyroid weight and induced transient increases in thyroid proliferation.

Study quality and assessment: This study is allocated a reliability rating of 2, reliable with restrictions. The study is generally well described but chemical purity and CAS number is not reported. Whether toxicity was monitored, and clinical signs observed is not reported either.

McKim et al. 1998

Summary: The purpose of this study was to investigate the effects of D4 on hepatic cytochrome P450 (CYP) enzyme expression. Animals (3-9/group) were exposed to D4 vapor at concentrations of 70 and 700 ppm, via whole body inhalation for 6 h/day, 5 days/week for 4 weeks. Animals were euthanized on exposure days 3, 7, 14, 21, and 28. Microsomal fractions were prepared from fresh liver by differential centrifugation. Enzyme activity as well as immunoreactive protein levels of several enzymes were assessed.

Repeated inhalation exposure to D4 induced hepatic CYP enzymes and epoxide hydrolase in a manner like that observed for phenobarbital (PB).

Study quality and assessment: This study is at present not considered relevant for the assessment of the endocrine disrupting potential of D4.

Burns-Nass et al. (2002)

Summary: The objective of the study was to investigate the sub-chronic toxicity of D4 in a 3-month inhalation study. Male and female rats (approximately 10 weeks old) were exposed to 0, 35, 122, 488 or 898 ppm for 6h/day, 5 days a week for 3 months (20/sex/group) to D4 by nose-only inhalation. A recovery study was performed with 10 rats per group exposed to 0 or 898 ppm as described previously for 3 months and with a following recovery period of 1 month. Blood was collected for hematologic and clinical chemistry, urine analysis was performed, and organs were weighed, including liver, spleen, heart, lung, thymus, ovaries, testes, kidneys, adrenals, and brain. Histopathological assessment of a full set of organs (including the thyroid gland) was conducted.

Body weights of both male and female rats in the 898-ppm group were slightly less (4% to 6%) than controls over the course of the study, however, not statistically significant. Slight indications of systemic toxicity were, however, observed as clinical signs were seen in one third of the females in the 898-ppm group. These clinical signs, consisting of hunched posture and a slightly stiff gait, were of minor severity and were recorded only occasionally during the first 8 days of exposure. Five females in the highest-exposure group died on days 2 (two animals), 3, 43, and 78. Gross necropsy was unrevealing, and the cause of death could not be determined. Furthermore, during the first 3 days of exposure food consumption was decreased in male (20%) and female (28%) rats in the 898 ppm groups. However, food consumption was generally not affected over the remainder of the study and no statistically significant effects on this endpoint were observed.

Changes in endocrine related endpoints were found. Testis weight was increased (117% of control) in the 488 ppm group but not at the highest dose (898 ppm), and the change was considered not to be exposure related. Ovary weight was decreased (by 38% compared to controls) in the highest exposure group. Histopathological evaluation of the ovaries showed an increased incidence of hypoactivity seen as a lack of active corpora lutea in the 898-ppm dose-group after a 3-month exposure period (in 9/20 rats compared to 1/20 in control). No statistical analysis was carried out. The effect was not observed after 1 month recovery period (10 animals/group). Follicular development appeared normal. The morphological changes indicated a decreased ovulatory activity of the ovaries, and the reduced ovary weight was most likely due to the lower number of corpora lutea. After the 1-month recovery period, the ovaries showed normal follicular development. Increased incidence and thickness of mucification of the vaginal mucosa was seen in the uterus in females exposed to 898 ppm for 3 months (19/20 rats compared to 9/20 in control). This effect was not observed after 1 month recovery.

*Study quality and assessment: This study is allocated a reliability rating of 1, reliable without restrictions. The study is well-described and is assessed to be of high quality. However, t*hyroid gland histopathology results were not mentioned in the manuscript. A full study report would be needed to evaluate if any relevant effects were seen on this endpoint.

Jean and Plotzke (2017)

This study presents data of a chronic toxicity and oncogenicity study of D4. In the oncogenicity part of the study, 7-8 weeks old male and female Fischer 344 rats (60/sex/group) were exposed to D4 (0, 10, 30, 150 or 700 ppm) for 6 hours per day, 5 days/week for 24 months in whole-body inhalation chambers. Organ weights were recorded, and histologic examination of organs was performed.

6 months (n=6), 12 months (n=10 for chronic tox, n=20 for recovery group (12 months recovery)), 24 months (n=60).

Exposure had no effect on body weight or body weight gain with the exception of 700 ppm males in the 24-month exposure group (data not shown). Mean body weight for this group remained approximately 6% lower than controls for the last few months on study. The decrease was statistically significant only for week 97 and at necropsy (month 24).

Organ weights:

Treatment-related increases in organ weight were evident for kidney, liver, testes, and uterus.

There was a consistent and clear statistically significant increase in kidney weight for both males and females in subgroups 12 months and 24 months at the 700-ppm exposure concentration. The increased weight was apparent based on both absolute and relative weight (kidney-to-body weight and kidney-to-brain weight). This was true also for the female recovery group at 700 ppm. A similar effect on the recovery subgroup males was only observed for the relative weights. The noted increases in kidney weight may be related to a treatment related increased severity in chronic nephropathy (see histology section).

Liver weight increases were evident at 6, 12, and 24-months exposure for both sexes. For males, the liver weight increases were associated with exposure to ≥ 30 ppm D4 at 6 months, ≥150 ppm at 12 months, and 700 ppm D4 at 24-months. Group mean liver absolute weight, relative-to-body weight, and relative-to-brain weight ratios for recovery group males across the exposure levels were generally higher than the control values, although only the relative–to-body weight ratio at 700 ppm was statistically significant. Female liver weight increases were statistically significant at all three time points (6, 12, and 24 months) at 700 ppm and at 12 and 24 months with exposure to 150 ppm D4. Increases were seen in absolute, relative–to-body weight and relative-to-brain weight ratios. There was no effect of treatment among the recovery group females.

Uterine weight absolute and relative to body weight) were increased (24%) (ss) in animals in the 700-ppm group after 24 months. Group mean uterine weights among exposure groups at 12 months were generally increasing with exposure levels ≥30 ppm however, none were statistically different from control. There was no indication of a treatment-related effect on uterine weight among the recovery group females.

Testes weight increase was observed at 12- and 24-month exposure periods for males exposed to 700 ppm D4. The increase was discernible on an absolute (8.6% at 12 months, 21% at 24 months) as well as on a relative-to-body weight (8.5% at 12 months, 28% at 24 months) basis, but only the testis-to-body weight ratio was statistically significant. Increased testes weight was present in the recovery group as an absolute value (67%) as well as relative-to-body weight (67.5%) and brain weight (66.5%) at 700 ppm D4.

Histopathology:

Treatment-related, non-neoplastic findings in the liver included an increased incidence of centrilobular hypertrophy in males and a reduction in basophilic foci in females at 700 ppm at 12 and 24 months.

Chronic nephropathy was a common finding, observed in males (80%-100%) and females (28%–92%) in all groups including controls at 12 and 24 months. No treatment-related differences in incidence or severity were evident at 12 months or in recovery groups. The incidence of chronic nephropathy was elevated at 24 months for females at ≥ 30 ppm (statistically significant). Although not statistically significant, the severity scores were generally increasing with increasing exposure concentration for both males and females at 24 months.

A focused and detailed histomorphological review of the ovaries, uterus, vagina, pituitary and mammary gland from the control and high treatment group females was conducted with the purpose to assess the potential for treatment-related alteration of estrous cycle synchronicity among these tissues. Abnormal estrous cycle (lack of synchronicity among the tissues) was present in both control and 700 ppm D4 group.

An increased incidence of uterine cystic endometrial hyperplasia (from 19 % in controls to 50 % in treated females) was found in females exposed to 700 ppm D4.

Although few high-dose females had cervical squamous epithelial hyperplasia and/ or ovarian atrophy, the incidence of these changes (5 % and 6.7 %, respectively) was statistically significantly increased compared to controls (0 % and 1.7 %, respectively). These low incidence findings were not considered toxicologically significant by the authors.

The severity of the changes in uterus and ovaries were increased by increasing exposure, but no information on statistical significance of this is given.

A statistically significant increase in the incidence of testicular interstitial cell hyperplasia was observed after 24 months of exposure in the two highest exposure groups (150 ppm: 21.7% (13/60) and 700 ppm: 26.7% (16/60), controls 11.7%). Severity score was increasing with exposure, but this was not assessed statistically. No statistically significant effects were observed on incidence of testis interstitial cell adenomas, uterine neoplasia, or thyroid gland C-cell neoplasia.

Study assessment: This study is allocated a reliability rating of 1, reliable without restrictions. The study is very well described and assessed to be of high quality.

Jean et al. (2017)

This study describes the effects of chronic exposure to 700 ppm of D4 on markers of reproductive senescence in female rats (49-50 weeks of age). The animals (50 per group) were exposed through whole-body inhalation from 11 to 24 months of age. Estrous cycle was monitored throughout the study and blood samples were collected monthly (for measurement of prolactin, progesterone, estradiol and corticosterone). At necropsy, blood samples were collected (FSH, estradiol and estradiol metabolites), organ weights were recorded (adrenal glands, pituitary glands, uterus, ovaries with oviducts and other non-endocrine related organs) and histopathology (of ovaries, uterus and vagina) was performed. Histological assessment of reproductive organs was also used to evaluate the estrous cycle and the types of corpora lutea and to record the number of atretic and healthy antral follicles and primordial follicles.

No treatment-related effects in body weights were observed.

Of the organs investigated liver (33%) and kidney (8%) weight increases (ss) were noted after D4 exposure.

Increased (ss) cumulative number (6966) or percent (range: 28–62%) estrogen-predominant days (proestrous and estrous) in the estrous cycle was found in D4 treated animals compared to controls (days: 4152, percent range: 12.5-34.9%). The mean estrous cycle incidence was also increased (ss) in D4 group compared to controls and increased proestrous/estrous phase durations (2.4–3.5 days/cycle) were observed for the D4 exposure group in intervals 1–6 (out of 9 in total, each interval was 45 days) compared to control (1-2 days/cycle).

Progesterone levels were elevated (approximately 40% increase compared to control, assessed from graph) (ss) in exposed rats 2-10 weeks after start of treatment and estradiol was reduced (maximally around 167% increase compared to control, assessed from graph) (ss) over the total study period after exposure start. Consequently, lower estradiol:progesterone ratios compared to controls were found in D4 treated females. Corticosterone concentrations were increased (ss) in exposed animals during almost the entire study period (10-30% during weeks 2-51 out of 57 weeks).

The authors mention that they measure FSH, but the results are not reported in this article. In supplementary table S10 the values are included where D4-exposed animals had 12% lower FSH levels at necropsy compared to control but no statistical differences were found.

Histological analyses of the ovary, uterus and vagina was performed; however, the results were not subjected to statistical analysis. An increased incidence of cystic endometrial hyperplasia was observed (14/37 compared to control with 8/37). There was also a marked decrease (37%) in the incidence of antral-size atretic follicles in the ovaries. For the vagina, an increase in severity was observed for epithelial thickness. D4-exposed animals also had a slightly lower incidence of

vaginal mucification than controls – but for those animals in the D4-group that did, display mucification there was a shift to moderate and severe severity.

Study assessment: This study is allocated a reliability rating of 2, reliable with restrictions. The study is well described and thorough but information on chemical CAS-number and statistical assessment of histopathological findings are not included. The study is assessed to be of high quality.

Quinn et al. (2007b)

The objective of the study was to investigate the effects of D4 on ovulation and on reproductive hormones, including luteinizing hormone (LH). Two studies in female rats (13 weeks old) were performed with whole-body vapor inhalation exposure (generally 6 hours per day) to D4 at 700 or 900 ppm for 3 days from the day of diestrus until proestrous. In the first study, called phase I (n=24, 22 and 27 for controls, 700 and 900 ppm, respectively), rats were euthanized on the day of proestrous. Blood was collected for hormone analysis (Follicle stimulating hormone (FSH), 17β-estradiol, estrone and progesterone). In the second study, called phase II (n=138 in total at study start), blood was collected at 2, 4, 6, 8 and 10 p.m. on the day of proestrous and the animals were euthanized on the day of estrous. The serial blood samples from phase II were analyzed for luteinizing hormone (LH) and prolactin levels. On the day of necropsy body weight and weight of uterus, ovaries and brain were assessed and the number of ova in the oviducts was counted in both studies/ phases. Step sections of ovaries from phase II were evaluated histologically for assessment of large follicles, new corpora lutea and atretic antral follicles for classification of ovulators and non-ovulators. Terminal blood samples were used for hormone analysis (FSH, estradiol, estrone and progesterone).

Body weight was reduced in both treatment groups in phase I (3-4%) and in the 900 ppm group in phase II (4%). The absolute ovary and relative ovary and uterus weights were reduced in the highest exposure group in phase II as well as the ovary relative to brain weight (% changes could not be determined from the article). More non-ovulating females were seen in the treatment groups (n=26, 12 and 10 ovulating females in controls, 700 ppm and 900 ppm groups, respectively compared to n=9, 19, 23 non-ovulating females in the controls, 700 ppm, 900 ppm groups, respectively). In general, plasma LH levels and the peak levels of LH were lower compared to controls (~40-57% decrease compared to control), but the changes in LH peaks were related to the ovulatory status, i.e., lower mean levels of LH were related to a higher number of non-ovulators in the treatment groups. However, in ovulating females, prolactin levels were reduced at 2 p.m. in the 900-ppm group (~67% decrease). In phase I and II, estrone hormone levels were increased in both treatment groups (~15-19%) and in phase II, 17β-estradiol levels were elevated in both exposure groups, both in ovulating (~75%) and in non-ovulating (~136-163%) rats. The ratio between estrone and 17β- estradiol was reduced in the 900-ppm group in non-ovulating females. FSH was decreased (20-40% decrease compared to control) in both treatment groups. In phase I,

progesterone was increased (~62%) in the highest exposure group on the day of proestrous. Histological assessment of phase II ovaries showed a tendency to increased number of large antral follicles, which correlated well with the increased estradiol levels. The number of ova found in the oviducts was reduced in the treated groups compared to controls, but not in the ovulating females. Overall, there were no signs of follicular toxicity, but D4 appeared to disrupt the LH surge and decreased the portion of ovulating females.

Study assessment: This study is allocated a reliability rating of 2, reliable with restrictions. The study is well described and the interpretation of data and relations between the different observed effects are well elucidated. The study is assessed to be of high quality although no CAS-number of D4 is given.

Meeks et al. (2007)

The aim of this study was to investigate which phases of the female reproductive cycle are affected by D4. A study design allowing investigation of the complete female reproductive cycle from pre-mating and throughout gestation was performed. Several different cohorts were performed in female rats exposed by whole-body vapor inhalation for 6 hours per day. In the first experiment, female rats were exposed to D4 (0, 70, 300, 500 or 700 ppm) from 28 days prior mating to GD19 (n=24/ group) or for a shorter period covering the ovarian phase, the fertilization phase, or the implantation phase. In these experiments, females were exposed to 700 ppm of D4 from 31 days prior to mating to 3 days before mating (n=26 and 50 pregnant dams in controls and exposed groups, respectively), from 3 prior to mating until GD3 (n=28 and 19 pregnant dams in controls and exposed groups, respectively) or from GD2-GD5 (n=23 pregnant females/ group), respectively. In the second experiment with focus on the fertilization and implantation phases, females were exposed for 1 day only on days 1, 2, 3 or 4 prior to mating or daily either for 3 days prior to mating or from 3 days prior to mating until GD3 (n=11-40 pregnant dams). Necropsies were performed on GD20 in the first experiment and on GD8 in the second experiment. The body weight gain, number of corpora lutea (CLs) in ovaries, number of fetuses, early resorptions and implantation sites and weight of uterus, ovaries, thyroid gland, adrenal glands, and brain were assessed in all the studies.

Exposure concentrations were found to be very close to the targeted exposure levels, e.g., in the 700 ppm group, mean exposure concentrations were found to range between 689 and 702 ppm in the different studies.

Overall, the paper shows effects of D4 on female fertility. In the first experiment, effects on the investigated parameters were seen in the study with exposure to D4 during the fertilization phase and in the study with exposure during the whole mating to gestation period. In the second study, decreased fertility was seen in females exposed at a time-point close to mating.

In the females, exposed from before mating and throughout gestation reduced body weight gain during gestation and increased weight of adrenal glands was seen in the highest exposure group. The number of CLs was reduced by D4 in the three highest exposure-groups, but only statistically significantly, in the 300 and 500 ppm groups and increased pre-implantation loss was seen in the two highest dose-groups of 500 and 700 ppm. The number of viable fetuses was lower compared to controls because of lower numbers of CLs and the higher level of pre-implantation losses. The gravid uterine weight was reduced in the highest exposure group due to a lower number of viable fetuses. In the fertilization phase, pregnant females exposed to 700ppm of D4 had a lower body weight gain, a lower number of CLs and an increased percentage of pre-implantation loss. The number of implantation sites, the number of viable fetuses and the uterine weight were reduced. No effects were found in females exposed during the ovarian or the implantation phases. In the second study, a reduced number of pregnant dams were found among the confirmed mated females exposed to D4 1 day prior to mating. Fewer CLs decreased uterine weight and an increased number of small implantation sites were seen in females exposed from 3 days before mating until GD 3.

Study assessment: This study is allocated a reliability rating of 2, reliable with restrictions. The study is well-described and although the paper includes many sub-studies with different exposure periods it is clear how it was done, what the results are and the link between the different results is well explained. The study is assessed to be of high quality even though no CAS-number of D4 is given.

Siddiqui et al. (2007)

The aim of the study was to evaluate the effects of D4 on reproductive function in F0 and F1 animals and on survival, growth, and development of the offspring. A two-generation toxicity study with whole-body vapor inhalation of 0, 70, 300, 500 or 700 ppm of D4 for 6 hours per day was performed in rats. Male and female rats (F_0, 165 per sex, 44-45 days old) were exposed from 70 days before mating and until weaning of the pups (F_1, n=23-27 litters per group). The F_1 offspring were exposed in utero and through lactation and directly exposed from weaning throughout adulthood with at least 70 days of exposure prior to first mating. F_1 offspring were mated twice delivering two litters (F_{2a} and F_{2b}, n= 17-29 and n=12-26 litters per group, respectively) and male offspring were mated a third time with untreated females while males were still exposed. Exposure was interrupted for 5 days at the time of delivery from GD20 until PND4 for all pregnant female rats.

General toxicity (food consumption, body weight gain and weight and histology of liver, kidney, and lung) was assessed. Reproductive (e.g., mating and fertility index, number of implantation sites, pups born per litter etc.) and developmental parameters (e.g., sperm parameters, AGD and sexual maturation) were assessed.

Food consumption in F_0 males was reduced compared to controls in the 700ppm group during weeks 1-2 which was consistent with a reduced weight gain observed in these males at that time-point. Increased organ weights were seen in livers (absolute and relative weights in F_0 and F_1 males and females), kidneys (absolute and relative weights in F_0 males and F_1 females and relative weights in F_1 males) and pituitary glands (absolute weights in F_1 females and relative weights in F_0 males and F_1 females). Histologically, changes were seen in livers, kidneys, and lungs. Hepatocytic hypertrophy was increased in F_1 females in 500 and 700 ppm groups and in F_1 males in the 700-ppm group. In the F_1 males in the 700-ppm group, increased bile duct hyperplasia and bile pigment were observed. Tubular mineralization was seen in the kidneys of F_0 and F_1 males in the 700-ppm group.

Reproductive parameters were also affected. Female body weight gain was reduced during GD14-20 in the 700ppm group (F_0 and F_1), a change considered to be related to a decreased number of fetuses in the uterus, as a reduced number of pups were born in this group. The percentage of mated rats with successful delivery of litters was reduced and the gestation length was increased in F_1 offspring in the 700ppm treatment group. The estrous cycle length was increased in F_1 females in the highest exposure-group. Most (8 out of 9) females with extended diestrus did not show evidence of mating and this led to decreased mating indices (number of successful matings out of number of animals used for mating) during the second mating in F_1 animals. Histologically, the number of corpora lutea was decreased in F_1 females and half of the females (15 out of 30) in the highest exposure group were anovulatory or had disturbed estrous cycles. In males, no effects were seen on sperm parameters. The number of litters was reduced in the 700ppm group for the first mating in the F_1 generation and in the two highest dose-groups for the second mating. The number of live pups per litter was reduced in the 500 and 700ppm groups in the F_0 and F_1 generations and the number of implantation sites was reduced in the 700ppm group in F_0 females. Pup survival on PND 0 was reduced in F_{2b} pups from the 700ppm group.

Study assessment: This study is allocated a reliability rating of 1, reliable without restriction. The study was conducted according to EPA OPPTS Health Effects Test guideline (which is equivalent or similar to the OECD guideline 416) was conducted in compliance with Good Laboratory Practice (GLP) Regulations. It looks like there is a mistake in table 1 showing data on reproductive and developmental parameters from F_0, F_{1a}, F_{1b} and F_{1c} animals. The table covers more than one page, and it is probably data from F_{1b} and F_{1c} that are shown on page 2 and not data from F_0 and F_{1a} as stated in the first line of the table on page 2. Apart from this detail, the study is well-described and thorough, and the study is assessed to be of high quality.

Study summaries; Registration Dossier - ECHA:

The ECHA dissemination site contains multiple entries. A total of 152 study reports are included under toxicological information. Of these, more than 30 deal with D4 kinetics while at least 115 may contain information relevant for assessment of the endocrine disrupting potential of D4 in relation to human health. Around 20 of the entries are references to the published papers that have been presented in this report and to conference abstracts, while the majority are unpublished study reports. Access to these would be needed to include the data properly in the "lines of evidence" and evaluate their relevance for the ED assessment.

The reviews by Franzen et al 2017 and Dekant et al 2017 introduce many of these unpublished in vivo toxicity studies, thus providing evidence for their existence. Based on the information in these two review papers, a list of references has been compiled that includes 17 repeated dose toxicity studies, 25 reproductive toxicity studies, 6 liver enzyme induction studies, and 9 in vitro studies. This list of studies, including some of the published papers, is presented in this document as Annex 1.

3. "Line of Evidence" for adverse effects on female reproduction.

Lines of evidence – EAS modalities

The lines of evidence (LoE) for the modalities EAS and other modalities sensitive to, but not diagnostic of, EAS are assembled in the Excel file "Appendix E (D4_EDGD_Appendix-E1_EDdata)". This tabulation of all the available relevant data was used for making LoE for both *in vitro*, *in vivo* mechanistic and *in vivo* adverse effects, also included in the excel file.

In the present report, only the lines of evidence for *in vivo* adversity (female) via EAS modalities has been included and are presented below:

Table 2 Lines of evidence for adverse effects *in vivo* via EAS modalities (Effect target: Female reproductive system).

The colour codings for this table are: Orange= reduction red=increase green=no effect, refor to Appendix E1 to find study references according to study IDs.

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence
8	EATS-mediated	Age at Vaginal opening	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation		ppm	No effect (No effect, Absolute)	1/1 study found no effect on pubertal timing after developmental exposure (70-700 ppm).	**Evidence for effects on female reproduction** including effects on estrous cycle, ovary histopathology and weight, uterus histopathology, vagina histopathology, pregnancy parameters, and fertility.
8	EATS-mediated	Ano-Genital distance	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation		ppm	No effect (No effect, Absolute)	1/1 study found no effect on AGD after developmental exposure (70-700 ppm).	
4	EATS-mediated	Cervix histopathology	Rat	24	Months	Inhalation	700	ppm	Increase 700 (cervical squamous epithelial hyperplasia was observed in 5% (3/60) of high dose females at 24 months (compared to 0% in controls)., Absolute)	1/1 study observed effects on cervix histopathology after 24 months exposure. No systemic toxicity (bw) was observed in the females from this study.	

5	EATS-mediated	Estrus cyclicity	Rat	14	Months	Inhalation	700	ppm	Increased cumulative number of days (6966) in estrogen-predominant days (proestrous and estrous) in the estrous cycle in D4 group compared to controls (4152). Percent also increased.	2/2 studies observed effects on the estrous cycle. Increased cycle incidence and number of estrogen-predominant days in aging rats as well as abnormal estrous cycles (longer and irregular) in F1 females after developmental and continued adult exposure were observed. No effects were observed in F0 rats after adult exposure. No systemic toxicity was observed in these studies (bw, clinical signs, and food consumption).	
5	EATS-mediated	Estrus cyclicity	Rat	14	Months	Inhalation	700	ppm	Increased mean estrous cycle incidence compared to control		
5	EATS-mediated	Estrus cyclicity	Rat	14	Months	Inhalation	700	ppm	Increased duration of proestrous/estrous phases (2.4-3.5 days/cycle compared to control with 1-2 days/cycle)		
8	EATS-mediated	Estrus cyclicity	Rat	107-125 (F0)	Days	Inhalation		ppm	No evidence of exposure-related effects in F0 rats		
8	EATS-mediated	Estrus cyclicity	Rat	≥138 days (F1)	Days	Inhalation	700	ppm	15/30 F1 females in the 700 ppm group were anovulatory or had longer estrous cycles (5.3 days compared to 4.2 in controls)		

8	EATS-mediated	Estrus cyclicity	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation		ppm	The number of F1 non-pregnant rats with estrous cycle irregularities was 2/4, 8/10, 5/9, 10/12, and 15/18 in control, 70, 300, 500, and 700 ppm groups, respectively. No statistical assessment was made.		
8	EATS-mediated	Mammary gland histopathology (female)	Rat	107-125 (F0)	Days	Inhalation		ppm	No effect (No evidence of exposure-related effects, Absolute) (F0)	1/1 study found histopathological changes in mammary glands in F1 females after developmental and continued adult exposure, however, no statistical assessments were made, and no effects were observed in F0 females. No systemic toxicity was observed in this study (bw, clinical signs, food consumption).	
8	EATS-mediated	Mammary gland histopathology (female)	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation		ppm	(Number of mammary gland changes with ductal/acinar and/or lobular proliferation with secretory activity was 1, 6, 3, 5, 11 and number of rats with duct ectasia galactocele was 0, 3, 3, 1, 6 in control, 70, 300, 500, 700 ppm D4, respectively (out of groups of 30 animals). No statistical analysis was made, Absolute) (F1)		

5	EATS-mediated	Ovary histopathology	Rat	14	Months	Inhalation	700	ppm	Decrease 700 (Decrease (37%) in the incidence of antral-size atretic follicles in the ovaries, Absolute)	4/5 studies found histopathological changes in the ovaries of D4-exposed females. Changes occurred after adult exposure (24 months and 3 months but not after 4 months in the 2-gen. study or 3 days) and in aging rats as well as after developmental and continued adult exposure.	
4	EATS-mediated	Ovary histopathology	Rat	24	Months	Inhalation	700	ppm	Increase 700 (ovarian atrophy presented in 6.7% (4/60) of high dosed females at 24 months (compared to 1.7% in controls), Absolute)		
9	EATS-mediated	Ovary histopathology	Rat	3	Months	Inhalation	898	ppm	Increase 898 (Ovarian hypoactivity, meaning absence of corpora lutea; normal follicular development present, was increased in incidence in female rats from the 898-ppm exposure group (9/20 compared to 1/20 in control). No statistical analysis was carried out. The effect was not observed after 1 month recovery period (10 animals/group). Absolute)		
7	EATS-mediated	Ovary histopathology	Rat	3	Days	Inhalation		ppm	No change in the number of Corpora lutea was detectable among ovulating rats		
8	EATS-mediated	Ovary histopathology	Rat	107-125 (F0)	Days	Inhalation		ppm	No effect (No evidence of exposure-related effects, Absolute)		

8	EATS-mediated	Ovary histopathology	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation		ppm	Increase (Number of non-pregnant rats with abnormal ovaries and devoid of corpora lutea were increasing with exposure from 2/4, 6/10, 4/9, 5/12 and 13/18 in the control, 70, 300, 500, and 700ppm groups, respectively. No statistical analysis was performed. , Absolute)		
5	EATS-mediated	Ovary weight	Rat	14	Months	Inhalation	700	ppm	No effect 700 (No effect, Absolute and relative)	3/5 studies reported decreased ovary weight after ≥700 ppm D4 exposure. No effects were observed in a study with aging rats (5), or in a 2-generation study (8). In adult rats, short diestrus and/or proestrus exposure decreased ovary weight if exposure continued during proestrus (7) and 3 months repeated exposure also decreased ovary weight (9) - an effect that, however, was not observed after 1 month recovery. Lastly, 6 days of exposure for 3 days before mating and until GD3 decreased ovary weight (bw gain was also decreased in these animals), whereas other exposure scenarios (28 days pre-mating to GD19, 31 days pre-mating to 3	
9	EATS-mediated	Ovary weight	Rat	3	Months	Inhalation	898	ppm	Decrease 898 (Decreased in the 898 ppm exposure group (62% of control). But no effect was observed in animals that was allowed 1 month recovery (10 animals), Absolute and relative to brain weight)		
7	EATS-mediated	Ovary weight	Rat	2.1	Days	Inhalation		ppm	No effect (No effect, Absolute and relative)		
7	EATS-mediated	Ovary weight	Rat	3	Days	Inhalation	900	ppm	Decrease 900 (Decreased ovarian weight was observed in the 900 ppm group, both absolute and relative to body weight and brain weight, Absolute and relative)		

6	EATS-mediated	Ovary weight	Rat	37	Days	Inhalation		ppm	No effect (No effect, Absolute and relative)	days pre-mating, GD2-5) in the same study (6) did not affect ovary weights (despite affecting bw gain as well).	
6	EATS-mediated	Ovary weight	Rat	28	Days	Inhalation		ppm	No effect (No effect, Absolute and relative)		
6	EATS-mediated	Ovary weight	Rat	6	Days	Inhalation	700	ppm	Decrease 700 (Mean absolute maternal ovary weight was decreased in the 700 ppm group by 10.1% compared to control, Absolute)		
6	EATS-mediated	Ovary weight	Rat	4	Days	Inhalation		ppm	No effect (No effect, Absolute and relative)		
8	EATS-mediated	Ovary weight	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation		ppm	No effect (No effect on ovary weight, Absolute and relative)		
7	EATS-mediated	Oviduct histopathology	Rat	3	Days	Inhalation	700	ppm	Reduction in the mean number of ova in the oviducts on the day of estrus in both exposure groups (4.8±0.97 and 2.8±0.71 ova/rat) relative to controls (8.7±0.78 ova/rat)	1/1 study reported effects on oviduct histopathology.	
5	EATS-mediated	Uterus histopathology (with cervix)	Rat	14	Months	Inhalation	700	ppm	Increase 700 (An increased incidence of cystic endometrial hyperplasia was observed (14/37 compared to control with 8/37), Absolute)	2/4 studies reported effects on uterus histopathology. Both studies reported an increased incidence of cystic endometrial hyperplasia in adult (24 months exposure) and	

4	EATS-mediated	Uterus histopathology (with cervix)	Rat	24	Months	Inhalation	700	ppm	An increased incidence of uterine cystic endometrial hyperplasia (from 19 % in controls to 50 % in treated females) was found in females exposed to 700 ppm D4.	aging rats (14 months exposure), respectively. After 24 months adult exposure endometrial adenomas were also observed, those were not observed in the control group. 3-4 months exposure did not induce effects on uterine histopathology.	
4	EATS-mediated	Uterus histopathology (with cervix)	Rat	24	Months	Inhalation		ppm	No effect (No statistically significant effect of D4 on incidence of uterine neoplasia, Absolute) But an increased incidence of endometrial adenoma was observed (4/60 in 700 ppm group vs 0/60 in the controls) this effect was not statistically significant.		
9	EATS-mediated	Uterus histopathology (with cervix)	Rat	3	Months	Inhalation		ppm	No effect (No effect, Absolute)		
8	EATS-mediated	Uterus histopathology (with cervix)	Rat	107-125 (F0)	Days	Inhalation		ppm	No effect (No evidence of exposure-related effects, Absolute)		
5	EATS-mediated	Uterus weight (with cervix)	Rat	14	Months	Inhalation	700	ppm	No effect 700 (No effect, Absolute and relative)	2/3 studies reported effects on uterine weight. 24 months adult exposure increased the uterine weight whereas	

4	EATS-mediated	Uterus weight (with cervix)	Rat	24	Months	Inhalation	700	ppm	Increase 700 (Uterine weight (absolute and relative) was increased (24%) in the 700 ppm exposure group at 24 months, Absolute and relative)	14 months in aging rats did not. In contrast 3 days exposure during diestrus and proestrous decreased the relative uterine weight, whereas 2.1 days did not. In this last study, bw was also decreased.	
7	EATS-mediated	Uterus weight (with cervix)	Rat	2.1	Days	Inhalation		ppm	No effect (No effect, Absolute and relative)		
7	EATS-mediated	Uterus weight (with cervix)	Rat	3	Days	Inhalation	900	ppm	Decrease 900 (Decreased uterus/body weight in 900 ppm group compared to control, Relative)		
5	EATS-mediated	Vagina histopathology	Rat	14	Months	Inhalation	700	ppm	Increase in severity was observed for epithelial thickness compared to controls	2/3 studies showed effect on vagina histopathology. Aging and adult rats showed increased mucification and epithelial thickness.	

5	EATS-mediated	Vagina histopathology	Rat	14	Months	Inhalation	700	ppm	Change 700 (D4-exposed animals also had a slightly lower incidence of vaginal mucification than controls – but for those animals in the D4-group that did display mucification there was a shift to moderate and severe severity.)	In adult rats this was concomitant with an increase in number of diestrus rats. In the 2 gen study this effect was not observed in F0 females after 4 months adult and gestational exposure.	
9	EATS-mediated	Vagina histopathology	Rat	3	Months	Inhalation	898	ppm	More female rats from the 898-ppm exposure group appeared to be in the diestrus stage of the rat estrous cycle (18/20 rats compared to 9/20 in control). No statistical analysis was made. At the end of the 1-month recovery phase, the estrous cycle, as inspected by the vaginal changes in the control and highest-exposure group, were similar		

9	EATS-mediated	Vagina histopathology	Rat	3	Months	Inhalation	898	ppm	Increase 898 (Mucification of the vaginal mucosa was increased in incidence and the thickness of the mucinous cell layer was more prominent in the 898ppm-exposure group (19/20 rats compared to 9/20 in control). No statistical analysis was made. After 1 month recovery no effects were observed (10 animals/group), Absolute)		
8	EATS-mediated	Vagina histopathology	Rat	107-125 (F0)	Days	Inhalation		ppm	No effect (No evidence of exposure-related effects, Absolute)		
6	Sensitive to, but not diagnostic of, EATS	Fertility (mammals)	Rat	37	Days	Inhalation		ppm	No effect (No effect of D4 on fertility indices compared to control, Absolute)	2/2 studies reported effects on fertility index. The first study (6) showed that a specific exposure at 1 day before mating decreased the fertility index, whereas other exposure scenarios (28 days pre-mating to GD19, 4 days pre-mating to GD1/3 or GD0-2) did not. In the 2-gen study, fertility and number of pregnant rats decreased only in the F1 females after	
6	Sensitive to, but not diagnostic of, EATS	Fertility (mammals)	Rat	1	Day	Inhalation	700	ppm	Decrease 700 (Fertility index decreased after D4 exposure 1 day before mating (64.7%) compared to control (95.5%), Absolute)		

6	Sensitive to, but not diagnostic of, EATS	Fertility (mammals)	Rat	4-6	Days	Inhalation		ppm	No effect (No effect on fertility index (%), Absolute)	developmental and continued adult exposure.	
6	Sensitive to, but not diagnostic of, EATS	Fertility (mammals)	Rat	1-3	Days	Inhalation		ppm	No effect (No effect of short post-mating exposure on fertility index (%), Absolute)		
8	Sensitive to, but not diagnostic of, EATS	Fertility (mammals)	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation	700	ppm	Decrease 700 (Fertility indices (%) were not affected in F0 rats but were decreased in 700 ppm F1 males and females (77% compared to 100% in controls), Absolute)		
8	Sensitive to, but not diagnostic of, EATS	Fertility (mammals)	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation		ppm	Increase (Number of non-pregnant F1 rats were increasing with exposure (4/30, 10/30, 9/30, 12/30, 18/30 in control, 70, 300, 500, and 700 ppm group respectively). No statistical assessment was made. Absolute)		

8	Sensitive to, but not diagnostic of, EATS	Gestation length	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation	700	ppm	Increase 700 (Gestation length was increased in F1 700ppm rats (22.1 days compared to 21.6 days in controls), but not in F0 rats., Absolute)	1/1 study showed an increased gestational length in F1 rats after developmental and continued adult and gestational exposure.	
8	Sensitive to, but not diagnostic of, EATS	Litter size	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation	500	ppm	Decrease 500 (Reductions in total litter sizes and live pups/litter were observed in the 500 and 700 ppm groups in the F0 generation and for the F1 animals in the first mating. No effects were observed on litter size when the F1 males were paired with unexposed females., Relative (per litter))	1/1 study showed a reduced litter size in F0 and F1 females. Bw gains (but not general bws) were also decreased in this study in accordance with fewer fetuses.	
6	Sensitive to, but not diagnostic of, EATS	Litter viability	Rat	37	Days	Inhalation	500	ppm	Decrease 500 (Number of viable fetuses was decreased in 500 and 700 ppm groups, Absolute)	2/2 studies showed effects on litter viability. Number of viable litters decreased following exposure from 28 days pre-mating to GD19 and 3 days pre-mating to GD3, but not 31 days pre-mating to 3 days pre-mating or GD2-5. In a 2-gen study 5 litter losses were observed in the 500 ppm F0 females. Bw gains (but	
6	Sensitive to, but not diagnostic of, EATS	Litter viability	Rat	28	Days	Inhalation		ppm	No effect (No effect, Absolute)		
6	Sensitive to, but not diagnostic of, EATS	Litter viability	Rat	6	Days	Inhalation	700	ppm	Decrease 700 (Decreased number of viable fetuses in D4 group (61% of control)., Absolute)		

6	Sensitive to, but not diagnostic of, EATS	Litter viability	Rat	4	Days	Inhalation		ppm	No effect (No effect, Absolute)	not general BWs) were also decreased in these studies in accordance with fewer viable fetuses.	
8	Sensitive to, but not diagnostic of, EATS	Litter viability	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation	500	ppm	Increase 500 (Litter loss: The number of total litter losses was comparable to controls in the F0 litters and all F1 litters (F2a, F2b, and F2c) except in the F0 500 ppm group where 5 litter losses were observed, Relative (per litter))		
6	Sensitive to, but not diagnostic of, EATS	Number of implantations, corpora lutea	Rat	37	Days	Inhalation	300	ppm	Decrease 300 (Decreased number of corpora lutea in 300 (90% of control) and 500 (87% of control) ppm groups. Decreased number of implantation sites in 500 (79% of control) and 700 (68% of control) ppm groups, Absolute)	2/2 studies observed effects on number of implantations/corpora lutea. Number of corpora lutea was decreased following exposure from 28 days pre-mating to GD19 and 3 days pre-mating to GD3, but not 31 days pre-mating to 3 days pre-mating, GD0-2, day 4/3/2/1 pre-mating, or GD2-5. In a 2-gen study F0 females had lower number of implantation sites whereas F1 females had fewer corpora lutea.	
6	Sensitive to, but not diagnostic of, EATS	Number of implantations, corpora lutea	Rat	28	Days	Inhalation		ppm	No effect (No effect, Absolute)		

6	Sensitive to, but not diagnostic of, EATS	Number of implantations, corpora lutea	Rat	6	Days	Inhalation	700	ppm	Decrease 700 (Number of corpora lutea decreased in D4 group (85% of control). Number of implantation sites decreased (69% of control), Absolute)		
6	Sensitive to, but not diagnostic of, EATS	Number of implantations, corpora lutea	Rat	4	Days	Inhalation		ppm	No effect (No effect, Absolute)		
6	Sensitive to, but not diagnostic of, EATS	Number of implantations, corpora lutea	Rat	1	Day	Inhalation		ppm	No effect (No effect of exposure on corpora lutea or number of implantation sites, Absolute)		
6	Sensitive to, but not diagnostic of, EATS	Number of implantations, corpora lutea	Rat	4-6	Days	Inhalation	700	ppm	Decrease 700 (Decreased number of corpora lutea in 'day -3 to GD3' exposure group (87% of control). No statistically significant effect on number of implantation sites. , Absolute)		
6	Sensitive to, but not diagnostic of, EATS	Number of implantations, corpora lutea	Rat	1-3	Days	Inhalation		ppm	No effect (No effect of short post-mating exposure on number of corpora lutea or number of implantation sites, Absolute)		

8	Sensitive to, but not diagnostic of, EATS	Number of implantations, corpora lutea	Rat	≥138 days (F1)	Days	Inhalation		ppm	Decrease (Number of rats with corpora lutea of pregnancy was 26/30, 20/30, 21/30, 18/30, and 12/30 rats in the control, 70, 300, 500, and 700 ppm groups, respectively. No statistical analysis was made., Absolute)		
8	Sensitive to, but not diagnostic of, EATS	Number of implantations, corpora lutea	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation		ppm	(Number of non-pregnant rats with reduced numbers or only old corpora lutea was present in all exposure groups but not in control (0/4, 2/10, 1/9, 5/12, 2/18 for control, 70, 300, 500, 700 ppm, respectively). No statistical analysis was made., Absolute)		
8	Sensitive to, but not diagnostic of, EATS	Number of implantations, corpora lutea	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation	700	ppm	Decrease 700 (Number of implantation sites was lower in 700 ppm F0 females (73% of control), whereas this parameter was not affected in F1 females., Absolute)		

8	Sensitive to, but not diagnostic of, EATS	Number of ovarian follicles	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation		ppm	No effect (No treatment-related differences in ovarian primordial follicle counts or number of sections with growing follicles present., Absolute)	0/1 studies found effects on follicle counts.	
6	Sensitive to, but not diagnostic of, EATS	Post implantation loss	Rat	37	Days	Inhalation		ppm	No effect (No statistically significant effect on % post-implantation loss in D4 groups, but absolute number increased with exposure from 500 ppm D4 (control 7.8%, 30 ppm D4 6.1%, 300ppm D4 7.5, 500 ppm D4 11.3%, 700 ppm D4 16.6%), Absolute)	1/1 study found increased post-implantation loss if exposure was from 3 days pre-mating to GD3 but not pre-mating exposure only, GD2-5 only, or from 28 days pre-mating to GD19.	
6	Sensitive to, but not diagnostic of, EATS	Post implantation loss	Rat	28	Days	Inhalation		ppm	No effect (No effect, Absolute)		
6	Sensitive to, but not diagnostic of, EATS	Post implantation loss	Rat	6	Days	Inhalation	700	ppm	Increase 700 (Increased post-implantation loss in D4 group (18%) compared to control (6.4%)., Absolute)		
6	Sensitive to, but not diagnostic of, EATS	Post implantation loss	Rat	4	Days	Inhalation		ppm	No effect (No effect, Absolute)		

6	Sensitive to, but not diagnostic of, EATS	Pre implantation loss	Rat	37	Days	Inhalation	700	ppm	Increase 700 (Increased % pre-implantation loss in 700 ppm group, Absolute)	1/1 study found increased pre-implantation loss if exposure was from 3 days pre-mating to GD3 or from 28 days pre-mating to GD19, but not pre-mating exposure only, GD2-5, GD0-2, or 3 days pre-mating to GD1/3	
6	Sensitive to, but not diagnostic of, EATS	Pre implantation loss	Rat	28	Days	Inhalation		ppm	No effect (No effect, Absolute)		
6	Sensitive to, but not diagnostic of, EATS	Pre implantation loss	Rat	6	Days	Inhalation	700	ppm	Increase 700 (Increased in D4 group (28.4%) compared to control (9.8%)., Absolute)		
6	Sensitive to, but not diagnostic of, EATS	Pre implantation loss	Rat	4	Days	Inhalation		ppm	No effect (No effect, Absolute)		
6	Sensitive to, but not diagnostic of, EATS	Pre implantation loss	Rat	1	Day	Inhalation		ppm	No effect (No effect on % pre-implantation loss, Absolute)		
6	Sensitive to, but not diagnostic of, EATS	Pre implantation loss	Rat	4-6	Days	Inhalation		ppm	No effect (No effect on % pre-implantation loss, Absolute)		
6	Sensitive to, but not diagnostic of, EATS	Pre implantation loss	Rat	1-3	Days	Inhalation		ppm	No effect (No effect of short post-mating exposure on % pre-implantation loss , Absolute)		

8	Sensitive to, but not diagnostic of, EATS	Pup survival index	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation	700	ppm	Decrease 700 (Pup survival of F2b pups was significantly reduced ($p < 0.05$) in the 700 ppm offspring at PND 0 (87.6% compared to 97.8% in control). No other relevant effects on pup survival were noted, Relative (per litter))	1/1 study found reduced pup survival in the F2 generation.	
6	Sensitive to, but not diagnostic of, EATS	Time to mating	Rat	37	Days	Inhalation		ppm	No effect (No effect, Absolute)	0/2 studies found effects on time to mating.	
6	Sensitive to, but not diagnostic of, EATS	Time to mating	Rat	28	Days	Inhalation		ppm	No effect (No effect, Absolute)		
8	Sensitive to, but not diagnostic of, EATS	Time to mating	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation		ppm	No effect (No effect, Absolute)		
6	[Not in list]	[Not in list]	Rat	37	Days	Inhalation	700	ppm	Decrease 700 (Gravid uterine weight decreased after 700 ppm D4 exposure, Absolute)	1/1 study showed decreased gravid uterine weights following exposure from 3 days pre-mating to GD3 or from 28 days pre-mating to GD19, but not pre-mating exposure only, or GD2-5, or GD0-2.	
6	[Not in list]	[Not in list]	Rat	6	Days	Inhalation	700	ppm	Decrease 700 (Gravid uterine weight decreased after 700 ppm D4 exposure, Absolute)		

6	[Not in list]	[Not in list]	Rat	4	Days	Inhalation		ppm	No effect (No effect on gravid uterine weight, Absolute)		
6	[Not in list]	[Not in list]	Rat	1	Day	Inhalation		ppm	No effect (No effect on gravid uterine weight, Absolute)		
6	[Not in list]	[Not in list]	Rat	4-6	Days	Inhalation	700	ppm	Decrease 700 (Decreased gravid uterine weight in 'day -3 to GD3' exposure group, Absolute)		
6	[Not in list]	[Not in list]	Rat	1-3	Days	Inhalation		ppm	No effect (No effect of short post-mating exposure on gravid uterine weight, Absolute)		
6	[Not in list]	[Not in list]	Rat	28	Days	Inhalation		ppm	No effect (No effect on gravid uterine weight, Absolute)		
7	[Not in list]	[Not in list]	Rat	3	Days	Inhalation	700	ppm	Decrease 700 (Exposure to D4 at 700 and 900 ppm significantly decreased the proportion of rats that ovulated (79% of controls, 42% of 700 ppm, and 31% of 900 ppm exposed rats)., Absolute)	1/1 study showed decreased number of ovulating rats following short diestrus and proestrous exposure.	

8	[Not in list]	[Not in list]	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation	700	ppm	Decrease 700 (Mating indices (%) were not affected in F0 rats but in F1 males and females in the 700 ppm group they were decreased (down to 53-55% compared to 93% in controls). , Absolute)	1/1 study reported decreased mating indices in F1 males and females following developmental and continued adult exposure.	
8	[Not in list]	[Not in list]	Rat	107-125 (F0) or ≥138 days (F1)	Days	Inhalation	500	ppm	Decrease 500 (Litter number: The number of litters produced in the F1 generation in the 700 ppm group for the first mating and in the 500 and 700 ppm groups for the second mating were significantly reduced (59%, 65% and 46% of control, respectively)., Relative (per litter))	1/1 study reported decreased number of litters produced in an F1 generation after developmental and continued adult exposure.	

4. Discussion of "human relevance"

Dekant et al. 2017 have reviewed the human relevance of the D4-findings in rats. In the last section of their manuscript, they summarize the arguments presented in their paper in a section called "Synthesis regarding endometrial proliferative findings".
The arguments are posted as bullet points in the paper. We have provided responses to all of them below.

Argument: *corpus luteum function in rodents relies on adequate prolactin secretion by the pituitary gland, while prolactin is normally not involved in menstrual cyclicity in women. To the extent that D4 interferes with prolactin production through a dopaminergic mechanism, it would be expected to interfere with estrous cyclicity in rats but not in the menstrual cyclicity in women.*
Response: If we could be certain that the adverse reproductive effects seen in D4 exposed female rats were caused solely by disruption of the dopaminergic system (ultimately leading to altered prolactin secretion), then the argument that the effects are only relevant for rats and not humans could be correct. However, there is no available data to support the argument; that the adverse effects on female reproduction caused by D4 exposure is solely due to disrupted dopamine signaling, ultimately leading to altered prolactin secretion. In fact, there is data showing that D4 does not bind directly to the dopamine receptor and that any effects on this system caused by D4, are therefore more likely indirect. Dekant et al. 2017 even state that **"Studies performed to characterize the interaction of D4 with this pathway indicated that D4 is neither a potent nor a direct agonist at the dopamine receptor; however, the results suggested an indirect interaction with the dopamine pathway distal to the receptor".**

At the same time there are *in vitro* studies showing ERα binding at concentrations of 0.45 and 40 µM (He et al. 2003, Quinn et al. 2007a), ERα activation at a concentration of 10 µM (Quinn et al. 2007a), and uterotrophic studies in ovariectomized mice and rats (He et al. 2003, Quinn et al. 2007a) showing increased uterine weights after 3-4 days of D4 exposure. Such effects were also seen in immature SD rats where the HPG axis is not yet fully functioning (McKim et al. 2001a). This indicates that D4 has a clear estrogenic potential and that it may very well be mediated directly through binding and activation of the estrogen receptor (ER).

Argument*: It is likely that cycle disruption occurred over time in F344 females exposed to D4 due to either an inhibition by D4 of pituitary prolactin production and/or through modulation of the LH surge leading to an increased endogenous E2 signal to the uterus. Neither mechanism would be relevant to human risk due to differences between rat and human in pituitary control of the female reproductive cycle.*
Response: As explained above, there is no data to support that disruption of prolactin production is the main mechanism responsible for altered reproductive effects seen after D4 exposure, even though it may be a contributing factor. The D4-induced effects on the LH surge are not irrelevant in terms of potential human risk. Even though there may be species differences in how various hormones control the menstrual/estrous cyclicity, LH-secretion also plays a crucial role in

regulating the human menstrual cycle. Therefore, chemicals that cause disruption of LH-secretion can potentially also affect reproductive capacity in women.

***Argument**: No tumors were associated with chronic D4 exposure of male F344 rats and no organs other than the uterus developed treatment-related proliferative lesions in female F344 rats following chronic D4 exposure.*
Response: while it is correct that no tumors were seen in the male rats, clear adverse effects were seen in the testes of male rats exposed for two years. The effects were reported as increased incidence of interstitial cell hyperplasia at 24 months of 21.7% (13/60) after exposure to 150 ppm D4, and 26.7% (16/60) after exposure to 700 ppm D4. Controls showed an incidence of 11.7%. Thus, it was not only the female's reproductive system that was affected by the long-term exposure to D4, but the male reproductive system as well.

***Argument:** Uterine cystic endometrial hyperplasia and adenoma in the female F344 rat arose during the 2nd year of exposure, a period of marked changes in physiology and onset of a reproductive senescence that is unique to the F344 rat, distinctly different from human, and often associated with increased endogenous E2 from ovarian cysts;*
Response: Adverse effects seen in the period of reproductive senescence in rats should not automatically be viewed as irrelevant for humans. While the specific mechanisms and reproductive hormones controlling reproductive senescence do differ between rats and humans, women also experience menopause mediated by changes in reproductive hormones. Furthermore, reproductive aging in the rats is not in itself a plausible explanation for the increased incidence of uterine adenomas, as control rats went through the same processes of reproductive aging, but none of them developed endometrial adenomas. The failure to recognize this fact (as in comparing data against the only proper control) is misleading.

***Argument**: The affinity of D4 for the estrogen and progesterone receptors is low to non-existent. It is unlikely that the demonstrated weak estrogenicity of D4 was involved in the uterine effects that developed in the aging F344 rat in response to D4 exposure either in the chronic bioassay or in animals treated from 11 to 25 months of age, because there were no other indications of a weak estrogenic response in either males or females in the chronic bioassay or a two-generation study (Batelle, 2004; Siddiqui et al., 2007; Jean et al., 2016a*);
Response: while it is correct that D4 does not seem to bind to the progesterone receptor, this is not the case for the estrogen receptor. Several studies have shown binding to, and activation of, ERα at concentrations of 0.45 and 40 µM (He et al. 2003, Quinn et al. 2007a). While the binding affinity of D4 is lower than that of estradiol, it does not necessarily mean that it is weak. Studies have shown that D4 has comparable potency for binding to the ERα as e.g., BPA or butylparaben – compounds that presently are identified and regulated as endocrine disrupting chemicals in the EU, through an estrogenic mode of action. In Quinn et al. 2007a the potency of D4 was compared to that of BPA in the ERα-reporter gene assay, and here 10µM of D4 caused a 5-fold induction of the reporter gene compared to control, while 10µM of BPA resulted in an approximately 7-fold induction. Also, uterotrophic studies with D4 show an estrogen–mediated

effect, with increasing uterine weights and altered expression of estrogen-regulated genes and proteins (He et al. 2003, Lee et al. 2015, McKim et al. 2001a, Quinn et al. 2007a). Furthermore, it is incorrect to conclude that there were no other indications of estrogenic responses in the chronic bioassay or in the two-generation study. Males in the chronic study showed an increased incidence of interstitial cell hyperplasia in the testes (Jean & Plotzke 2017), an effect that could be mediated through an endocrine mode of action. Since there are no indications from the available studies that D4 acts as an anti-androgen, the adverse histopathological effects observed in the testes could be mediated through an estrogenic mechanism. In the two-generation study, a series of adverse effects were observed in both P and F1 females. These included decreased fertility and mating indices, increased gestation length, altered estrous cycle length, anovulation and histopathological findings in ovaries and mammary glands (Siddiqui et al. 2007). No effects were seen on anogenital distance (AGD) in the offspring. This is not surprising since most clear AGD reductions are caused by exposure to anti-androgenic chemicals, and there are no indications that D4 acts through an anti-androgenic mechanism of action (Schwartz et al. 2019). Age at sexual maturation was not affected either in the two-generation study. While developmental exposure to estrogenic chemicals can lead to perturbation in the timing of sexual maturation, this is not always the case, as it is not a very sensitive endpoint (Johansson et al. 2021). Therefore, lack of statistically significant effect on this endpoint cannot be used to dismiss the findings. In conclusion, we find that it is biologically plausible that many of these adverse effects seen in the 2-generation study can be mediated by an estrogenic mode of action.

Argument*: Although D4 is not a direct dopamine agonist, there were slight alterations in the dopamine activation pathway and modulation of prolactin concentrations following exposure to D4 that are suggestive of some interference with this pathway.*
Response: We agree with this statement but have not seen any data that would support the argument that dopamine activation by D4 is the main mechanism behind the observed adverse effects on female reproduction. Therefore, while it may be a contributing factor, we find it plausible that the direct estrogenic response caused by D4 exposure is the main driver of the observed effects.

Argument*: D4 exposure inhibits ovulation and can prolong exposure of the endometrium to endogenous estrogen in the rat. In addition, in aged rats (Jean et al., 2016b), D4 exposure produced a higher percentage of days for which the vaginal lavages exhibited a more estrogenic character. The higher percentage of days in proestrus/estrus in the D4 group appeared to be the result of prolonged estrogenic phases during the first half of the study (consistent with the LH surge study) followed by increased cycling (i.e., greater numbers of times in proestrus/estrus) during the second half of the study. If alteration of the LH surge with subsequent prolonged exposure of the uterine endometrium to endogenous estrogen is responsible for cystic endometrial hyperplasia and adenomas, it is unlikely this effect would occur in humans due to the marked differences in reproductive function, brain regulation of LH secretion, and the mechanism of reproductive aging and the hormonal environment of reproductive senescence in the rat versus human.*

Response: Arguments against all these points have been stated above.

Argument: *Cystic hyperplasia without atypia in female humans is not a cancer precursor, and there is no endometrial lesion in women that is directly analogous to endometrial adenoma in the rat.*
Response: While this may be correct, the present argument is not whether D4 should be classified as a carcinogen, but rather as an endocrine disrupter. While the specific form of endometrial cancer observed in the animals may not occur in the same form in women that does not exclude this from being an adverse effect in rats caused by hormonal imbalances induced by D4 exposure.

Argument: *The uterine effects were only seen following exposure to the highest exposure concentration of D4 (700 ppm). Sarangapani et al., 2003 developed a pharmacodynamic extension to a physiologically based pharmacokinetic (PBPK) model to characterize dose-response behaviors of cytochrome P450 induction following inhalation exposure to D4. This evaluation showed that at exposures greater than 300 ppm there was an apparent saturation of liver enzymes with subsequent decreasing liver metabolism suggesting that the high doses of D4 used in the toxicity studies may have exceeded the rats' physiological capacity to handle the chemical. The effects seen above this exposure concentration are of questionable toxicological relevance when compared to actual human exposures*.
Response: The use of 700 ppm as the top dose is not of questionable toxicological relevance. The registrant has chosen to perform more than 50 animal studies using inhalation exposure, and in the majority of them, a dose of 700 ppm has been included. At this dose, severe signs of systemic toxicity are only very rarely seen, and in most studies, no mortality and no significant body weight reductions are seen. Some studies show increased liver weights at this dose, but the authors themselves argue that this is a compensatory adaptation and not an adverse effect. Therefore, there is no data to support that this exposure should exceed the rats' physiological capacity to handle the chemical.

Argument: *In summary, the available information suggests that the induction of benign proliferative endometrial lesions in the rat after chronic D4 inhalation has no relevance for human risk characterization. Due to the absence of genotoxicity of D4 and absence of any appreciable direct hormonal activity of D4, the induction of cystic endometrial hyperplasia and the significant trend for an increased incidence of uterine endometrial adenoma observed across D4 dose levels in the two-year inhalation study are likely due to interferences of D4 with rat estrous cycle control that are only seen at doses that exceed the metabolic capacity of animals and not relevant to women*
Response: Replies to all these statements have been provided in the above responses.

Additional incorrect statements in the manuscript (Dekant et al. 2007)
Dekant et al. 2007 (page 45): No developmental effects of D4 were observed in the developmental toxicity studies, and reproductive effects induced by D4 inhalation were not observed in male animals in any of the studies.
Comment: This is clearly incorrect, since an increased incidence of interstitial cell hyperplasia was seen in the testes of male rats exposed for two years, and developmental effects in the form of decreased number of live pups per litter and decreased pup survival was seen in the 2-generation study.

Dekant et al. 2007 (page 45): The timing and duration of exposure of female rats to D4 required to induce female-specific reproductive toxicity is limited to a very narrow time-window immediately prior to ovulation since a single 6-h exposure to D4 on the day prior to mating resulted in a statistically significant reduction in fertility (Meeks et al., 2007).
Comment: It is correct that adverse effects on the female reproductive system have been observed after an only 6-h exposure prior to ovulation. This, however, does not mean that the adverse effects caused by D4 exposure are necessarily limited to this narrow time window. Uterotrophic effects have been reported in both immature and ovariectomized females, so these are definitely not caused by exposure in the short pre-ovulatory time window. The observed adverse effects on uterus, cervix, vagina, and ovaries in sub-chronic and chronic studies, may be in part caused by altered ovulatory regulation, but could just as well be caused by other mechanisms**.**

Dekant et al. 2007 (page 46): Although D4 has weak estrogenic/anti-estrogenic activity (He et al., 2003; Quinn et al., 2007b; McKim et al., 2001b), there were no reported indications of estrogenic or anti-estrogenic effects in male rats, in estrogen-sensitive tissues in females, or in hormone-related developmental landmarks, including anogenital distance, in rat pups in a two-generation reproductive developmental study with D4. It is unlikely that the very weak activity of D4 in estrogenic assays is responsible for the increase in the endometrial proliferative lesions seen in the 2-year chronic bioassay
And repeated on (page 48): D4 possesses only very weak estrogenic and anti-estrogenic activity in rats and has a low affinity for estrogen receptor-a (He et al., 2003)…A direct hormonal effect of D4 on endometrial cells is unlikely as a mode of action for D4-associated endometrial hyperplasia and adenoma in the aging F344 rat.

Comment: Providing our response to the latter statements is not necessary, as our counterarguments have already been presented in the answers to the arguments above.

Additional similar arguments

As seen below additional similar arguments have been presented in papers by Meeks et al 2007, Quinn et al 2007b and Franzen et al 2017.

– These are listed below, but our responses have not been provided in order to avoid repetition.

Meeks et al 2007 (page 198): While D4 has been shown to be a very weak partial estrogen agonist, the relative potency of D4 was several orders of magnitude less than other estrogenic compounds such as ethinylestradiol, diethylstilbestrol, and several times less than coumestrol (D4 was ~0.6 million times less potent in SD rats when compared to ethinylestradiol) [13]. However, the observations on the reproductive studies with D4 are not consistent with an estrogenic mode of action. For example, there were no effects on male or female reproductive organs or male accessory sex organs, sperm counts, sperm production rate, sperm motility or morphology, vaginal patency, balanopreputial separation, or anogenital distance or male reproductive performance [8]. Endpoints such as vaginal patency and balanopreputial separation [14,15] are endpoints that are typically affected with exposures to estrogenic compounds. Further, the lack of effects on male reproductive organs and sperm counts, sperm production rate, sperm motility or morphology also calls into question estrogenicity as a mode of action for the D4 reproductive effects.

Meeks et al 2007 (page 200): Analogous mechanisms control ovulation in both rats and humans. In both species, ovulation is triggered following a surge of LH. However, the control mechanisms for the timing and release of LH from the pituitary are quite different in rodents and in humans [23,24]. The LH surge in humans is much broader than in rats, and the LH release, ovulation, and mating behavior are all intimately linked and critically timed in rats, but not in humans [25]. Thus, it is possible, the effect of a shift in humans would not have the impact that it would in rats.

Meeks et al 2007 (page 200): In rodents, the LH surge is dependent on hypothalamic GnRH release via afferent noradrenergic nerves [27]. Administration of -adrenergic antagonists, such as phenoxybenzamine, or barbiturates such as phenobarbital, can attenuate or completely block the LH surge in rodents [27]. Conversely, in humans, noradrenergic neurons do not play a role in the release of LH, and -adrenergic antagonists or sedating doses of phenobarbital are not associated with disruption of LH release [28]. Therefore, it is possible that in rodents D4 will act in a similar manner to phenobarbital by disrupting norepinephrine-mediated neurotransmission, with a subsequent inhibition of GnRH release and a disruption of the LH surge. If D4 acts through this pathway, it is unlikely that D4 would disrupt the human LH surge.

Quinn et al 2007b (page 539): There is evidence to suggest that D4 can elicit weak estrogenic and/or antiestrogenic activity both in vitro and in vivo [20,21] although the relative potency was several orders of magnitude less than other estrogenic compounds, such as ethinyl estradiol, diethylstilbestrol and several times less potent than coumestrol. Additionally, data from reproductive studies conducted to date do not support a mode of action involving a direct

estrogenic affect [22,23]. For example, no effects were seen on estrogen-sensitive developmental endpoints, such as anogenital distance, time to either vaginal patency or balanopreputial separation in a two-generation reproductive toxicity study. It is improbable, therefore, that the estrogenic activity of D4 can account for the effects seen in the one- and two-generation studies.

Quinn et al 2007b (page 539): D4 may be acting as a dopamine D2 receptor agonist. Recent experiments indicate that D4 exposure can result in a decrease in prolactin levels following a 6 h exposure in reserpine treated animals [27]. A small decrease in prolactin was seen in this study at the 2 p.m. time point. This is relevant as a prolactin peak proceeds the LH surge in rats and may have some effect on follicle maturation. Agents which could alter prolactin levels could impact the LH surge and subsequent ovulation within the context of the relatively short 3-day exposure scenario outlined in this report.

Quinn et al 2007b (page 539): It is also possible that the effect on the LH profile seen in this report is a general short term response to an irritating or stressful inhalation exposure, which may result in an interruption in the normal LH surge and associated hormonal profile.
Rats have a precisely timed, light and stress sensitive, brain regulated pre-ovulatory LH surge, whereas the primate pre-ovulatory LH surge is controlled by the ovary and is neither light sensitive nor as precisely timed [30,31]. When considering the impact on human health, the control mechanisms for the timing and release of LH from the pituitary are quite different in rodents compared to humans. Rodents have a critical period for the pre-ovulatory LH surge, whereas humans do not. The D4 mediated effects demonstrated here, whether due to a phenobarbital-like action, alterations in GnRH release, a dopamine-like effect or a generalized stress mechanism, would result in an attenuation of the release of LH which may have little impact, if any, in humans compared to the rodent model.

Franzen et al 2017 (page 17): The decrease in female rat reproductive capability after inhalation of D4 is consistent with impaired ovulation due to a shift in preovulatory LH surge. This effect might be due to inhibition of preovulatory prolactin (Quinn et al., 2007a). As reported in Dekant et al. (2017), D4 might act as a dopamine agonist, perhaps downstream of receptor activation, and might thereby reduce prolactin release. Whereas prolactin is required for normal ovulation in rats, it does not appear to play a role in human ovulation. Indeed, bromocriptine appears to facilitate ovulation even in women with normal plasma prolactin concentrations (Porcile et al., 1990; Yasui et al., 1990). Therefore, the impairment of fertility in female rats exposed by inhalation to D4 is of questionable relevance for human reproductive risk assessment.

Franzen et al 2017 (page 18): In summary, the affinity of D4 for the estrogen and progesterone receptors is low to non-existent as determined in various in vivo studies. Dekant et al. (2017) concluded that "it is unlikely that the very weak activity of D4 in estrogenic assays is responsible for the increase in the endometrial proliferative lesions seen in the 2-year chronic bioassay".

Franzen et al 2017 (page 19): Clinical studies with dopamine agonists show no effect on follicle stimulating hormone, luteinizing hormone, estrogen levels, progesterone levels or endometrial histopathology in women. Based on the lack of effects observed in the clinical studies, the tumorigenic effect of dopamine agonists in female rats is considered a species-specific effect with no risk to human health (Burek et al., 1988). In summary, the studies conducted to investigate the direct interaction of D4 with the dopamine receptor have not provided evidence of a precise mode of action, and the subtlety of the effects observed may prevent further assessment (Dekant et al., 2017). While D4 was not a direct dopamine agonist, slight alterations in the dopamine activation pathway and modulation of prolactin levels after D4 exposure are suggestive of some indirect interference with this pathway (Dekant et al., 2017).

Franzen et al 2017 (page 19): The reproductive effects reported in the female rats in the two generation reproductive study (Siddiqui et al., 2007) and the additional studies (Quinn et al., 2007a,b; He et al. 2003; Lee et al. 2015) conducted to assess the potential endocrine activity of D4 have suggested that D4 has very weak estrogenic and antiestrogenic activity. However, there are observations in the reproductive studies that don't support the direct effect of D4 as a weak estrogen and that are inconsistent with this activity (Siddiqui et al., 2007), thus indicating the very weak hormonal potency of D4. A more relevant explanation for the reproductive toxicity is induction of a delay of the LH surge necessary for optimal timing of ovulation (Quinn et al., 2007a,b). An insufficient or blocked pre-ovulatory LH surge fails to induce complete ovulation in the rat and results in the reduced litter size observed following exposure. However, the current understanding of estrous cyclicity and neural/hormonal regulation of ovulation in humans suggests that the effects of D4 on fertility as observed in the rat are unlikely to be relevant to humans (Plant, 2012; Dekant et al., 2017).

Franzen et al 2017 (page 19): The remaining treatment-related endpoint identified following inhalation exposure to D4 was the statistically significant increase in the incidence of endometrial epithelial hyperplasia and a significant positive trend for the incidence of benign endometrial adenomas observed only in female rats exposed to the highest concentration tested (700 ppm). Dekant et al. (2017) examined the biological relevance and possible modes of action for these effects observed in the F344 rat following chronic inhalation exposure to D4. They reported that endometrial adenomas are an unusual lesion in rats, have no malignant potential and there is no endometrial lesion in women that is directly analogous to the endometrial adenoma in the rat. They further hypothesize that an alteration in the estrous cycle in the aging F344 rat was the most likely mode of action for the observed uterine effects following chronic inhalation exposure to D4. It should also be noted that although the mode of action for the induction of uterine adenomas in the female F344 rat has not been specifically defined, the available data suggest that the observed benign tumors are not relevant to human health (Dekant et al., 2017). D4 has not been shown to be mutagenic or genotoxic in in vitro or in vivo experimental models, therefore the observed tumors likely occur by a non-genotoxic mechanism. In addition, no tumors were observed in male F344 rats and no proliferative lesions reported in any other hormone dependent tissues other than the uterus of female F344 rats following chronic D4 exposure.

4. Preliminary conclusion on the endocrine disrupting properties of D4

The ED list report (Hass et al. 2018) concluded that there was: "*Strong evidence for an estrogenic mechanism of action of D4, and strong evidence for adverse effects on the female reproductive system that may be related to this estrogenic mode of action of D4*".

In the present assessment, we have tabulated all the relevant information from the publicly available studies and made lines of evidence for all observed ED-related mechanisms and adverse effects, including those on female reproduction. We assess that in order to make any firm conclusions regarding the ED effects of D4, it would also be necessary to include in the lines of evidence the information from all the unpublished study reports. This was however not a part of the present project, and furthermore a "mode of action analysis" was not performed, so the argumentation for ED effects of D4 has not been fully developed here. Despite these limitations, we find that there seems to be sufficient evidence to conclude that D4 exposure, via an estrogenic mechanism of action, can lead to adverse effects on female reproduction.

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Appendix 1

References collected from review articles by Franzen et al 2017 and Dekant et al 2017

Studies that stand next to each other and where the entire title is colored in the same color (red, blue, orange, green), are proposed to be the same study - but with a small difference in the title which means that we have included both.

Repeated dose toxicity studies

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DCC, 1992. Dow Corning Corporation—A 14-day oral gavage study of D4 in female rabbits. Report No. 1992-I0000-37117, April 28, 1992.

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DCC, 1995. Dow Corning Corporation, RCC Group—One-month repeated dose inhalation toxicity study with D4 in rats. Report No. 1995-I0000-40168, March14, 1995.

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