Comments on DECOS draft document on the reproductive hazards of tretinoin

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SECTION & PARAGRAPH	COMMENT
General Comments	This document aims to review the effects of tretinoin on reproduction/adverse reproductive outcomes. This includes male and female fertility, *in utero* development, development during the post-natal period, and the effects on lactation. This evaluation fails to make a clear distinction between all-trans retinoic acid, which is naturally produced in our bodies after Vitamin A intake, and tretinoin, which, even though it is a retinoic acid, usually refers to the drug preparation. The lack of differentiation and explanation between the two is a source of confusion in this document. The terms should not be used interchangeably, and the committee should be careful to not summarize every exposure as "tretinoin" and should instead use the language that is originally used in the referenced papers. The title of this document refers to toxicity of tretinoin. The authors have spent a considerable amount of time discussing toxicity to isotretinoin exposure, and since they are isomers of one another (trans- and cis- retinoic acid) their structures are different. This needs to be clarified further for the reader. It would be very helpful in the beginning of this document to discuss what Vitamin A is, why humans need it and how it is supplemented since it is not naturally produced, and then discuss the derivatives of Vitamin A such as retinol and retinoids, and ultimately retinoic acid. Then it needs to be made very clear that tretinoin and isotretinoin are the names given to medications that are retinoic acids. It would make this document much stronger and easier to follow. The document summarizes many studies both in humans and animals. The summaries should be more streamlined and not verbatim from the articles unless discussing experimental parameters. There are often too many details which makes understanding the actual point of the summary difficult. The reader can always reference the citation if they want all the details. In looking at all the summaries of the papers, the committee starts every sentence with the author's

	name and year, followed by a sentence that "X" was investigated", and this is very repetitive. It would look cleaner if it were something like this (example from pages 16-17): *Topical Exposure* a. Louerio *et al*. (2005) A prospective study was conducted…. *b.* Shapiro *et al. (1997)* The effects of tretinoin exposure…. c. Jick *et al.* (1993) The effects of first trimester exposure…. In article summaries, sometimes the committee will say "data not shown," or "maternal toxicity not described." While the data may not have been shown in the original article, there is no point to repeat that in the summary or mention the lack of maternal toxicity. Throughout the document, the authors use the phrase "effects on or via lactation." Stating "effects on lactation" is enough. The authors have referenced several studies, in which retinoic acid was purposefully used as a positive control to induce a neural tube defect (i.e., spina bifida), MMC, cleft palate, clubfoot, etc. When retinoic acid is used this way, these studies don't necessarily pose the question of whether retinoic acid is teratogenic or not or has any effect on development. The authors in these studies are specifically using retinoic acid to induce toxicity, to answer very specific questions or look for biomarkers. It is a very fine distinction to make, but I do not believe they should be included. Alternatively, if the committee wishes to include them, these studies in which RA is purposefully being used to induce toxicity should have their own section.

Page 5, Lines 11-12	This opening sentence is misleading and needs to be re-written for clarity. The term tretinoin is used when referencing drug preparations, not the endogenously produced retinoic acid, which is a derivative of Vitamin A. The authors should also clarify that Vitamin A is not produced by humans, and since it is essential to our health, intake occurs either through foods or supplementation. It is also misleading to say that tretinoin itself plays a crucial role in cellular processes during embryogenesis. Retinoids, however, which are chemicals that are structurally and/or functionally like retinol (Vitamin A), are essential for embryonic development.
Page 5, Line 15	Mention that the use of tretinoin for promyelocytic leukemia is in the form of chemotherapy, whereas for other cancer types, it is often used in combination with other medications.
Page 5, Lines 16-17	Suggested re-wording: "Occupational exposure to tretinoin can occur in laboratory, pharmacy, and hospital settings."
Page 5, Lines 19-20	Suggested re-wording: "The committee has evaluated the effects of tretinoin exposure on both male and female fertility, *in utero* development, and the development of the offspring during the post-natal period. The committee also evaluated the effects of exposure on lactation."
Page 5, Line 33	After reading the document in its entirety and then returning to this section on the committee's classifications, the following stands out: Does a distinction need to be made between tretinoin and isotretinoin for classification purposes? Or perhaps use a broader term such as Retinoic Acid. "….the committee recommends not classifying the effects tretinoin/isotretinoin exposure on male or female fertility due to a lack of studies and findings." It needs to be re-worded somehow so that both male and female fertility is included.
Page 6, Lines 1-3	I believe that there are enough data presented so that retinoic acid can be categorized as Category 1A, a known human reproductive toxicant. The committee itself has cited many incidents of cleft palate, clubfoot, skeletal malformations, hindlimb malformations, etc. in animals, and the FDA also

	refers to Accutane (isotretinoin) as "highly teratogenic." One could argue that the classification should even be H360Fd instead. Though data are lacking on lactation, I think that it needs to be mentioned somewhere that caution should be exercised. Not sure if also using H362 would be too conservative.
Page 6, Lines 4-5	Remove "or via"
Page 7, Lines 21-22	Remove "or via"
Page 9, Line 23	A starting date should be included (the date of your oldest reference), and then re-worded to say "Literature searches were conducted……and CAPLUS between Month, Year and August 2012."
Page 9, Line 25	Instead of handbook, use "textbook", or "relevant textbooks." Where did the collection of most recent reviews come from? Were they not part of the original literature search?
Page 9, Line 26-27	This sentence ("Additional searches…") should go after the end of the first sentence of this section. In doing so, it would keep all the websites used together. Why are there two separate searches listed for Toxline? First it is stated on Line 23 that a Toxline search was done from "X to August 2012" and then again from September 2012 to October 2019. A suggestion would be to only list Toxline once, using a timeframe of "X to October 2019."
Page 9, Line 26	Were these websites from reliable sources? Was it toxicology in general? Reproductive toxicology? This needs to be more specific, and list the websites used.
Page 9, Lines 28-40	Suggestion: In the opening sentence of Section 1.4, the online databases listed in order are Toxline, Medline, CAPLUS, and then PubMed (Line 27). The search criteria should appear in the same order, so Toxline would be first, and then PubMed. Why are only the search terms for PubMed and Toxline mentioned, when MEDLINE and CAPLUS were also used? For the search terms used, why wasn't "Tretinoin AND Reproduction" included? Even if the search term was limited to the past 5 years, it

	yields 273 results which may include relevant publications that were missed in more specific searches such as "prenatal exposure delayed effects." The search term in PubMed "Tretinoin AND Male Fertility" also yields 10 results which appear to be relevant to this evaluation.
Page 10, Line 1	Replace the word "Describes" with "evaluated."
Page 10, Lines 2-3	It is unclear how the document quality was assessed and use of the word "quality" is repetitive if the sentence is left as-is. This sentence should be simplified. The words "as well" can be removed after "Annex A."
Page 10, Lines 5-7	The purpose of this document is to evaluate the effects of tretinoin on male/female reproduction. It is understood that the toxic effects that occur would be as a result of using tretinoin for "therapeutic" reasons such as to remove wrinkles, dark spots, acne, etc. This sentence seems unnecessary and is confusing.
Page 12, Line 2	Is there another word that can be used in place of "essentiality"? After reading the rest of the paragraph, maybe use "background", or "introduction."
Page 12, Lines 3-4	It is misleading to say that tretinoin is "found endogenously." The term would be retinoic acid.
Page 12, Lines 6-7	After a thorough review of this reference, the word tretinoin is only mentioned once, and that is in the references section. Even then, it's isotretinoin. To make the statement that tretinoin is vital for maintenance of pregnancy based on the referenced Spiegler et al. paper is inaccurate. What this paper *does* state, is that the developing mammalian embryo requires retinoic acid (first sentence of the abstract).
Page 12, Line 8-10	The correct phrasing would be that all-trans retinoic acid can be isomerized to 13-cis retinoic acid and vice versa. However, the literature seems to suggest that the isomerization of 13-cis retinoic acid to all-trans retinoic acid is specific to certain cell types. This sentence needs to be clarified further. Reference: Tsukada, M. et al. "13-cis Retinoic acid exerts its specific activity on human sebocytes

	through selective intracellular isomerization to all-trans retinoic acid and binding to retinoid acid receptors" (2000) Journal of Investigative Dermatology Volume 115, Issue 2 Pages 321-327
Page 12, Line 11	The reference that is used to show that isotretinoin is teratogenic is from more than 30 years ago. If this reference is the first to show that isotretinoin is a teratogen, then that needs to be specified. There are newer references that explain teratogenicity as related to isotretinoin. References: 1. Melink, B.C. "Apoptosis May Explain the Pharmacological Mode of Action and Adverse Effects of Isotretinoin, Including Teratogenicity" (2017) Journal: Acta Derm Venereol Volume: 97 Pages: 173-181 2. Bauer et al. "Isotretinoin : controversies, facts, and recommendations" (2016) Journal: Expert Review of Clinical Pharmacology Volume 9, Issue 11 Pages 1435-1442
Page 12, Lines 11-13	The authors need to be more specific. What caused the malformations? Was it exposure to isotretinoin during a certain trimester of pregnancy? Throughout pregnancy?
Page 12, Lines 15-16	Is this still in reference to the citation from 1985?
Page 12, Lines 17-18	Are the authors referring to retinoids?
Page 12, Lines 18-20	Reference 5, the title of this paper specifically says Retinoids. This reference also discusses differences in levels of retinol, and the concentrations of all-trans retinoic acid and 13-cis retinoic acid. The phrases "tretinoin" and "isotretinoin" in reference to serum is never mentioned.
Page 12, Lines 21-22	1. Reference 15 refers to pediatric patients, so it would be an inaccurate reference if referring to women. Moreover, reference 15 specifically refers to the treatment of patients with all-trans retinoic acid tablets. The discussion does briefly mention the conversion of all-trans retinoic acid to 13-

	cis retinoic acid (page 404), but not isotretinoin.
Page 12, Entire "Isomerization" section	The beginning of this section mentions that isomerization of tretinoin to isotretinoin is important because isotretinoin is a known human teratogen. There is the mention of one reference in which birth defects were detected. The remainder of this section inaccurately describes treatment of patients with tretinoin, and it fails to establish a link between 13-cis retinoic acid (isotretinoin) and birth defects or any other reproductive hazards. It is not clear why there is an entire section dedicated to the isomerization of all-trans retinoic acid to 13-cis retinoic acid.
Page 13, Lines 1-2	It is an established fact that isomerization takes place, which is not solely based on the references cited.
Page 13, Lines 2-5.	Because this section only makes one statement to one reference from 1985 concerning the teratogenicity of isotretinoin, this sentence makes some very broad and unverified claims. It is also of concern that the authors seem to have taken these references, in which patients were specifically exposed to all-trans retinoic acid, or in the case of pregnant women, where serum levels of retinoids were measured, and substituted these words with "tretinoin" and "isotretinoin." These phrases are not interchangeable in this context.
Page 13, Lines 6-15 (Kinetics)	The purpose of this document is to identify the potential reproductive hazards associated with tretinoin exposure. This section on kinetics is based off Reference 6, which is a review of the teratogenicity of isotretinoin. Nowhere in lines 6-15 on page 13 is there mention of the teratogenicity of isotretinoin. I would suggest to either re-write this entire section so that it keeps the focus on the reproductive hazards of tretinoin/isotretinoin as related to toxicokinetics and metabolism or eliminate it entirely.
Page 13, Lines 16-23 (Fetal/Maternal Ratio)	This paragraph could be re-worded so that it is clear what the committee is trying to convey.

	Some of the major findings of this study include: 1. Levels of serum retinol are relatively stable throughout gestation. 2. Compared to controls, serum levels of retinols were lower in pregnant and parturient women. 3. All-trans retinoic acid is the only retinoid which seems to increase in the 3rd and 4th months of pregnancy. 4. Newborns had significantly lower levels of serum retinol concentrations than their mothers, as well as lower levels of the active metabolite all-trans retinoic acid in the placental cord serum when compared to their mothers. 5. It is likely that the teratogenic effects of retinoids come mainly from all-trans retinoic acid. If re-worded, this section would be more relevant, and is necessary to this document. Also, the units are missing concerning blood.
Page 13, Lines 25-26	The literature that exists is on too much or too little Vitamin A, not tretinoin. To make this claim, you would need references.
Page 13, Line 27	What is meant by "other side of maternal exposure spectrum: high exposure"?
Page 13, line 29	Same comment as above, concerning high exposure. Do the authors mean chronic exposure? Or do they mean "high concentrations of tretinoin"?
Page 13, Entire section of "Implications for this advisory report"	This paragraph should summarize and bring Section 2.2 together. It is strange that the authors don't mention anywhere that tretinoin is often prescribed as a gel, which comes in different concentrations, for the treatment of acne. That is the main use of tretinoin, followed by cancer therapy. It is listed on the properties section on Page 11 under "uses", but the authors have not made a case as to why the reproductive toxicity of tretinoin is being studied, and that is because the terms for endogenous retinoic acid and tretinoin have been used interchangeably.
Page 14, Line 3-5	Combine the sentences: In 1985, Vogt and Ewers examined the sperm of 20 men with acne conglobata, that were treated

	with an oral dose of isotretinoin (1 mg/kg bodyweight) for 12 weeks. Was this treatment once a week? Once a day? "At the end of 12 weeks, it was determined that the sperm concentration per ejaculate (as determined by X) was significantly increased when compared to concentrations prior to treatment. Of the 20 initial participants, 12 were available for a follow-up 12 weeks after treatment had ended. It was found that their sperm concentrations per ejaculate were not significantly different when compared to concentrations before the start of treatment." My only concern is that this document is titled "tretinoin." While tretinoin and isotretinoin are related, they are in fact, different. Tretinoin for acne is in topical form, whereas isotretinoin (previously sold as Accutane, which I also think should be mentioned somewhere in this document) is usually orally administered. The document should be re-named if the focus will be on tretinoin and isotretinoin, or a more inclusive "Retinoic Acid." Also, perhaps at the very beginning of section 2.3, include a statement that significance is defined as $P < 0.05$ moving forward, so it does not need to be written all the time throughout the document.
Page 14, Line 10	Even though this document is being published in the Netherlands, the committee fails to cite the "iPledge" regulatory program in the United States, which is aimed to prevent teratogenicity associated with isotretinoin therapy. Reference: Charrow et al. "Differences in isotretinoin start, interruption, and early termination across race and sex in the iPLEDGE era" (2019) Journal: PLoS ONE Volume 14, Issue 3 Pages: e0210445 The references suggested in the commentary of Page 12, Line 11 would also be helpful. The first two references are from over 30 years ago.

Page 14, Lines 19-21	"Cinar et al investigated the effects of oral isotretinoin treatment in 81 males (age X-X) that were diagnosed with severe or refractory acne vulgaris. Patients received a total dose of 120 mg/kg over the course of 6 months, and spermiogram parameters were assessed both prior to, and after treatment. …" And then the authors can discuss the findings of the study.
Page 14, Lines 26-33	"Cinar et al also investigated the effect of ….." In what increments were the doses increased? Why is the reference to the dose coming from a different study? (Reference 24).
Page 15, Line 3	A very recent reference to include would be: Suuberg A. Psychiatric and Developmental Effects of Isotretinoin (Retinoid) Treatment for Acne Vulgaris (2019) Journal: Current Therapeutic Research Volume 90 Pages 27-31
Page 15, Lines 5-6	End the sentence after the words "first trimester."
Page 15, Lines 5-40	Not only for this study review, but for all of them, the authors need to condense their article summaries and mention what is most relevant. Below is an example of a summary that gets straight to the point of toxicity associated with exposure for the Lammer et al. references: To investigate the teratogenicity of retinoic acids such as isotretinoin, pregnant women (n=154) that reported oral use of this drug between 5 days prior to, and 70 days post-conception were eligible for this study (daily dose was between 0.5-1.5 mg/kg body weight). Of the 154 pregnancies, 36 were categorized as a prospective study, and the remaining 118 were retrospective. The outcomes of this study were as follows: (a) <u>Retrospective Study</u>: There was a total of 95 elective, first-trimester abortions. Of the remaining 23 pregnancies, 4 resulted in spontaneous abortions in the first trimester, in which the fetus was deemed non-viable. From the remaining 19 pregnancies, the outcomes were 2 stillborn infants with malformations, 14

	live infants with malformations, and 3 infants born with no malformations. (b) Prospective Study: Of the 36 pregnancies in this category, there were 8 spontaneous first-trimester abortions, 1 malformed stillborn infant, 4 live-born infants with at least 1 major malformation, and 23 without any major malformations. Within the context of this study, the structural morphogenesis of either the cranium and face, heart, thymus, or central nervous system (CNS) were most affected. The authors of this study were able to establish a statistical relationship between exposure to isotretinoin during early pregnancy and a characteristic pattern of malformation in infants. In addition to the aforementioned reference, other investigators have also reported on CNS malformations in infants born to mothers that were exposed to isotretinoin either before conception or during early pregnancy (Rosa et al. reference). I also suggest giving a more detailed definition of what holoprosencephaly is since it comes up several times in the following paragraphs.
Page 16, Lines 9-24	The De Wals et al. study is summarized as having looked at an association between holoprosencephaly and retinoid exposure, but it is concluded that the use of either oral or topical retinoids was not identified in any case that resulted in this CNS malformation. It is probably best to exclude this study. Since retinoid use is not a factor, it is irrelevant.
Page 16, Line 25	Be consistent throughout the document, either start with the oldest studies and progress toward the most recent, or vice versa.
Page 16, "Topical exposure" section	This seems like a good place to put the following reference: 1. Veraldi et al. "Are Topical Retinoids Teratogenic?" (2016) This reference is listed in the kinetics section on page 11 which is fine, but this section would be a place to refer back to this.

Page 16, Line 28	The sentence can end with "…of pregnant women to tretinoin between 1983 and 2003."
Page 16, Line 29	Should be "outcomes."
Page 16, Line 33	This sentence is unnecessary "Women in the exposed group…"
Page 16, Line 36	The dates could be moved up and combined with the first sentence of this paragraph.
Page 17, Line 4	Instead say "Infants from both groups were examined for malformations characteristic of retinoic acid embryopathy such as flat nasal bridge, micrognathia, cleft uvula, etc… but no significant differences were found." This review can end after this sentence. The rest of the information is not necessary for a streamlined summary.
Page 17, Line 12	Since the topic of this section is "topical exposure," perhaps it would be better to say "…investigated the effects of maternal topical exposure to tretinoin on gestation (or gestational outcomes) …."
Page 17, Line 22	"The 94 tretinoin-exposed women and the 133 women that were not exposed were similar with respect to…"
Page 17, Line 24	"No significant differences were detected between the tretinoin-exposed and control groups in terms of live births, miscarriage, elective termination of pregnancy and major malformations amongst live-born infants."
Page 18, Lines 1-2	Does this mean that the difference is significant or not significant between the groups?
Page 18, Lines 3-7	Since the facts are not exactly clear here, and there is some speculation involved, this can be left out.
Page 18, Line 8	Replace "girl" with "infant."
Page 18, Line 12	Replace "girl" with "infant."
Page 18, Lines 12-16	Find a way to make this sentence shorter; don't necessarily have to list every condition. The maternal use of tretinoin should be explained first, and then the pregnancy outcome.
Page 18, Lines 19-27	Since some of this is repetitive, just keep the portion on topical application.
Page 18, Line 29	Remove "or via."
Page 18, Lines 31-34	"Selected animal studies involving tretinoin exposure are listed below. A detailed overview with experimental parameters can be found in Annex A." Again, be consistent with the way studies are listed (oldest to newest, or newest to oldest).

Page 19, Line 1	Are the authors trying to say, "exposed five groups of pregnant rats"? In this reference, Ikemi et al. only uses the term "all-trans- retinoic acid."
Page 19, Line 2	Gestational day is the correct term, and should be corrected throughout the document, and abbreviated with GD.
Page 19, Line 4	"Reproductive parameters" is very vague, what does this mean? Is this referring to reproductive outcomes?
Page 19, Lines 1-8	This paragraph is very confusing. The authors state that the rats were sacrificed on GD 14 and fetal palates were examined. This has to do with facial structure. Then the authors mention maternal weight and food consumption between the groups, which is not necessary. Finally, there is no mention on whether there were any significant differences in fetal palates between groups, and the numbers of corpeus lutea and implantation sites seem to be very out of place when written as such. The review of Ikemi et al. needs to be re-written so that the focus on fertility is clear.
Page 19, Lines 8-15	This is not about the effect of pre-implantation. Is the committee trying to say, "the effects of tretinoin exposure *on* implantation?" If this study is focusing on implantation sites, there is no need to mention maternal toxicity, especially since it was not described in the study. "The presence of implantation sites did not differ between control animals and those exposed to tretinoin." Use this to replace the second to last sentence.
Page 19, Lines 17-21	Selected studies on the effects of tretinoin exposure on *in utero* development in several animal species are listed below.
Page 19, Lines 22/23	Not necessary, it's already stated that these studies focus on developmental problems in animals. "Primates" is the only necessary classification here.
Page 19, Lines 32-34	Instead of "animal" please specify that this is in reference to the exposed mother/exposed monkey/exposed pregnant animal.
Page 19 Line 37-Page 20 Line 1	This summary is confusing because the committee states that hysterotomies were performed on GD 100. Based on this information, how is it possible to know that resorption occurred so early on in

	gestation as listed on Page 20, Lines 1-4? A key piece of information in the methods seems to be missing.
Page 20, Line 11	The committee states that 5/7 viable foetuses in the 5 mg/kg group had an extra rib on the 7th cervical vertebrate, along with variations in vertebrate count and morphology. But earlier, on Line 6, they state that no malformations were found in the foetuses of the 5mg/kg group, which is contradictory. Did the control animals all have normal pregnancies and deliveries?
Page 20, Line 20	The first sentence is not necessary. The paragraph can start with "Groups of 12…"
Page 20, Lines 20-23	The committee has left out a key piece of information in this summary. The Nolen paper states that in the first set of experiments, rats were supplemented with 25,0000 IU/kg of Vitamin A palmitate for the first 8 days of their pregnancy (controls were not). After the 8 days, animals received either 5 or 10mg/kg all-trans retinoic acid on GD 9 (acute exposure). In the second set of experiments, pregnant rats were once again supplemented with Vitamin A (except for the controls). In addition to this, they were also exposed to either 4 or 8 mg/kg retinoic acid from GD 6-15 (chronic exposure). Also, the Nolen paper states that animals were exposed to all-trans retinoic acid. The committee cannot say tretinoin, if that is not mentioned in the paper. In the abstract of this paper, the authors state that high systemic levels of background Vitamin A increased teratogenicity in one group, while appearing to decrease it in the other. The summary of this paper given by the committee is inaccurate.
Page 21, Lines 1-6	"In order to determine if maternal exposure to retinoic acid causes palatal rugae and cleft palate format in the offspring, pregnant Sprague Dawley rats were exposed to either 1.25, 5, 20, or 80 mg/kg bodyweight of retinoic acid in corn oil on GD 14. Rats were euthanized on GD 20, and

	foetuses removed by cesarean section. The number of corpora lutea, implantation sites, and both live and dead foetuses were recorded. Ikemi *et al*. found that dose did not appear to be a factor for average number of late resorption sites, or still-born foetuses. However, the weight of foetuses in the 80mg/kg/day group was significantly lower than that of controls, and Palatal abnormalities and syndactyly were also found in this group. It was concluded that …. (fill in conclusions)"
Page 21, Lines 9-10	The opinion of the committee does not belong here.
Page 21, Lines 17-27	The Astroff reference should not be included. This is not a paper that focuses on tretinoin toxicity, but rather on comparing tissue fixation methods.
Page 21, Line 29	In looking at the Yu reference, they state in the abstract that for rats exposed to 125 mg/kg bodyweight of all-trans retinoic acid n=17. The committee has it listed as n=5.
Page 22, Line 5	The committee needs to clarify the exposure parameters, as shown in Table 1 of this paper. There was a total of 13 pregnant animals; 5 received R.A., and the rest were controls.
Page 22, Line 6	The committee also has the wrong gestational days. The actual paper has GD 7.5-11.5 and 14.5. The committee should not round gestational days.
Page 22, Lines 9-10	The committee should not include their opinion.
Page 22, Line 12	Should clarify that animals were euthanized on GD 21.
Page 22, Line 13-14	The committee seems to have misinterpreted this paper. Though there were 12 embryos from groups 1 and 2 (treated), the committee makes no distinction between 9 embryos in group 1, and 3 embryos in group 2. Their exposures were different, so it is incorrect to lump the sum of their embryos together.
Page 22, Lines 14-16	The committee should explain what anophthalmia, exencephaly, and carioschisis mean.
Page 22, Lines 29-30	Cell activity needs to be more specific.
Page 22, Lines 31-32	This paper clearly states in the abstract that the purpose of this study was to examine the effect of retinoic acid exposure on TGF beta expression in the developing cerebral cortex in the rat. To simply state "timing of exposure on the developing cortex" is incorrect.

Page 22, Lines 32-33	The committee needs to clarify the exposure parameters of the 3 groups of rats. As shown in the paper: Group 1- controls, Group 2- exposure on GD 8, Group 3, exposure on GD 12, and the route of exposure was gavage. The committee seems to be summarizing these papers in ways that miss very important distinctions, and tend to group exposures together, which is not accurate.
Page 23, Lines 5-9	Though the Colakoglu and Kukner paper makes it clear why they are looking at TGF beta, the committee does not. To simply regurgitate TGF beta results seems very out of context the way it is written. The reader will want to know why TGF beta was studied, which the committee failed to explain in the beginning of this summary.
Page 23, Lines 10-12	The committee has incorrectly summarized the exposure parameters. According to the paper, there were 7 groups of rats. Each group had 5 rats, except for the control group, which had 6. The way the committee summarizes this, it seems as if there were only 5 rats in the entire study, and they received anywhere between 40-80 mg/kg bw retinoic acid, which is incorrect. Also, the paper refers to retinoic acid here as Isotretinoin, not tretinoin. The committee did not adequately explain exposure to folic acid. As written, it would seem as if these animals only received folic acid, which is not the case. Some received retinoic acid prior to folic acid. The committee also makes no mention of the results in rats given folic acid. When the committee references the control group, are they referring to corn oil or folic acid? There were 2 control groups.
Page 23, Lines 26-27	This is a very broad generalization of the actual exposure parameters. The individual exposure groups had different numbers of animals, ranging from 3 to 38/group, and need to be explained as such, along with how many animals received each dose.
Page 23, Line 29	Replace “like” with “such as.”

Page 23, Lines 29-30	There are two control groups, one that only received olive oil, and one that was untreated. The distinction between the 2 needs to be made clear.
Page 23, Line 34	31 exposed dams had combined exencephaly and MMC-like defects.
Page 23, Lines 36-40	If no defects were observed in the control group, along with the 20 and 40 mg/kg bw group, then the dose-dependant toxicity starts with the 50 mg/kg bw group and should be described as such.
Page 24, Lines 3-4	On the previous page, the committee states that the observation of malformations happened in a dose-dependant manner (starting at 50 mg/kg bw). However, the 70 mg/kg treatment group had 0 animals with curly tail, so to say "dose dependence" is misleading.
Page 24, Lines 8-25	This Danzer study should not be included. The point of this paper was to use retinoic acid to induce myelomeningocele and study amniotic fluid levels of GFAP. The authors are looking for potential markers of spinal cord toxicity here.
Page 24, Lines 26-33	The committee makes no mention of the fact that exposure to retinoic acid could disturb the notochord and tail bud development in the process of primary and secondary neurulation in rat embryos, which cause lumbosacral NTDs including myeloschisis and hamartoma. This was the take-home message.
Page 24, Lines 34 (Entire Liu study)	The authors of this paper state that they are studying both fetal skeletal and hindlimb retardation, not just skeletal as mentioned in line 34. The authors of this paper also state that exposure to retinoic acid (in the abstract) delayed fetal increases in body weight and skeletal ossification development. The committee states on Page 25, Lines 3-4 that it caused a decrease in skeletal ossification. There is a difference between a decrease, and a delayed increase.
Page 25, Line 7	The committee says n=5, which would indicate that a total of 5 rats were used in this study. There were different exposure groups, each with 5 pregnant rats. This makes a difference. This paper makes no reference to the word "tretinoin." The committee needs to use what was stated in the paper. In this case, it would be all-trans-retinoic acid.

	The first sentence here is very misleading. There were two separate rat studies, each using different doses. Here the committee makes no distinction between the two.
Page 25, Line 13	No need to specify when data are not shown.
Page 25, Lines 14-25	This needs to be re-written for clarity, as there were two separate rat studies, and this has all been lumped together.
Page 25, Lines 26-27	The committee should explain that WEC is a potential alternative for in vivo studies. Also, the authors of this study do not use the term tretinoin, so the committee should not either.
Page 25, Line 28	Fix "n=3." More than 3 rats total were used in this study.
Page 25, Line 30	Delete the maternal toxicity statement. This section of the review is on fetal development.
Page 25, Lines 32-40	The Robinson et al. paper is extremely detailed and a very in-depth study. The committee makes some very broad generalizations and conclusions here, and they need to be more specific, especially in relation to gene expression. There is no mention of the genes affected.
Page 26, Lines 1-8	This reference should not be included, because retinoic acid was specifically used to induce spina-bifida aperta.
Page 26, Line 10	Does n=12 refer to all animals used in this study?
Page 26, Line 12	No need to mention maternal toxicity.
Page 26, Line 12	What is NO referring to? This is a very brief summary that mentions NO and glutathione, with no background explanation.
Page 26, Lines 15-22	This study should not be included. The authors of this study specifically used retinoic acid to induce neural tube defects.
Page 26, Line 23	This study should also not be included, as spina bifida is intentionally being caused, with retinoic acid being the agent to do so. This is not a true study of whether or not retinoic acid causes developmental toxicity.
Page 26, Lines 32-38	For the same reasons stated above, this study should not be included. They are purposefully using retinoic acid to induce clubfoot.
Page 27, Lines 1-6	This study should not be included. The Agarwal paper uses RA to specifically induce MMC. This is not a question of whether RA will induce MMC, which would be in line with what this review is trying to prove. Please see "general comments" section.

Page 27, Lines 7-10	This description is unnecessary. Just say "Mice" and then list the studies.
Page 27, Line 11	It is important that the committee differentiate between cis- and trans- retinoic acid, as the whole point of this paper is to determine if there is a difference in toxicity between the two. To simply state "tretinoin" is inaccurate.
Page 27, Line 13	No need to state that maternal toxicity was not described. Is the committee referring to cis- or trans- retinoic acid for the 60 mg/kg results?
Page 27, Line 16	Is the committee referring to cis- or trans- RA?
Page 27, Line 31	The committee needs to use the same wording in the paper. Mice were exposed to all-trans retinoic acid.
Page 27, Line 33	Maternal toxicity not necessary, unless the committee wants to include a separate section on this.
Page 27, Line 33-34	No effect as a result of exposure to both the 40 and 60 mg/kg group? Needs to be specific.
Page 27, Line 33-38	A suggestion would be to discuss toxicity observed at 40mg/kg first, followed by 60 mg/kg, and then both. As written, the committee goes back and forth between the two doses, and it is confusing.
Page 28, Lines 4-5	Nugent et al. tested the teratogenic effects of retinoic acid exposure in TGF-B2 knockout mice. TGF-B2 is of interest because…..(explain why).
Page 28, Line 9	Remove reference to maternal toxicity.
Page 28, Lines 9-12	The first paragraph of the "results" section of the paper gives a very good breakdown of the results, which the committee should use to summarize this study.
Page 28, Lines 17-19	Maternal information not necessary. Use "retinoic acid" if that is what is stated in the paper.
Page 28, Line 35	Change to retinoic acid.
Page 29, Lines 5-13	The committee fails to mention that this study looked at the induction of cleft palate, and other malformations in mice with altered expression of TGFα and the EGF receptor. This paper review needs to be re-written.
Page 29, Line 14	Change tretinoin to retinoic acid.
Page 29, Line 15	Change skin development to epidermal morphogenesis.
Page 29, Line 19	Remove reference to maternal toxicity.
Page 29, Line 27	The committee should include the last sentence from the abstract of this paper, which states that

	prolonged in utero effect of RA exposure might play a role in adult skin disease.
Page 29, Line 28	Use retinoic acid, as stated in the paper.
Page 29, Line 34	Remove reference to maternal toxicity.
Page 29, line 37	The paper says "all trans retinoic acid."
Page 30, Line 5	Paper uses retinoic acid.
Page 30, Line 9	Remove reference to maternal toxicity.
Page 30, Lines 9-15	The Billington reference states that mice carrying a TSWG1 mutation are sensitized to retinoic acid teratogenesis. The committee has not accurately summarized this study.
Page 30, Lines 16-28	In the Yan study, gene expression of heat shock proteins is the focus. While the committee does report on malformations in animals exposed to all-trans retinoic acid, there is no mention of heat shock proteins, so the summary is somewhat misleading.
Page 30, Lines 29-35	The focus of this study was to determine whether folic acid would have antiteratogenic effects on retinoic acid-induced cleft palate. This reference should not be included. Refer to the general comment on page 3, last paragraph.
Page 31, Lines 2-4	Maternal toxicity not necessary.
Page 31, Lines 4-9	Needs to be re-worded for clarity; it is confusing.
Page 31, Lines 10-16	This study should not be included since retinoic acid was specifically used to cause cleft palate, so that the authors could study LncRNA H-19.
Page 31, Lines 17-23	This paper should not be included, as the authors specifically used retinoic acid to induce neural tube defects to study the protective role of Taurine. Again, if the committee wishes to include papers in which the authors are specifically using retinoic acid to induce toxicity to study something else, then those papers should have their own section.
Page 31, Lines 24-29	Same comment as above. The authors use retinoic acid as a positive control to induce cleft palate to study Notch2 signalling.
Page 31, Line 32	The rabbits were split into groups of 4, not a total of n=4 as suggested here.
Page 32, Lines 7-23	Suggestion would be to not include this reference; it is almost 50 years old. If included, remove maternal toxicity in Lines 13-14. Using LD_{50} studies compared by gestational day (Lines 15-16) does not make sense. If LD_{50} doses are going to be compared, then the gestational day needs to be held constant.

Page 33, Lines 1-2	Needs to be more specific.
Page 35, Line 21	Remove "or via." Re-word "in either humans or animals."
Page 35, Line 24	Suggest not to start this paragraph with data that do not exist. Instead, start with what was found in this review and what is currently known about male/female fertility in relation to tretinoin/retinoic acid exposure.
Page 35, Line 29	First sentence needs to be more specific with examples of what is increased and what is decreased.
Page 35, Lines 29-33	Be careful to distinguish more clearly between tretinoin and isotretinoin.
Page 35, Lines 32-33	The committee cited 4 papers concerning human fertility, two with tretinoin, and two with isotretinoin. Should these also be distinguished when classifying fertility? I agree that there are very limited data on the effects of tretinoin on fertility and a classification at this time would be difficult.
Page 47, Line 3	GD= Gestational day
Page 47-63	The presentation of the table needs to be fixed. The table should be a very quick summary. If the reader wants full details, she can always consult either the committee's earlier summary, or the original study. There is no need to include the "n" in the table. There is no need to include general toxicity, just focus on reproduction to reduce clutter. In the table below, I have included the reference as a subscript number next to the species. That takes away from having to have an additional column in the table. Use bullet points for the outcomes, and keep the outcomes short. Distinguish between tretinoin and isotretinoin and trans and cis retinoic acid.

Table 1. Fertility studies in animals (group all of the same kinds of animals together)

Species	Exposure	Dose	Route	Outcome
Sprague- Dawley Rat [x] (all other rat studies should follow)	GD 14	0, 1.25, 5, 20, or 80 mg/kg b.w.	Oral gavage	• Corpea lutea and implantation sites not affected at any dose
CD-1 mouse [x] (all other mouse studies should follow)	GD 3-10	1, 5, 10 mg/kg b.w.	Oral Gavage	• Main Outcome #1 • Main Outcome #2