Department of Defense (DoD)
Comments on the Interagency Science Consultation
Draft IRIS Toxicological Review of Perfluorohexanesulfonic Acid (PFHxS)
January 2023
(Date Received February 3, 2023)

Section	Pages	Comment	Suggested Action, Revision and References (if necessary)
Abbreviations and Acronyms	ix	Missing Abbreviations and Acronyms. For example, "PFHxS" and osRfD are not present.	Add missing acronyms to this list.
Executive Summary	xii (line 14)	The text indicates PFAS have *"… resistance to heat, oil, stains, grease, and water."* More correctly, many PFAS are resistant to heat and are used to confer resistance of products (e.g., textiles) to stains by repelling oil, grease, and water. PFAS are also used in a wide range of other applications, including as electrical insulation and to confer frictionless coatings onto surfaces. The text, as currently written, does not identify the property (e.g., surfactant) that caused some PFAS to be used in AFFF formulations.	Please consider more clearly describing the properties of PFAS that make them useful to industry (e.g., surfactant properties) and in commercial products (e.g., to reduce friction).
Executive Summary	xii (lines 14-15)	How are PFAS "linked" to industrial sites, military fire training areas, wastewater treatment plants and commercial products? The text is overly vague. How are PFAS associated with military fire training areas? Is it through AFFF use/release? Is this use/release limited to training or does it also include AFFF use in suppression of fires? Is it more correct to describe PFAS contaminated environmental media at, and adjacent to military facilities, as resulting primarily from the use/release of AFFF at these locations? More direct and informative statements might also be made concerning industrial releases, wastewater pass thorough at treatment plants, and the use and disposal of commercial products containing PFAS.	Please consider clarifying how PFAS are "*linked*" to sources and areas. The identification of a source (e.g., an industrial site) should be consistent with its known use of PFAS. For example, the text might identify industrial sites as sources of PFAS (e.g., chrome plating facilities), note that municipal wastewater treatment plants are not currently capable of destroying all of the PFAS entering the wastewater stream, and that PFAs can be placed in landfills as disposed of products (e.g., pizza boxes and popcorn bags).

Executive Summary	xii (footnote)	The footnote touches on a subject that requires additional attention elsewhere in the document. For example, prior to 2005, it was known that while PFHxS used in a variety of products was typically composed of the linear isomers, but also that it typically included 4 to 5 % branched isomers. While branched isomers were present in historical preparations of PFHxS, a result of the method used in PFHxS formation, they may not be quantified in current analyses of environmental samples since branched isomers generally have a shorter half-life and are more prone to degradation than are linear isomers.	Additional detail concerning the physical makeup of PFHxS used in historical applications is warranted.
Executive Summary	xiii (lines 9 and 17)	It is unclear what is meant by *"… or studies per health outcome.*" Is the authors' intent to indicate that the number of studies is not sufficiently large to draw meaningful conclusions?	Please clarify. Be specific about what it is about the "studies" that prevent drawing meaningful conclusions.
Executive Summary	xiii (lines 15-17)	The text, as written, suggests that the only limitations identified in animal studies are those noted. There seem to be several limitations to the use of PFAS animal studies that are not identified here. The selection of the subject test animal species is a potential limitation of animal studies. Rats and mice are not good models of human lipid metabolism. Because PFAS can act like fluorinated fatty acids, the test animal species utilized should be one that most closely models likely human responses (e.g., monkeys and guinea pigs, which have lipid metabolism more similar to humans).	Please clarify and consider discussing more limitations of the animal studies noted.
Executive Summary	xiii (line 18)	Does the available evidence indicate that PFHxS exposure is likely to cause thyroid and immune effects? This statement suggests that there is both epidemiological and animal evidence in support this statement. The following paragraph suggests that the statement is referring to the available evidence in animal studies indicates that PFHxS exposure may result in thyroid effects. The immune effects are determined in human children. Where authors list a critical effect, the text should clearly inform the reader of the nature of that effect and the strength of the evidence for that effect.	Please clarify. Given that the subsequent paragraph (lines 25 to 30) specifically addresses epidemiological evidence, the reader might assume that the statement made on lines 18 and 19 refers ONLY to the animal evidence. Additionally, please consider addressing what exactly is the health impact of the effects reported. It's

			unclear whether there is evidence that lower antibody levels result in greater disease incidence or greater disease severity Specifically, whether diphtheria and tetanus disease incidence and severity change with PFHxS levels in environmental media over the last 30 years is not addressed. Tetanus and Diphtheria incidence and severity (morbidity) information is available from the CDC.
Executive Summary	xiii (lines 28-30)	The purpose of comparison with a non-RfD derived for a POD for developmental effects is unclear. It appears the author may be suggesting that even if developmental effects were used to determine an RfD, this RfD would not result in a greater estimate of toxicity (a lower RfD) than was determined for immune effects.	Please clarify.
Executive Summary	xiii (lines 31-34)	Metabolic Syndrome (MeS), discussed by Costello et al (2022), and Ducatman and Fenton (2022), is not identified anywhere in this paragraph as an effect, at least explicitly.	Please consider updating the text to more specifically reference metabolic syndrome, to include the adequacy of the evidence. See also the following references: • Costello et al. 2022. Exposure to Per- and Polyfluoroalkyl Substances and Markers of Liver Injury: A Systematic Review and Meta-Analysis. Environmental Health Perspectives. 130(4):046001. DOI: 10.1289/EHP10092. • Ducatman and Fenton. 2022. Invited Perspective: PFAS and Liver Disease: Bringing All the Evidence Together. Environmental Health Perspectives. 130(4):041303. https://doi.org/10.1289/EHP11149.

Executive Summary	Table ES-1	First Column, First Row; The Organ/System identified is "Developmental Immune." This is different from the text on the preceding page that indicates only immune effects. Is the effect identified here a result of developmental exposure (in utero)? Is there an on-going exposure after birth (lactation)? How did the authors of the key study link a decrease in antibody level to an external or internal PFHxS dose? Intuitively, it seems inappropriate to identify a lifetime osRfD, or even a subchronic osRfD, from a study identifying developmental effects from a developmentally relevant dose. This is because such effects ONLY occur over a very short window of exposure. Was C_{MAX} used?	Please clarify. Is the Organ/System immune effect a result of developmental or a systemic exposure? Over what period (i.e., duration and timing) was exposure to PFHxS known to occur? How was this exposure quantified/extrapolated to become a lifetime and subchronic osRfD. Why is the developmental exposure not adjusted by a UF_S of 10 to extrapolate from a less than chronic to a chronic exposure?
Executive Summary	xiv (lines 2-8)	The authors assume that the measured change in antibody levels to tetanus and diphtheria toxins, is associated with an adverse health outcome. It is not. The CDC reports that there has been no change, up or down, regarding tetanus and diphtheria disease incidence or severity since 1990 – this covers the window of increased PFHxS exposures in the general population up to about 2020 and the subsequent decline of PFHxS exposure after 2020 (see; Norwegian Environmental Agency 2018 and NHANES for details on PFHxS levels in people). Is there information from the study population in the Faroe Islands that suggests a different PFHxS distribution over time? Children in the Faroe Islands population are also known to also be impacted by PCBs (Shih et al. 2021). Mercury is also known to be present in the maternal diet and may also confound the determination of PFAS-related effects. While the authors dismiss the potential confounding of their results due to these co-exposures, they do not provide an analysis supporting their statement. • Shi h et al. 2021. Serum vaccine antibody concentrations in adults exposed to per- and polyfluoroalkyl substances: A birth cohort in the Faroe Islands. Journal of Immunotoxicology. 18(1):85-92. https;//doi.org/10.1080/1547691X.2021.1922957.	Please consider additional justification for the use of the reported key studies as identifying a "critical effect."
Executive Summary	xv (lines 19-23)	The basis for a finding of "medium" confidence for RfD and chronic RfD is unclear. On page 1-11 (lines 24-25), medium confidence is defined as "*possible deficiencies or concerns were noted, but the limitations are unlikely to be of a notable degree or to have a notable impact on the results*."	Please consider adding more supporting information to this section.

		How does one have medium confidence in an external exposure without knowing how exposure occurred or how exposure may have varied over the course of the developmental exposure period? Was maternal exposure measured? How does maternal exposure inform in utero fetal exposure? How was lactational exposure (presumed to have been present) used to determine developmental exposure? How were developmental exposures extrapolated to a lifetime and subchronic osRfD? It seems that there are sufficient limitations in the data to warrant finding a less than medium level of confidence. For immunological effects in the Faroe Islands cohort, it is inappropriate to offer a "Good" rating for the confounding domain, which (page 1-12; lines 30-32) *"… is reserved for situations in which there is minimal concern for substantial confounding across PFAS **as well as other sources of confounding***." The known impact of dietary mercury and PCBs on this population should not be given a "Good" rating., regardless of what unsubstantiated comments the authors make in the text of their paper.	
1.1 Background Information on PFHxS	Table 1-1	Table 1-1 describes the basic chemistry of these compounds. It's difficult reconciling some of these numbers like the pKa, logP and water solubility, and how they would feed into analyses of partitioning into tissues (as in the Ruark et al., 2014 paper). Note - some of these chemical properties are predicted (i.e. not measured) values. Specifically, a pKa of 0.14 (i.e. < 1) means that PFHxS is a strong acid (is this compound really that strong, comparable to sulfuric or hydrochloric acid?), which would pretty much totally dissociate in water. That would be consistent with the logP (or logKow) being pretty much the same for the acid and the salts, as in the Table. But a high logP of 2-4 for all of these compounds means that they vastly prefer lipids, even in ionized (or salt) form. This seems unusual behavior. I would guess it's because of all those fluorides, producing amphiphilic compounds (see below). But then the water solubility for the salts is very high for the (ionic or charged) salt forms (2-4 x 10-1 mol/L), but much lower for the (presumably highly dissociated, and hence also ionic) acid (6 x 10-4 mol/L). This needs to be reconciled.	Given the unusal properties of PFHxS, this should be explained further in this section and the pharmacokinetics section, or the reader may be left wondering.
1.1 Background Information on PFHxS	Table 1-1	Is the "Bioconcentration Factor" the ratio of serum: drinking water PFHxS concentration? If so, the sodium salt has a significantly different BCF than the free acid or the potassium/ammonia salts, which could impact the estimation of external dose based on the serum POD.	Please specify
1.1 Background Information on PFHxS	Table 1-1	Footnotes do not match the cell data. For example, the PKa for PFHxS with CASRN 335-46-4 is referenced to [b]CASRN 3871-99-6. This is not the same CASRN. The correct CASRN is identified by a footnote as "ND".	Please correct the errors noted in Table 1-1. If the only source of information for Table 1-1 is limited to the CompTox

		The soil adsorption coefficient (L/kg) is typically identified as the K_{OC}. Is there a rationale for why it is not identified as such in this Table? The CompTox Dashboard includes information that is not correct. For example: the CompTox Dashboard predicts the PFHxS (CASRN 355-46-4) **PK Half Life** in humans as 789.6 hours or 0.09 years. No other predicted or measured value is provided. This is much lower than the 5-to-14-year half-lives that might be obtained from a number of published sources. The PKa is defined as the negative log of the dissociation constant (Ka) for the compound of interest. The predicted PKa of 0.14 is very small and indicates a very strong acid. However, this makes no sense since PFHxS does not act like a very strong acid. The predicted value is likely the result of an algorithm designed to work with Carbon-Hydrogen bonds and not the stronger Carbon-Fluoride bonds. Does the algorithm used to estimate the PKa for a compound made up of carbon-fluoride bonds provide an accurate value? Is the predicted PKa value of 0.14 a realistic estimate of PFHxS acidity?	Dashboard, then please consider being consistent and use all relevant information provided from that source, including the short 789.6 hour half-life in humans. Selective use of physicochemical information requires justification. The information provided in Table 1-1 should include the best available data and not just the data provided from one EPA source (U.S. EPA, 2018a). The predicted PKa value (ECHA) also requires validation.
1.1.1. Physical and Chemica Properti es	1-1 to 1-3	Table 1-1 describes the basic chemistry of these compounds. It is difficult reconciling some of these numbers like pKa, logP and water solubility and how they would feed into analyses of partitioning into tissues (as in the Ruark et al., 2014 paper). Note: some of these chemical properties are predicted (i.e, not measured) values. Specifically, a pKa of 0.14 (i.e.<1.0) means that PFHxS is a strong acid (is this compound really that strong, comparable to sulfuric or hydrochloric acid?), which would totally dissociate in water. That would be consistent with the logP (or logKow) being very similar for the acid and the salts, as presented in the Table. But a high logP of 2-4 for all of these compounds, means that they vastly prefer lipids, even in the ionized (or salt) form. These seem unusual behavior, which can be assumed to be because of all those fluorides, producing amphiphilic compounds.	There isn't much discussion in the Review about basic chemistry of these compounds. Amphiphilicity, for example, does not appear to be mentioned at all, yet would appear to be key in understanding their behavior, both in the environment and in the body. (In particular, it may go some way to understanding why the PBPK models are having difficulties consistently describing their behavior under different conditions - see next comment on pharmacokinetics.) It would be extremely valuable for understanding what's going on with the biology and would suggest that this as an important addition to the Review.
1.1.2 Sources, Production, and Uses	1-4 (lines 5-6)	Limiting reviews to a single source will often miss important information. The Norwegian Environmental Agency (2018) referenced by EPA in the subject document provides information on the consumption of PFHxSF in various sectors, applications and includes market research reports on the production capacity of PFHxS from 2011 to 2016 (pages 7-8).	Recommend that the authors consider including more production data sources in this section.

1.1.3 Environmental Fate and Transport	1-4 (lines 13-23)	The persistence of PFHxS in various environmental media is known (EPA cites ATSDR 2018a, and Harbison et al., 2015, Kim and Kannan, 2007), but the values indicating its level of persistence (i.e., typically half-lives in the environmental media of interest) are not identified in this paragraph.	Please consider including estimates of environmental persistence to support the statements made in the paragraph.
1.1.4. Potential for Human Exposure and Populations with Potentially Greater Exposure	1-4 (lines 29-30)	The text notes that "*this conclusion is based on several studies that have investigated the various routes of PFAS exposure (Klaunig et al., 2015)*", but then only references one study.	Please clarify whether Klaunig et al (2015) is a review of a group of studies or there are additional studies available to support the statement made.
	Table 1-2	Just as important as the "Value" reported in this table is the additional information provided by NHANES that indicates the variation (95th CI) about the "Value". CDC reports the geometric mean of sample analyses with a 95th CI. For example, the Total Population geometric mean from NHANES 2013-2014 is reported as 1.35 (1.20-1.52).	Please consider adding pertinent information to Table 2-1 that provides the reader with important information about "Value" variance. Please also note that Table footnote [a] should provide additional detail concerning the conditions under which the CDC calculates a geometric mean from sample analyses. For example, CDC notes that if 40% or more of the total proportion of results for each PFAS compound is below the LOD a geometric mean is not calculated.
1.1.4. Potential for Human Exposure and Populations with Potentially Greater Exposure	1-6 (line 15)	Where one reports the range of analyses in an environmental media, as between the LOD and some maximum value, the concentration of the LOD, like the maximum value, should be identified. This is because the LOD can vary depending upon the method used for analysis, the media analyzed, the analyte, and the presence of matrix interferences.	Please consider including the magnitude of the limit of detection here and elsewhere in the document where such information would be pertinent.

1.1.4. Potential for Human Exposure and Populations with Potentially Greater Exposure	1-7 (lines 5-6)	"*PFHxS was detected in 45% of the samples at maximum concentrations of 3.5 ng/g.*" This statement is unclear as to whether these samples in which PFHxS was detected were all at the maximum concentration or whether a portion of them were at the maximum concentration. It is also unclear whether the statement refers to the work of Stahl et al (2014).	Please clarify the points referred to in the comment.
1.1.4. Potential for Human Exposure and Populations with Potentially Greater Exposure	1-7 (lines 6-12)	Concerning ***Other Exposures***, additional information can be found in FDA Market Basket studies including: • Ruffle et al. 2020. Perfluoroalkyl Substances in U.S. market basket fish and shellfish. Environmental Research. 190:109932. November. https://doi.org/10.1016/j.envres.2020.109932	Please consider rewriting this section to clearly reflect what is known about PFHxS in various food items and diets.
1.1.4. Potential for Human Exposure and Populations with Potentially Greater Exposure	1-7 (lines 13-23)	There is a difference between current (ongoing) and historical exposure to PFHxS. PFHxS has not been used in commercial/industrial applications for several years. As a result of its long half-life in the human body, however, PFHxS blood levels (i.e., the metric of PFHxS exposure) may be the result of both current and historical occupational and non-occupational exposures.	Additional detail can be incorporated into this text: • Levasseur et al. 2022. Characterizing firefighter's exposure to over 130 SVOCs using silicone wristbands: A pilot study comparing on-duty and off-duty exposures. Science of the Total Environment. 834:155237. http://dx.doi.org/10.1016/j.scitotenv.2022.155237.
1.1.4. Potential for Human Exposure and Populations with Potentially Greater Exposure	1-8 (line 4)	*"PFHxS was detected in the serum of at least 30% of the NHANES participants, and after adjusting for demographic characteristics shellfish consumption was associated with elevated levels of PFHxS."* A citation is required here.	Please include a citation.
3.1 Pharmacokinetics	3-1 to 3-6	PFHxS is an acid with a very low pKa, so it is apparently a very strong acid and dissociates pretty completely. What does this imply for PK (in particular, membrane permeability and tissue partitioning)? Also, log Kow is 2.20 (and even higher, paradoxically, for the salts), suggesting a definite preference for partitioning into lipids (but see Amphiphilicity above;	Please discuss the implications of the low pKa and high log Kow on membrane permeability and tissue partitioning. See Ruark's or Schmitt's

		again, the physical chemistry of these compounds should be discussed in relation to the measured and predicted parameter values in Table 1-1).	analyses of effect of dissociation on tissue partition coefficients (PCs). Reference provided below. These studies provide a detailed understanding of the processes leading to the distribution of both the neutral and ionized forms of the chemical in the lipid and aqueous phases of specific tissues: • Ruark CD, et al. Predicting Passive and Active Tissue:Plasma Partition Coefficients: Interindividual and Interspecies Variability. J Pharm Sci, 103, 2189-2198, 2014 Schmitt W. 2008. General approach for the calculation of tissue to plasma partition coefficients. Toxicol In Vitro 22:457–467.
3.1 Pharmacokinetics	3-3 (line 4)	More correctly, the statement *"… and human blood PFHxS levels to a human equivalent PFHxS dose."* might be re-worded to read *"… and human blood PFHxS levels to a human equivalent external oral dose."* Added text is underlined.	Please clarify as to whether human plasma levels are converted (extrapolated) to an external oral dose.
3.1 Pharmacokinetics	3-3 (lines 8-10)	The authors correctly identify the fact that rats and mice may not be useful models from which to obtain PK data relating to PFHxS. The authors state *"…. to what extent might significant differences between PK in male and female rats be predictive of possible sex differences in humans? Differences or similarities between rats and monkeys can likewise be indicative of the comparison between rats and humans."* The authors, however, in the following section (3.1.1. Absorption and following ADME sections), appear to focus on rat absorption as the best evidence for ADME in humans. Is rodent data the best information from which a model PFHxS ADME in humans? Although Butenhoff et al. (2004) reports on PFOA in monkeys, the information reported might be useful to support PFHxS PK values obtained from other sources.	Please consider in the ADME sections of the document the information provided by the limited number of primate studies as supportive or not supportive of the rat studies used. • Butenhoff et al. 2004. Pharmacokinetics of Perfluorooctanoate in Cynomolgus Monkeys. Toxicological Sciences. 82(2):394-406. https://doi.org/10.1093/toxsci/kfh302. Other references of potential usefulness might include the following: • Cui et al. 2019. Per- and Polyfluoroalkyl Substances (PFASs) in the Blood of Two

			Colobine Monkey Species from China: Occurrence and Exposure Pathways. Science of the Total Environment.674:524-531. https://doi.org/10.1016/j.scitotenv.2019.04.118 • Gannon et al. 2016. Absorption, distribution, metabolism, excretion, and kinetics of 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoic acid ammonium salt following a single dose in rat, mouse, and cynomolgus monkey. Toxicology. 340:1-9. • Loccisano et al. 2011. Evaluation and prediction of pharmacokinetics of PFOA and PFOS in the monkey and human using a PBPK model. Regulatory Toxicology and Pharmacology. 59(1):157-175. https://doi.org/10.1016/j.yrtph.2010.12.004 • Russel et al. 2013. Elimination kinetics of perfluorohexanoic acid in humans and comparison with mouse, rat and monkey. Chemosphere. 93(10):2419-2425. https://doi.org/10.1016/j.chemosphere.2013.08.060 • Xu et al. 2006. N-Glucuronidation of Perfluorootanesulfonamide by Human, Rat, Dog, and Monkey Liver Microsomes and by Expressed Rat and Human UDP-Glucuronosyltransferases. Drug metabolism and Disposition. 34(8):1406-1410. DOI: 10.1124/dmd.106.009399
3.1.1 Absorption	3-4 (lines 28=31)	It is unclear why it is reasonable to assume that uptake in monkeys and humans is fairly efficient in the absence of supporting information.	Please consider adding information to support this statement.
3.1.1 Absorption	3-5 (lines 17-19)	Because of the clear differences in lipid metabolism and ADME (i.e., sex related differences) between primate and rodent species, additional discussion on these topics is warranted. Several monkey PFAS studies are available for comparison.	Please consider including discussion of how primate studies support or refute this observation.

3.1.2 Distribution	3-6 (lines 12-13)	Is there paired liver and serum PFAS data in primates? If information in primates is available, it should be used to compare, contrast, and/or support the use of similar information in rodent species as a useful model of ADME in humans.	Please consider including discussion of primate studies, and how they might affect the use of similar information in rodent species.
3.1.2 Distribution	3-6 and 3-7 (lines 38 and lines 1-2)	While active transport processes arguably contribute to re-uptake of PFHxS in both the gut and kidney, the high distribution of PFHxS in the liver and other tissues is likely a result of PFHxS binding affinity for those tissue proteins. The half-life of PFHxS in humans is likely a result of both active resorption and binding avidity to tissue proteins (see: Kim et al 2018b). This and perhaps other mechanisms result in a relatively long half-life of PFHxS in humans. Is the half-life of PFHxS in rats in any way similar to that in monkeys and/or humans?	Add an appropriate citation, quote the author's text, or provide additional detail regarding the importance of protein binding and reabsorption (i.e., enterohepatic GI reabsorption and renal reabsorption) in determining human PFHxS half-life. Species specific differences in PFHxS protein binding between rats and humans is provided by Kim et al (2018b). (See page 3-10; lines 4-7). This information suggests that just a fraction of 1 percent of the total PFHxS in the body is free (unbound) at any one time, something that likely results in limited loss from the body.
3.1.2 Distribution	3-7	Line 19: Reported values of Vd are listed in Table 3-1, grouped by species and sex. No data are available to determine Vd in humans. While Vd in rodents for several PFAS have generally been found to be less than 1,000 mL/kg (1 L/kg), the reported values vary considerably. In the absence of data on human Vd, there is substantial uncertainty in pharmacokinetics.	Recommend adding this uncertainty to the discussion and consider it appropriately.
3.1.2 Distribution	Table 3-2	A measure of variance in the mean of medians and the mean of means gives the reader an appreciation for the variation in the median and mean values reported in the Table.	The standard deviation (SD) on the mean of means is 0.16, which might be reported as 0.60±0.16. The SD of the mean of medians is 0.14 (53±0.14).

			The fact that the mean of medians is not statistically different from the mean of means suggests a relatively normal distribution of this data (i.e., the median and mean are about the same). Please add a more detailed explanation to this statement.
3.1.2 Distribution	3-8 (lines 6-13)	Is the Volume of distribution (Vd) different for different PFAS compounds? If so, are the results of Sundstrom et al (2012) reported in Table 3-1 specific to PFHxS? If the Tabled values are not specific for any one PFAS, why is the monkey Vd (PFOA), reported by Butenhoff et al (2004) not included in this Table?	Please clarify. Add additional primate information where applicable. Consider describing why different Vds are indicative of different disposition of the subject material within the body.
3.1.2 Distribution	3-10 (lines 15-21)	Additional justification for considering Monroy et al. (2008) unreliable is required. The author's statement "*It could be that the greater mean value in the censored data was an artifact resulting from the high variance in the minority of the data above the limit of detection"* needs further justification. Statistically, because of the high variance associated with the mean values reported, there is no statistically important difference between the mean PFHxS levels in cord blood and serum levels measured by Monroy et al. This is a fact that might impact the confidence on the study.	Please re-evaluate the finding that the Monroy et al. (2008) findings are unreliable and provide a stronger justification for decision.
3.1.2 Distribution	3-10 (line 35)	More correctly, the hematocrit is the fraction of whole blood that is red blood cells.	Recommend changing the text in line 35 to read *"…cells ~~and~~ to whole blood."*
3.1.2 Distribution	3-12 (lines 1-13)	The evaluation of the median ratio (serum:whole blood) provided by Poothong et al (2017) would be more meaningful if the normal range of hematocrit values (i.e., an upper and lower bound range of normal Hct values) were used. Fp requires definition.	According to *Topping and Cembrowski. 2013. Within-Individual Hematocrit Variations and Self-Monitoring of Blood Glucose. Journal of Diabetes Science and Technology.*

			7(1):190-192. https://doi.org/10.1177%2F193229681300700124 the average within-individual variation of hematocrit is between 2.9 and 3.3 percent. Given the range of values (ratios) presented (1.88 to 1.6) and the normal variation in individual hematocrit values, are the number of significant figures presented in the Fp (1.74) appropriate? Please justify. Fp should be defined and added to the acronym list.
3.1.2 Distribution	3-13 (lines 1-14)	How do the Vd's reported here compare to those reported for other monkey studies, and other PFAS compounds? Why are these study results not discussed earlier within the ADME sections? Are monkey data preferred over the rat data?	What values are carried forward and used? Is there a justification for assuming in humans (or primates) that the distribution of PFHxS to fat and muscle tissues is substantially less than to internal organs (as determined by Kim et al. in rats)? Please provide further justification.
3.1.2 Distribution	3-16 (12-16)	Why is the larger blood volume considered an "opposing factor?" Is the Vd different because of the larger blood volume? If not, is the concentration of PFHxS different because it is bound to proteins in the blood instead of somewhere else in the body? Is the author's intent to indicate that the blood PFHxS concentration is more dilute because of the higher newborn blood volume? How does this impact dosimetry?	Additional discussion/clarification and definition is required.

3.1.2 Distribution	3-16 (10-11)	What is the range of uncertainty for distribution?	Additional information regarding the uncertainty in the distribution is required for such a strong statement.
3.1.2 Distribution	3-16 (31-32)	"Karrman et al. (2010) did compare liver and serum 30 levels, but "using blood concentrations previously determined in Catalonia," presumably (Kärrman 31 et al., 2007), i.e., from different individuals." Sentence needs clarification.	Please provide clarification.
3.1.4 Excretion	3-17 (lines 23-33) 3-18 (lines 1-5)	Why focus on rat (page 3.1.7) and mouse (page 3.1.8) studies when there is data in monkeys? Do monkey studies (page 3.1.8; lines 5-8) provide useful excretion kinetics? Are monkey excretion kinetics more similar to excretion kinetics of humans than mice and rats? Information regarding the excretion in rats should be used in support of what is available from primates. Do the authors expect humans to have sex-specific differences in urinary excretion kinetics (a reason to use rats)?	Recommend revising the order of paragraphs to focus (highlight) the information of interest (e.g., primate excretion kinetics) and then respond to user questions and concerns. Additional information is provided in previously cited monkey studies. Rat and mouse studies should be used to support the use of primate excretion kinetics.
3.1.4 Excretion	3-18 (lines 27-29)	The authors write, "*A key assumption in this classical* [PK] *analysis is that distribution and elimination are independent of the exposure route, and the EPA interpreted these discordant empirical results as indicating that this assumption is incorrect.*" The EPA's subsequent analysis suggests that urinary excretion might be slower after IV dosing. Is there any supporting evidence for this, and the statements above?	Additional analysis is required. Are there other explanations for the difference between oral and IV dosing AUCs? • Given the predicted PKa for PFHxS, does passage through the acid environment of the stomach facilitate availability for protein binding in the blood? EPA's analysis should report on any known differences in PFHxS

			excretion in animals dosed orally and by IV.
3.1.4 Excretion	Table 3-3	Why is the only one monkey study reported in Table 3-3 that performed by Sundstrom et al. and not all the previously identified in the text (e.g., Goldsmith and Egeghy 2012)?	Suggest reporting all the available information regarding excretion kinetics in primates.
3.1.4 Excretion	3-19 (lines 1-7)	Why was the Kim et al. (2018b) study used to determine fecal clearance/elimination in humans? If the *"… higher fraction eliminated in feces after oral dosing was attributed in part to incomplete absorption by that route*" is the oral absorption of PFHxS much less (e.g., 84.9%) than the earlier assumption of 100%. If you use the average value of 8% from IV data in humans should you also assume an absorption of 92%?	Please clarify.
3.1.4 Excretion	3-21 (lines 23-24)	How does a log-linear plot showing a decline in in serum PFHxS levels indicate little effect of ongoing exposure in test subjects? The fact that PFHxS has such a long half-life in humans means that any ongoing exposures to PFHxS would be difficult to tease apart from PFHxS clearance. This is a known problem for PFOA, whose half-life in humans is much less than that ascribed to PFHxS.	Please clarify.
3.1.4 Excretion	3-22 (line 33) 3-23 (lines 1-2)	The fact that the overall population GM (geometric mean) clearance level is close to that observed by Fu et al. does not make it "correct." It simply provides greater confidence that the true value lies close by these two values. The fact that Zhang et al. GM values (men and women) are different, but within the same order of magnitude as the overall population GM provides confidence that the true value is within an order of magnitude of those reported.	Please clarify.
3.1.4 Excretion	3-23 (lines 12-14)	What is the justification for using rat fecal excretion rates to adjust total excretion/clearance estimates in humans?	Please clarify.
3.1.4 Excretion	3-24 (lines 5-6)	The average rate of clearance (0.030mL/kg-day) that corresponds to the median monthly loss (mL/month) should be reported here too. These values are provided later in this paragraph as appoint of comparison between Lorber et al (2105) and Hallberg et al (1966).	Please report a common metric (mL/month?) for use in comparing menstrual loss.

		When estimating an average year total serum loss, why is the average (female) human weight assumed to be 80 kgs? Assuming the standard default female body weight of 60 kg results in a substantial increase in the dal loss (i.e., 0.040 mL/kg-day).	Justify the use of 80 kgs in the calculation or use the standard US EPA default value of 60 kgs.
3.1.4 Excretion	Table 3-4	An indication of the variance about the reported clearance levels should be reported.	Report the weighted geometric mean clearance (mL/kg-day) and its standard deviation (a measure of the variation about the geometric mean).
3.1.4 Excretion	3-26 (lines 28-30)	Given the statement earlier on this Page (lines 2-4), *"… renal excretion may not be proportional to BW across all lifestages, for example if it varies in proportion to body surface area with age, and the volume of distribution may also vary with age."*. Why does EPA compare human clearance levels to those values predicted using allometric scaling ($BW^{3/4}$)? Is there a reason why clearance rates in monkeys were not used in this analysis?	Recommend adding a topical sentence indicating to the reader the importance of the analysis described in the paragraph. For example, an appropriate topical sentence might be "*Allometric scaling appears to overpredict human clearance rates from rodent species*."
3.1.4 Excretion	3-28	The report states that for the Kim et al. model to match the observed rate of decline in blood as well as the observed accumulation in urine and feces would require an assumption of another route of excretion, for which there are no data. Kim et al., 2018 reports that the partition coefficients (Kp's) used in their model were obtained from an in vivo study in which male rats were administered a dose of 10 mg/kg PFHxS and females were given 0.5-10 mg/kg IV. Assuming the Kp's were derived from the clearance phase of the IV kinetics, but given it was a single dose and PFHxS has a long half-life, it may be possible that the parameters do not represent steady state partition coefficients and therefore may be underestimated. If so, some stores of PFHxS may not be accounted for.	Although it is not likely possible to provide this, a comparison of predicted Kp's, based on physio-chemical properties, derived as described in Ruark et al., 2014, with those derived by Kim et al., from in vivo data, would be interesting. It would be reasonable, however, to add this to the limitations of the models discussed in the report.
3.1.4 Excretion	Table 3-5	The table should include an estimate of the confidence EPA has in the two clearance rates reported. By the number of studies used in its derivation/support, is there greater confidence in the human geometric mean clearance rate of 0.031 mL/kg-day than the other?	Assess, justify, and report the level of confidence in the results. What clearance rate is to be used? Was the use of the 80 kg female body weight justified?

3.1.6 Empirical PK Analysis	Table 3-6	Assuming the same Vd between monkeys and humans adds uncertainty to the estimation of half-life.	Recommend adding this uncertainty to the discussion and consider it appropriately.
3.2.1 Thyroid Effects	3-39 (lines 24-29)	The text states that changes in prenatal and maternal T4 have been shown to be biologically significant in the absence of changes in TSH (15 reference citations). However, there is no indication as to what magnitude of T4 change (total or free) in prenatal or maternal serum was required to cause biologically significant change.	Include information to indicate the magnitude of the change in (total and free) T4 levels associated with significant biological effects in test species and humans. This is important given the functional reserve of thyroid hormone present in humans (more than rats) that must be changed in order to cause a significant biological effect.
3.2.1 Thyroid Effects	Figure 3-4	The figure does not provide information on the form of T4 or T3 measured.	This information is critical to determining the potential impact of the change in thyroid hormones.
3.2.1 Thyroid Effects	3-42 (lines 1-23)	The results for T4 and T3 are mixed at best, with as many or more studies showing no effect as those showing a positive effect on hormone status. The data concerning TSH is even less clear.	Please provide a better explanation of how thyroid hormone effects rise to a level of concern where the effect is identified as a critical effect for use in the derivation of an RfD
3.2.1 Thyroid Effects	3-48	The EPA draft document stated that "the available evidence indicates that oral exposure to perfluorohexane sulfuric acid [PFHxS] and its related salts is likely to cause adverse thyroid and immune effects in humans on the basis of the evidence presented in human and animal toxicity studies" (p. 3-48). Regarding human toxicity studies, this is inconsistent with a previous statement in the draft document that "overall, the evidence for the association between PFHxS exposure and thyroid effects is inconsistent… Given these concerns about sensitivity, the findings across this set of [epidemiology] studies are difficult to interpret". This conclusion aligns with the ATSDR toxicological profile on PFAS which stated that "[although some associations between serum PFOA, PFOS, PFHxS, PFNA, PFDA, and PFUnA and thyroid stimulating hormone (TSH), triiodothyronine (T3), or thyroxine (T4) levels or thyroid disease have been found, the results are not consistent across studies and a larger	Please address this shortfall in the discussion.

		number of studies have not found associations" (ATSDR, 2021; p. 264[1]). Thus, it does not appear that there is strong epidemiological evidence in support of thyroid effects as an endpoint of interest for PFHxS. • Agency for Toxic Substances and Disease Registry (ATSDR). 2021. Toxicological Profile for Perfluoroalkyls. ATSDR, US Department of Health and Human Services, Atlanta, GA. Available online at: https://www.atsdr.cdc.gov/toxprofiles/tp200.pdf	
3.2.1 Thyroid Effects	3-50 (lines 1-16)	Are rats a good model of PFAS-caused thyroid hormone levels in humans? The fact that (line 16) "… *no overt toxicity was observed at any of the highest doses tested in any of the available studies*", along with conflicting evidence in humans (page 3-61; line 1-2) is not a meaningful rationale for the selection of thyroid hormone changes used as the critical effect in the derivation of the RfD.	Please provide additional justification for the use of changes in thyroid hormone levels as a critical effect in RfD development.
3.2.1 Thyroid Effects	3-62 (lines 3-4)	What does EPA mean by "… given sufficient exposure conditions."? What is present in Table 3.8 (Page 3-63) that addresses the sufficient exposure conditions? Dose and the nature of exposure are not identified as an exposure condition in Table 3-8.	Please provide a better explanation
3.2.2 Immune Effects	3-65 (lines 3-16)	More recent guidance is available regarding the assessment of immune system hazard. Germolec et al (2022) notes that the relationship between suppression of functional immune measures and clinical disease remains uncertain at the lower end of the curve.	Recommend using the consensus Key Characteristics identified in Germolec et al. (2022) as a basis for identifying immunotoxic agents for hazard identification. • Germolec et al. 2022. Consensus on the Key Characteristics of Immunotoxic Agents as a Basis for Hazard Identification. Environmental Health Perspectives. 130(10):105001-1. https://doi.org/10.1289/EHP10800.
3.2.2 Immune Effects	3-65 (lines 17-33)	Antibody response outcomes are not useful as adverse outcomes from which to derive an RfD.	Provide better justification for selecting this critical effect. • Chang et al 2016. A critical review of perfluorooctanoate and

		According to Chang et al (2016), an immunodeficiency should not be presumed to exist when there is no evidence of a clinical abnormality. Shih et al (2021) noted, in their study of PFAS on vaccine antibody concentrations in the Faroe Islands cohort, that overall, there was limited evidence for inverse associations between PFAS (including PFHxS) and antibody concentrations at a significance level of 0.05 threshold.	perfluorooctanesulfonate exposure and immunological health conditions in humans. Critical Reviews in Toxicology. 46(4):279-331. Http://dx.doi.org/10.3109/10408444.2015.1122573. Shih et al. 2021. (Full reference provided earlier)
3.2.2. Immune Effects	3-65	In a prior publication co-authored by Grandjean (Heilmann et al., 2006), the authors evaluated vaccine antibody response in the same population of Faorese children. They concluded that "[i]ncreased perinatal exposure to PCBs may adversely impact on immune responses to childhood vaccinations" in all matrices (maternal serum, breastmilk, and in children at 18 months or 7.5 years old), with exposure measured as a sum of congeners CB-138, CB-153, and CB-180 multiplied by 2 and congeners CB-105, CB-118, and CB-156 weighted using dioxin toxicity equivalency factors. Associations were observed for tetanus and diphtheria in both groups evaluated. In the analysis of PFAS, Grandjean et al. (2012) controlled for PCB exposure via the sum of congeners CB-138, CB-153, and CB-180 multiplied by 2, but it does not appear they considered other congeners or dioxin-like equivalency toxicity factors. It is noted that these PCB serum measurements were collected "as previously described" prenatally and postnatally but is unclear whether confounding was assessed at all timepoints. The supplementary tables mention only adjustment for milk PCBs and in child serum at age 5. Grandjean et al. (2012) noted that "PCB exposures in the Faroes are higher than in the United States, **but associations with PFC concentrations were weak"** but that adjustment for PCB exposure did not "appreciably change the results." Considering the previous study reporting an association between PCBs and decreased vaccine antibody response and given the poor reporting and possibly less rigorous control of PCB exposure, is possible that there is residual confounding by PCBs in the reported associations with PFAS in Grandjean et al. (2012). • Heilmann, C., Grandjean, P., Weihe, P., Nielsen, F., & Budtz-Jørgensen, E. (2006). Reduced antibody responses to vaccinations in children exposed to polychlorinated biphenyls. PLoS medicine, 3(8), e311. https://doi.org/10.1371/journal.pmed.0030311.	We recommend that EPA carefully review the Grandjean studies for issues such as residual confounding.

3.2.2 Immune Effects	3-65 to 3-70	EPA did not include a recent study of vaccine response in children in Guinea-Bissau (Timmermann et al., 2020). This is a critical omission, because while a much smaller study than the Faroe Islands cohort, there are so few studies of PFAS and vaccine response that each study is important in the assessment of the overall weight of evidence for immunosuppression. Timmerman et al. (2020) used data from a subset of 422 children in a randomized controlled trial of measles vaccination in Guinea-Bissau and evaluated PFAS exposure and vaccine response after one or two vaccine doses at 4-7 and 9 months, respectively. Antibody levels were measured again at age 2. Serum concentrations of all measured PFAS (PFOS, PFOA, PFNA, PFDA, PFHxS and PFUnDA) were low, 0.10-0.77 ng/mL, but all were above the LOQ except for PFUnDA in one child. Statistically significant inverse associations were found for PFOS and PFDA at 9 months of age, but these associations went no longer present at 2 years of age. In fact, PFOS was associated with *higher* measles antibodies in boys at age 2. No statistically significant associations were observed at any time point for PFHxS and serum antibody concentration for measles. • Timmermann, C. A. G., Jensen, K. J., Nielsen, F., Budtz-Jørgensen, E., van der Klis, F., Benn, C. S., Grandjean, P., & Fisker, A. B. (2020). Serum Perfluoroalkyl Substances, Vaccine Responses, and Morbidity in a Cohort of Guinea-Bissau Children. Environmental health perspectives, 128(8), 87002. https://doi.org/10.1289/EHP6517	Recommend addressing the lack of statistical associations in the discussion or eliminating the data.
3.2.2 Immune Effects	3-65 to 3-70	EPA also did not include a recent study of response to vaccines in 101 infants in Germany (Abraham et al., 2020). There were no statistically significant associations between PFHxS and antibodies for diphtheria, tetanus and Haemophilus influenzae type B (HiB), after adjusting for exposure to numerous other substances including PCBs, pesticides, mercury, and several other metals. The Timmerman et al. (2020) and Abraham et al. (2020) studies should be included in the assessment of the weight of the evidence for PFHxS and vaccination response.	We recommend that EPA carefully evaluate Timmermann et al. (2020) and Abraham et al. (2020) and include a discussion of these studies the revised IRIS draft, noting that these studies may affect EPA's overall weight of evidence conclusions.
3.2.2 Immune Effects	3-67 (1-38)	The Grandjean et al. (2012) and Budtz-Jorgensen and Grandjean (2018) studies identify a reduced response (lower antibody levels) to tetanus and diphtheria toxins in young children and then suggest that this is in some way a significant loss in their ability to fight off disease or reduce the severity of disease. These authors use this limited effect to inappropriately use of a 5% BMR in dose response modeling. It is not warranted. Small changes in antibody levels are unlikely to result in a clinical measure of vaccine effectiveness. In fact, the Advisory Committee on Immunization Practices (ACIP) in General Best Practice Guidelines for Immunization (2021) report that "*The effectiveness of any vaccine is uncertain*	The limited effects identified in Grandjean et al. (2012) and Budtz-Jorgensen (2018) do not rise to the level of a critical effect from which an RfD is derived. Please provide better justification for the use of this endpoint.

		if it depends only on the humoral immune response [antibody producing]." That and the fact that the CDC does not identify any change in disease incidence or disease severity for either tetanus or diphtheria since 1990 (CDC 2022a,b), suggests that there is no clinical relevance to the findings of these authors. Consequently, the reduction of antibodies should not, by itself, be used as the critical effect from which an RfD is derived.	• ACIP. 2021. General Best Practice Guidelines for Immunization. Appendix A-25. Vaccination of persons with primary and secondary immunodeficiencies. Available Online at: https://www.cdc.gov/vaccines/pubs/pinkbook/downloads/appendices/a/immuno-table.pdf. • CDC. 2022a. Tetanus Secular Trends in the United States. Center for Disease Control and Prevention. Available Online at: https://www.cdc.gov/vaccines/pubs/pinkbook/tetanus.html#epidemiology CDC. 2022b. Diphtheria Secular Trends in the United States. Center for Disease Control and Prevention. Available Online at: https://www.cdc.gov/vaccines/pubs/pinkbook/dip.html#secular
3.2.2	3-67 to 3-68, Table 3-9	Table 3-9 does not include all the many analyses Grandjean et al. conducted across the three publications. Grandjean et al. (2017a) performed follow-up studies of the original 1997-2000 Faroese population and Grandjean et al. (2017b) conducted analyses of the original and a second cohort recruited 2007-2009. Grandjean et al. (2017a) reported no statistically significant associations between PFHxS and *diphtheria* antibody concentrations in most analyses. Total cohort analyses were not significant for serum PFHxS at 7 or 13 years, except for one analysis - serum PFHxS at 7 in the "No ER visit" group was associated with lower diphtheria antibody concentrations at 7 and 13 years of age (Indirect effect: Change= -16.2; p-value=0.042 and Total effect: Change= -19.5; p-value=0.043). When the authors used structural equation modeling to adjust for the effects of other PFAS, the statistically significant inverse associations with PFHxS disappeared for all groups and analyses, including the no ER visit, which showed an increase in antibody levels (25% change) in the total effect estimate; there was an increase in antibodies for the total cohort as well. In contrast to the 2012 results, there were no statistically significant associations between PFHxS and tetanus antibody concentrations for any group (p-value >0.05 for all groups). Grandjean et al. (2017a) does not discuss the differences across their studies; it is unclear why a general immunosuppressive effect would not cause similar effects across all antigens. Further, it is unknown whether it biologically, we would expect inconsistency across different age groups.	We recommend that the EPA re-evaluate its study evaluations of and overall confidence in the Grandjean studies. Further, EPA should reflect on whether the conflicting evidence across the numerous Grandjean et al. studies of the same population should be considered as a factor that "reduces" confidence in the overall hazard judgment for immune effects.

		Grandjean et al. (2017b) conducted analyses of the earlier 1997-2000 cohort but also a similar but later cohort of Faroese children (the 2007-2009 cohort). There were no statistically significant associations between PFHxS measured at birth, 18 months, or 60 months and antibody levels for diphtheria or tetanus measured at age 5 in either cohort. Interestingly, for PFHxS measured at the later ages, diphtheria vaccine antibodies were *increased* at age 5**,** albeit not statistically significantly, with increasing infant serum PFHxS in the 2007-2009 cohort. It should be noted that given the many analyses conducted across these studies and considering the inconsistencies in findings and the fact that we do not expect PFHxS has a protective effect on the immune system, some of these results are likely due to chance or possibly, confounding. EPA acknowledged the inconsistency across studies stating, "**There are some results in the opposite direction for sub-analyses of the Faroe Island cohorts (Grandjean et al., 2017b; Grandjean et al., 2017a; Grandjean et al., 2012). No biological rationale has been identified as to whether one time period is more predictive of an overall immune response" (p-3-67).** • Grandjean, P; Heilmann, C; Weihe, P; Nielsen, F; Mogensen, UB; Budtz-Jørgensen, E. (2017a). Serum vaccine antibody concentrations in adolescents exposed to perfluorinated compounds. Environ Health Perspect 125: 077018. http://dx.doi.org/10.1289/EHP275. • Grandjean, P; Heilmann, C; Weihe, P; Nielsen, F; Mogensen, UB; Timmermann, A; Budtz-Jørgensen, E. (2017b). Estimated exposures to perfluorinated compounds in infancy predict attenuated vaccine antibody concentrations at age 5-years. J Immunotoxicol 14: 188-195.	
3.2.2. Immune Effects	Table 3-9	Table 3-9 in the Draft demonstrates that there is a lack of consistently decreased antibodies across multiple types of antigens, including diphtheria, tetanus, measles, rubella, Hib vaccine, flu, and mumps vaccines (with no positive associations reported for flu, measles, Hib, or mumps vaccines in any study). There is no clear biological reason for such inconsistency. It also is notable that an in-press systematic review and meta-analysis (Crawford et al., 2023) found no statistically significant associations between PFHxS and antibody response for "all vaccines" combined, and for all ages and for children only. "Rubella in children" was the only	Recommend that the EPA conduct a supplementary literature search to identify potentially relevant literature that have been published more recently.

		statistically significant association, and this was based on two studies. Further, the risk of bias was rated as “moderate” or “serious” for most of the studies of PFHxS, indicating limitations in the underlying body of evidence. • Crawford, L; Halperin, SA; Dzierlenga, M; Skidmore, B; Linakis MW; Nakagawa, S.; Longnecker, MP. 2023. Systematic review and meta-analysis of epidemiologic data on vaccine response in relation to exposure to five principal perfluoroalkyl substances, Environment International, *in press*.	
3.2.8 Female Reproductive Effects	3-224 to 3-225 and 3-229	For both male and female reproductive histopathological analysis, assembled IRIS authors are advised to re-assess the pathology images in all stated articles to increase confidence in appropriate interpretation of the images/data. Interpretation of the female reproductive histopathology (reported on page 3-224) appears somewhat arbitrary and subjective in the absence of clearly stating that such images/data were interpreted against the standards provided by the International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) of the Society of Toxicological Pathology for all citations referenced. Similarly, while it was also found that in male SD rats, exposure to PFHxS for 28 to 44 days at doses ranging from 0.3 to 10 mg/kg-day did not affect the histopathology of the testes, preputial glands, epididymis, or seminal vesicles, this data/imaging found in the cited articles needs to be critically determined to the International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) standards of the Society of Toxicological Pathology for all citations referenced.	Suggest reevaluating the histopathological examination of both the male and female reproductive histopathology data to strengthen confidence in appropriate and accurate interpretation. Please refer to the following helpful resources: • Mann PC, Vahle J, Keenan CM, et al. International Harmonization of Toxicologic Pathology Nomenclature: An Overview and Review of Basic Principles. Toxicologic Pathology. 2012;40(4_suppl):7S-13S. doi:10.1177/0192623312438738 • Dixon D, Alison R, Bach U, Colman K, Foley GL, Harleman JH, Haworth R, Herbert R, Heuser A, Long G, Mirsky M, Regan K, Van Esch E, Westwood FR, Vidal J, Yoshida M. Nonproliferative and proliferative lesions of the rat and mouse female reproductive system. J Toxicol Pathol. 2014;27(3-4 Suppl):1S-107S. doi: 10.1293/tox.27.1S. PMID: 25516636 • Creasy D, Bube A, Rijk E de, et al. Proliferative and Nonproliferative

			Lesions of the Rat and Mouse Male Reproductive System. Toxicologic Pathology. 2012;40 (6_suppl): 40S-121S. doi:10.1177/0192623312454337
5. Derivation of Toxicity Values	5-6	EPA states that, *"Inverse associations, indicating immunosuppression, were generally observed between PFHxS exposure and antibody levels across different combinations of timing of exposure and outcome measures, and similar findings were reported for other long-chain PFAS" (pg. 5-6).* While it is true that some inverse associations were observed, there were also several relationships evaluated where this was not the case. In Grandjean et al. (2012), maternal and postnatal exposures to PFHxS were not associated with any significant changes in diphtheria antibodies (Grandjean et al., 2012). Furthermore, while there were significant decreases in tetanus antibodies in children of exposed mothers at ages 5 and 7 for PFHxS, adjustment at age 7 for age 5 appeared to attenuate the degree of significance of the association at year 7.	There remains uncertainty in the strength of the evidence for the cohort data relied upon to derive the RfD. Please consider addressing this shortfall.
5. Derivation of Toxicity Values	5-6	*"Consistent findings of reduced antibody responses from human epidemiological studies provide moderate human evidence of immunosuppression with PFHxS exposure."* EPA's conclusion that they are "consistent findings" across human epidemiological studies is not supported by the underlying literature, and it is unclear if there is a causal association between PFHxS and immunosuppression in humans. Grandjean et al. (2012) stated that "Although all of the 5 PFCs [including PFHxS] measured showed negative associations with antibody levels, the overlapping confidence intervals and the lack of comparative toxicology studies prevent inference in regard to causal attribution". As discussed further in the next comment, there were null findings for PFHxS for some antigens and age groups. The draft IRIS document itself acknowledges that "*there are a minority of combinations **for which positive associations (higher antibody levels with higher PFHxS exposure)** were observed (not statistically significant). This heterogeneity in results does not have a clear biologic explanation and the relevant etiologic window of exposure for this outcome is not known…"* [emphasis added] Therefore, there is currently no consistent evidence of an adverse, consistent association between PFHxS and immunosuppression. Overall, the evidence does not support the hazard conclusion of "moderate" evidence of an immunosuppressive effect, a classification that the 2022 IRIS Handbook states is used when there is "moderate human evidence supporting an effect and moderate-to-indeterminate	We recommend that the EPA change the evidence integration judgment for immunosuppression to "evidence inadequate." This classification would prevent this endpoint from being used as the basis for an RfD.

		animal evidence." Given there is no evidence of immunosuppression in animals and a paucity of information, accompanied by only slight human evidence, this classification is not supported. Similarly, the classification of "evidence suggests" can be applied for slight evidence in both human and animal evidence; however, the evidence does not rise to these standards, either.	
5.2.1. Oral Reference Dose (RfD) Derivation	5-2, Appendix D	The Grandjean studies (2012, 2017a, 2017b) did not report a POD for immunosuppressive effects; thus, EPA relied on the results of Budtz-Jorgensen and Grandjean (2018) to derive the RfD. Budtz-Jorgensen and Grandjean (2018) used data from the cohort studies in Grandjean et al. (2012) to perform benchmark dose modeling (BMD). The modeling is more sophisticated compared to traditional BMD analysis, as it incorporated individual data and allowed for control of study confounders, such as sex and age. Each of the 5 measured PFAS were modeled separately, and then in adjusted models where each of the 5 were modeled with the other four as additional covariates. However, the overall presentation and justification for the two linearized models – one linear and one a two-piece spline model – is lacking. The authors do not discuss how the models fit the underlying data, and there are no statistics to help visualize the fit; the authors failed to report the R^2 for the models. For the two-piece spline model, the authors did not appear to evaluate where the inflection point was likely to be and simply used the median. The authors should also have run other model types, including non-linear models, and more carefully evaluated the likely inflection point in the two-piece model. Further, the authors selected a BMR of 5%, which while often used by EPA for epidemiological data, must be carefully considered given the questions surrounding adversity and clinical significance of this particular outcome (decreased antibody levels). A BMR should be near the low end of the observable range of response or tied to assay sensitivity. If you have an outcome that is not necessarily adverse, the BMR is usually less sensitive than 5%; in other words, the BMR chosen in this study was possibly overly conservative given the potential lack of adversity in the outcomes measured.	Please consider clarifying how the models fit the underlying data and including appropriate justification for approaches used in the modeling efforts.
5.2.1. Oral Reference Dose (RfD) Derivation	5-2, Table 5-4, Appendix D	Despite the uncertainties in the Budtz-Jorgensen and Grandjean (2018) analysis, EPA appeared to wholly accept the methods and results. EPA then calculated the regression coefficients (β) and their standard errors (SE) from the publication's BMDs and BMDL based on a BMR of ½ SD in log2(diphtheria antibody concentrations) and in log2(tetanus antibody concentrations) to consider for the RfD derivation. Ultimately, The POD_{HED} for immune effects	We recommend that the EPA consider alternative approaches, e.g., using 1 SD as the BMR for modeling for immune changes, keeping in mind that it is

		for tetanus and diphtheria antibody concentrations were 5.4×10^{-9} mg/kg-day versus 5.7×10^{-9} mg/kg-day, respectively, and EPA chose POD_{HED} of 5.4×10^{-9} mg/kg-day for decreased serum anti-tetanus antibody concentrations at age 7 for the derivation of the osRfD. The EPA acted counter to its own guidance in using a BMR based on ½ SD from the control mean. "When no information is readily available that allows for determining a minimally biological significant response, the BMD Technical Guidance (U.S. EPA, 2012) recommends a BMR based on 1 SD for continuous endpoints when biological information is not sufficient to identify the BMR." EPA states in Appendix D, "…decrements in the ability to maintain effective levels of tetanus antitoxins following immunization may be indicative of wider immunosuppression in these children exposed to PFHxS. By contrast, a BMR of 1 SD may be more appropriate for an effect that would be considered "minimally adverse." EPA does not support that the lowered antibody levels were minimally observe, and in fact acknowledges the uncertainty in the 0.1 IU/mL cutoff referenced by Grandjean et al. In fact, as noted in comment X above, it is likely that many of these exposed children had "effective levels" of tetanus antitoxins and anti-diphtheria antibodies.	recommended that an alternative POD be selected for the RfD. If these data are still used for the final IRIS assessment, EPA should at a minimum perform its own fully independent BMD analysis of the Grandjean et al. data rather than basing its analysis off an existing published BMD analysis with critical limitations and insufficient information.
5.2.1. Oral Reference Dose (RfD) Derivation	5-11	EPA's confidence in an immunotoxic hazard and selection of this endpoint as the critical effect to derive its RfD for PFHxS differs from approaches by other health agencies. The Minnesota Department of Health (MNDOH) derived an RfD of 9.7 ng/kg/day for PFHxS based on decreased FT4 modeled from the NTP (2018) study (BMDL of 20% decrease). MNDOH did not derive an RfD for immunotoxicity, noting that, several epidemiology studies have examined the potential association between PFHxS and suppression of the immune system. Inverse or no associations were observed in these studies. In general, available studies have not found an association between PFHxS and infectious disease resistance or with hypersensitivity outcomes." Rather, MNDOH applied a UFD of 10 to account for uncertainties pertaining to immunotoxic effects. The same RfD was used by the states of Washington (2021) and Michigan (2019) for drinking water guidance derivation. Neither of these state reviews identified immunotoxicity as a co-critical effect. Illinois based their health advisory level on the ATSDR's Maximum Risk Level of 2×10^{-5} mg/kg-day, which was based on thyroid follicular damage in rats reported in Butenhoff et al. (2009). **Comparison of EPA's Lifetime RfD Derivation to other Agencies**	We recommend that the EPA consider putting forth the thyroid hormone-based RfD as the recommended toxicity value (or perhaps it should be the only RfD presented), considering that there are fewer uncertainties and higher confidence in the thyroid toxicology studies relative to the epidemiological studies of immune effects.

Endpoint	Study	Strain/ species/ sex	POD_{HED} (mg/kg-d)	UFA	UFH	UFS	UFL	UFD	UFC	Candidate value (mg/kg-d)
EPA										
Decreased vaccine antibody response	Budtz-Jorgenson et al. (2018)	Humans	5.40×10^{-9}	1	10	1	1	3	30	2×10^{-10}
↓ Total T4	Ramhøj et al. (2018)	Wistar rat, M+F F1 (PND 16/17)	1.15×10^{-5}	3	10	1	1	3	100	1×10^{-7}
MN DOH										
↓ FT4	NTP (2018)	Sprague-Dawley, Male (Adult)	0.00292	3	10	1	1	10	300	9.7×10^{-6}
ATSDR MRL/IL										
Thyroid follicular hypertrophy	Butenhoff et. al. (2009)	Sprague-Dawley, Male (Adult)	0.0047	3	10	1	1	10	300	2×10^{-5}

EPA should consider whether there is sufficient confidence in the weight of the evidence for PFHxS and immunosuppression to support derivation of an RfD based on immunotoxicity.

- MNDOH. 2020. Toxicological Summary for: Perfluorohexane sulfonate. Available online at https://www.health.state.mn.us/communities/environment/risk/docs/guidance/gw/pfhxs.pdf
- https://doh.wa.gov/sites/default/files/2022-02/331-673.pdf

5.2.1 Oral Reference Dose (RfD) Selection	5-13 and 5-18	Deriving a RfD from epidemiological data suggesting developmental immune impacts is overly conservative (with 3x higher UF, osRfDs for thyroid are 3000x higher than those for immune).	As described on 5-18, the EPA has high confidence in the thyroid data (rodents) compared to medium

			confidence or developmental immune (humans). The rodent work also doesn't have the issue of confounding with other PFAS. The more scientifically justified endpoint is decreased T3 and T4.
5.2.2. Subchronic Toxicity Values for Oral Exposure	5-22	With regard to the study choice for the thyroid organ-specific RfD, EPA states "Ultimately, similar to the datasets advanced for the lifetime thyroid osRfD derivation, the PODs from the Ramhøj et al. (2018) study were advanced over the PODs from the *high* confidence NTP 21 (2018a) study because the Ramhøj et al. (2018) study included PFHxS exposure during what is interpreted as a critical window for effects on the developing thyroid system (i.e., gestation), and because it resulted in a lower POD" (pg. 5-22). It appears that the main reason Ramhøj et al. (2018) was selected over NTP (2018) was because the resulting RfD was lower. In this study, there were only two doses: 0.05 and 5 mg/kg/day. The NOAEL was reported to be 0.05 mg/kg-day and the LOAEL at 5 mg/kg-day. With a difference of two orders of magnitude between the NOAEL and LOAEL, it is difficult to ascertain where the true POD lies with a large range of values. As the Massachusetts Department of Environmental Protection (MassDEP) noted in its Technical Support Document for PFAS drinking water toxicity values, *"While [Ramhøj et al. (2018)] supports the other animal data that show PFHxS to be toxic to the thyroid and liver, the dose spacing makes it hard to determine a reliable NOAEL or LOAEL."* • MassDEP. 2019. Per- and Polyfluoroalkyl Substances (PFAS): An Updated Subgroup Approach to Groundwater and Drinking Water Values. Available online at: https://www.mass.gov/files/documents/2019/12/27/PFAS%20TSD%202019-12-26%20FINAL.pdf	We recommend that EPA consider using NTP (2018) to derive the RfD for thyroid effects.
Appendix D	D-1 to D-3	EPA utilized modeling from Budtz-Jorgensen and Grandjean (2018) to derive the RfCs for PFHxS. EPA noted that in BMD modeling, the effects of PFHxS were significant in single pollutant modeling but attenuated by 25% when controlling for PFOS and PFOA, suggesting possible confounding. However, EPA also noted that controlling for these exposures actually	We recommend that the EPA re-evaluate and select the critical effect with the least uncertainty for derivation of the RfD. It is

		induced confounding. EPA concluded, “The reasons for the change in main effect size for PFHxS are not known. For this reason, there is uncertainty in knowing which point estimate is the best representation of any effect of PFHxS” (pg. D-2). Considering the results of the BMD modeling in concert with the inconsistent results and many null findings in the underlying Grandjean et al. cohort studies (2012, 2017) calls into question the use of the immune data to derive the RfD.	recommended not to use immune effects as a candidate critical effect. At a minimum, the benchmark modeling approach should be refined and re-run using a BMR of 1 SD.
Multiple (3.2.1, 3.2.3, 3.2.4, 3.2.8, 3.2.9)	3-39, 3-90, 3-131, 3-213, 3-233	For all histopathology data, especially where there is concern over the appropriate interpretation of that data, and (low, medium, high) confidence in that data, it is ill-advised to take author's word for histopathology interpretation of that data found in the cited articles. It would lend strengthened confidence and add an additional layer of objective analysis if contributing and key IRIS authors refer to the International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) of the Society of Toxicological Pathology for all citations referenced. This will require additional interpretation, but oftentimes, histopathological image data is poorly or incorrectly analyzed and interpreted. On doing so, and documenting doing so, would effectively remove any doubts about the objective and independent scientific interpretation of the histopathology data for all organ systems assessed in both male and female animal models.	Please refer to the following detail guidance articles, and please document them in the bibliography of this (and all future IRIS assessment) IRIS assessment of PFHxS. It is hoped that by referencing the below guidance articles, that standard nomenclature across this IRIS assessment for histopathological imaging analysis will be adopted and documented clearly in the report. • Mann PC, Vahle J, Keenan CM, et al. International Harmonization of Toxicologic Pathology Nomenclature: An Overview and Review of Basic Principles. Toxicologic Pathology. 2012;40(4_suppl):7S-13S. doi:10.1177/0192623312438738 • Brändli-Baiocco A, Balme E, Bruder M, et al. Nonproliferative and Proliferative Lesions of the Rat and Mouse Endocrine System. J Toxicol Pathol. 2018;31(3 Suppl):1S-95S. doi:10.1293/tox.31.1S • Thoolen B, Maronpot RR, Harada T, et al. Proliferative and Nonproliferative Lesions of the Rat and Mouse Hepatobiliary System. Toxicologic Pathology. 2010;38(7_suppl):5S-81S. doi:10.1177/0192623310386499

			• Frazier KS, Seely JC, Hard GC, et al. Proliferative and Nonproliferative Lesions of the Rat and Mouse Urinary System. Toxicologic Pathology. 2012;40(4_suppl):14S-86S. doi:10.1177/0192623312438736 5) Berridge BR, Mowat V, Nagai H, Nyska A, Okazaki Y, Clements PJ, Rinke M, Snyder PW, Boyle MC, Wells MY. Non-proliferative and Proliferative Lesions of the Cardiovascular System of the Rat and Mouse. J Toxicol Pathol. 2016;29(3 Suppl):1S-47S. doi: 10.1293/tox.29.3S-1. Epub 2016 Jul 29. PMID: 27621537; PMCID: PMC5013710.