Case study from SEURAT-1: Perfluoroalkyl acids: direct acting toxicant category supported by SEURAT-1 data

SEURAT-1 PERFLUOROALKYL ACIDS READ-ACROSS CASE STUDY CONSIDERED IN CONTEXT OF THE ECHA RAAF

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NOTE: This assessment document has been prepared to facilitate the discussion at the Topical Scientific Workshop and does not represent ECHA's position.

Case Study: **Read-across for 90-Day Rat Oral Repeated-Dose Toxicity for Selected Perfluoroalkyl Acids: A Case Study**
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PFAA Category (C7-C10) Case Study Read-Across Hypothesis:
Read-across is proposed to fill data gaps for 90-day oral repeated dose toxicity of selected perfluoroalkyl acids (PFAA) category members. Category members and additional analogues flanking the category (C6, C11, and C12 PFAAs) for which repeated dose data exist have been shown to elicit liver toxicity as the critical effect. The read-across hypothesis is that the PFAA category members all have the potential to directly (i.e. parent chemical, biotransformation not required) elicit liver toxicity. Based on the quantitative variations in effects (potency) observed across category members and flanking analogues, a worst-case approach is used. A 90-day oral repeated dose toxicity study for the category member (C8 PFAA) exhibiting the highest degree of liver toxicity is proposed for use in read-across to fill the data gaps for the other category members (C7, C9, and C10).

Scenario according to ECHA Read-Across Assessment Framework (RAAF):
This read-across is consistent with RAAF read-across scenario # 4 – i.e. category approach with a read-across hypothesis based on different compounds having the same type of effect and where there are variations in the strength of effects which follow a regular pattern across source substances. In this scenario, the predicted property is either based on the established regular pattern or a worst-case approach. For this PFAA case study, the prediction (and read-across) is based on a worst-case approach. Data from additional flanking analogues of the category (C6, C11, and C12 PFAA) are pivotal to this read-across justification, particularly with regard to establishing the regular pattern which enables identification of the worst-case category member. In the end, the 90-day study for C8 PFAA is proposed as the read-across source for target chemicals C7, C9, and C10 PFAA.

Evaluation of the PFAA Case Study according to ECHA RAAF:
All relevant read-across 'Assessment Elements' (i.e. crucial scientific aspects of the read-across justification) for RAAF Scenario #4 are evaluated and an 'Assessment Option' (i.e. score based on strength of the information/evidence provided) is proposed for each Element. This is done first based on only consideration of the traditional data and then again including consideration of the new approach methods (NAM) data, in order to elucidate the utility of the NAM data to strengthen the read-across. Finally, based on the totality of 'Assessment Option' responses to all elements, the read-across is judged for acceptability.

The table below represents consideration of the PFAA case study read-across (RA) justification in the context of the ECHA RAAF. Contributions of NAM data are in red text, and how the additional information improves the Assessment Options are highlighted in grey.

RAAF SCENARIO #4		PFAA Case Study		RAAF Assessment Option	
AE#	**Assessment Element/** Details	**Page# Lines**	**Relevant Text and Tables in Case Study Report**	**AO#**	**Rationale**
C.1	**Identify/characterize substances which are members of the category, including impurity profile**	p4 123-126 p5 130-131	Annex Table 1 list of category members "A purity/impurity profile for the analogues listed in 2.4 is unknown. However, since the category is so limited structurally, the potential impact of any impurities on the endpoint being evaluated is considered very small."	2	No impurity profile provided. *To more directly address the RAAF: Add % purity and list any expected impurities.*
C.2	**Describe the structural similarity and allowable differences for category**	p3 85-88 p4 120-121 p13 383-384 p13 391-393 p21 658-659	Annex Table 3 the C7 – C10 PFAAs form a consistent category…. highly fluorinated chemicals that consist of a straight- chain hydrocarbon backbone and a single terminal carboxylate moiety. The applicability domain for this read-across is confined to straight-chain perfluorinated carboxylic acids of C7 to C10. Structurally, the only difference is the length of the C-atom backbone. As shown in Table 3 of Annex I, all the PFAAs included in the category have common constituents in the form of: 1) a single key substituent, $-CO_2H$, 2) structural groups, $-CF_3$ and $-CF_2-$, 3) extended structural fragment $-CF_2CO_2H$. While PFAAs vary from C4 to C18, by design, the category is limited to C7 to C10 analogues.	5	Structural similarity and allowable differences are clearly stated. Similarity is based on carboxylic acids with fluorine saturated alkyl backbones and allowable differences are limited to the length of the fluorine substituted alkyl backbone.
C.3	**Explain the link between the structural similarities/differences and the proposed prediction of property**	p3 89-91 p4 104-107	Annex Table 2 PFAAs are absorbed by the gut, bind to albumin and other proteins and are not metabolized in the liver. Their persistence is markedly influenced by reabsorption in the kidneys. Because of strong carbon-fluorine bonds, PFOA, similar to other PFAAs, is resistant to environmental degradation and biotransformation. Extensive data in humans and animals demonstrate PFOA is readily absorbed and distributed throughout mammals	5	Sufficient evidence is provided to link the structure of category members (fluorinated linear C7-C10 alkyl chain carboxylic acids) to the predicted property (liver toxicity):

			via non-covalent binding to plasma proteins.		- structural similarity to linear fatty acids generally known to cause similar liver effects - strong C-Fl bonds are resistant to biotransformation - ionized carboxylate and Fl partial negative charges promote electrostatic interactions with proteins - protein binding facilitates absorption, transport, and bioaccumulation -bioaccumulation is increased by efficient reabsorption in the kidneys
		p5 136-140	In PFAAs, charge repulsion of the partially negative F-atoms and steric factors give preference to the lowest energy conformer being a near linear molecular shape. This most likely conformer is highly similar to the preferred conformation of corresponding fatty acid analogues. The ionized carboxylate grouping and the F-atom's partial negative charges promote electrostatic interactions between PFAAs and positively charged surfaces on macromolecules, especially proteins.		
		p5 149-156	PFAAs are resistant to biotransformation. Therefore, toxicity of the parent compound and not that of a metabolite is of concern. Due to their impact on receptors and other cellular proteins, PFAAs have the ability to alter intermediate metabolism and transformation of dietary molecules by altering enzyme activities and transport kinetics. In general, the rate of elimination is enhanced with decreasing C-atom chain length. However, the body-burden, especially in primates but also in rats is increased by efficient reabsorption of PFOA in the kidneys and thus retention in the body. The net effect is clearance rates that are both species- and analogue-dependent with lowest total clearance expected to be for PFDA (Han et al., 2012; Fujii et al., 2015).		
		p6 177-181	Several studies in rats and mice have examined PFAAs to determine the potential impact of the carbon chain length (C6-C9) on hepatic toxicity and peroxisome proliferation. Results suggest the difference in accumulation of these compounds in the liver was responsible for the different hepatic responses observed between PFAAs with different C-atom chain length. In any case, it is generally believed that the potency of PFAAs increases with increasing C-atom chain length up to C8 (Wolf et al., 2012).		
		P14 422-427	Evidence suggests that PFAAs circulate in the body by non-covalent binding to plasma proteins. For example, rat, human, and monkey plasma proteins bind > 95% of PFOA added at concentrations ranging from 1-500 ppm (Kerstner-Wood et al., 2003). Serum albumin, the most common serum protein and a common carrier of hydrophobic materials in the blood including short and medium chain fatty acids, carried the largest portion of the PFAAs among the protein components of plasma.		
		p14 433-436	Weiss et al. (2009) screened 30 perfluorinated compounds, differing by C-chain length (C4-C18), fluorination degree, and polar groups for potential protein binding. They concluded that binding affinity is highest for fully fluorinated materials and compounds having at least eight C-atoms.		

C.4	**Demonstrate/discuss consistency of effects in data matrix**			3	Sufficient evidence is provided in text descriptions of studies to demonstrate liver is consistent low dose target; however, insufficient detail in ordered data matrix to make determination. *To more directly address the RAAF: Include more descriptive detail on in vivo studies in data matrix so consistency of effects can be fully evaluated from review of the matrix. Inclusion of observed effects and LOAELs in addition to NOAELs would facilitate evaluation of consistency AND any potency differences across category members. (**See attached ANNEX 1 as example**.) Also add discussion to text address occurrence of other observed effects (e.g. hematological).*
	Documentation includes discussion of consistency of data for predicted property, and any inconsistencies are explained	P5 157-160 p7 199-373 p12 376-377	Annex Table 8 A first examination of mammalian toxicity data supports the contention that repeated-dosage oral exposure to PFAAs is linked to liver toxicity. PFAAs, in rat oral repeated-dose testing, exhibit liver toxicity typically in the form of hepatocyte necrosis and increased liver weight. While there are 90-day oral repeated-dose toxicity data for the octanoic and hexanoic derivatives, there are data gaps for other PFAA analogues in the category. *NOTE: Descriptions in text provided for repeat dose studies for PFOA and other PFAAs (C6, C11, and C12) indicated consistent effects on liver.* Oral repeated dose data for the 6C, 11C and 12C PFAA derivatives are in qualitative agreement with that reported for PFOA.		
	Documentation includes discussion of occurrence of any other relevant effects (than predicted property)	p7 195-197 p7 199-355 p12 357-378	Chronic toxicity data for PFOA are summarised in Table 1. There is a substantive body of evidence that liver toxicity is prevalent at higher doses of PFOA and whilst other organ level effect occur, those to the liver dominate. *NOTE: In one 2-yr study and one 28-day study hematological effects were also noted at higher doses (LOAELs of 16 and 30 mkd and NOAELs of 1.6 and 10 mkd, respectively) than the identified LOAELs for liver effects (14 and 0.3 mkd, respectively).*		
	Consistency between predicted and related properties is demonstrated	p6 164-166	In addition to hepatotoxicity, PFAAs are linked to developmental toxicity (Lau et al., 2004; Wolf et al., 2010) and immunotoxicity (DeWitt et al., 2012)		
	Characterize any clustering of effects across structural features of category (or subcategories)	*NA*	*No clustering observed so not addressed in Case Study text*		
C.5	**Demonstrate adequacy of the source data to meet the info requirements**			4	Sufficient evidence is provided in cited source describing study design and test material. *To more directly address the RAAF: Directly cite read-across source study*
	Read-across source study is of adequate design	p12 374-375 p35 Table 2	there are *in vivo* data of sufficient quality and quantity for PFOA to be a source chemical and read across to fill the data gaps for the rat oral 90-day-repeated-dose endpoint of other analogues in the category High quality empirical data from standard test guidelines for the stated regulatory endpoint exists for PFOA.		
	Test material used represents	*None*	*No information found in Case Study text*		

	source				*and state test material and study design details in read-across justification text.*
	Results of read-across source study are sufficient for C&L purposes	p21 663-665	The NOAEL for PFOA of 0.06 mg/kg bw/d (based on hepatocyte necrosis and hepatocellular hypertrophy, and increased liver weight in males and females, respectively (EFSA, 2008) is read across to the other three analogues in the category		
4.1	**Identify all compounds to which the test organism is exposed**			4	Lack of metabolism of PFAAs is clearly explained but metabolism data is only provided for PFOA and METEOR predictions suggest phase II metabolism. *To more directly address the RAAF: Include metabolism data (or discussion to address lack thereof) for other PFAAs and provide rationale (PFAAs or predictive tool limitations) to dismiss METEOR prediction.*
	Substances to which organism is exposed (for target and all sources) are identified, as well as how they are formed	p3 89-90 p4 104-105 p5 149-150 p15 473-475	Annex Table 5 <u>From a toxicokinetics standpoint PFAAs are absorbed by the gut, bind to albumin and other proteins and are not metabolised in the liver.</u> Because of strong carbon-fluorine bonds, <u>PFOA, similar to other PFAAs, is resistant to environmental degradation and biotransformation</u>. <u>PFAAs are resistant to biotransformation. Therefore, toxicity of the parent compound and not that of a metabolite is of concern</u>. METEOR reveals the potential for II phase: glucuronidation of carboxylic acid moiety, but overall the compounds are predicted not to be metabolised.		
	Supporting evidence of qualitative or quantitative kinetics is provided	p13 403-405 p13 410	Annex Table 4 In mammalian studies, <u>PFAAs have been shown to be readily absorbed orally but poorly eliminated; they are not metabolized and undergo extensive uptake from enterohepatic circulation</u> (Lau et al., 2007; Bull et al., 2014; USEPA, 2014) <u>Steady-state serum levels of PFAAs are reached within a few weeks with oral dosing</u>.		
4.2	**Identify the common underlying mechanism , qualitative aspects**			5	The RA explanation provides sufficient evidence from traditional data to link the structure of category members to a common underlying mechanism for the predicted property (liver toxicity): - PFAAs are readily absorbed, bind plasma protein, and are distributed
	The mechanism that links the structures of the category members with the predicted property/effect is explained AND Supporting qualitative evidence from in vivo or in vitro studies or for uptake/kinetics (for negative read-across) is provided	p5 145-148 p6 167-168 p13	Annex Table 7 It is clear that there are species differences (e.g., half-lives in human vs. monkey/rodent) in the toxicokinetics of PFAAs. Typically, <u>PFAAs are readily absorbed following oral exposure and distributed mainly to the serum, kidney, and liver, with liver concentrations being several times higher than serum concentrations.</u> <u>Due to their structural similarity to naturally occurring fatty acids, mechanistic studies of PFAAs have focused on the peroxisome proliferator-activated receptor (PPAR) pathways leading to liver toxicity</u> <u>In general, the elimination is decreased by renal resorption thus, retention in the body is increased</u> (Andersen et al., 2006; Rusyn, 2015).		

		400-402 p14 428-429 p14 441-442 p15 446 p15 457-462 p16 483-488 p16 501-505 p16 506-513	 Wu et al. (2009) in examining the interaction of PFOA and human serum albumin demonstrated PFAA binding to the protein. Serum concentrations reached steady-state levels within four to six weeks in all dose groups. Urine PFOA concentrations reached steady-state after 4 weeks Fujii et al (2015) studied the toxicokinetics of six to fourteen carbon chain length PFAAs in both mice and humans. In mice, C6 and C7 PFAAs were eliminated rapidly in the urine, as compared to C8 to C14 which accumulated in the liver and were excreted slowly in faeces. Fujii also showed a large interspecies difference which was related to the sequestration volumes of the liver. Urinary clearance of PFFAs in humans also decreased with increasing alkyl chain lengths, while biliary clearances increased. The C9 to C10 derivatives had the smallest total clearance for both mice and humans. while the toxicology of perfluorinated chemicals is well-studied, the mechanistic pathways of toxicity of PFAAs are likely multiplicative. PFAAs have been associated with interference with lipid metabolism and bind to fatty acid-binding protein (Luebker et al., 2002; Zang et al., 2013), and also bind to human serum albumin (Chen and Gao, 2009). There is growing evidence that underlines liver PPAR ligand-dependent activation as a key MIE in the elicitation of liver steatosis (Al Sharif et al., 2014). In addition to PPARs dysregulation, studies of PFAAs' toxicities have reported other potential molecular mechanisms. Specifically, previous studies assessed the binding potency of PFAAs with several proteins, including other nuclear receptors such as the oestrogen receptor (Benninghoff et al., 2011; Gao et al., 2013), as well as transport proteins such as Transthyretin (Ren et al., 2015). Bjork et al. (2011) concluded that multiple nuclear receptors are activated by PFAAs. While there is evidence supporting PFOA-induced liver toxicity and adenomas via a PPARα agonist mode of action in rodents, there is also some evidence that hepatomegaly may be associated with a PPARα independent mode of action (Rosen et al., 2008). It is likely this is a PPARγ-mediated mode of action. While PPARα is more likely related to fatty acids oxidation, PPARγ is the main regulator of adipocyte differentiation, stimulating the expression of lipogenic proteins (i.e., transporters, fatty acid synthesizing enzymes, enzymes related to triglyceride synthesis and lipid droplet associated proteins). Therefore, the liver enlargement observed in PPARα null mouse		- efficient renal resorption (which increases w/ increasing chain length) increases body retention - liver concentrations are higher than serum concentrations - linear fatty acid-like structure binds to PPAR and fatty-acid binding protein - activation of PPAR and perturbing lipid metabolism/transport can induce peroxisome proliferation and result in liver toxicity - both PPARα (fatty acid oxidation) and PPARγ (stimulates expression of lipogenic proteins) are activated.

			may be due to the accumulation of lipid droplets or the accumulation of PFOA in the liver and PPARγ and adipose differentiation-related protein (ADRP).		
		p17 514-515	the largest categories of induced genes are those involved in metabolism and transport of lipids, particularly fatty acids (Rosen et al., 2008).		
		p17 518-524	NMR-based metabonomic results for both liver tissues and serum revealed that exposure to PFDoA leads to hepatic lipidosis, which is characterized by a severe elevation in hepatic triglycerides and a decline in serum lipoprotein levels. Moreover, results from transcriptomic changes induced by the C12 derivative confirm these results as changes in gene transcript levels associated with fatty acid homeostasis. It was concluded that PFDoA induces hepatic steatosis via perturbations to fatty acid uptake, lipogenesis, and fatty acid oxidation. A dose-dependent increase in the expression of both PPARα and PPARγ and also of CD36 (fatty acid translocase), which is a common target of the two receptors is reported (Ding et al., 2009).		
		p19 573-575	Further consideration of Ding et al. (2009), specifically the increase in the expression of PPARγ and its role in fatty acids and triglyceride synthesis, together with the stimulation of accumulation in lipid droplets, suggest a synergistic action of PPARα and PPARγ in the liver pathology of PFAAs.		
		p21 660-662	While there is evidence that PFAAs activate a multiplicity of nuclear receptors, PPARα and/or PPARγ interactions are the most likely initiating events leading to repeated-dose, liver toxicity.		
		p22 688-689	Toxicogenomic studies of PFAAs reveal the largest group of induced genes is those involved in transport and metabolism of lipids, particularly fatty acids.	**5**	***NAM data adds to WOE and further increases confidence in the mechanism:*** *- PFAAs are identified by in silico profilers as positive for nuclear receptor binding and PPAR agonism* *- largest category of induced genes were involved in lipid metabolism and transport* *- Gene profiling in HepaRG cells*
		p22 692-695	Preliminary data from targeted gene expression profiling in metabolically-competent HepaRG cells shows activity in several PPAR / CAR/ PXR genes (ACOX1, CYP2B6, CYP2C19, CYP2C8, CYP3A4, CYP3A7, IL6, IL6R, PDK4), showing up-regulation of many of these.		
		p23 714-715	In dual luciferase reporter assays using transiently transfected HepG2 cells, PFAAs acted as *h*PPARγ agonists with potency correlating with *h*PPARγ-LBD binding affinity.		
		p23 722-727	In thyroid hormone (TH)-responsive cell proliferation assays, PFHxA and perfluorooctadecanoic acid exhibited agonistic activity by promoting cell growth. Within the same study, molecular docking analysis revealed that most of the tested perfluorinated compounds efficiently fit into the T3-binding pocket in TR and formed a		

			hydrogen bond with arginine 228 in a manner similar to T3. The combined *in vitro* and computational data (Ren et al., 2015) strongly suggest that some PFAAs disrupt the normal activity of TR pathways by directly binding to TR.		*demonstrates upregulation in several PPAR/CAR/PXR genes* *- C6-C11 PFAAs induced activity in ToxCast assays that target PPAR, PXR/CAR, and ER* *- C6 and C8 PFAA acted as TH receptor agonists by promoting cell growth in a thyroid hormone-responsive cell proliferation assay* *- molecular docking confirmed that most PFAAs fit into the T3-binding pocket*
		p23 728-730	PFAAs were screened with a variety of *in silico* profilers. The potential for full PPARγ agonism is predicted by a virtual screening procedure		
		p24 736-742	In addition, more profilers for nuclear receptor binding were run to identify potential binding to the following nuclear receptors; PPAR, AR (androgen receptor), AHR (aryl hydrocarbon receptor), ER (estrogen receptor), GR (glucocorticoid receptor), PR (progesterone receptor), FXR (farnesoid X receptor), LXR (Liver X receptor), PXR (pregnane X receptor), THR (thyroid hormone receptor), VDR (vitamin D receptor) as well as RXR (retinoic acid receptor). Some of these receptors are associated with the development of hepatosteatosis, so chemicals likely to induce hepatic steatosis are highlighted.		
		p24 746-748	C8-C10 PFAA are profiled as positive for PPAR with the nuclear receptor binding profilers, with the C11 and C12 derivatives predicted as full agonist binders for PPARγ.		
		p24 756-757 760-762	The USEPA ToxCast program screened the PFAA molecules with chain length 6-11 in up to 800 separate *in vitro* assays. Specifically, activity was seen in several target classes including PPAR, PXR/CAR, FXR, ER (estrogen receptor), AR (androgen receptor), cell stress pathways and a number of enzymes and G-protein-coupled receptors (GPCRs).		
4.3	**Describe the quantitative aspects of the common underlying mechanism**			3	Sufficient evidence is provided to demonstrate general trends across chain lengths; however, there are limited and some contradictory traditional data to address why C8 PFAA can be expected to be worst case (i.e. peak potency). This is critical to the read-across justification since both lower and higher chain lengths
	Prediction model, which defines the independent variable (structural feature or physchem property), is clearly stated and based on either a regular pattern or worst case. Explanation is provided for how chemical structures influence kinetics and/or potency to determine the differences in strength of effects across category AND Supporting evidence for the		*NOTE: Prediction model is based on PFOA as worst case.*		
		p5 152-156	In general, the rate of elimination is enhanced with decreasing C-atom chain length. However, the body-burden, especially in primates but also in rats is increased by efficient reabsorption of PFOA in the kidneys and thus retention in the body. The net effect is clearance rates that are both species- and analogue-dependent with lowest total clearance expected to be for PFDA (Han et al., 2012; Fujii et al., 2015		
		p6 180-181	In any case, it is generally believed that the potency of PFAAs increases with increasing C-atom chain length up to C8 (Wolf et al., 2012).		
		p15 460-462	Urinary clearance of PFFAs in humans also decreased with increasing alkyl chain lengths, while biliary clearances increased. The C9 to C10 derivatives had the smallest		

	explanation and prediction model is provided. Any uncertainty for targets at the boundary of the category is addressed and/or worst-case approach is justified.		total clearance for both mice and humans. p18 543-544 Wolf et al. (2008) concluded in general: 1) PFAAs of increasing C-atom chain length, up to C9, induce increasing activity of the mouse and human PPARα p18 547-550 additional work on the *in vitro* activity of PFAAs with mouse and human PPARα. They note that PPARα activity exhibits a bell-shaped curve, with PFOA being the strongest activator. Moreover, longer C-atom chain PFAAs (i.e., > C10) are relatively less potent and some do not activate human PPARα. p18 554-557 PFAAs cause a concentration- and chain length-dependent increase in expression of gene targets related to cell injury and PPARα activation in primary rat hepatocytes, and 2) The sulfonates are less potent than the corresponding carboxylates in stimulating PPARα-related gene expression in rat hepatocytes. p18 558-563 Liver fatty acid binding protein (L-FABP) is highly expressed in hepatocytes. Perfluorinated substances, including PFAAs, may bind with FABP and change their toxicokinetics and toxicity profile. Zhang et al. (2013) examined the binding interaction of 17 structurally diverse perfluorinated substances with human L-FABP in an effort to assess their potential to disrupt fatty acid binding. The binding affinity of 12 PFAAs, as determined by fluorescence displacement assay, increased significantly with their carbon number from C4 to C11 and decreased slightly when the C-number was > 11. p21 637-643 All analogues or category members are considered, from a toxicokinetic standpoint, to be similar. Regardless of the species of mammals, all four category members are judged to be readily absorbed orally, not metabolized, and with similar distributions and similar elimination mechanisms. However, there are sex-, species and chain length-dependent difference in the rates of key processes. While there is evidence that PFNA and PFDA have lower clearance in both rodents and humans than PFOA this is not considered to be significant to the read-across. Limiting the read-across to rats and narrowing the range of C-atoms for the applicability domain limits increases the similarity of ADME-related features, especially clearance rates. p21 658-665 While PFAAs vary from C4 to C18, by design, the category is limited to C7 to C10 analogues. This limitation assures that the impact of toxicokinetic and toxicodynamic uncertainties is minimal. While there is evidence that PFAAs activate a multiplicity of nuclear receptors, PPARα and/or PPARγ interactions are the most likely initiating events leading to repeated-dose, liver toxicity. The NOAEL for PFOA of 0.06 mg/kg bw/d (based on hepatocyte necrosis and hepatocellular hypertrophy, and increased liver weight in males and females,		are targets for read-across. Traditional data demonstrates: -potency increases with increasing chain length up to C8 -NOAELs for flanking analogues C11 and C12 are lower than C8 -clearance decreases with increasing chain length but biliary clearance increases -PFAAs of increasing chain length up to C9 increase PPARα activity with C8 being strongest activator -increasing affinity for liver fatty acid binding protein with increasing chain length *To more directly address the RAAF: more clearly distinguish the TK and TD data for the category and explain how together they result in peak potency for liver toxicity at C8 PFAA (PFOA).*

			respectively (EFSA, 2008) is read across to the other three analogues in the category.		
		p22 668-672	Endpoint specific factors affecting the prediction include the uncertainty associated with is the true nature of the molecular mechanism of PFAA-induced liver toxicity and how exactly the length of the fluorocarbon-backbone impacts repeated-dose toxic potency. However these uncertainties are considered low to moderate, especially since the lower and higher molecular weight derivatives are not included in the category. No endpoint non-specific factors affecting the predictions have been identified		
		p22 692-695	Preliminary data from targeted gene expression profiling in metabolically-competent HepaRG cells shows activity in several PPAR / CAR/ PXR genes (ACOX1, CYP2B6, CYP2C19, CYP2C8, CYP3A4, CYP3A7, IL6, IL6R, PDK4), showing up-regulation of many of these. The signal is strongest in PFOA, followed by the heptanoic, nanonoic and decanoic derivatives (Judson, personal communication).	5	**NAM data provides evidence to demonstrate:** - PFAAs upregulate several PPAR / CAR/ PXR genes in metabolically competent HepaRG cells with the order of signal strength being C8, C7, C9, C10 - the binding interactions of PFAAs to human PPARγ and thyroid receptor increased with increasing carbon number and were optimal at >C10 - in ToxCast assays on C6-11 PFAAs, PFOA had the most evidence for PPAR activity in concentration ranges not also associated with cytotoxicity - in ToxCast assays on C6-11 PFAAs, PXR activity is only seen in the middle length range, with PFOA being the only PFAA with consistent activity outside of the cytotoxicity range.
		p23 699-701	Wang et al. (2014) studied the inhibitory effect of thirteen PFAAs on lysine decarboxylase (LDC) activity *in vitro*. The inhibitory effect (i.e., inhibition constants obtained in fluorescence enzyme assays) of PFAAs increased significantly with chain length (C7-C18), whereas the PFAAs of < C7 did not show any effect.		
		p23 708-711	Zhang et al. (2014) also examined the binding interactions between PFAAs and PPARγ. Specifically, the *in vitro* binding of eleven PFAAs to human PPARγ ligand binding domain (*h*PPARγ-LBD) and their activity on the receptor in cells were investigated. The results showed that the binding affinity increased with carbon number from C4 to C11 and then decreased slightly.		
		p23 717-722	Ren et al. (2015) investigated the binding interactions of 16 structurally diverse perfluorinated compounds with human TR and their activities on TR in cells. Specifically, in fluorescence competitive binding assays, most of the 16 perfluorinated compounds were found to bind to TR, with relative binding potency in the range of 0.0003-0.05 compared to triiodothyronine (T3) (Ren et al.,2015). A structure-binding relationship was observed, where fluorinated alkyl chain length longer than ten and an acid end-group were optimal for TR binding.		
		p24 756-759	The USEPA ToxCast program screened the PFAA molecules with chain length 6-11 in up to 800 separate *in vitro* assays. They note an increasing trend in cytotoxicity with C-atom chain length as measured in a set of 37 cell-proliferation decrease and cytotoxicity assays. The ranges of AC50 values are given in Table 8 in Annex I. Limited cytotoxicity was seen up to C8, but above C8, many assays while activated were concentration limited.		

		p24 762-771 p25 774-776	PFOA, in the middle of the length range, had the most evidence for PPAR activity in concentration ranges not also associated with cytotoxicity. Longer chains activated the PPAR assays at about the same concentration as does PFOA, but they are all cytotoxic in that range. Shorter chain length variants activate PPAR at higher concentration, or not at all in the concentration range tested (up to 100 ⍰M). PXR activity is only seen in the middle length range, with PFOA, again being the only one of these analogues with consistent activity outside of the cytotoxicity range. There is some evidence of FXR activity for PFOA and PFDA. For ER and AR, there are several active assays, with the most evidence for receptor-mediated activity being in PFOA. However, in a more complete analysis of a large number of ER and AR assays, the weight of evidence points towards this activity being non-receptor-mediated, and likely due to some assay-interference process (Judson, personal communication)[3]. the Activity in Cell proliferation depression and cytotoxicity assays from ToxCast and demonstrates that as the chain length increases, the evidence for cytotoxicity increases and the concentration tends to decrease.		**Together, the traditional and NAM data provide sufficient TK and TD evidence to support read-across from C8 PFAA to both C7 as well as C9 and C10.**
4.4	**For any compounds not linked to the prediction (i.e. non-common compounds such as intermediates, metabolites, impurities of category members), characterize (or demonstrate no) influence on the prediction**			NA or 5	Read-across justification provides sufficient explanation that no metabolites impact the prediction.
	Documentation indicates whether other compounds not linked to the prediction are present	p5 130	Annex Table 5 A purity/impurity profile for the analogues listed in 2.4 is unknown.		
	Explanation is provided for how/why any other compounds formed lack influence on the predicted property	p5 130-131 p5 149-150 p15 473-475	since the category is so limited structurally, the potential impact of any impurities on the endpoint being evaluated is considered very small. PFAAs are resistant to biotransformation. Therefore, toxicity of the parent compound and not that of a metabolite is of concern. Results of *in silico* metabolism simulations are presented in Table 5 of Annex I. METEOR reveals the potential for II phase: glucuronidation of carboxylic acid moiety, but overall the compounds are predicted not to be metabolised.		
	Supporting evidence based on kinetics or lack of other effects in data matrix is provided (at minimum for target and RA source)	*None*	*No information found in Case Study text*		

4.5	**Characterize (or demonstrate no) occurrence of other effects than those covered by the read-across hypothesis and justification**			4	Read-across justification does not include discussion of other effects mentioned in case study text. *To more directly address the RAAF: Increase descriptive detail to characterize occurrence (at what dose levels) of other effects of PFAAs.*
	Documentation indicates whether other effects not linked to the prediction are present	p6 165-166	Annex Table 8 In addition to hepatotoxicity, PFAAs are linked to developmental toxicity (Lau et al., 2004; Wolf et al., 2010) and immunotoxicity (DeWitt et al., 2012).		
	Any other effects are evaluated on a case-by-case basis and it is explained why they are either irrelevant OR possibly indicative of additional mechanisms not identified in the hypothesis	*None* p7 199-355 p12 357-378	*No information found in Case Study text* *NOTE: In one 2-yr study and one 28-day study hematological effects were also noted at higher doses (LOAELs of 16 and 30 mkd and NOAELs of 1.6 and 10 mkd, respectively) than the identified LOAELs for liver effects (14 and 0.3 mkd, respectively).*		
	Any uncertainty arising from possibility of additional mechanisms is addressed	*None*	*No information found in Case Study text*		
4.6	**Demonstrate there is no bias influencing the prediction**			5	Read-across justification and case study text indicate worst-case study is used.
	Criteria used in selection of sources is described and no otherwise suitable members have been excluded (or if so a justification is provided)	p4 123-126	Annex Table 1 *list of category members* *No information found in Case Study text to verify/describe search strategy used to identify category members*		
	Conservative 'worst case' (highest concern) studies available on source(s) are used in RA or if not a justification is provided	p7 199-355 p12 357-378 p21 663-665 p22 673-674	Annex Table 8 *Descriptions of individual repeat dose studies available for category members are provided and indicate the worst-case study is being used as read-across source.* The NOAEL for PFOA of 0.06 mg/kg bw/d (based on hepatocyte necrosis and hepatocellular hypertrophy, and increased liver weight in males and females, respectively (EFSA, 2008) is read across to the other three analogues in the category. Since the NOEAL value for the source substance, PFOA is supported by data for the C11 and C12 derivatives, a quantitative read-across is possible. *NOTE: Information in Table 8(Data Matrix) for repeat dose toxicity NOAELs does not support that PFOA is most potent category member and is in conflict with the text where potency and worst-case study being used as read-across source is demonstrated.*		

SUMMARY

READ-ACROSS RATIONALE	KEY SUPPORTING INFORMATION	RAAF SCORE
PFAAs are highly fluorinated chemicals that consist of a straight- chain hydrocarbon backbone and a single terminal carboxylate moiety. They are structurally similar to fatty acids generally known to cause liver effects. The strong C-Fl bonds are resistant to biotransformation (PFAAs are not metabolized) and the ionized carboxylate and the Fl partial negative charges promote electrostatic interactions with proteins. Protein binding facilitates absorption, transport, and bioaccumulation which is increased by efficient reabsorption in the kidneys. PFAAs cause liver toxicity via multiple inter-related mechanisms that perturb lipid (particularly fatty acids with which PFAAs share structural similarity) metabolism and transport. PFAAs bind PPARs and other nuclear receptors. In ToxCast assays, PFAAs have shown activity in several target classes including PPAR, PXR/CAR, FXR, ER, and TH. In addition, both PPARα and PPARγ binding have been demonstrated in vitro. PPARα activity exhibits a bell-shaped curve, with C8 chain length PFAA (PFOA) being the strongest activator and longer chain PFAAs being less potent. This was also demonstrated in HT ToxCast assays where PFOA, in the middle of the PFAA carbon chain length range, had the most evidence for PPAR activity. Available in vivo data on PFAAs demonstrate consistent repeat dose effects and support the read-across hypothesis that C8-PFAA (PFOA) is the most potent member of the category.	PFAAs are highly fluorinated chemicals that consist of a backbone of 4 to 18 C-atoms and a single terminal carboxylate moiety. PFAAs are resistant to biotransformation. Therefore, toxicity of the parent compound and not that of a metabolite is of concern. PFAAs circulate in the body by non-covalent binding to plasma proteins. PFAAs are readily absorbed following oral exposure and distributed mainly to the serum, kidney, and liver, with liver concentrations being several times higher than serum concentrations. In general, the elimination is decreased by renal resorption thus retention in the body is increased. Urinary clearance of PFFAs in humans decreased with increasing alkyl chain lengths, while biliary clearances increased. Total clearance with rats was observed to be greatest for the C6 analogues and then decreased significantly with minimal clearance for PFDA. The mechanistic pathways of toxicity of PFAAs are likely multiplicative. Bjork et al. (2011) concluded that multiple nuclear receptors are activated by PFAAs. PFAA-induced liver toxicity is considered to be mediated via PPARs While there is evidence that PFAAs activate a multiplicity of nuclear receptors, PPARα and/or PPARγ interactions are the most likely initiating events leading to repeated-dose, liver toxicity. PFAAs of increasing C-atom chain length, up to C9, induce increasing activity of the mouse and human PPARα. PPARα activity exhibits a bell-shaped curve, with PFOA being the strongest activator. Moreover, longer C-atom chain PFAAs (i.e., > C10) are relatively less potent and some do not activate human PPARα. The increase in the expression of PPARγ and its role in fatty acids and triglyceride synthesis, together with the stimulation of accumulation in lipid droplets, suggest a synergistic action of PPARα and PPARγ in the liver pathology of PFAA. The USEPA ToxCast program screened the PFAA molecules with chain length 6-11 in up to 800	**2** (as is – due to lack of impurity profile) **3** (after addition of impurity profile and only considering traditional data) **5** (after addition of impurity profile and adding detailed data matrix, and considering both traditional and NAM data)

Collectively, the available information and data support the read-across of rat repeat dose data on PFOA to fill rodent repeat dose data gaps for the other members of the category (C7, C9, and C10 PFAA).	separate *in vitro* assays. Specifically, activity was seen in several target classes including PPAR, PXR/CAR, FXR, ER (estrogen receptor), AR (androgen receptor), cell stress pathways and a number of enzymes and G-protein-coupled receptors (GPCRs). PFOA, in the middle of the length range, had the most evidence for PPAR activity in concentration ranges not also associated with cytotoxicity. Longer chains activated the PPAR assays at about the same concentration as does PFOA, but they are all cytotoxic in that range. Shorter chain length variants activate PPAR at higher concentration, or not at all in the concentration range tested (up to 100 □M). Preliminary data from targeted gene expression profiling in metabolically-competent HepaRG cells shows activity in several PPAR / CAR/ PXR genes (ACOX1, CYP2B6, CYP2C19, CYP2C8, CYP3A4, CYP3A7, IL6, IL6R, PDK4), showing up-regulation of many of these. The signal is strongest in PFOA, followed by the heptanoic, nanonoic and decanoic derivatives (Judson, personal communication). Toxicogenomic studies of PFAAs reveal the largest group of induced genes is those involved in transport and metabolism of lipids, particularly fatty acids.	

ANNEX 1

Table 8: Comparison of Toxicologically Relevant *In Vivo, In Vitro* and *Ex Vivo* Data

	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUA	PFDoA
Name	Perfluoro hexanoic acid	Perfluoro-heptanoic acid	Perfluoro-octanoic acid	Perfluoro-nonanoic acid	Perfluoro-decanoic acid	Perfluoro-undecanoic acid	Perfluoro-dodecanoic acid
Subacute Repeat Dose			28-day rat gavage at doses from 0.3 to 30 mkd. Effects: ↓ BW; ↓ hematology values; ↑reticulocytes and hematopoiesis; ↓ cholesterol and triglycerides; ↑ abs and rel liver wt; ↑ incidence hepatocellular hypertrophy and necrosis. LOAEL = 0.3 mkd based on ↓ cholesterol and triglycerides. [6] 28-day rat gavage at doses of 5 and 20 mkd. Effects: ↑incidence of hypertrophy, fatty degeneration, lesions/congestionin the liver. LOAEL = 5 mkd based on liver changes. [7] Two 28-day rat dietary studies at doses of either 19 or 23 mkd. Effects: ↓				

	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUA	PFDoA
			BW; ↑ abs and rel liver wt. LOAEL = 19 or 23 mkd based on ↑liver wts. [8] 21-day mouse drinking water study at doses of 2, 10, 50, and 250 ppm (equivalent to 0.49, 2.64, 17.73, and 47.21 mkd in mice). Effects: ↓BW ; ↓FC and WC; ↑liver enzymes; ↑liver wts; and hepatocellular hypertrophy and necrosis. LOAEL = 0.49 mkd based on↑liver wts. [13] 14-day rat and mouse gavage studies at doses of 0.3, 1, 3, 10, and 30 mkd. Effects: ↓BW, ↓FC ↓cholesterol and triglycerides; ↑abs and rel liver wts; and ↑hepatic peroxisomal β-oxidation. LOAEL = 0.3 mkd based on ↓lipids and ↑rel liver wt.[14]				
Subchronic Repeat Dose	90-day rat gavage at doses of 10, 50, and 200 mkd. Effects: ↑liver wts; ↑peroxisome β-		90-day rat dietary at doses from 0.56/0.74 to 63.5/76.5 mkd. Effects: ↓ BW; ↑ rel kidney wt; ↑abs and rel			42-day rat gavage (OECD 422) at doses of 0.1, 0.3, and 1.0 mkd. Effects: ↓BW; ↓fibrinogen and APPT; ↑ BUN;	45-day rat gavage (OECD 422) at doses of 0.1, 0.5, and 2.5 mkd. Effects: ↓BW;

	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUA	PFDoA
	oxidation; hepatocellular hypertrophy. NOAEL = 50 mkd LOAEL = 200 mkd based on ↑liver wts and hepatocellular hypertrophy [9] 90-day rat gavage at doses of 20, 100, and 500 mkd. Effects: ↓BW; changes in red cell parameters; ↑reticulocytes;; ↑thyroid parameters; nasal lesions; thyroid hypertrophy; and hepatocellular hypertrophy. NOAEL = 20 mkd LOAEL = 100 mkd based on ↑liver wts and nasal lesions. [15]		liver wt; and hepatocellular hypertrophy and necrosis. NOAEL = 0.56 mkd LOAEL = 1.72 mkd based on liver necrosis in males. [1] 90-day rat dietary at doses from 0.06 to 6.50 mkd. Effects: ↓ BW; no hormone (estradiol, testosterone, luteinizing hormone) level changes; ↑abs and rel liver wt; hepatocellular hypertrophy; and ↑ hepatic palmitoyl CoA oxidase activity. NOAEL = 0.06 mkd LOAEL = 0.64 mkd based on liver hypertrophy. [2,3]			↓protein ; ↑ liver enzymes; ↑ liver wts; and hepatocellular hypertrophy and necrosis. NOAEL = 0.1 mkd LOAEL = 0.3 mkd based on hypertrophy.[11]	↓FC; ↓ hematopoiesis; ↑ liver enzymes; inflammatory cholestasis; ↑liver wts; and hepatocellular hypertrophy and necrosis. NOAEL = 0.1 mkd LOAEL = 0.5 mkd based on cholestasis liver hypertrophy and necrosis. [10]
Chronic Repeat Dose			2-yr rat dietary at doses of 1.3 and 14.2 mkd. Effects: ↓ BW gain; ↓ hematology values at high dose only; and ↑incidence of liver lesions including necrosis and hyperplasia. NOAEL = 1.3 mkd LOAEL = 14.2 mkd based on liver histological changes. [4]				

	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUA	PFDoA
			2-yr rat dietary at a dose of 13.6 mkd. Effects: ↓ BW; ↑rel liver wt; ↑hepatic beta oxidation activity; ↑abs testis wt. LOAEL = 13.6 mkd based on liver changes.[5] 26-week monkey oral capsule at doses of 3, 10, and 20 mkd. Effects: ↓BW; ↓FC; ↑liver wt; and liver damage. LOAEL = 3 mkd based on ↑liver wt. [12] 2-year rat dietary at 30 and 300 ppm (1.5 and 15 mkd). Effects: ↓ BW gain; ↓ hematology values at high dose only; ↑liver enzymes; ↑rel liver wts; and ↑incidence of liver lesions including necrosis and hyperplasia LOAEL = 1.5 mkd based on ↑liver enzymes and rel liver wts. [18]				

	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUA	PFDoA
Reproductive Toxicity	NOAEL = 100 mkd LOAEL = 500 mkd based on reduced pup wt. [15]		NOAEL=5 mkd LOAEL=10 mkd based on reduced # live births.[16] NOAEL = 30 mkd No repro effects noted so no LOAEL.[17]			NOAEL=1.0 mkd No repro effects noted so no LOAEL.[11]	NOAEL=0.5 mkd LOAEL= 2.5 mkd based on male sperm effects and female estrous changes and toxicity.[10]
Developmental and maternal toxicity	NOAEL = 100 mkd LOAEL = 500 mkd based on reduced maternal and pup BW. [15]		NOAEL = 1 mkd LOAEL = 3 mkd based on growth deficits. [16] NOAEL=10 mkd LOAEL=30 mkd based on increased pup mortality and reduced birth wt. [17]	NOAEL=3 mkd LOAEL=5 mkd based on reduced neonatal survival. [19]		NOAEL=0.3 mkd LOAEL= 1.0 mkd based on reduced pup wt.[11]	NOAEL=0.5 mkd LOAEL= 2.5 mkd based on reduced live births and reduced pup wt.[10]

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