Read-Across for 90-Day Rat Oral Repeated-Dose Toxicity for Selected Perfluoroalkyl Acids:
A Case Study

Terry W. Schultz¹, Claire Mellor², Katarzyna Przybylak², Sylvia Escher³, Richard Judson⁴, Ivanka Tsakovka⁵ and Andrea Richarz²

¹The University of Tennessee, College of Veterinary Medicine, 2407 River Drive, Knoxville, TN 37996-4543 USA; ²Liverpool John Moores University, Byrom Street, L33AF Liverpool, United Kingdom; ³Fraunhofer ITEM, Nikolai-Fuchs-Str. 1, 30625 Hannover, Germany; ⁴U.S. EPA, National Center for Computational Toxicology; Office of Research and Development 109 T.W. Alexander Drive Research Triangle Park, NC 27709, USA; ⁵Department of QSAR & Molecular Modelling, Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, 105 G. Bontchev St. 1113 Sofia, Bulgaria

Executive Summary: Grouping of chemicals and read-across of properties to fill data gaps is a valuable method used in the safety assessment of chemicals. However, there is still insecurity about the practical application and regulatory acceptance. A previously devised strategy (Schultz et al. 2015) is used to structure and report this case study which constructs a read-across line of reasoning for selected perfluoroalkyl acids (PFAAs). This group of compounds represents a scenario of chemicals with no or very slow metabolism. Based on similarities in chemistry, toxicokinetics, especially clearance, and toxicodynamics, especially peroxisome proliferator-activated receptor (PPARα and/or PPARγ) activation, a small congeneric series (i.e., C7-C10) of straight-chain PFAAs is proposed as a read-across category. Perfluoronated octanoic acid (PFOA) is identified as the source substance. It was demonstrated that in vivo oral repeated-dose exposure of rats to PFAAs gives rise to a standard set of symptoms, including liver toxicity. Specifically, hepatocellular injury is accompanied by oxidative stress and inflammatory response, as well as alteration in lipid transport and metabolism. While there is evidence that PFAAs activate a multiplicity of nuclear receptors, peroxisome proliferator-activated receptors (PPARα and/or PPARγ) activation are the most likely initiating events leading to rat oral repeated-dose, liver toxicity. Following the “traditional” group formation for read-across basis on in vivo, in vitro and structure-activity data, similarity and uncertainty for the category were assessed. Chemical uncertainty is deemed low. Uncertainty associated with the fundamentals of chemical transformation/toxicokinetic similarity is deemed low to moderate. Uncertainty associated with the fundamentals of toxicodynamic similarity is also deemed to be deemed low to moderate. Lastly, uncertainty associated with mechanistic relevance and completeness of the read-across is judged to be low to moderate. Following the consideration of information derived “new methods” approaches, including results of the US EPA ToxCast programme, uncertainty and
weight-of-evidence associated with transformation/toxicokinetic were unchanged; however, uncertainty and
weight-of-evidence associated with toxicodynamic similarity were reduced and increased, respectively. It is
concluded that the rat oral 90-day NOAEL for PFOA, 0.06 mg/kg body weight (bw)/day (d) (based on
hepatocyte necrosis and hepatocellular hypertrophy and increased liver weight), may be read across to the
untested C9 and C10 analogues with low uncertainty and used to inform regulatory decisions. It is worth
noting, this conclusion is supported by the in vivo data (i.e., NOAELs of 0.1 mg/kg bw/d) for the C11 and C12
derivatives. Further it is concluded that the oral 90-day NOAEL for PFOA can be read across to the C7
derivative as the most conservative argument.

1. Introduction

The underlying beliefs of a toxicological read-across are that chemicals which are similar in structure will have
similar chemical properties and thereby have similar toxicokinetic and toxicodynamic properties. For this
reason, experimentally-derived toxicokinetic and toxicodynamic properties from one compound, the source
chemical, can be read across to fill the data gap for the second compound, the target chemical, which has a
similar chemical structure. The grouping of organic chemicals, with the intention of conducting read-across, is
a valuable method which has application to a number of regulatory decisions. Despite this fact, one still finds a
number of the challenges, especially in regard to gaining more universal acceptance of read-across predictions
(Cronin et al., 2013).

Recently, a “Strategy for Structuring and Reporting a Read-Across” was proposed by Schultz et al. (2015). To
examine the utility of this strategy, we have undertaken the first of a series of case studies as proofs-of-concept.
The case study follows the “Workflow for Reporting a Read-Across Prediction” as detailed in Appendix C of
Schultz et al. (2015). In addition to identifying the target and source substances, the Workflow involves
completing up to eight tables to compare the similarity of the molecules and two tables addressing uncertainty
detailed in Appendices A and B of Schultz et al. (2015), respectively. Ideally each table is associated with a
narrative evaluating the various aspects of the evidence leading to an overall conclusion (Schultz et al., 2015).

In addition to applying the Schultz et al. (2015) Workflow, case study was undertaken in a two-step process.
The purpose of the two-step process is to demonstrate the usefulness of new approach data in reducing the
uncertainty in the read-across prediction of the toxicological properties and thereby improve the likelihood that
it will be accepted for regulatory use. The initial assessment is a “traditional” read-across using established in
vivo data supplemented, as applicable, with conventional in vivo, in vitro and structure-activity relationship
information. The second assessment supplemented the initial exercise with “new-approach” data, including
high-throughput molecular screening, novel in vitro methodology and/or toxicogenomics information combined
in a rational manner (Whelan and Schwarz, 2011; Gocht et al., 2014, Sturla et al., 2014).

The case described in this study involves substances with no or very slow metabolism. In this scenario,
chemicals are able to reach the target organ with no evidence of producing toxic metabolites, typically in the
liver. The aim of this investigation was to undertake, report and justify, using the Workflow and templates
provided by Schultz et al. (2015), the grouping of perfluoroalkyl acids (PFAAs) and the read-across of 90-day
rat oral repeated-dose toxicity from perfluorooctanoic acid (PFOA) to other compounds in this category. The
read-across was performed initially without “new-approach” data. Subsequently new methods data were
considered to determine if they have any significant impact on the uncertainty associated with the read-across.

2. Approach

This study reports efforts to form a category for the perfluoroalkyl acids (PFAAs), as an illustration of one of
the potential read-across scenarios reported by Berggren et al. (2015). Following initial problem formulation,
the read-across was performed according to the Workflow described in Appendix C of Schultz et al. (2015).
Templates describing similarity and uncertainties, as provided in Appendices A and B of Schultz et al. (2015)
were completed.

2.1 Resources utilised

Every effort was made to use freely available resources to undertake this study, where possible. The specific
resources utilised are described in Supplementary Information Tables Suppl.A.1-8.

2.2. Premise

The premise for this case study is for read-across of rat oral repeated-dose toxicity, the C7 – C10 PFAAs form a
consistent category. Specifically, the hypothesis for this read-across is:

• PFAAs are chemically very similar. For example, highly fluorinated chemicals that consist of a straight-chain hydrocarbon backbone and a single terminal carboxylate moiety.

• From a toxicokinetics standpoint PFAAs are absorbed by the gut, bind to albumin and other proteins and are not metabolised in the liver. Their persistence is markedly influenced by reabsorption in the kidneys. In rats, measured half-lives for C7 to C10 PFAAs are consistent.

• Toxicodynamically, PFAAs are direct-acting toxicants (i.e., where metabolism is not a factor) with similar modes of action, (MoA), most likely a combination of PPARα/PPARγ interactions, leading to rat oral repeated-dose hepatotoxicity via perturbations to fatty acid uptake, lipogenesis, fatty acid
oxidation, and centrilobular hepatocellular hypertrophy correlated with higher liver weights. Repeated-dose toxicity data for PFOA (the proposed source chemical) are supported by those for the C6, C11 and C12 derivatives, which reside outside the applicability domain of the proposed category.

- The molecular mechanism of PFAA-induced liver toxicity is not completely characterised. Toxicogenomics studies suggest that PFAAs suppress immunity and induce fatty acid transport and metabolism, as well as inflammation. While PFAAs have been shown to be involved in several mechanistic-relevant events, the length of the fluorocarbon-backbone has not been shown to have an impact on mechanism of action.

2.3. Justification

PFOA is the best studied analogue of the category. 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 via non-covalent binding to plasma proteins. Oral rat acute toxicity (LC50) for PFOA is between 400 and 700 mg/kg bw (EFSA, 2008). Recent reviews of the literature (Bull et al., 2014; USEPA, 2014) have shown the liver is a key site of action, or target organ, with increased liver weight in laboratory animals being one of the earliest and lower-dose manifestations of exposure. At least three transport families seem to play a role in PFOA absorption, distribution, and excretion: organic anion transporters (OATs), organic anion transporting peptides (OATPs) and multidrug resistance-associated proteins (MRPs). As evidenced by the half-life of 2.3 years, PFOA is not readily eliminated from humans. In contrast, the half-life in the monkey and rodent is in the 10 to 20 day range.

PFOA is known to activate the peroxisome proliferator activated receptor (PPAR) pathway by increasing transcription of proteins involved in mitochondrial and peroxisomal lipid metabolism, sterol, and bile acid biosynthesis and retinol metabolism. However, based on transcriptional activation of many genes in PPARα null mice, the effects of PFOA involve more than activation of PPARα (e.g., PPARγ, the constitutive androstane receptor (CAR), farnesoid receptor (FXR), and pregnane X receptor (PXR)).

2.4. Applicability domain

The applicability domain for this read-across is confined to straight-chain perfluorinated carboxylic acids of C7 to C10.

2.5. Analogues or category members

1) Perfluoroheptanoic acid PFHpA 375-85-9 C7F13O2
2) Perfluorooctanoic acid PFOA 335-67-1 C8F15O2
3) Perfluorononanoic acid PFNA 375-95-1 C9F17O2
4) Perfluorodecanoic acid PFDA 335-76-2 C10F19O2
The chemical structures of the category members are given in Table 1 in Annex I.

2.6. Purity/impurities

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.

3. Report on the rat oral 90 day repeated-dose toxicity and related information on PFAAs

3.1. Short characterisation of perfluoroalkyl acids

PFAAs are highly fluorinated chemicals that consist of a backbone of 4 to 18 C-atoms and a single terminal carboxylate moiety. 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.

The acid dissociation constants, pKa, for PFAAs are very low; the result is in most biological fluids, excluding gastric secretions, PFAAs are primarily in the anion form. This is an important feature governing absorption and membrane transport.

While the toxicokinetics of PFOA is well-studied, the toxicokinetic understanding of other PFAAs is incomplete. It is clear that there are species differences (e.g., half-lives in human vs. monkey/rodent) in the toxicokinetics of PFAAs. Typically, 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. Oral absorption is mainly mediated by transporter proteins or mechanisms other than simple diffusion (Launay-Vacher et al., 2006; Zaïr et al., 2008). 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).

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.

Besides hepatocellular adenomas in rats, PFAAs are associated with liver enlargement (hepatomegaly) in rodents and non-human primates. Based in large part on studies of perfluorinated octanoic acid, PFOA (e.g., Son, 2008; EFSA, 2008; Chu et al., 2009; Bull et al., 2014; Yang et al., 2014; USEPA, 2014), PFAAs are considered to be direct-acting toxicants which act via multiple receptor interactions, including PPARs. In addition to hepatotoxicity, PFAAs are linked to developmental toxicity (Lau et al., 2004; Wolf et al., 2010) and immunotoxicity (DeWitt et al., 2012).

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; however, other molecular mechanism have also been implicated. There has been a sharp increase in the number of toxicological studies of perfluorinated chemicals since 2008; however, the mechanistic pathways of toxicity of PFAAs are far from clear.

The molecular mechanisms of PFAA-induced liver toxicity include binding to PPARs. Specifically, PPARα and/or PPARγ binding is considered to mediate many toxic effects of PFAAs (van den Heuvel et al., 2006; Takacs and Abbott, 2007; Rosen et al., 2008; Wolff et al., 2008; 2012; Bjork and Wallace, 2009). In addition to PPARs, other studies of PFAAs’ toxicities have reported other potential molecular mechanisms. These findings have led Bjork et al. (2011) to conclude there is a multiplicity of nuclear receptor activation by PFAAs.

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).

3.2. Statement target substance(s) and the regulatory endpoint(s) to be read across

This read-across is for the perfluoroalkyl acids (PFAAs). Specifically a category of four PFAAs has been formed (further described in Supplementary Information Table Suppl.A.1). The PFAAs range in carbon number from seven to ten. The endpoint to be read across is 90-day rat oral repeated-dose toxicity.

While there are several 28-day rodent oral gavage studies of various PFAAs, there are not many 90-day rat oral in vivo repeated-dose data for PFAAs. Whilst not included in this category, it should be noted that there are (OECD Test Guideline 422, Combined Repeat Dose and Reproductive/Developmental Toxicity Screening Test) data for perfluoroundecanoic acid (PFUA) [2058-94-8] and perfluorododecanoic acid (PFDoA) [307-55-1], and
90-day oral repeated-dose for perfluorohexanoic acid (PFHxA) [307-24-4]. More to the point of this exercise, there is extensive high quality 90-day oral repeated-dose data for PFOA [335-67-1]. Hence, an evaluation was conducted to determine the suitability of PFOA as a source substance with the NOEAL data being read across to fill the data gaps for the other analogues in the category.

3.3. Existing Relevant Toxicity Data for PFOA

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.

3.4. 90-day Rat Oral Repeated-dose Studies of PFOA

In a dietary study, Goldenthal (1978) administered ChR-CD rats (5/sex/group) PFOA at concentrations levels equivalent to doses of 0, 0.56, 1.72, 5.64, 17.9, and 63.5 mg/kg bw/d in males, and 0.74, 2.3, 7.7, 22.36 and 76.47 mg/kg-day in females. Body weight and food consumption were recorded weekly. Blood and urine samples were collected during the pre-test period and at 1 and 3 months of the study for haematology, clinical chemistry and urinalysis. At necropsy, the organs from the control, and the three highest dose groups were weighed and examined for histopathological lesions; livers from all dose groups were also examined microscopically. There were neither treatment-related deaths nor changes in behaviour or appearance. There was a decrease in body weight gain for male rats at the two highest dose levels. At 13 weeks, mean body weight of males in the highest does group was significantly less than that of controls. There were no treatment related effects on the haematological, biochemical or urinary parameters were observed. Relative kidney weights were significantly increased in males in the three highest dose groups. However, absolute kidney weights were comparable among dose groups, and there were no histopathological lesions. Absolute liver weights were significantly increased in males in the higher dose groups and in females in the highest dose group. The mean absolute liver weight of each group (i.e., 13.4, 14.3, 19.1, 18.6, 20.1, and 19.2 g, respectively) increased with dosage. Relative liver weights were significantly increased in males in the two highest dose groups and in females in the highest dose group. Hepatocellular hypertrophy (focal to multifocal in the centrilobular to mid-zonal regions) was observed in 4/5, 5/5, and 5/5 males in the 5.64, 17.9, and 63.5 mg/kg bw/d groups, respectively. Hepatocyte necrosis was observed in 2/5, 2/5, 1/5, and 2/5 males in the 1.72, 5.64, 17.9, and 63.5 mg/kg bw/d groups, respectively. Under the conditions reported above, the Lowest Observed Adverse Effect Level (LOAEL) for males is 1.72 mg/kg bw/d based on liver effects and the No Observed Adverse Effect Level (NOAEL) is 0.56 mg/kg bw/d; the LOAEL for females is 76.5 mg/kg bw/d based on increased liver weight, and the NOAEL is 22.4 mg/kg bw/d.
In another dietary study (Perkins et al., 2004; see EFSA, 2008), male ChR-CD rats in groups of ≈ 50 were administered concentrations of 1, 10, 30, or 100 ppm PFOA for 13 weeks (i.e., doses equivalent to 0.06, 0.64, 1.94, and 6.50 mg/kg bw/d). There were two control groups, a non-pair-fed control group and a control group pair-fed to the 6.50 mg/kg bw/d dose group. Following the 13-week exposure period, 10 animals per group were fed a basal diet for an 8-week recovery period. The animals were observed for clinical signs of toxicity, and body weights and food consumption were recorded. In the analysis of the relevant data, animals in groups exposed to feed at 0.06, 0.64, 1.94, and 6.50 mg/kg bw/d PFOA were compared to the control animals in the non-pair-fed group, while the data from the pair-fed control animals were compared to animals exposed to 6.50 mg/kg bw/d PFOA. With diets at 6.50 mg/kg bw/d, significant reductions in body weight and body weight gain were seen compared to the pair-fed control group during week 1 and the non-pair-fed control group during weeks 1-13. Body weight data in the other dosed-groups were comparable to controls. At feeds with 0.64 and 1.94 mg/kg bw/d, mean body weight gains were significantly lower than the non-pair-fed control group at week 2. These differences in body weight and body weight gains were not observed during the recovery period. Animals fed the 6.50 mg/kg diet consumed significantly less food during weeks 1 and 2, when compared to the non-pair-fed control group. Overall, there was no significant difference in food consumption. A total of 15 animals per group were sacrificed following 4, 7, or 13 weeks of treatment; in addition, 10 animals were sacrificed after 13 weeks of treatment and following the 8 week recovery period. Serum samples collected from 10 animals per group at each scheduled sacrifice during treatment and from 5 animals per group during recovery were analyzed for estradiol, total testosterone and luteinizing hormone. There were no significant differences among the groups for any of the hormones evaluated. Weights of selected organs were recorded, and these tissues were examined histologically. In addition, some organs were prepared for electron microscopic examination. Significant increases in absolute and relative liver weights and hepatocellular hypertrophy were observed at weeks 4, 7, or 13 in the groups fed 0.64, 1.94, and 6.50 mg/kg bw/d PFOA. There was no histological evidence of any degenerative changes. Hepatic palmitoyl CoA oxidase activity was significantly increased at weeks 4, 7, and 13 in the groups fed the two highest concentrations. At 0.64 mg/kg bw/d level, hepatic palmitoyl CoA oxidase activity was significantly increased at week 4 only. During recovery, however, no liver effects were observed, indicating that the treatment-related liver effects were reversible. EFSA (2008), report a LOAEL of 0.64 mg/kg bw/d) based on increases in absolute and relative liver weights with hepatocellular hypertrophy and a NOAEL of 0.06 mg/kg bw/d.

Butenhoff et al. (2012) investigated the chronic toxicity of PFOA in a two year study with Sprague-Dawley (Crl:CD BR) rats. Briefly, large groups (i.e., 50/sex) were fed diets containing 0, 30 or 300 ppm PFOA (0, 1.3, and 14.2 mg/kg/d for males; 0, 1.6, and 16.1 mg/kg/d for females). Additionally, groups (15/sex) were fed 0 or 300 ppm PFOA and evaluated at one year. All Observations included body weights and feed consumption,
haematology, serum chemistry, urinalysis, gross pathology, organ weights, and histopathology. There were no
significant differences in mortality between the treated and untreated groups (Butenhoff et al., 2012). The
authors further report there were dose-related decreases in body weight gains in male and female rats as
compared to the controls with statistical significance attained in both sexes for the high-dose group. Since feed
consumption increased during the study, the body weight changes (decrease in gain) were considered treatment-
related. Additionally, significant decreases (as compared to control values) in haematological-related values
were observed at the high-dose for both sexes. No dose- or treatment-related differences in absolute and
relative organ weights were found between the treated and control groups at two years (Butenhoff et al., 2012).

Significantly increased incidences of lesions in the liver were observed in the high-dose male group (Butenhoff
et al., 2012). Specifically, at 1 year, diffuse hepatomegalocytosis, portal mononuclear cell infiltration and
hepatocellular necrosis were reported. At 2-years, significant increases in megalocytosis were observed for
both sexes in the high-dose group. Hepatic cystoid degeneration and localized hepatic parenchymal hyperplasia
were also significantly increased in high-dose males. Among the high dose males histological changes were
noted in tissues other than the liver.

Butenhoff et al. (2012) report under the conditions of the studies a LOAEL for male rats of 14.2 mg/kg bw/d
based on a decrease in body weight gain and histological changes, especially in the liver; the LOAEL for female
rats is 16.1 mg/kg bw/d based on decreased body weight gain and haematologic effects. The NOAEL is 1.3
mg/kg bw/d and 1.6 mg/kg bw/d for females.

Biegel et al. (2001) conducted a two-year mechanistic study. Briefly, male Crl:CD BR (CD) rats (156/group)
were fed a diet containing 0 or 300 ppm PFOA (0 or 13.6 mg/kg/d), sacrifices were conducted every three
months up to 21 months and quantification of liver weight and testes weight, peroxisome proliferation, and cell
replication reported. Body weight was significantly decreased from days 8 to 630 in PFOA-exposed rats
(Biegel et al., 2001). Further, when compared to the controls, relative liver weights and hepatic β-oxidation
activity were statistically significantly increased at all time points between 1 and 21 months. Absolute testis
weights were significantly increased, but only at end of the experiment.

Effects of PFOA on cell proliferation in various organs were reported (Biegel et al., 2001). No hepatic or
Leydig cell proliferation was observed at any sampling times. The incidence of Leydig cell hyperplasia was
significantly increased in PFOA-exposed rats (46% vs. 14% control). Pancreatic acinar cell proliferation was
significantly increased at 15, 18, and 21 months. The incidence of pancreatic hyperplasia was higher in PFOA-
exposed animals than in controls 39% and 18%, respectively.

3.5. 28-day Rat Oral Repeated-dose Studies of PFOA
Currently, 28-day repeated-dose toxicity studies are limited. Loveless et al. (2008) administered PFOA by oral gavage at doses of 0, 0.3, 1, 10, or 30 mg/kg bw/d to male CD rats in groups of ten for 29 days. Body weight was recorded. At necropsy, haematology, clinical chemistry, and corticosterone measurements were made. Tissues were collected for weight and histopathological examination. Body weight, weight gain, haematocrit, and haemoglobin were reduced at \( \geq 10 \) mg PFOA/kg/day. Increased reticulocytes and haematopoiesis were observed in the rats dosed with 30 mg/kg bw/d. Total and non-HDL cholesterol were significantly reduced at 0.3 and 1 mg/kg/day compared to control. HDL cholesterol was significantly decreased at 0.3, 1, and 10 mg/kg/day. Triglyceride levels were significantly decreased at all doses except 1 mg/kg. Absolute liver weight \( (\geq 1 \text{ mg/kg bw/d}) \) and relative liver weight \( (\geq 10 \text{ mg/kg bw/d}) \) were significantly increased, minimum to mild (0.3, 1 mg/kg bw/d) and moderate \((\geq 10 \text{ mg/kg/day})\) hepatocellular hypertrophy, and focal necrosis \((\geq 10 \text{ mg/kg/day})\) were observed. Although not statistically significant, serum corticosterone was increased at \( \geq 10 \) mg/kg/day.

Cui et al. (2009) exposed male Sprague-Dawley rats in groups of ten by gavage once daily to PFOA at 0, 5, or 20 mg/kg/day for 28 days. The rats dosed with 5 mg/kg/day exhibited hypoactivity, decreased food consumption and lethargy, during the third week of the study. Rats dosed with 20 mg/kg/day also exhibited enhanced sensitivity to external stimuli. All rats were sacrificed after the final exposure. Hepatic, renal, gonadal weight/animal’s body weight was calculated to evaluate hyperplasia, swelling, or atrophy in the PFOA-treated animals compared to controls. In the liver, treatment with 5 or 20 mg/kg bw/d hepatic hypertrophy, fatty degeneration, acidophilic lesions as well as gross dilation and congestion in the hepatic sinusoid or central vein were reported. No effects were observed in the kidneys of the low dose animals, however turbidity and swelling in the epithelium of the proximal convoluted tubule was observed at the 20 mg/kg bw/d dose. Under the conditions of this study, the LOAEL was 5 mg/kg bw/d based on liver effects; no NOAEL was established.

Elcombe et al. (2010) exposed male Sprague-Dawley rats in groups of ten by diets containing 0 or 300 ppm PFOA for 1, 7, or 28 days in two studies. The mean daily intake for study 1 and study 2 were 19 and 23 mg/kg bw/d, respectively. In addition, a group of rats (i.e., the positive control) was fed diets containing 50 ppm of the PPAR\(_\alpha\) agonist, Wyeth 14,643. The animals were observed daily and body weights and food consumption were recorded. At necropsy, day 2, day 8, or day 29, the organs were weighed, examined for gross pathology and preserved for histopathology. Liver DNA content and concentration were determined, and plasma was collected for analysis (study 1 only) of liver enzymes, cholesterol, triglycerides, and glucose. Hepatic cell proliferation and apoptosis were also determined. In both studies, body weight significantly decreased after 7 and 28 days on the PFOA diet. Body weight was not affected by Wyeth 14,643. Absolute liver weight was significantly increased in rats fed PFOA diets for 7 days in the first study and in rats treated for 7 and 28 days in the second study. The liver-to-body-weight ratio was significantly higher in rats fed PFOA diets for 7 and 28
11 days in both studies. Absolute liver weight and liver-to-body-weight ratio were significantly increased in Wyeth 14,643 diet fed rats in both studies.

3.6. Other Oral Repeated-dose Studies of PFOA

Thomford (2001) and Butenhoff et al. (2002) reported results from small groups of male Cynomolgus monkeys orally administered PFOA by capsule containing 0, 3, 10, or 30/20 mg/kg body weight (bw)/day (d) for 26 weeks. Because of succession of feeding (i.e., low food consumption, decreased body weight, and decreased faeces) the dosing of animals in the 30 mg dose group ceased after day 12. Subsequently, the dose was decreased to 20 mg/kg bw/d and restarted on day 22.

At sacrifice (90 days), the mean absolute liver weight was significantly increased in all dose groups, and the relative liver-to-body weight ratio was significantly increased for the high dose group. The cause of the increase in liver weights was suggested to be due to hepatocellular hypertrophy (indicated by decreased hepatic DNA content) which was hypothesized to result from mitochondrial proliferation based on an increase in hepatic succinate dehydrogenase activity (Butenhoff et al., 2002). Repeated-dose studies for short durations in both rats and mice also reported reductions in body weight, increases in liver weight with hepatocellular hypertrophy. Specifically, Son et al. (2008) evaluated male ICR mice exposed continuously to 0, 2, 10, 50 and 250 ppm of PFOA in drinking water for 21 days. They report food and water consumption decreased in mice exposed to the highest concentration of PFOA. Mean body weight gain was reduced in mice exposed to both 50 and 250 ppm of PFOA. Most notably, the size and relative weight of the liver increased dose-dependently in PFOA-treated mice. In the histopathological evaluation, the liver of PFOA-treated mice showed hepatocytomegaly and acidophilic cytoplasm; at the high doses of PFOA, diffuse hepatic damage by multifocal coagulation and liquefaction necrosis were further noted (Son et al., 2008).

Similar findings were reported by Yang et al. (2014) who studied the induction of hepatic effects in mice orally administered different concentrations of PFOA (i.e., 2.5, 5, or 10 mg/kg bw/d) for 14 consecutive days. Histological examination showed that the exposure to PFOA led to serious hepatocellular injury and obvious inflammatory cell infiltration.

Yahia et al. (2010) gavage dosed pregnant ICR mice in groups of 5 with PFOA at doses of 0, 1, 5, or 10 mg/kg bw from gestation day 0-17 or 18. Maternal liver, kidney, brain, and lungs were examined histologically. Serum was collected for clinical chemistry and lipid analysis. Body weight was significantly decreased in dams receiving 10 mg/kg. Maternal absolute liver weight was significantly increased at doses ≥ 5 mg/kg and relative liver and kidney weights were significantly increased at all doses. At the two lower doses, hepatic hypertrophy was localized to the centrilobular region; at the highest dose, hepatic hypertrophy was diffuse. At all doses,
renal cells in the outer medullar and proximal tubule were slightly hypertrophic. Treatment at the highest dose caused a significant increase in cytosolic enzymes and a significant decrease in total serum protein, albumin, globulin, triglycerides, phospholipids, total cholesterol, and free fatty acids. At the intermediate dose (i.e., 5 mg/kg bw/d), total serum protein and globulin were significantly decreased, and phosphorus was increased. Based on increased relative liver and kidney weight, the maternal LOAEL in this study is 1 mg/kg bw/d; no NOAEL was established.

3.7. Repeated-dose Studies of other PFAAs

There are no other repeated-dose data available for members of the PFAA category with carbon number from seven to ten. However, rat, oral, 90-day, repeated-dose NOAEL for the 6C derivative (i.e., 50 mg/kg bw/d) is based on centrilobular hepatocellular hypertrophy correlated with higher liver weights and slightly higher peroxisome β-oxidation activity with zero evidence for metabolism (Chengelis et al., 2009). Zhang et al. (2008) demonstrates that 12C derivative exhibits hepatotoxicity in male rats. Specifically, male rats (gavaged for 14 days with 0, 1, 5, or 10 mg/kg bw/d) exhibited diminished absolute liver weights with the relative liver weights significantly increased in the 5 and 10 mg doses. Additionally, a combined repeated-dose and reproductive/developmental toxicity screening study for C12 derivative was conducted in accordance with OECD guideline 422 (Kato et al., 2014). Dosing at 0.5 and 2.5 mg/kg bw/d for 42-47 days mainly affected the liver, in which hypertrophy, necrosis, and inflammatory cholestasis were noted. The NOAEL of the 12C PFAA was concluded to be 0.1 mg/kg bw/d. Takahashi et al. (2014) conducted a combined repeated-dose and reproductive/developmental toxicity screening study for the C11 derivative in accordance with OECD guideline 422. Specifically male and female rats were gavaged at dose of 0, 0.1, 0.3, or 1.0 mg/kg bw/d. Body weight gain was inhibited in both sexes were observed at 1.0 mg/kg/day. Liver weight was increased in males at 0.3 mg/kg/day and above and in females at 1.0 mg/kg/day. In both sexes, centrilobular hypertrophy of hepatocytes was observed at 0.3 mg/kg/day and above and focal necrosis was observed at 1.0 mg/kg/day. The NOAEL for the 11C PFAA were concluded to be 0.1 mg/kg bw /d.

In summary, 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. Oral repeated dose data for the 6C, 11C and 12C PFAA derivatives are in qualitative agreement with that reported for PFOA. Moreover, the data for the 11C and 12C derivatives are in quantitative with that reported for PFOA.

4. Data matrices for assessing similarity

The data supporting the similarity argument for the four analogues listed above are reported in Annex I.

4.1. Structural similarity
As demonstrated in Table 1 of Annex I, all the PFAAs included in the category are structurally highly similar. Specifically, they possess common molecular scaffolding, straight-chain C-atom backbone. Structurally, the only difference is the length of the C-atom backbone.

4.2. Chemical property similarity

As demonstrated in Table 2 of Annex I, all the PFAAs included in the category have physico-chemical properties that typically are either constant or show a trend in values related to carbon chain length. Specifically, all category members exhibit high molecular weights (i.e., >300), while hydrophobicity (log Kow) increases with size, and ionization (pKa) is largely unaffected by size.

4.3. Chemical constituent similarity

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, -CO₂H, 2) structural groups, -CF₃ and -CF₂-, 3) extended structural fragment -CF₂CO₂H. Further, all belong to the same chemical class and sub-class, straight-chain perfluoronated carboxylic acids of with a limited range of C-atoms.

4.4. Toxicokinetic similarity

As demonstrated in Table 4 of Annex I, the toxicokinetic understanding of PFAAs is incomplete. With that said, PFOA is well-studied. It is clear that there are in the toxicokinetic (i.e., ADME-related) differences within the PFAAs. These differences are animal species- and chemical analogue-related (e.g., half-lives or clearance rates). 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 (Andersen et al., 2006; Rusyn, 2015).

In mammalian studies, PFOA have been shown to be readily absorbed orally but poorly eliminated; they are not metabolized and undergo extensive uptake from enterohepatic circulation (Lau et al., 2007; Bull et al., 2014; USEPA, 2014). For example, 24 hours post administration of a single dose of ¹⁴C-PFOA (averaging 11.4 mg/kg bw) by gavage to 3 male 10-week old CD rats, > 90% of total carbon-14 was absorbed (Gibson and Johnson, 1979). In another study, male Sprague-Dawley rats (10/group) were exposed to PFOA (96% a.i.) at 0, 5, and 20 mg/kg bw/d once daily by gavage for 28 days. It was demonstrated that >92% of both doses was absorbed (Cui et al., 2010).

Steady-state serum levels of PFAAs are reached within a few weeks with oral dosing. For example, the steady-state serum levels for PFOA in monkeys dosed with oral capsules containing 3, 10, or 20 mg/kg bw for 6 months were reached within 4-6 weeks (Butenhoff et al., 2004b). Kennedy et al. (2004) observed that peak blood levels of PFOA were attained 1-2 hours following a 25 mg/kg bw dose to male and female rats.
Moreover, it was noted that blood levels of PFOA over time were similar in rats given a single dose of 25 mg PFOA/kg as compared to a rat given 10 daily doses of 25 mg PFOA/kg (Kennedy et al., 2004). In another study (Elcombe et al., 2010), plasma PFOA concentrations in male Sprague-Dawley rats fed a diet containing 300 ppm PFOA for 1, 7, or 28 days were noted to be 259, 234, and 252 µg/mL, respectively.

In rats, a marked gender difference in serum and tissue levels exists following PFOA administration. Specifically, males consistently have much higher levels than females with the difference maintained and becoming more pronounced over time. Correspondingly, female rats show much greater urinary excretion of PFOA than do male rats with serum half-life values in hours for females compared with days for males.

Distribution of orally absorbed PFAAs includes vascular transport from the gut to other tissues. 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.

Wu et al. (2009) in examining the interaction of PFOA and human serum albumin demonstrated PFAA binding to the protein. Specifically, within 4 hours in the absence of albumin, 98% of the dissolved PFOA was found in the dialysate. In the presence of albumin, the amount of PFOA detected in the dialysate after 4 hours decreased; reductions were in direct proportion to the albumin concentration.

MacManus-Spencer et al. (2010) using a variety of approaches to quantify the binding of PFOA to serum albumin suggest the presence of primary and secondary binding sites on albumin. 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.

Studies of tissue distributions are available for several species including monkeys, rats, and mice. Butenhoff et al. (2002; 2004b) studied the fate of PFOA in monkeys in a six-month oral exposure study. Briefly, groups of 4-6 male monkeys each were administered PFOA daily via oral capsule at dose rates of 0, 3, 10, or 20 mg/kg bw. Serum, urine and faecal samples were collected at two-week intervals and were analyzed for PFOA concentrations. Liver samples were collected at time of sacrifice. Serum concentrations reached steady-state levels within four to six weeks in all dose groups. The mean serum concentration of PFOA in control monkeys was 0.134 µg/mL. The serum steady-state concentrations of PFOA, which varied markedly between animals were 77, 86, and 158 µg/ml after six weeks (Butenhoff et al., 2002) and 81, 99, and 156 µg/ml (Butenhoff et al., 2004b) after six months for the 3, 10, and 20 mg/kg bw, respectively.
Urine PFOA concentrations reached steady-state after 4 weeks and were 53, 166, and 181 µg/ml with SD of 50% in the 3, 10, and 20 mg/kg dose groups, respectively. Liver PFOA concentrations at terminal sacrifice in the 3 mg/kg and 10 mg/kg dose groups were similar and ranged from 6.29 to 21.9 µg/g. Liver PFOA concentrations in two monkeys exposed to 20 mg/kg were 16.0 and 83.3 µg/g. Liver PFOA concentrations in two monkeys dosed with 10 mg/kg-day at the end of a 13-week recovery period were 0.08 and 0.15 µg/g (Butenhoff et al., 2004b).

Han et al (2012) reviewed the clearance of PFOA with an emphasis on renal clearance. Human clearance is clearly demonstrated to be much longer than in other species (e.g. rodents, monkeys) with half-life values of 2.3 – 3.5 years reported (Olsen et al., 2007; Bartell et al., 2010). Total clearance with rats was observed to be greatest for the C6 analogues and then decreased significantly with minimal clearance for PFDA. With the exception of PFDA, total clearance was less in male rats than female rats.

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 PPFAs 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.

Ng and Hungerbühler (2014) examined the two prevailing hypotheses for the mechanisms that control the bioaccumulation of PFAAs. The first assumes that partitioning to membrane phospholipids, which have a higher affinity for charged species than neutral storage lipids, can explain the high bioaccumulation potential of these compounds. The second assumes that interactions with proteins, including serum albumin, liver fatty acid binding proteins (L-FABP), and organic anion transporters determine the distribution, accumulation and half-lives of PFAAs. After consideration of: 1) observed patterns of tissue distribution in the laboratory and field, 2) the relationship between perfluorinated chain length and bioaccumulation, and 3) species- and gender-specific variation in elimination half-lives, it was concluded that the two models need not be mutually exclusive, but that protein interactions are needed to explain some important features of PFAA bioaccumulation.

4.5. Metabolic similarity

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.

4.6 Toxicophore similarity
As demonstrated in Table 6 of Annex I, based on *in silico* predictions the PFAAs are considered Cramer class III compounds. While none of the other profilers in the OECD QSAR Toolboxv3.3 are triggered, some of the newly developed COSMOS profilers are activated. In particular, PFAAs are associated by full agonism prediction *in silico* with PPARγ ligand-dependent activation, see section 6.

4.7. Mechanistic plausibility similarity

As summarised in Table 7 of Annex I, the PFAAs included in the category are associated with a number of molecular mechanisms of toxicity. As previously noted, 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). PFAA-induced liver toxicity is considered to be mediated via PPARs, especially PPARα binding (Takacs and Abbott, 2007; Rosen et al., 2008; Wolff et al., 2008; 2012; Bjork and Wallace, 2009).

While several perfluorinated compounds can activate PPARα, they may also induce peroxisome proliferation by perturbing lipid metabolism and transport. This may be significant, as the subsequence of key events for perturbing lipid metabolism and transport is not consistent with only a PPARα agonist mode of action.

PPARs are members of the steroid-thyroid hormone super-family of ligand-activated transcription factors. Because of their role in glucose and lipid homeostasis, they are well studied. The mechanisms of PPAR genomic activity (transactivation and transrepression) involve the nuclear receptor forming a heterodimer with retinoid X receptor alpha (RXRα) and binding to specific DNA sequences within the promoter regions of target genes. PPARs bind and respond to a diverse set of endogenic lipid metabolites, including eicosanoids and fatty acids. Briefly, the activation of PPARs is brought about by specific conformational changes associated with ligand binding. Subsequently, these changes release corepressors and allow for the recruitment of coactivators.

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, PPAR\(\gamma\) 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 PPAR\(\alpha\) null mouse may be due to the accumulation of lipid droplets or the accumulation of PFOA in the liver and PPAR\(\gamma\) and adipose differentiation-related protein (ADRP). Supporting this argument is gene expression profile data for rat liver treated with PFOA, where the largest categories of induced genes are those involved in metabolism and transport of lipids, particularly fatty acids (Rosen et al., 2008).

The above findings are supported by the findings of Ding et al. (2009). Ding et al. (2009) studied the dosage-dependent metabonomic and transcriptomic responses of male rats’ exposure to the C12 derivative for 110 days. 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 PPAR\(\alpha\) and PPAR\(\gamma\) and also of CD36 (fatty acid translocase), which is a common target of the two receptors is reported (Ding et al., 2009). Its over-expression is one of the most probable key intermediate events outlined in the prosteatotic PPAR\(\gamma\)-mediated mode of action recently proposed (Al Sharif et al., 2014).

A potential mode of action is thought to be agonism of peroxisome proliferator receptors. For example, APOE*3-Leiden.CETP mice were fed a high-fat diet consistent with food eaten in Western parts of the world with perfluorobutanoic sulfonate, perfluorohexanoic sulfonate or perfluorooctanoic sulfonate (30, 6, and 3 mg/kg bw/day, respectively) for 4-6 weeks (Bijland et al., 2011). Whereas perfluorobutanoic sulfonate modestly reduced only plasma triglycerides, perfluorohexanoic sulfonate and perfluorooctanoic sulfonate markedly reduced triglycerides, non-HDL- cholesterol, and HDL- cholesterol (Bijland et al., 2011). Hepatic gene expression profiling data indicated that these effects were the combined result of PPAR\(\alpha\) and pregnane X receptor (PXR) activation.

It is worth noting, the PXR induces lipogenesis is via activation of CD36, PPAR\(\gamma\), SCD1, and FAE gene expression. The PXR inhibits fatty acid \(\beta\)-oxidation through its suppression of PPAR\(\alpha\) and thiolase gene expression (López-Velázquez et al., 2012).

\textit{In vitro} studies have evaluated the ability of numerous PFAAs to induce mouse and human PPAR\(\alpha\) activity in a transiently transfected COS-1 cell assay (Wolf et al., 2008). Specifically, COS-1 cells were transfected with either a mouse or human PPAR\(\alpha\) receptor-luciferase reporter plasmid. After 24 hours, cells were exposed to
either negative controls (water or dimethyl sulfoxide); positive control (the PPARα agonist WY-14643); PFOA
or PFNA at 0.5-100µM; perfluorobutanoic acid, PFHxA, or PFDA at 5-100µM. Following 24 hours of
exposure, luciferase activity from the plasmid was measured. 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, 2) Carboxyl derivatives are stronger activators of mouse and human PPARα than the corresponding
sulfonate derivatives, and 3) The mouse PPARα is more sensitive to PFAAs than the human PPARα.

A more recent study by Wolf et al. (2012) further reported 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.

Bjork and Wallace (2009) conducted a structure-activity relationship study of the transcriptional activation of
peroxisome proliferation in primary rat liver cell cultures for perfluoronated carboxylic and sulfonic derivatives
of varying C-atom chain length. Moreover, they examined whether this activity can be translated to human
liver cells in culture. They concluded: 1) 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.

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. While perfluorinated sulfonic acids exhibited similar
affinity, no binding was detected for perfluorinated alcohols. Molecular docking experiments show that the
driving forces for the binding of PFAAs with FABP are predominantly hydrophobic and hydrogen-bonding
interactions, and the binding geometry is dependent on both the size and rigidity of the PFAAs.

The mechanism proposed by Ding et al., 2009, notes that the activation of PPARα triggers oxidation of fatty
acids, which increases the pool of acetyl-CoA. In animals, acetyl-CoA and other acyl-CoA coenzymes are
essential to the balance between carbohydrate and lipid metabolism. Under normal circumstances, acetyl-CoA
from fatty acid metabolism feeds into the citric acid cycle, contributing to the cell’s energy supply. In the liver,
when levels of circulating fatty acids are high, the production of acetyl-CoA from the catabolism of lipids
exceeds cellular energy requirements. From the excess acetyl-CoA, ketone bodies are produced, which can then
enter the circulation. 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.

4.8. Other endpoint similarity

In addition to the mammalian in vivo data noted above, other data has been gathered with the purpose of strengthening the similarity hypothesis.

4.8.1. Fish toxicity

PFAAs have been tested in fish. PFAAs also have been associated with oxidative stress and mitochondrial toxicity (Hagenaars et al., 2013; Huang et al., 2013). PFOA also acts as a mixed-type enzyme inducing agent with inductions of CYP2B2, CYP3A4, and CYP4A1 in liver microsomes. Specifically, male Japanese medaka (Oryzias latipes) were exposed to the nominal concentrations of 10, 50, 100 mg/L PFOA for 7 days. There was no impact on survival, relative liver and gonad size, or condition factor (measure of growth) at any concentration tested. Peroxisomal acyl-CoA oxidase (ACO) activity was elevated at the highest dose. The increase of ACO activity was paralleled by the significant up-regulation of PPAR-α expression at the same dose. PFOA also induced a significant inhibition of catalase activity at high doses but showed no changes of superoxide dismutase or glutathione peroxidase activities in the liver. These results suggest that PFOA may induce peroxisomal fatty acid oxidation and impose the oxidative stress through the alteration of cellular oxidative homeostasis in the liver. PFOA also increased the mRNA levels of proinflammatory cytokines IL-6, TNF-α and IL-1β. The latter indicates that inflammation may be involved.

Hagenaars et al. (2013) studied male and female zebrafish exposed to nominal concentrations of 0.1, 0.5 and 1 mg/L PFOA for 4 and 28 days. They described the general mode of action of PFOA as an increase of the mitochondrial membrane permeability followed by an impairment of aerobic ATP production. This mitochondrial dysfunction further resulted in effects on oxidative stress and apoptosis at the gene transcript and protein level.

Benninghoff et al. (2011) studied the structural characteristics of PFAAs which elicit oestrogen-like activity in juvenile rainbow trout and determined, using in vitro species comparisons whether these chemicals interact directly with the oestrogen receptor (ER). PFAAs of C8 to C11 are inducers of the oestrogen-responsive biomarker vitellogenin in vivo. These in vivo findings were corroborated by in vitro mechanistic assays for trout and human ER. All PFAAs tested weakly bound to trout liver ER, with half maximal inhibitory concentration (IC50) values of 15.2-289mM. Additionally, they significantly enhanced human ERα-dependent transcriptional activation at concentrations ranging from 10-1000nM. Overall, these data support the contention that intermediate size PFAAs are weak environmental estrogens.
4.8.2. In vitro toxicity

PFAAs have been evaluated in vitro. Viability tests were performed here at varying time-exposures on C6-C18 PFAAs with human colon carcinoma (HCT116) cells (Kleszczyński et al., 2008). A chain length-dependent correlation was observed for EC50 values for C6 to C14 derivatives. Responses to further increases in C-atoms were non-linear and even partially reversed. The latter observation is likely due to reduced bioavailability due to protein binding. It was concluded that PFAAs are not acutely toxic at the cellular level; however, they can trigger cell apoptosis (Kleszczyński et al., 2008).

Wallace et al. (2013) examined the structure-activity relationships by which PFAAs interfere with mitochondrial respiration in vitro. Briefly, freshly isolated rat liver mitochondria were incubated with one of 16 different PFAAs, including perfluorinated carboxylic, acetic, and sulfonic acids, sulfonamides and sulfamidoacetates, and alcohols. The effect on mitochondrial respiration, measured at five concentrations and dose-response curves, was used to describe the effects on state 3 and 4 respiration and respiratory control. The data for carboxylic acids support prior evidence that the perfluorinated carboxylic and acetic acids induce the mitochondrial permeability transition. In contrast, the sulphonamides are protonophoric uncouplers of oxidative phosphorylation. In both cases, potency increased with increasing number of C-atoms, with a prominent inflection point between C6 and C8.

In the study of PFOA by Haung et al. (2013), flow cytometry analysis demonstrated that PFOA induced oxidative stress, cell cycle arrestment and apoptosis in L-02 (a human non-tumour hepatic cell line). Furthermore, alterations in protein profile within L-02 cells exposed to PFOA suggest involvement of p53 activation, which triggers apoptosis in L-02 cells.

5. Statement of uncertainty in similarity

In Annex II, the assessment of uncertainty is presented. Data uncertainty associated with chemical similarity is judged to be low. Data uncertainty and weight-of-evidence associated with the fundamentals of chemical transformation/toxicokinetic similarity is judged to be low to moderate. Data uncertainty and weight-of-evidence associated with the fundamentals of toxicodynamic similarity is also judged to be deemed low to moderate. Finally, uncertainty associated with mechanistic relevance and completeness of the read-across is judged to be low to moderate. In terms of chemistry, the narrowly defined applicability domain of this category leads to all analogues or category members being highly similar chemically. Specifically, the key feature, perfluoronated carboxylic acid, relevant for toxicity is common within the category. While there are differences among the category members with respect to physicochemical properties, these differences are not considered toxicologically relevant outside of their impact on bioavailability.
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.

The greatest toxicodynamic uncertainty for the PFAAs included in the category is mechanistic plausibility. Specifically, PFAAs are experimentally associated with a number of molecular mechanisms of toxicity. It is unclear if the repeated-dose toxicity is related to one mechanism or the combination of more than one mechanism.

All analogues or category members are considered, from a toxicodynamic standpoint, to be moderate in similarity. Specifically, from a qualitative standpoint, all analogues or category members exhibit highly similar toxicological profiles for \textit{in vivo} liver adverse effects, as well as specific \textit{in vitro} endpoints. In contrast, potency either shows a linear trend with increased in C-number or a bell-shaped trend with the apex at C8.

6. Statement of the conclusions from traditional data

\textit{In vivo} oral repeated-dose exposure to PFAAs, especially PFOA, gives rise to a standard set of symptoms, considering specifically liver toxicity, including an increased liver weight, hepatocyte hypertrophy, hepatic triglyceride accumulation, multifocal coagulation, and liquefaction necrosis. This hepatocellular injury is accompanied by inflammatory cell infiltration. Therefore, \textit{in vivo} hepatic toxicity may be involved in oxidative stress and inflammatory response, as well as alteration in lipid transport and metabolism. 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, PPAR\textalpha and/or PPAR\textgamma 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, respectively (EFSA, 2008) is read across to the other three analogues in the category.

The chemical category under consideration, intermediate size PFAA, is an extremely well-studies group of compounds. There is extensive high quality \textit{in vivo} data for repeated-dose toxicity and ample \textit{in vitro} data,
especially for the source substance. 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.

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. The read across predictions are clearly relevant to priority setting, hazard identification, and classification and labelling. However, their applicability to risk assessment may be contested because of uncertainties in most relative toxic mechanism(s) and trend analysis of toxic potency.

7. New methods information

While read-across predictions are based on in vivo data, information gathered from alternative methods may improve the weaker aspects of the category similarity argument and thereby reduce the uncertainties association with the prediction (Schultz et al., 2015). Typically for repeated-dose effects, this information is targeted towards supporting mechanistic plausibility and refining toxicokinetic and toxicodynamic similarities. In addition, such data often add to the overall weight-of-evidence of the read-across prediction. The so called “new methods” information includes the new generation of in vitro molecular screening and toxicogenomics data (Sturla et al., 2013). In the current case study, there are two weak aspects of the similarity argument. The first is the mechanistic plausibility of PPAR activation as the most likely initiating events leading to repeated-dose liver toxicity and the impact of the derivative-specific clearance rate (i.e., renal reabsorption).

Toxicogenomic studies of PFAAs reveal the largest group of induced genes is those involved in transport and metabolism of lipids, particularly fatty acids. The largest groups of suppressed gene are those related to inflammation and immunity. For example, gene chip analysis of PFOA (Guruge et al., 2006) revealed approximately 106 and 38 genes were consistently up-regulated or down-regulated, respectively, in all treatment groups. 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).

Eriksen et al. (2010) examined the ability of a series of five PFAAs to generate reactive oxygen species and to induce oxidative DNA damage in HepG2 cells. The results show that PFAAs induce only modest production of reactive oxygen species and DNA damage in a cell line that represents the human liver.
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.

Circular dichroism spectroscopy results showed that PFAA binding induced significant protein secondary structural changes. Molecular docking revealed that the inhibitory effect could be rationalized well by the cleft binding mode, as well as the size, substituent group and hydrophobic characteristics of the PFAAs. At non-cytotoxic concentrations, three selected PFAAs inhibited LDC activity in HepG2 cells and subsequently, resulted in the decreased cadaverine level in the exposed cells, suggesting that LDC may be a possible target of PFAAs for their in vivo toxic effects.

Zhang et al. (2014) also examined the binding interactions between PFAAs and PPAR\(\gamma\). Specifically, the in vitro binding of eleven PFAAs to human PPAR\(\gamma\) ligand binding domain (hPPAR\(\gamma\)-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. Additionally, it was shown that the hPPAR\(\gamma\)-LBD binding affinity of perfluorinated sulfonic acids is stronger than their PFAA counterparts, while perfluorinated alcohols show no hPPAR\(\gamma\)-LBD binding. Circular dichroim spectroscopy showed that PFAA binding induced a distinctive structural change of the receptor. In dual luciferase reporter assays using transiently transfected HepG2 cells, PFAAs acted as hPPAR\(\gamma\) agonists with potency correlating with hPPAR\(\gamma\)-LBD binding affinity.

Perfluoroalkyl compounds, including PFAAs, have been shown to disrupt thyroid functions through thyroid hormone receptor (TR)-mediated pathways. Specifically, 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. 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.

Within the COSMOS project of SEURAT-1, PFAAs were screened with a variety of in silico profilers. These results are reported in Table 6 of Annex I. Specifically, the potential for full PPAR\(\gamma\) agonism is predicted by a virtual screening procedure, including docking with filtering by four PPAR\(\gamma\) pharmacophores (Tsakovska et al.,...
The pharmacophore models were developed by analysing 118 PPARγ 3D complexes from the Protein Data Bank (PDB), taking into consideration structural elements (e.g., hydrogen bonds, hydrophobic and aromatic) of the ligands essential for their interactions with the receptor. The key protein interaction of the most active agonists include hydrogen binding to 4/5 amino acids in the receptor pocket; the most active agonists interact directly with H12 residues. PPARγ active full agonists share at least four common pharmacophoric features; the most active ones have additional interactions. 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. The evaluation of potential binding to the receptors is based on structural fragments and physico-chemical features that have been identified as essential to bind to these nuclear receptors and induce a response. The profilers have been developed by studying the physico-chemical features of known nuclear receptor binders and elucidating the structural features needed for binding to the ligand binding pocket (using the Protein Data Bank and ChEMBL) (Mellor et al. 2015; Steinmetz et al. 2015). 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.

Other in silico profilers, developed within COSMOS, also were applied. The PFAAs were processed for their potential LXR binding by employing and combining different in silico approaches, including ensemble docking, pharmacophore matching, fingerprint-based similarity and a QSAR classification model (Fioravanzo et al. 2013). Similarly as for the LXR nuclear receptor profiler, no LXR binding potential was found. Furthermore, the substances were screened with profilers for potential hepatotoxicity, including sixteen structural alerts associated with observed human hepatotoxicity (Hewitt et al. 2013), for phospholipidosis (Przybylak et al. 2014) and for protein binding (Enoch et al. 2011). However, no positive responses were reported.

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. 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 µM). 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). There is activity in several cell-stress processes for C7 and above, leading to frank cytotoxicity by C9. GPCR activity is seen increasingly with C-atom length. It is worth noting that the ToxCast program has observed with their screening assays that surfactants can cause false activity, potentially through a protein-denaturation MoA. Table 8 in Annex I summarises 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.

Taken collectively, these new methods data support the premise that the molecular mechanism of action inducing repeated-dose liver toxicity of PFAAs is PPAR-linked; most likely a most likely a combination of PPARα/PPARγ interactions. The new methods data have no impact on the toxicokinetic uncertainty. However they reduce the uncertainty and increase the weight-of-evidence associated with the toxicodynamic similarity as well as the uncertainty associated with mechanistic relevance and completeness of the read-across.

8. Statement of conclusion after considering new methods data

Intermediate size PFAAs are a well-studied group of compounds. There is quality in vivo data for repeated-dose toxicity test schemes. Moreover, there are ample new methods data, especially from ToxCast. Specifically these data better clarify the PPAR-linked molecular mechanism of PFAA-induced liver toxicity. The new methods data add weight-of-evidence to the initial read-across prediction and the use of the NOAEL value for PFOA in quantitative read-across to other category members as the read across prediction is relevant to all regulatory decisions including risk assessment. The final conclusion of the data evaluation is that read-across may be conducted from the most studied analogue, PFOA. Specifically, the rat oral 90-day NOAEL for PFOA, 0.06 mg/kg bw/d (based on hepatocyte necrosis and hepatocellular hypertrophy and increased liver weight), may be read across to the untested C9 and C10 analogues with low uncertainty and used to inform regulatory decisions. It is worth noting, this conclusion is supported by the in vivo data (i.e., NOAELs of 0.1
mg/kg bw/d) for the C11 and C12 derivatives. Furthermore, it is concluded that the oral 90-day NOAEL for PFOA can be red across to the C7 derivative as the most conservative argument.

**Disclaimer**

This case study has been designed to illustrate specific issues associated with read-across and to stimulate discussion on the topic. It is not intended to be related to any currently ongoing regulatory discussions on this group of compounds. The background document has been prepared to facilitate the discussion at the Topical Scientific Workshop and does not necessarily represent ECHA’s position. The papers are not final publications and are solely intended for the purposes of the Workshop.

**Acknowledgement**

Authors acknowledge funding from the COSMOS Project which was funded by the European Community’s Seventh Framework Programme (FP7/2007-2013) under grant agreement number 266835 and the European Cosmetics Association Cosmetics Europe. This paper was also supported by a consultancy agreement with Cosmetics Europe.

**References**


Andersen, M.E., Clewell, H.J. 3rd, Tan, Y.M., Butenhoff, J.L. and Olsen, G.W. 2006. Pharmacokinetic modeling of saturable, renal resorption of perfluorooalkylic acids in monkeys--probing the determinants of long plasma half-lives. Toxicology 227: 156-64


Elcombe, C.R., Elcombe, B.M., Foster, J.R., Farrar, D.G. Jung, R., Chang, S-C., Kennedy, G.L. and Bunthoff, J.L. 2010. Hepatocellular hypertrophy and cell proliferation in Sprague-Dawley rats following dietary exposure to ammonium perfluorooctanoate occurs through increased activation of the xenosensor nuclear receptors PPARα and CAR/PXR. Arch. Toxicol. 69: 244-257.


Judson, R. 2015. personal communication.


Rusyn, I., 2015, personal communication, upcoming IARC Monograph, DRAFT NOT TO BE CITED


### Table 1. Data Uncertainty and Weight-of-Evidence Associated with the Fundamentals of Chemical, Transformation/Toxicokinetic and Toxicological Similarity

<table>
<thead>
<tr>
<th>Similarity Parameter</th>
<th>Data Uncertainty a (empirical, modelled)</th>
<th>Strength of Evidence b (low, medium, high)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance Identification, Structure and Chemical Classifications</td>
<td>low</td>
<td>high</td>
<td>All category members have CAS numbers, similar 2D structure and belong to the same chemical class and subclasses.</td>
</tr>
<tr>
<td>Physio-Chem &amp; Molecular Properties</td>
<td>Empirical: low</td>
<td>high</td>
<td>All category members are appropriately similar with respect to key physicochemical and molecular properties. Where appropriate (e.g., log Kow) changes in values are linked to changes in C-atom chain length. There is a high degree of consistency between measured and model estimated values.</td>
</tr>
<tr>
<td></td>
<td>Modeled: low</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substituents, Functional Groups, &amp; Extended Structural Fragments</td>
<td>low</td>
<td>high</td>
<td>Substituents, functional groups and extended structural fragments are consistent across all category members.</td>
</tr>
<tr>
<td>Transformation/Toxicokinetics and Metabolic Similarity</td>
<td>Empirical: In vivo: low</td>
<td>medium</td>
<td>Based on in vivo data for multiple category members, there is evidence for similar toxicokinetics and metabolic pathways within a species. However, bioavailability and excretion different are observed. Comparison of results from empirical studies and model predictions indicate similar metabolism (i.e., no metabolism) among all category members.</td>
</tr>
<tr>
<td></td>
<td>In vitro: none</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Simulated: low</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potential Metabolic Products</td>
<td>low</td>
<td>high</td>
<td>Based on in silico metabolic simulations, no potential metabolic products are predicted to be produced by any of the category members.</td>
</tr>
<tr>
<td>Toxophores/Mechanistic alerts</td>
<td>medium</td>
<td>medium</td>
<td>Based on in silico profilers, outside of PPAR-binding, no category member contains any established toxophores.</td>
</tr>
<tr>
<td>Mechanistic plausibility and AOP-Related Events</td>
<td>medium</td>
<td>medium</td>
<td>Although no AOP is currently available for the hypothesized toxicity pathway, many category members have been tested for what is accepted as mechanistically-relevant events.</td>
</tr>
<tr>
<td>Other relevant, in vivo, in vitro and ex vivo endpoints</td>
<td>low</td>
<td>high</td>
<td>Although not part of the hypothesized toxicity pathway, many category members have been tested in vivo in fish for oxidative stress, oestrogen-like activity and mitochondrial toxicity. In addition, many category members have been tested in vitro for cell viability, mitochondrial toxicity and thyroid hormone receptor binding. There is general agreement in the trend of the reported EC50 values.</td>
</tr>
</tbody>
</table>

Similarity of chemistry within the category is high. Within the category data similarity and weight-of-evidence associated with the fundamentals of chemical, transformation/toxicokinetic is moderate to high and uncertainty, mainly related to excretion, is low to moderate. Within the category data similarity and weight-of-evidence associated with toxicodynamics is moderate to high and uncertainty mainly related, to the molecular mechanism inducing repeated-dose liver toxicity, is low to moderate. Uncertainties associated with mechanistic relevance and completeness of the read-across (i.e., uncertainty in the predictions) are reduced with the addition of new methods data and by reading-across from the best studied and most potent analogue, perfluorinated octanoic acid.

Summary: Key features of chemistry are highly similar within the structurally-limited category. With noted differences between species and analogues, key features of toxicokinetics are common within the category. Category members are considered mechanistically similar. Category members exhibit a similar toxicological profile with respect to in vivo toxicity.

---

*Uncertainty associated with underlying information/data used in the exercise

*Consistency within the information/data used to support the similarity rational and prediction
Table 2. Template for Assessing Uncertainty Associated with Mechanistic Relevance and Completeness of the Read-Across

<table>
<thead>
<tr>
<th>Factor</th>
<th>Uncertainty (low, medium, high)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>The problem and premise of the read-across</td>
<td>Low</td>
<td>The endpoint to be read across, oral 90-day repeated-dose toxicity, for straight chain perfluorinated carboxylic acids, is well-studied but not well-understood. The scenario of the read-across hinges on no metabolism, similar clearance or excretion and LOAEL of hepatocellular hypertrophy, higher liver weights and hepatic inflammation.</td>
</tr>
<tr>
<td>In vivo data read across</td>
<td>Number of analogues in the source set</td>
<td>Low; 1 of 4 tested</td>
</tr>
<tr>
<td>Quality of the in vivo apical endpoint data read across</td>
<td>Low; consistent phenotypic expression of toxicity.</td>
<td>High quality empirical data from standard test guidelines for the stated regulatory endpoint exists for PFOA. Similar non-standard test data exists for PFUA and PFDoA. All these data are consistent in regards to qualitative description of effects</td>
</tr>
<tr>
<td>Severity of the apical in vivo hazard</td>
<td>Low.</td>
<td>Potency data for the in vivo apical endpoint is the NOAEL of 0.06 mg/kg bw/d based on hepatocyte necrosis (males) and hepatocellular hypertrophy and increased liver weight (females) for PFOA (EFSA, 2008). This is supported by the in vivo data (i.e., NOAELs of 0.1 mg/kg bw/d) for the C11 and C12 derivatives.</td>
</tr>
<tr>
<td>Evidence to the biological argument for RA</td>
<td>Robustness of analogue data set</td>
<td>Low; numerous endpoints reveal the same structure-activity relationships.</td>
</tr>
<tr>
<td>Concordance with regard to the intermediate and apical effects and potency data</td>
<td>Medium; limited by lack of mechanistic plausibility.</td>
<td>While data is limited, there appears to be good agreement between the sequences of biochemical and physiological events leading to the in vivo liver toxicity. There is consistency and high specificity for the association between in vivo symptoms and in vitro endpoints, and the structural domain of the category. There is general agreement among the dose-response relationships of the tested category members for relevant in vitro event.</td>
</tr>
<tr>
<td>Weight of Evidence</td>
<td>Low to medium</td>
<td>Overall the available information is generally consistent with the stated premise. The sharp structural limitations on the category strengthen the WoE. While the toxicokinetics data is limited, the lack of inconsistencies and lack of metabolism adds to the WoE. However, the variability in clearance rates do to reabsorption in the kidney reduces the WoE. The fact the source substance in vivo data is supported by similar data for two other analogues adds to the WoE. The fact that there is consistent relevant in vitro data for most if not all the category members strengthens the WoE. The lack of consistent mechanistic plausibility weakens the WoE. However, this is offset by the consistency in results from molecular interaction assays. New method data is consistent with the premise of the read across.</td>
</tr>
</tbody>
</table>

The overall uncertainty associated with this read-across prediction is judged to be acceptable to use read-across-based predictions to fill data gaps for regulatory decisions.