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

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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 i*n 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

34 weight-of-evidence associated with transformation/toxicokinetic were unchanged; however, uncertainty and
35 weight-of-evidence associated with toxicodynamic similarity were reduced and increased, respectively. It is
36 concluded that the rat oral 90-day NOAEL for PFOA, 0.06 mg/kg body weight (bw)/day (d) (based on
37 hepatocyte necrosis and hepatocellular hypertrophy and increased liver weight), may be read across to the
38 untested C9 and C10 analogues with low uncertainty and used to inform regulatory decisions. It is worth
39 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
40 derivatives. Further it is concluded that the oral 90-day NOAEL for PFOA can be red across to the C7
41 derivative as the most conservative argument.

42

43 **1. Introduction**

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

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

59 In addition to applying the Schultz et al. (2015) Workflow, case study was undertaken in a two-step process.
60 The purpose of the two-step process is to demonstrate the usefulness of new approach data in reducing the
61 uncertainty in the read-across prediction of the toxicological properties and thereby improve the likelihood that
62 it will be accepted for regulatory use. The initial assessment is a "traditional" read-across using established *in*
63 *vivo* data supplemented, as applicable, with conventional *in vivo*, *in vitro* and structure-activity relationship
64 information. The second assessment supplemented the initial exercise with "new-approach" data, including

65 high-throughput molecular screening, novel *in vitro* methodology and/or toxicogenomics information combined
66 in a rational manner (Whelan and Schwarz, 2011; Gocht et al., 2014, Sturla et al., 2014).

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

74

75 **2. Approach**

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

81 *2.1 Resources utilised*

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

84 *2.2. Premise*

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

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

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

92 • Toxicodynamically, PFAAs are direct-acting toxicants (i.e., where metabolism is not a factor) with
93 similar modes of action, (MoA), most likely a combination of PPARα/ PPARγ interactions, leading to
94 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

127 The chemical structures of the category members are given in Table 1 in Annex I.
128
129 *2.6. Purity/impurities*

130 A purity/impurity profile for the analogues listed in 2.4 is unknown. However, since the category is so limited
131 structurally, the potential impact of any impurities on the endpoint being evaluated is considered very small.

132

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

134 *3.1. Short characterisation of perfluoroalkyl acids*

135 PFAAs are highly fluorinated chemicals that consist of a backbone of 4 to 18 C-atoms and a single terminal
136 carboxylate moiety. In PFAAs, charge repulsion of the partially negative F-atoms and steric factors give
137 preference to the lowest energy conformer being a near linear molecular shape. This most likely conformer is
138 highly similar to the preferred conformation of corresponding fatty acid analogues. The ionized carboxylate
139 grouping and the F-atom's partial negative charges promote electrostatic interactions between PFAAs and
140 positively charged surfaces on macromolecules, especially proteins.

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

144 While the toxicokinetics of PFOA is well-studied, the toxicokinetic understanding of other PFAAs is
145 incomplete. It is clear that there are species differences (e.g., half-lives in human vs. monkey/rodent) in the
146 toxicokinetics of PFAAs. Typically, PFAAs are readily absorbed following oral exposure and distributed
147 mainly to the serum, kidney, and liver, with liver concentrations being several times higher than serum
148 concentrations. Oral absorption is mainly mediated by transporter proteins or mechanisms other than simple
149 diffusion (Launay-Vacher et al., 2006; Zaïr et al., 2008). PFAAs are resistant to biotransformation. Therefore,
150 toxicity of the parent compound and not that of a metabolite is of concern. Due to their impact on receptors and
151 other cellular proteins, PFAAs have the ability to alter intermediate metabolism and transformation of dietary
152 molecules by altering enzyme activities and transport kinetics. In general, the rate of elimination is enhanced
153 with decreasing C-atom chain length. However, the body-burden, especially in primates but also in rats is
154 increased by efficient reabsorption of PFOA in the kidneys and thus retention in the body. The net effect is
155 clearance rates that are both species- and analogue-dependent with lowest total clearance expected to be for
156 PFDA (Han et al., 2012; Fujii et al., 2015).

157 A first examination of mammalian toxicity data supports the contention that repeated-dosage oral exposure to
158 PFAAs is linked to liver toxicity. PFAAs, in rat oral repeated-dose testing, exhibit liver toxicity typically in the

159 form of hepatocyte necrosis and increased liver weight. While there are 90-day oral repeated-dose toxicity data
160 for the octanoic and hexanoic derivatives, there are data gaps for other PFAA analogues in the category.

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

167 Due to their structural similarity to naturally occurring fatty acids, mechanistic studies of PFAAs have focused
168 on the peroxisome proliferator-activated receptor (PPAR) pathways leading to liver toxicity; however, other
169 molecular mechanism have also been implicated. There has been a sharp increase in the number of
170 toxicological studies of perfluorinated chemicals since 2008; however, the mechanistic pathways of toxicity of
171 PFAAs are far from clear.

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

177 Several studies in rats and mice have examined PFAAs to determine the potential impact of the carbon chain
178 length (C6-C9) on hepatic toxicity and peroxisome proliferation. Results suggest the difference in
179 accumulation of these compounds in the liver was responsible for the different hepatic responses observed
180 between PFAAs with different C-atom chain length. In any case, it is generally believed that the potency of
181 PFAAs increases with increasing C-atom chain length up to C8 (Wolf et al., 2012).

182 *3.2. Statement target substance(s) and the regulatory endpoint(s) to be read across*

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

186 While there are several 28-day rodent oral gavage studies of various PFAAs, there are not many 90-day rat oral
187 *in vivo* repeated-dose data for PFAAs. Whilst not included in this category, it should be noted that there are
188 (OECD Test Guideline 422, Combined Repeat Dose and Reproductive/Developmental Toxicity Screening Test)
189 data for perfluoroundecanoic acid (PFUA) [2058-94-8] and perfluorododecanoic acid (PFDoA) [307-55-1], and

190 90-day oral repeated-dose for perfluorohexanoic acid (PFHxA) [307-24-4]. More to the point of this exercise,
191 there is extensive high quality 90-day oral repeated-dose data for PFOA [335-67-1]. Hence, an evaluation was
192 conducted to determine the suitability of PFOA as a source substance with the NOEAL data being read across
193 to fill the data gaps for the other analogues in the category.

194 *3.3. Existing Relevant Toxicity Data for PFOA*

195 Chronic toxicity data for PFOA are summarised in Table 1 There is a substantive body of evidence that liver
196 toxicity is prevalent at higher doses of PFOA and whilst other organ level effect occur, those to the liver
197 dominate.

198 *3.4. 90-day Rat Oral Repeated-dose Studies of PFOA*

199 In a dietary study, Goldenthal (1978) administered ChR-CD rats (5/sex/group) PFOA at concentrations levels
200 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
201 76.47 mg/kg-day in females. Body weight and food consumption were recorded weekly. Blood and urine
202 samples were collected during the pre-test period and at 1 and 3 months of the study for haematology, clinical
203 chemistry and urinalysis. At necropsy, the organs from the control, and the three highest dose groups were
204 weighed and examined for histopathological lesions; livers from all dose groups were also examined
205 microscopically. There were neither treatment-related deaths nor changes in behaviour or appearance. There
206 was a decrease in body weight gain for male rats at the two highest dose levels. At 13 weeks, mean body
207 weight of males in the highest does group was significantly less than that of controls. There were no treatment
208 related effects on the haematological, biochemical or urinary parameters were observed. Relative kidney
209 weights were significantly increased in males in the three highest dose groups. However, absolute kidney
210 weights were comparable among dose groups, and there were no histopathological lesions. Absolute liver
211 weights were significantly increased in males in the higher dose groups and in females in the highest dose
212 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)
213 increased with dosage. Relative liver weights were significantly increased in males in the two highest dose
214 groups and in females in the highest dose group. Hepatocellular hypertrophy (focal to multifocal in the
215 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
216 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,
217 17.9, and 63.5 mg/kg bw/d groups, respectively. Under the conditions reported above, the Lowest Observed
218 Adverse Effect Level (LOAEL) for males is 1.72 mg/kg bw /d) based on liver effects and the No Observed
219 Adverse Effect Level (NOAEL) is 0.56 mg/kg bw/d; the LOAEL for females is 76.5 mg/kg bw/d based on
220 increased liver weight, and the NOAEL is 22.4 mg/kg bw/d.

221 In another dietary study (Perkins et al., 2004; see EFSA, 2008), male ChR-CD rats in groups of ≈ 50 were
222 administered concentrations of 1, 10, 30, or 100 ppm PFOA for 13 weeks (i.e., doses equivalent to 0.06, 0.64,
223 1.94, and 6.50 mg/kg bw/d). There were two control groups, a non-pair-fed control group and a control group
224 pair-fed to the 6.50 mg/kg bw/d dose group. Following the 13-week exposure period, 10 animals per group
225 were fed a basal diet for an 8-week recovery period. The animals were observed for clinical signs of toxicity,
226 and body weights and food consumption were recorded. In the analysis of the relevant data, animals in groups
227 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
228 non-pair-fed group, while the data from the pair-fed control animals were compared to animals exposed to 6.50
229 mg/kg bw/d PFOA. With diets at 6.50 mg/kg bw/d, significant reductions in body weight and body weight gain
230 were seen compared to the pair-fed control group during week 1 and the non-pair-fed control group during
231 weeks 1-13. Body weight data in the other dosed-groups were comparable to controls. At feeds with 0.64 and
232 1.94 mg/kg bw/d, mean body weight gains were significantly lower than the non-pair-fed control group at week
233 2. These differences in body weight and body weight gains were not observed during the recovery period.
234 Animals fed the 6.50 mg/kg diet consumed significantly less food during weeks 1 and 2, when compared to the
235 non-pair-fed control group. Overall, there was no significant difference in food consumption. A total of 15
236 animals per group were sacrificed following 4, 7, or 13 weeks of treatment; in addition, 10 animals were
237 sacrificed after 13 weeks of treatment and following the 8 week recovery period. Serum samples collected from
238 10 animals per group at each scheduled sacrifice during treatment and from 5 animals per group during
239 recovery were analyzed for estradiol, total testosterone and luteinizing hormone. There were no significant
240 differences among the groups for any of the hormones evaluated. Weights of selected organs were recorded,
241 and these tissues were examined histologically. In addition, some organs were prepared for electron
242 microscopic examination. Significant increases in absolute and relative liver weights and hepatocellular
243 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.
244 There was no histological evidence of any degenerative changes. Hepatic palmitoyl CoA oxidase activity was
245 significantly increased at weeks 4, 7, and 13 in the groups fed the two highest concentrations. At 0.64 mg/kg
246 bw/d level, hepatic palmitoyl CoA oxidase activity was significantly increased at week 4 only. During
247 recovery, however, no liver effects were observed, indicating that the treatment-related liver effects were
248 reversible. EFSA (2008), report a LOAEL of 0.64 mg/kg bw/d) based on increases in absolute and relative liver
249 weights with hepatocellular hypertrophy and a NOAEL of 0.06 mg/kg bw/d.

250 Butenhoff et al. (2012) investigated the chronic toxicity of PFOA in a two year study with Sprague-Dawley
251 (Crl:CD BR) rats. Briefly, large groups (i.e., 50/sex) were fed diets containing 0, 30 or 300 ppm PFOA (0, 1.3,
252 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
253 300 ppm PFOA and evaluated at one year. All Observations included body weights and feed consumption,

254 haematology, serum chemistry, urinalysis, gross pathology, organ weights, and histopathology. There were no
255 significant differences in mortality between the treated and untreated groups (Butenhoff et al., 2012). The
256 authors further report there were dose-related decreases in body weight gains in male and female rats as
257 compared to the controls with statistical significance attained in both sexes for the high-dose group. Since feed
258 consumption increased during the study, the body weight changes (decrease in gain) were considered treatment-
259 related. Additionally, significant decreases (as compared to control values) in haematological-related values
260 were observed at the high-dose for both sexes. No dose- or treatment-related differences in absolute and
261 relative organ weights were found between the treated and control groups at two years (Butenhoff et al., 2012).

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

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

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

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

284 *3.5. 28-day Rat Oral Repeated-dose Studies of PFOA*

285 Currently, 28-day repeated-dose toxicity studies are limited. Loveless et al. (2008) administered PFOA by oral
286 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
287 was recorded. At necropsy, haematology, clinical chemistry, and corticosterone measurements were made.
288 Tissues were collected for weight and histopathological examination. Body weight, weight gain, haematocrit,
289 and haemoglobin were reduced at ≥10 mg PFOA/kg/day. Increased reticulocytes and haematopoiesis were
290 observed in the rats dosed with 30 mg/kg bw/d. Total and non-HDL cholesterol were significantly reduced at
291 0.3 and 1 mg/kg/day compared to control. HDL cholesterol was significantly decreased at 0.3, 1, and 10
292 mg/kg/day. Triglyceride levels were significantly decreased at all doses except 1 mg/kg. Absolute liver weight
293 (≥1 mg/kg bw/d) and relative liver weight (≥10 mg/kg bw/d) were significantly increased, minimum to mild
294 (0.3, 1 mg/kg bw/d) and moderate (≥10 mg/kg/day) hepatocellular hypertrophy, and focal necrosis (≥10
295 mg/kg/day) were observed. Although not statistically significant, serum corticosterone was increased at ≥ 10
296 mg/kg/day.

297 Cui et al. (2009) exposed male Sprague-Dawley rats in groups of ten by gavage once daily to PFOA at 0, 5, or
298 20 mg/kg/day for 28 days. The rats dosed with 5 mg/kg/day exhibited hypoactivity, decreased food
299 consumption and lethargy, during the third week of the study. Rats dosed with 20 mg/kg/day also exhibited
300 enhanced sensitivity to external stimuli. All rats were sacrificed after the final exposure. Hepatic, renal,
301 gonadal weight/animal's body weight was calculated to evaluate hyperplasia, swelling, or atrophy in the PFOA-
302 treated animals compared to controls. In the liver, treatment with 5 or 20 mg/kg bw/d hepatic hypertrophy, fatty
303 degeneration, acidophilic lesions as well as gross dilation and congestion in the hepatic sinusoid or central vein
304 were reported. No effects were observed in the kidneys of the low dose animals, however turbidity and
305 swelling in the epithelium of the proximal convoluted tubule was observed at the 20 mg/kg bw/d dose. Under
306 the conditions of this study, the LOAEL was 5 mg/kg bw/d based on liver effects; no NOAEL was established.

307 Elcombe et al. (2010) exposed male Sprague-Dawley rats in groups of ten by diets containing 0 or 300 ppm
308 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
309 bw/d, respectively. In addition, a group of rats (i.e., the positive control) was fed diets containing 50 ppm of the
310 PPARα agonist, Wyeth 14,643. The animals were observed daily and body weights and food consumption were
311 recorded. At necropsy, day 2, day 8, or day 29, the organs were weighed, examined for gross pathology and
312 preserved for histopathology. Liver DNA content and concentration were determined, and plasma was
313 collected for analysis (study 1 only) of liver enzymes, cholesterol, triglycerides, and glucose. Hepatic cell
314 proliferation and apoptosis were also determined. In both studies, body weight significantly decreased after 7
315 and 28 days on the PFOA diet. Body weight was not affected by Wyeth 14,643. Absolute liver weight was
316 significantly increased in rats fed PFOA diets for 7 days in the first study and in rats treated for 7 and 28 days in
317 the second study. The liver-to-body-weight ratio was significantly higher in rats fed PFOA diets for 7 and 28

318 days in both studies. Absolute liver weight and liver-to-body-weight ratio were significantly increased in
319 Wyeth 14,643 diet fed rats in both studies.

320 *3.6. Other Oral Repeated-dose Studies of PFOA*

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

326 At sacrifice (90 days), the mean absolute liver weight was significantly increased in all dose groups, and the
327 relative liver-to-body weight ratio was significantly increased for the high dose group. The cause of the
328 increase in liver weights was suggested to be due to hepatocellular hypertrophy (indicated by decreased hepatic
329 DNA content) which was hypothesized to result from mitochondrial proliferation based on an increase in
330 hepatic succinate dehydrogenase activity (Butenhoff et al., 2002).

331 Repeated-dose studies for short durations in both rats and mice also reported reductions in body weight,
332 increases in liver weight with hepatocellular hypertrophy. Specifically, Son et al. (2008) evaluated male ICR
333 mice exposed continuously to 0, 2, 10, 50 and 250 ppm of PFOA in drinking water for 21 days. They report
334 food and water consumption decreased in mice exposed to the highest concentration of PFOA. Mean body
335 weight gain was reduced in mice exposed to both 50 and 250 ppm of PFOA. Most notably, the size and relative
336 weight of the liver increased dose-dependently in PFOA-treated mice. In the histopathological evaluation, the
337 liver of PFOA-treated mice showed hepatocytomegaly and acidophilic cytoplasm; at the high doses of PFOA,
338 diffuse hepatic damage by multifocal coagualation and liquefaction necrosis were further noted (Son et al.,
339 2008)

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

344 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
345 bw from gestation day 0-17 or 18. Maternal liver, kidney, brain, and lungs were examined histologically.
346 Serum was collected for clinical chemistry and lipid analysis. Body weight was significantly decreased in dams
347 receiving 10 mg/kg. Maternal absolute liver weight was significantly increased at doses ≥ 5 mg/kg and relative
348 liver and kidney weights were significantly increased at all doses. At the two lower doses, hepatic hypertrophy
349 was localized to the centrilobular region; at the highest dose, hepatic hypertrophy was diffuse. At all doses,

350 renal cells in the outer medullar and proximal tubule were slightly hypertrophic. Treatment at the highest dose
351 caused a significant increase in cytosolic enzymes and a significant decrease in total serum protein, albumin,
352 globulin, triglycerides, phospholipids, total cholesterol, and free fatty acids. At the intermediate dose (i.e., 5
353 mg/kg bw/d), total serum protein and globulin were significantly decreased, and phosphorus was increased.
354 Based on increased relative liver and kidney weight, the maternal LOAEL in this study is 1 mg/kg bw/d; no
355 NOAEL was established.

356 *3.7. Repeated-dose Studies of other PFAAs*

357 There are no other repeated-dose data available for members of the PFAA category with carbon number from
358 seven to ten. However, rat, oral, 90-day, repeated-dose NOAEL for the 6C derivative (i.e., 50 mg/kg bw/d) is
359 based on centrilobular hepatocellular hypertrophy correlated with higher liver weights and slightly higher
360 peroxisome β-oxidation activity with zero evidence for metabolism (Chengelis et al., 2009). Zhang et al. (2008)
361 demonstrates that 12C derivative exhibits hepatotoxicity in male rats. Specifically, male rats (gavaged for 14
362 days with 0, 1, 5, or 10 mg/kg bw/d) exhibited diminished absolute liver weights with the relative liver weights
363 significantly increased in the 5 and 10 mg doses. Additionally, a combined repeated-dose and
364 reproductive/developmental toxicity screening study for C12 derivative was conducted in accordance with
365 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
366 liver, in which hypertrophy, necrosis, and inflammatory cholestasis were noted. The NOAEL of the 12C PFAA
367 was concluded to be 0.1 mg/kg bw/d. Takahashi et al. (2014) conducted a combined repeated-dose and
368 reproductive/developmental toxicity screening study for the C11 derivative in accordance with OECD guideline
369 422. Specifically male and female rats were gavaged at dose of 0, 0.1, 0.3, or 1.0 mg/kg bw/d. Body weight
370 gain was inhibited in both sexes were observed at 1.0 mg/kg/day. Liver weight was increased in males at 0.3
371 mg/kg/day and above and in females at 1.0 mg/kg/day. In both sexes, centrilobular hypertrophy of hepatocytes
372 was observed at 0.3 mg/kg/day and above and focal necrosis was observed at 1.0 mg/kg/day. The NOAEL for
373 the 11C PFAA were concluded to be 0.1 mg/kg bw /d.

374 In summary, there are *in vivo* data of sufficient quality and quantity for PFOA to be a source chemical and read
375 across to fill the data gaps for the rat oral 90-day-repeated-dose endpoint of other analogues in the category.
376 Oral repeated dose data for the 6C, 11C and 12C PFAA derivatives are in qualitative agreement with that
377 reported for PFOA. Moreover, the data for the 11C and 12C derivatives are in quantitative with that reported
378 for PFOA.

379 **4. Data matrices for assessing similarity**

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

381 *4.1. Structural similarity*

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

385 *4.2. Chemical property similarity*

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

390 *4.3. Chemical constituent similarity*

391 As shown in Table 3 of Annex I, all the PFAAs included in the category have common constituents in the form
392 of: 1) a single key substituent, $-CO_2H$, 2) structural groups, $-CF_3$ and $-CF_2-$, 3) extended structural fragment -
393 CF_2CO_2H. Further, all belong to the same chemical class and sub-class, straight-chain perfluoronated
394 carboxylic acids of with a limited range of C-atoms.

395 *4.4. Toxicokinetic similarity*

396 As demonstrated in Table 4 of Annex I, the toxicokinetic understanding of PFAAs is incomplete. With that
397 said, PFOA is well-studied. It is clear that there are in the toxicokinetic (i.e., ADME-related) differences within
398 the PFAAs. These differences are animal species- and chemical analogue-related (e.g., half-lives or clearance
399 rates). PFAAs are readily absorbed following oral exposure and distributed mainly to the serum, kidney, and
400 liver, with liver concentrations being several times higher than serum concentrations. In general, the
401 elimination is decreased by renal resorption thus, retention in the body is increased (Andersen et al., 2006;
402 Rusyn, 2015).

403 In mammalian studies, PFOA have been shown to be readily absorbed orally but poorly eliminated; they are not
404 metabolized and undergo extensive uptake from enterohepatic circulation (Lau et al., 2007; Bull et al., 2014;
405 USEPA, 2014). For example, 24 hours post administration of a single dose of ^{14}C-PFOA (averaging 11.4
406 mg/kg bw) by gavage to 3 male 10-week old CD rats, > 90% of total carbon-14 was absorbed (Gibson and
407 Johnson, 1979). In another study, male Sprague-Dawley rats (10/group) were exposed to PFOA (96% a.i.) at 0,
408 5, and 20 mg/kg bw/d once daily by gavage for 28 days. It was demonstrated that >92% of both doses was
409 absorbed (Cui et al., 2010).

410 Steady-state serum levels of PFAAs are reached within a few weeks with oral dosing. For example, the steady-
411 state serum levels for PFOA in monkeys dosed with oral capsules containing 3, 10, or 20 mg/kg bw for 6
412 months were reached within 4-6 weeks (Butenhoff et al., 2004b). Kennedy et al. (2004) observed that peak
413 blood levels of PFOA were attained 1-2 hours following a 25 mg/kg bw dose to male and female rats.

414 Moreover, it was noted that blood levels of PFOA over time were similar in rats given a single dose of 25 mg
415 PFOA/kg as compared to a rat given 10 daily doses of 25 mg PFOA/kg (Kennedy et al., 2004). In another study
416 (Elcombe et al., 2010), plasma PFOA concentrations in male Sprague-Dawley rats fed a diet containing 300
417 ppm PFOA for 1, 7, or 28 days were noted to be 259, 234, and 252 µg/mL, respectively.

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

422 Distribution of orally absorbed PFAAs includes vascular transport from the gut to other tissues. Evidence
423 suggests that PFAAs circulate in the body by non-covalent binding to plasma proteins. For example, rat,
424 human, and monkey plasma proteins bind > 95% of PFOA added at concentrations ranging from 1-500 ppm
425 (Kerstner-Wood et al., 2003). Serum albumin, the most common serum protein and a common carrier of
426 hydrophobic materials in the blood including short and medium chain fatty acids, carried the largest portion of
427 the PFAAs among the protein components of plasma.

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

432 MacManus-Spencer et al. (2010) using a variety of approaches to quantify the binding of PFOA to serum
433 albumin suggest the presence of primary and secondary binding sites on albumin. Weiss et al. (2009) screened
434 30 perfluorinated compounds, differing by C-chain length (C4-C18), fluorination degree, and polar groups for
435 potential protein binding. They concluded that binding affinity is highest for fully fluorinated materials and
436 compounds having at least eight C-atoms.

437 Studies of tissue distributions are available for several species including monkeys, rats, and mice. Butenhoff et
438 al. (2002; 2004b) studied the fate of PFOA in monkeys in a six-month oral exposure study. Briefly, groups of
439 4-6 male monkeys each were administered PFOA daily via oral capsule at dose rates of 0, 3, 10, or 20 mg/kg
440 bw. Serum, urine and faecal samples were collected at two-week intervals and were analyzed for PFOA
441 concentrations. Liver samples were collected at time of sacrifice. Serum concentrations reached steady-state
442 levels within four to six weeks in all dose groups. The mean serum concentration of PFOA in control monkeys
443 was 0.134 µg/mL. The serum steady-state concentrations of PFOA, which varied markedly between animals
444 were 77, 86, and 158 µg/ml after six weeks (Butenhoff et al., 2002) and 81, 99, and 156 µg/ml (Butenhoff et al.,
445 2004b) after six months for the 3, 10, and 20 mg/kg bw, respectively.

446 Urine PFOA concentrations reached steady-state after 4 weeks and were 53, 166, and 181 µg/ml with SD of
447 50% in the 3, 10, and 20 mg/kg dose groups, respectively. Liver PFOA concentrations at terminal sacrifice in
448 the 3 mg/kg and 10 mg/kg dose groups were similar and ranged from 6.29 to 21.9 µg/g. Liver PFOA
449 concentrations in two monkeys exposed to 20 mg/kg were 16.0 and 83.3 µg/g. Liver PFOA concentrations in
450 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
451 (Butenhoff et al., 2004b).

452 Han et al (2012) reviewed the clearance of PFOA with an emphasis on renal clearance. Human clearance is
453 clearly demonstrated to be much longer than in other species (e.g. rodents, monkeys) with half-life values of 2.3
454 – 3.5 years reported (Olsen et al., 2007; Bartell et al., 2010). Total clearance with rats was observed to be
455 greatest for the C6 analogues and then decreased significantly with minimal clearance for PFDA. With the
456 exception of PFDA, total clearance was less in male rats than female rats.

457 Fujii et al (2015) studied the toxicokinetics of six to fourteen carbon chain length PFAAs in both mice and
458 humans. In mice, C6 and C7 PFAAs were eliminated rapidly in the urine, as compared to C8 to C14 which
459 accumulated in the liver and were excreted slowly in faeces. Fujii also showed a large interspecies difference
460 which was related to the sequestration volumes of the liver. Urinary clearance of PFFAs in humans also
461 decreased with increasing alkyl chain lengths, while biliary clearances increased. The C9 to C10 derivatives
462 had the smallest total clearance for both mice and humans.

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

472 *4.5. Metabolic similarity*

473 Results of *in silico* metabolism simulations are presented in Table 5 of Annex I. METEOR reveals the potential
474 for II phase: glucuronidation of carboxylic acid moiety, but overall the compounds are predicted not to be
475 metabolised.

476 *4.6 Toxicophore similarity*

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

481 *4.7. Mechanistic plausibility similarity*

482 As summarised in Table 7 of Annex I, the PFAAs included in the category are associated with a number of
483 molecular mechanisms of toxicity. As previously noted, while the toxicology of perfluorinated chemicals is
484 well-studied, the mechanistic pathways of toxicity of PFAAs are likely multiplicative.

485 PFAAs have been associated with interference with lipid metabolism and bind to fatty acid-binding protein
486 (Luebker et al., 2002; Zang et al., 2013), and also bind to human serum albumin (Chen and Gao, 2009). There
487 is growing evidence that underlines liver PPAR ligand-dependent activation as a key MIE in the elicitation of
488 liver steatosis (Al Sharif et al., 2014). PFAA-induced liver toxicity is considered to be mediated via PPARs,
489 especially PPARα binding (Takacs and Abbott, 2007; Rosen et al., 2008; Wolff et al., 2008; 2012; Bjork and
490 Wallace, 2009).

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

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

501 In addition to PPARs dysregulation, studies of PFAAs' toxicities have reported other potential molecular
502 mechanisms. Specifically, previous studies assessed the binding potency of PFAAs with several proteins,
503 including other nuclear receptors such as the oestrogen receptor (Benninghoff et al., 2011; Gao et al., 2013), as
504 well as transport proteins such as Transthyretin (Ren et al., 2015). Bjork et al. (2011) concluded that multiple
505 nuclear receptors are activated by PFAAs.

506 While there is evidence supporting PFOA-induced liver toxicity and adenomas via a PPARα agonist mode of
507 action in rodents, there is also some evidence that hepatomegaly may be associated with a PPARα independent
508 mode of action (Rosen et al., 2008). It is likely this is a PPARγ-mediated mode of action. While PPARα is

509 more likely related to fatty acids oxidation, PPARγ is the main regulator of adipocyte differentiation,
510 stimulating the expression of lipogenic proteins (i.e., transporters, fatty acid synthesizing enzymes, enzymes
511 related to triglyceride synthesis and lipid droplet associated proteins). Therefore, the liver enlargement
512 observed in PPARα null mouse may be due to the accumulation of lipid droplets or the accumulation of PFOA
513 in the liver and PPARγ and adipose differentiation-related protein (ADRP). Supporting this argument is gene
514 expression profile data for rat liver treated with PFOA, where the largest categories of induced genes are those
515 involved in metabolism and transport of lipids, particularly fatty acids (Rosen et al., 2008).

516 The above findings are supported by the findings of Ding et al. (2009). Ding et al. (2009) studied the dosage-
517 dependent metabonomic and transcriptomic responses of male rats' exposure to the C12 derivative for 110 days.
518 NMR-based metabonomic results for both liver tissues and serum revealed that exposure to PFDoA leads to
519 hepatic lipidosis, which is characterized by a severe elevation in hepatic triglycerides and a decline in serum
520 lipoprotein levels. Moreover, results from transcriptomic changes induced by the C12 derivative confirm these
521 results as changes in gene transcript levels associated with fatty acid homeostasis. It was concluded that
522 PFDoA induces hepatic steatosis via perturbations to fatty acid uptake, lipogenesis, and fatty acid oxidation. .
523 A dose-dependent increase in the expression of both PPARα and PPARγ and also of CD36 (fatty acid
524 translocase), which is a common target of the two receptors is reported (Ding et al., 2009). Its over-expression
525 is one of the most probable key intermediate events outlined in the prosteatotic PPARγ-mediated mode of action
526 recently proposed (Al Sharif et al., 2014).

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

535 It is worth noting, the PXR induces lipogenesis is via activation of CD36, PPARγ, SCD1, and FAE gene
536 expression. The PXR inhibits fatty acid β-oxidation through its suppression of PPARα and thiolase gene
537 expression (López-Velázquez et al., 2012).

538 *In vitro* studies have evaluated the ability of numerous PFAAs to induce mouse and human PPARα activity in a
539 transiently transfected COS-1 cell assay (Wolf et al., 2008). Specifically, COS-1 cells were transfected with
540 either a mouse or human PPARα receptor-luciferase reporter plasmid. After 24 hours, cells were exposed to

541 either negative controls (water or dimethyl sulfoxide); positive control (the PPARα agonist WY-14643); PFOA
542 or PFNA at 0.5-100μM; perfluorobutanoic acid, PFHxA, or PFDA at 5-100μM. Following 24 hours of
543 exposure, luciferase activity from the plasmid was measured. Wolf et al. (2008) concluded in general: 1)
544 PFAAs of increasing C-atom chain length, up to C9, induce increasing activity of the mouse and human
545 PPARα, 2) Carboxyl derivatives are stronger activators of mouse and human PPARα than the corresponding
546 sulfonate derivatives, and 3) The mouse PPARα is more sensitive to PFAAs than the human PPARα.

547 A more recent study by Wolf et al. (2012) further reported additional work on the *in vitro* activity of PFAAs
548 with mouse and human PPARα. They note that PPARα activity exhibits a bell-shaped curve, with PFOA being
549 the strongest activator. Moreover, longer C-atom chain PFAAs (i.e., > C10) are relatively less potent and some
550 do not activate human PPARα.

551 Bjork and Wallace (2009) conducted a structure-activity relationship study of the transcriptional activation of
552 peroxisome proliferation in primary rat liver cell cultures for perfluoronated carboxylic and sulfonic derivatives
553 of varying C-atom chain length. Moreover, they examined whether this activity can be translated to human
554 liver cells in culture. They concluded: 1) PFAAs cause a concentration- and chain length-dependent increase in
555 expression of gene targets related to cell injury and PPARα activation in primary rat hepatocytes, and 2) The
556 sulfonates are less potent than the corresponding carboxylates in stimulating PPARα-related gene expression in
557 rat hepatocytes.

558 Liver fatty acid binding protein (L-FABP) is highly expressed in hepatocytes. Perfluorinated substances,
559 including PFAAs, may bind with FABP and change their toxicokinetics and toxicity profile. Zhang et al.
560 (2013) examined the binding interaction of 17 structurally diverse perfluorinated substances with human L-
561 FABP in an effort to assess their potential to disrupt fatty acid binding. The binding affinity of 12 PFAAs, as
562 determined by fluorescence displacement assay, increased significantly with their carbon number from C4 to
563 C11 and decreased slightly when the C-number was > 11. While perfluorinated sulfonic acids exhibited similar
564 affinity, no binding was detected for perfluorinated alcohols. Molecular docking experiments show that the
565 driving forces for the binding of PFAAs with FABP are predominantly hydrophobic and hydrogen-bonding
566 interactions, and the binding geometry is dependent on both the size and rigidity of the PFAAs.

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

573 enter the circulation. Further consideration of Ding et al. (2009), specifically the increase in the expression of
574 PPARγ and its role in fatty acids and triglyceride synthesis, together with the stimulation of accumulation in
575 lipid droplets, suggest a synergistic action of PPARα and PPARγ in the liver pathology of PFAAs.

576 *4.8. Other endpoint similarity*

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

579 *4.8.1. Fish toxicity*

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

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

597 Benninghoff et al. (2011) studied the structural characteristics of PFAAs which elicit oestrogen- like activity in
598 juvenile rainbow trout and determined, using *in vitro* species comparisons whether these chemicals interact
599 directly with the oestrogen receptor (ER). PFAAs of C8 to C 11 are inducers of the oestrogen-responsive
600 biomarker vitellogenin *in vivo*. These *in vivo* findings were corroborated by *in vitro* mechanistic assays for
601 trout and human ER. All PFAAs tested weakly bound to trout liver ER, with half maximal inhibitory
602 concentration (IC50) values of 15.2-289mM. Additionally, they significantly enhanced human ERα-dependent
603 transcriptional activation at concentrations ranging from 10-1000nM. Overall, these data support the contention
604 that intermediate size PFAAs are weak environmental estrogens.

605 *4.8.2. In vitro toxicity*

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

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

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

625

626 **5. Statement of uncertainty in similarity**

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

637 All analogues or category members are considered, from a toxicokinetic standpoint, to be similar. Regardless
638 of the species of mammals, all four category members are judged to be readily absorbed orally, not metabolized,
639 and with similar distributions and similar elimination mechanisms. However, there are sex-, species and chain
640 length-dependent difference in the rates of key processes. While there is evidence that PFNA and PFDA have
641 lower clearance in both rodents and humans than PFOA this is not considered to be significant to the read-
642 across. Limiting the read-across to rats and narrowing the range of C-atoms for the applicability domain limits
643 increases the similarity of ADME-related features, especially clearance rates.

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

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

652

653 **6. Statement of the conclusions from traditional data**

654 *In vivo* oral repeated-dose exposure to PFAAs, especially PFOA, gives rise to a standard set of symptoms,
655 considering specifically liver toxicity, including an increased liver weight, hepatocyte hypertrophy, hepatic
656 triglyceride accumulation, multifocal coagulation, and liquefaction necrosis. This hepatocellular injury is
657 accompanied by inflammatory cell infiltration. Therefore, *in vivo* hepatic toxicity may be involved in oxidative
658 stress and inflammatory response, as well as alteration in lipid transport and metabolism. While PFAAs vary
659 from C4 to C18, by design, the category is limited to C7 to C10 analogues. This limitation assures that the
660 impact of toxicokinetic and toxicodynamic uncertainties is minimal. While there is evidence that PFAAs
661 activate a multiplicity of nuclear receptors, PPARα and/or PPARγ interactions are the most likely initiating
662 events leading to repeated-dose, liver toxicity.

663 The NOAEL for PFOA of 0.06 mg/kg bw/d (based on hepatocyte necrosis and hepatocellular hypertrophy, and
664 increased liver weight in males and females, respectively (EFSA, 2008) is read across to the other three
665 analogues in the category.

666 The chemical category under consideration, intermediate size PFAA, is an extremely well-studies group of
667 compounds. There is extensive high quality *in vivo* data for repeated-dose toxicity and ample *in vitro* data,

668 especially for the source substance. Endpoint specific factors affecting the prediction include the uncertainty
669 associated with is the true nature of the molecular mechanism of PFAA-induced liver toxicity and how exactly
670 the length of the fluorocarbon-backbone impacts repeated-dose toxic potency. However these uncertainties are
671 considered low to moderate, especially since the lower and higher molecular weight derivatives are not included
672 in the category. No endpoint non-specific factors affecting the predictions have been identified.

673 Since the NOEAL value for the source substance, PFOA is supported by data for the C11 and C12 derivatives, a
674 quantitative read-across is possible. The read across predictions are clearly relevant to priority setting, hazard
675 identification, and classification and labelling. However, their applicability to risk assessment may be contested
676 because of uncertainties in most relative toxic mechanism(s) and trend analysis of toxic potency.

677

678 **7. New methods information**

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

688 Toxicogenomic studies of PFAAs reveal the largest group of induced genes is those involved in transport and
689 metabolism of lipids, particularly fatty acids. The largest groups of suppressed gene are those related to
690 inflammation and immunity. For example, gene chip analysis of PFOA (Guruge et al., 2006) revealed
691 approximately 106 and 38 genes were consistently up-regulated or down-regulated, respectively, in all
692 treatment groups. Preliminary data from targeted gene expression profiling in metabolically-competent
693 HepaRG cells shows activity in several PPAR / CAR/ PXR genes (ACOX1, CYP2B6, CYP2C19, CYP2C8,
694 CYP3A4, CYP3A7, IL6, IL6R, PDK4), showing up-regulation of many of these. The signal is strongest in
695 PFOA, followed by the heptanoic, nanonoic and decanoic derivatives (Judson, personal communication).

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

699 Wang et al. (2014) studied the inhibitory effect of thirteen PFAAs on lysine decarboxylase (LDC) activity *in*
700 *vitro*. The inhibitory effect (i.e., inhibition constants obtained in fluorescence enzyme assays) of PFAAs
701 increased significantly with chain length (C7-C18), whereas the PFAAs of < C7 did not show any effect.
702 Circular dichroism spectroscopy results showed that PFAA binding induced significant protein secondary
703 structural changes. Molecular docking revealed that the inhibitory effect could be rationalized well by the cleft
704 binding mode, as well as the size, substituent group and hydrophobic characteristics of the PFAAs. At non-
705 cytotoxic concentrations, three selected PFAAs inhibited LDC activity in HepG2 cells and subsequently,
706 resulted in the decreased cadaverine level in the exposed cells, suggesting that LDC may be a possible target of
707 PFAAs for their *in vivo* toxic effects.

708 Zhang et al. (2014) also examined the binding interactions between PFAAs and PPARγ. Specifically, the *in*
709 *vitro* binding of eleven PFAAs to human PPARγ ligand binding domain (*h*PPARγ-LBD) and their activity on
710 the receptor in cells were investigated. The results showed that the binding affinity increased with carbon
711 number from C4 to C11 and then decreased slightly. Additionally, it was shown that the *h*PPARγ-LBD binding
712 affinity of perfluorinated sulfonic acids is stronger than their PFAA counterparts, while perfluorinated alcohols
713 show no *h*PPARγ-LBD binding. Circular dichroim spectroscopy showed that PFAA binding induced a
714 distinctive structural change of the receptor. In dual luciferase reporter assays using transiently transfected
715 HepG2 cells, PFAAs acted as *h*PPARγ agonists with potency correlating with *h*PPARγ-LBD binding affinity.

716 Perfluoroalkyl compounds, including PFAAs, have been shown to disrupt thyroid functions through thyroid
717 hormone receptor (TR)-mediated pathways. Specifically, Ren et al. (2015) investigated the binding interactions
718 of 16 structurally diverse perfluorinated compounds with human TR and their activities on TR in cells.
719 Specifically, in fluorescence competitive binding assays, most of the 16 perfluorinated compounds were found
720 to bind to TR, with relative binding potency in the range of 0.0003-0.05 compared to triiodothyronine (T3) (Ren
721 et al.,2015). A structure-binding relationship was observed, where fluorinated alkyl chain length longer than
722 ten and an acid end-group were optimal for TR binding. In thyroid hormone (TH)-responsive cell proliferation
723 assays, PFHxA and perfluorooctadecanoic acid exhibited agonistic activity by promoting cell growth. Within
724 the same study, molecular docking analysis revealed that most of the tested perfluorinated compounds
725 efficiently fit into the T3-binding pocket in TR and formed a hydrogen bond with arginine 228 in a manner
726 similar to T3. The combined *in vitro* and computational data (Ren et al., 2015) strongly suggest that some
727 PFAAs disrupt the normal activity of TR pathways by directly binding to TR.

728 Within the COSMOS project of SEURAT-1, PFAAs were screened with a variety of *in silico* profilers. These
729 results are reported in Table 6 of Annex I. Specifically, the potential for full PPARγ agonism is predicted by a
730 virtual screening procedure, including docking with filtering by four PPARγ pharmacophores (Tsakovska et al.,

731 2014). The pharmacophore models were developed by analysing 118 PPARγ 3D complexes from the Protein
732 Data Bank (PDB), taking into consideration structural elements (e.g., hydrogen bonds, hydrophobic and
733 aromatic) of the ligands essential for their interactions with the receptor. The key protein interaction of the
734 most active agonists include hydrogen binding to 4/5 amino acids in the receptor pocket; the most active
735 agonists interact directly with H12 residues. PPARγ active full agonists share at least four common
736 pharmacophoric features; the most active ones have additional interactions. In addition, more profilers for
737 nuclear receptor binding were run to identify potential binding to the following nuclear receptors; PPAR, AR
738 (androgen receptor), AHR (aryl hydrocarbon receptor), ER (estrogen receptor), GR (glucocorticoid receptor),
739 PR (progesterone receptor), FXR (farnesoid X receptor), LXR (Liver X receptor), PXR (pregnane X receptor),
740 THR (thyroid hormone receptor), VDR (vitamin D receptor) as well as RXR (retinoic acid receptor). Some of
741 these receptors are associated with the development of hepatosteatosis, so chemicals likely to induce hepatic
742 steatosis are highlighted. The evaluation of potential binding to the receptors is based on structural fragments
743 and physico-chemical features that have been identified as essential to bind to these nuclear receptors and
744 induce a response. The profilers have been developed by studying the physico-chemical features of known
745 nuclear receptor binders and elucidating the structural features needed for binding to the ligand binding pocket
746 (using the Protein Data Bank and ChEMBL) (Mellor et al. 2015; Steinmetz et al. 2015); . C8-C10 PFAA are
747 profiled as positive for PPAR with the nuclear receptor binding profilers, with the C11 and C12 derivatives
748 predicted as full agonist binders for PPARγ.

749 Other *in silico* profilers, developed within COSMOS, also were applied. The PFAAs were processed for their
750 potential LXR binding by employing and combining different *in silico* approaches, including ensemble docking,
751 pharmacophore matching, fingerprint-based similarity and a QSAR classification model (Fioravanzo et al.
752 2013). Similarly as for the LXR nuclear receptor profiler, no LXR binding potential was found. Furthermore,
753 the substances were screened with profilers for potential hepatotoxicity, including sixteen structural alerts
754 associated with observed human hepatotoxicity (Hewitt et al. 2013), for phospholipidosis (Przybylak et al.
755 2014) and for protein binding (Enoch et al. 2011). However, no positive responses were reported.

756 The USEPA ToxCast program screened the PFAA molecules with chain length 6-11 in up to 800 separate *in*
757 *vitro* assays. They note an increasing trend in cytotoxicity with C-atom chain length as measured in a set of 37
758 cell-proliferation decrease and cytotoxicity assays. The ranges of AC50 values are given in Table 8 in Annex I.
759 Limited cytotoxicity was seen up to C8, but above C8, many assays while activated were concentration limited.
760 Specifically, activity was seen in several target classes including PPAR, PXR/CAR, FXR, ER (estrogen
761 receptor), AR (androgen receptor), cell stress pathways and a number of enzymes and G-protein-coupled
762 receptors (GPCRs). PFOA, in the middle of the length range, had the most evidence for PPAR activity in
763 concentration ranges not also associated with cytotoxicity. Longer chains activated the PPAR assays at about

764 the same concentration as does PFOA, but they are all cytotoxic in that range. Shorter chain length variants
765 activate PPAR at higher concentration, or not at all in the concentration range tested (up to 100 μM). PXR
766 activity is only seen in the middle length range, with PFOA, again being the only one of these analogues with
767 consistent activity outside of the cytotoxicity range. There is some evidence of FXR activity for PFOA and
768 PFDA. For ER and AR, there are several active assays, with the most evidence for receptor-mediated activity
769 being in PFOA. However, in a more complete analysis of a large number of ER and AR assays, the weight of
770 evidence points towards this activity being non-receptor-mediated, and likely due to some assay-interference
771 process (Judson, personal communication)[3]. There is activity in several cell-stress processes for C7 and above,
772 leading to frank cytotoxicity by C9. GPCR activity is seen increasingly with C-atom length. It is worth noting
773 that the ToxCast program has observed with their screening assays that surfactants can cause false activity,
774 potentially through a protein-denaturation MoA. Table 8 in Annex I summarises the Activity in Cell
775 proliferation depression and cytotoxicity assays from ToxCast and demonstrates that as the chain length
776 increases, the evidence for cytotoxicity increases and the concentration tends to decrease.

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

782 *[3]Judson, R. 2015. Personal communication reference to a manuscript currently in development by the USEPA.*

783

784 **8. Statement of conclusion after considering new methods data**

785 Intermediate size PFAAs are a well-studied group of compounds. There is quality *in vivo* data for repeated-
786 dose toxicity test schemes. Moreover, there are ample new methods data, especially from ToxCast.
787 Specifically these data better clarify the PPAR-linked molecular mechanism of PFAA-induced liver toxicity.

788 The new methods data add weight-of-evidence to the initial read-across prediction and the use of the NOAEL
789 value for PFOA in quantitative read-across to other category members as the read across prediction is relevant
790 to all regulatory decisions including risk assessment. The final conclusion of the data evaluation is that read-
791 across may be conducted from the most studied analogue, PFOA. Specifically, the rat oral 90-day NOAEL for
792 PFOA, 0.06 mg/kg bw/d (based on hepatocyte necrosis and hepatocellular hypertrophy and increased liver
793 weight), may be read across to the untested C9 and C10 analogues with low uncertainty and used to inform
794 regulatory decisions. It is worth noting, this conclusion is supported by the *in vivo* data (i.e., NOAELs of 0.1

795 mg/kg bw/d) for the C11 and C12 derivatives. Furthermore, it is concluded that the oral 90-day NOAEL for
796 PFOA can be red across to the C7 derivative as the most conservative argument.

797

798 **Disclaimer**

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

804

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810

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Annex II: Template for Assessing Uncertainty for Read-Across

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

Similarity Parameter	Data Uncertainty [a] (empirical, modelled) (low, medium, high)	Strength of Evidence [b] (low, medium, high)	Comment
Substance Identification, Structure and Chemical Classifications	low	high	All category members have CAS numbers, similar 2D structure and belong to the same chemical class and subclasses.
Physio-Chem & Molecular Properties	Empirical: low Modeled: low	high	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.
Substituents, Functional Groups, & Extended Structural Fragments	low	high	Substituents, functional groups and extended structural fragments are consistent across all category members.
Transformation/Toxicokinetics and Metabolic Similarity	Empirical: In vivo: low In vitro: none Simulated: low	medium	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.
Potential Metabolic Products	low	high	Based on *in silico* metabolic simulations, no potential metabolic products are predicted to be produced by any of the category members.
Toxicophores /Mechanistic alerts	medium	medium	Based on *in silico* profilers, outside of PPAR-binding, no category member contains any established toxicophores.
Mechanistic plausibility and AOP-Related Events	medium	medium	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.
other relevant, *in vivo*, *in vitro* and *ex vivo* endpoints	low	high	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.

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.

[a] Uncertainty associated with underlying information/data used in the exercise

[b] Consistency within the information/data used to support the similarity rational and prediction

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Table 2. Template for Assessing Uncertainty Associated with Mechanistic Relevance and Completeness of the Read-Across

Factor	Uncertainty (low, medium, high)	Comment
The problem and premise of the read-across	Low	The endpoint to be read across, oral 90-day repeated-dose toxicity, for straight chain perfluoronated 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.
In vivo data read across		
Number of analogues in the source set	Low; 1 of 4 tested	There is only one suitable category member with *in vivo* apical endpoint data usable for read-across.
Quality of the *in vivo* apical endpoint data read across	Low; consistent phenotypic expression of toxicity.	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
Severity of the apical *in vivo* hazard	Low.	Potency data for the *in vivo* apical endpoint is the NOAEL of 0.06 mg/kg bw/d bases 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.
Evidence to the biological argument for RA		
Robustness of analogue data set	Low; numerous endpoints reveal the same structure-activity relationships.	The available data from *in vivo* studies for the category members is extensive with several assays being used and most if not all the analogues tested, especially the source analogue. The tests were judged to be reliable and conducted under the appropriate conditions.
Concordance with regard to the intermediate and apical effects and potency data	Medium; limited by lack of mechanistic plausibility.	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.
Weight of Evidence	Low to medium	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.
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.		

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