Summary of results

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21. Table 2 to Table 24 present summaries of in vitro data for 29 different PFAS, taken from nine published sources.

22. Taken together, the endpoints investigated provide valuable information towards understanding the mechanisms by which PFAS may exert their effects on THs and thyroid function. An indication of the relative potency of individual PFAS in causing the observed effects is also available from *in vitro* studies where multiple PFAS were studied in the same test system (Table 1). Ren et al. (2016) stated that concentrations tested in such *in vitro* studies were generally relevant to the range of serum levels known to arise in the general population.

Competitive binding studies

23. Weiss et al. (2009) and Ren et al. (2016) investigated the displacement of the natural ligand thyroxine (T4) from key functional proteins, as well as TTR (Ren et al., 2016; Weiss et al., 2009) and TBG (Ren et al., 2016). These two authors investigated binding affinities of different PFSAs, including PFBS, PFHxS, PFOS, L-PFDS, and the PFSIA L-PFOSi.

24. Using a fluorescence probe in a fluorescein-thyroxine (F-T4) competitive binding assay, Ren *et al*. (2016) measured the binding affinities of PFBS, PFHxS and PFOS, which all bound to wild type human TTR with potency values (relative to T4) ranging from 0.24 (PFOS) to 0.0002 (PFBS). These potency values were similar to those obtained by Weiss *et al*. (2009) who studied the binding affinities of PFSAs and PFSIAs (i.e. PFBS, PFHxS, PFOS, L-PFDS and L-PFOSi) to TTR using a radioisotope method. No activity was detected for L-PFDS, and binding affinities for the other three PFSAs ranged from 0.065 (PFHxS) to 0.003 (PFBS). In contrast to Ren et al. (2016), where PFOS had the highest relative potency of 0.24, in the study by Weiss *et al*. (2009) PFHxS had the highest relative potency of 0.085, followed by PFOS at 0.065. No competitive binding affinity of the three PFSAs to human TBG was reported by Ren *et al*. (2016) (see Table 1).

25. For the PFCAs, Weiss et al. (2009) and Ren *et al.* (2016) measured binding affinities for the same nine chemicals to TTR (PFBA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTeDA). In addition, Weiss *et al*. (2009) investigated 7H-PFHpA and 6:2FTUA, as well as N-MeFOSA, N-EtFOSA, PFOSA, N,N-Me2FOSE, N-MeFOSA and N-EtFOSE, while Ren *et al*. (2016) also investigated PFTrDA (see Table 1).

26. In both studies, all of the PFCAs displayed competitive binding activity to TTR, with the highest relative potencies (relative to T4), being for PFOA of 0.083 in the study by Ren *et al*. (2016) and 0.064 in the study by Weiss *et al*. (2009).

27. Of the PFAS studied, only two PFCAs (PFTrDA and PFTeDA) showed any affinity for binding to TBG, albeit both at a very low relative potency of 0.0002 (Ren et al., 2016).

28. For the four FOSE, none displayed competitive binding activity to TTR, and for the two FOSA only PFOSA (not N,N-Me2FOSA) displayed competitive binding to TTR.

29. Overall, binding affinities of PFAS to TTR were very much stronger than those of the same PFAS to TBG (Ren *et al*., 2016).

30. Ren et al. (2016) concluded that TTR binding potency was clearly associated with carbon chain length and the charged end group. Highest binding affinities to TTR were seen for PFAS with carbon chain lengths ranging from seven to 12 carbon atoms, and optimally at eight carbon atoms. They concluded this is due to the molecular sizes of long-chain perfluoroalkyl acids being larger than the volume of the T4 binding pocket. Potency was associated with the charged end group in the order of sulfonate > carboxylate > alcoholic hydroxyl.

31. In contrast, PFTrDA and PFTeDA, with an alkyl chain length longer than C12, bound to TBG with weak affinity (Ren *et al.*, 2016). The authors concluded that this is because perfluoroalkyl acids with carbon chains longer than C12 could fill the TBG ligand-binding pocket, whereas the short-chain PFASs could not.

32. These competitive binding studies have shown that PFAS can displace T4 from serum transport proteins (primarily TTR) and so provide a possible explanation of the TT4 decrease seen in in vivo studies in the absence of increases in thyroid-stimulating hormone (TSH) (see paper PFAS/2023/04 – in vivo studies paper). Relative binding potencies compared to the natural ligands have also provided an insight into which PFAS may have the greatest adverse effect upon these processes. PFAS with alkyl chain ranging from seven to 12 carbon atoms and with a sulphonate end group (i.e. the PFSAs) appear to be most potent in this respect when considering binding to the TH transport protein, TTR.

33. The relative potency for competitive binding to TTR, relative to T4, from the studies by Ren et al. (2016) and Weiss et al. (2009) are presented in Table 1 in descending order.

Table's 1 Relative potencies of PFAS, relative to T4, from competitive binding assays with TTR.

PFAS	**Relative potency (Ren et al., 2016)**
PFOS	0.24
PFOA	0.083

PFHxS	0.053
PFHpA	0.028
PFDA	0.019
PFNA	0.016
PFHxA	0.009
PDUnA	0.006
PFTrDA	0.006
PFTeDA	0.005
PFDoA	0.004
PFBS	0.002
PFBA	0.0003
6:2 FTOH	ND
8:2 FTOH	ND
10:2 FTOH	ND

PFAS	**Relative potency (Weiss et al., 2009)**

PFHxS	0.085
PFOS	0.065
PFOA	0.064
PFHpA	0.039
L-PFOSi	0.035
PFNA	0.022
FOSA	0.01
PFHxA	0.007
PFDA	0.007
7H-PFHpA	0.007
6:2 FTUA	0.007
PFUnA	0.003
PFBS	0.003
PFTdA	0.002
PFDoA	0.001
PFBA	ND

L-PFDS	ND
6:2 FTOH	ND
8:2 FTOH	ND
N-MeFOSE	ND
N-EtFOSE	ND
N,N-Me2FOSA	ND
N-MeFOSA	ND
N-EtFOSA	ND

AhR transactivation (TCDD comparison)

34. The effect of seven PFAS (PFOS, PFHxS, PFOA, PFNA, PFDA, PFUnA and PFDoA) on AhR transactivation in the AhR luciferase reporter gene bioassay was investigated by Long *et al*. (2013), using stably transfected mouse Hepa1.12cR cells (AhR-tact bioassay).

35. PFAS were tested alone (agonistic response) or co-treated with TCDD (competitive response). Compared to TCDD, only PFDoA weakly induced the AhR-tact in an agonistic response with a tentative AhR-relative potency of 5×10^{-6}; the other PFAS had no significant effect. In the presence of TCDD, only PFDA and PFDoA further increased the TCDD-induced AhR-tact in an antagonistic response. PFDA and PFDoA have 10 and 12 carbon atoms, respectively, and therefore may have a different toxic effect than the shorter PFOS and PFOA. Long *et al*. (2013) concluded that the two long chain PFAS studied interfered with AhR function and this may be one mechanism by which PFAS affect the endocrine system via interference with nuclear receptor pathways.

36. The results indicate, based on the five long-chain PFCAs and two long-chain PFSAs studied, that only the longer chain PFAS with alkyl chains of 10 to 12 carbon atoms appear to have the ability to interact with AhR and influence gene expression profiles of key factors involved in thyroid function.

Cell proliferation and cell viability studies

37. A number of studies have investigated the potential proliferative effects of PFAS on cell lines that are under the influence of THs, including GH3 cells (T-screen assay), FRTL-5 cells and NHT cells.

38. The T-screen assay employs the GH3 cell line in which cell growth is totally dependent on the active thyroid hormone triiodothyronine (T3), which, on interacting with various TH responsive elements, ultimately leads to gene expression and cell growth. Conflicting results on cell proliferation were found in the studies reviewed.

39. Long *et al*. (2013) studied the effect of seven PFAS (PFOS, PFHxS, PFOA, PFNA, PFDA, PFUnA and PFDoA) on GH3 cell proliferation in the absence (agonistic response) or presence (antagonistic response) of T3.

40. In the absence of T3, all seven PFAS significantly decreased GH3 cell proliferation, with PFOS, PFHxS, PFNA, PFDA and PFUnA decreasing such cell proliferation in a dose-dependent manner. No dose-response was seen with PFOA and PFDoA. Cytotoxicity was seen at the highest dose tested (1×10^5 nM) with PFUnA and PFDoA.

41. In the presence of T3, apart from PFOA where no significant effect on cell proliferation was reported, all six other PFAS significantly decreased T3-induced cell proliferation. Cytotoxicity was seen at the highest dose tested (1×10^5 nM) with PFUnA and PFDoA.

42. Deng *et al*. (2018) showed that a PFOS substitute, F-53B (described as a Chinese PFOS alternative with a similar structure), enhances proliferation of GH3 cells in a concentration-dependent manner, with a higher relative cell proliferation than T3. Cytotoxicity was not assessed. The authors concluded that F-53B may be considered a strong TH agonist.

43. Coperchini *et al*. (2021) evaluated the effect of in vitro exposure to PFOS and PFOA in both a thyroid cell proliferation assay and cell viability (WST-1) assay using FRTL-5 and NHT cells. Cell death was seen following exposure to

PFOS in FRTL-5 and NHT cells, but only in FRTL-5 cells following exposure to PFOA.

44. In this study, FRTL-5 cells cultured in the presence of PFOS up to 2 x10 ^{2} nM displayed a decrease in cell viability from 2 x 10^{1} nM (10 ng/mL) and a decrease in cell proliferation from 2 nM (1 ng/mL). In the presence of PFOA, cells displayed a decrease in cell viability from 2.41 x10^{1} nM (10 ng/mL) and a decrease in cell proliferation from 2.41 nM (1 ng/mL).

45. NHT cells cultured with PFOS showed a decrease in cell viability from 2 x 10^{-2} nM (0.01 ng/mL) and a decrease in cell proliferation from 2 nM (1 ng/mL). PFOA had no effect on cell viability or cell proliferation. 46. In the same study, a new generation PFAS (C6O4) was also tested in FRTL-5 and NHT cells. Treatment with C6O4 did not affect FRTL-5 or NHT cell viability or proliferation, in contrast to both PFOA and PFOS which reduced cell viability and cell proliferation in FRTL-5 cells, but only PFOA reduced cell viability and cell proliferation in NHT cells. The concentrations of PFAS used in these experiments were stated to be based on human serum data previously reported.

47. Evidence from in vitro studies shows that PFAS can decrease cell viability and cell proliferation, although this is not consistently seen across different cell types with the same PFAS.

Iodide accumulation and metabolism

48. Conti et al. (2020) evaluated the acute effects of PFOS and PFOA on iodide transport in FRTL5-YFP cells (a clonal population of FRTL-5 cells with stable expression of YFPH148Q/I152L) and human HEK-293 cells transiently expressing NIS, by monitoring changes in intracellular iodide concentration using live cell imaging. No cytotoxicity was reported up to the highest dose tested of 1 x 10^{5} nM (FRTL-5 cells) for PFOS or PFOA. PFOS, but not PFOA, acutely and reversibly inhibited iodide accumulation in FRTL5-YFP cells and HEK-293 cells. PFOS did not affect iodide efflux from thyroid cells nor the activation of efflux channels/transporters. However, the authors noted that effects occurred at PFOS concentrations that the general population are unlikely to be exposed to.

49. De Toni et al. (2022) investigated the effect of PFOA, PFOS and C6O4 on cell iodide-uptake induced by TSH in FRTL-5 cells. No cytotoxicity was reported up to the highest dose tested of 2 x 10^{2} nM or 2.41 x 10^{2} nM for PFOS and PFOA, respectively. Following exposure to PFOS and in the absence of TSH stimulation, no effect on basal iodide uptake was reported. However, following exposure to PFOA in the absence of TSH, a decrease in basal iodide uptake was seen. For both

PFAS, an increase in iodide update was seen following TSH stimulation.

50. Therefore, in contrast to effects reported by Conti et al. (2020) using FRTL5-YFP cells in the absence of TSH stimulation, PFOA, but not PFOS, was associated with significant impairment of iodide uptake in FRTL-5 cells. It is noted that the dose at which effects were seen with PFOS (Conti et al., 2020) were above the highest dose tested in the study by De Toni et al. (2022), although a lower dose of PFOA in the study by De Toni et al. (2022) did show an effect. In addition, a clonal population of FRTL-5 cells (FRTL5-YFP) was used in the study by Conti et al. (2020), whereas unmodified FRTL-5 cells were used in the study by De Toni et al. (2022). C6O4 was only associated with an increase in iodide update following TSH stimulation.

51. Disruption of iodide homeostasis in thyroid cells may be a one of the potential mechanisms for the anti-thyroid effects of some PFAS (Conti *et al*., 2020).

cAMP production and gene expression

52. Croce et al. (2019) assessed whether PFBS, PFOS, PFBA, C6 PFPA, PFPeA and PFOA interfered with endocrine function by inhibiting the TSH-induced production of cAMP in FRTL-5 cells. No cytotoxicity was reported in FRTL-5 cells for PFBS, PFBA, C6 PFPA, PFPeA and PFOA, up to the highest dose tested of 1 x 10^5 nM, with only PFOS being cytotoxic at high concentrations (from 1 x 10^5 nM), in a dose dependent manner. None of the six PFAS exerted a significant inhibition of TSH-induced cAMP production in FRTL-5 cells up to the highest doses tested (1 x 10^5 nM). The authors stated that the lower concentrations used were comparable to the concentration range found in sera of the general population and exposed workers, but the highest concentrations tested were higher than those ever reported in human sera.

53. De Toni et al. (2022) investigated the impact of PFOS, PFOA and C6O4 on intracellular cAMP levels induced by TSH. No cytotoxicity was reported up to the highest dose tested of 2 x 10^2, 2.41 x 10^2 or 2.9 x 10^2 nM for PFOS, PFOA and C6O4, respectively. TSH-stimulated FRTL-5 cells exposed for 24 hours to C6O4 or PFOS showed a significant decrease of intracellular cAMP levels but only at the highest concentration tested, whereas PFOA was associated with a dose-related decrease of intracellular cAMP levels at all concentrations tested.

54. As subsequent events mediated by cAMP drive the expression of downstream genes such as NIS and TPO, the effect of PFAS exposure on

expression of these genes was also investigated. Exposure to PFOA, in spite of having no effect on TSH-receptor gene expression, was associated with a significant reduction in both NIS and TPO gene expression upon TSH stimulation, but neither C6O4 or PFOS exerted any major alteration of the gene pattern.

55. There is variation in the ability of different PFAS to affect cell function in FRTL-5 cells, as shown with PFOS and PFOA in the studies by De Toni et al. (2022) and Croce *et al.* (2019) above. No effect on cAMP production was reported for PFOS and PFOA in the study by Croce *et al*. (2019), although exposure to both PFAS resulted in a decrease in cAMP levels at lower concentrations in the study by De Toni *et al*. (2022).