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Critical Review

S. Fenton et al.

Human health toxicity of per- and polyfluoroalkyl substances

Per- and Polyfluoroalkyl Substance Toxicity and Human Health Review: Current State of Knowledge and Strategies for Informing Future Research

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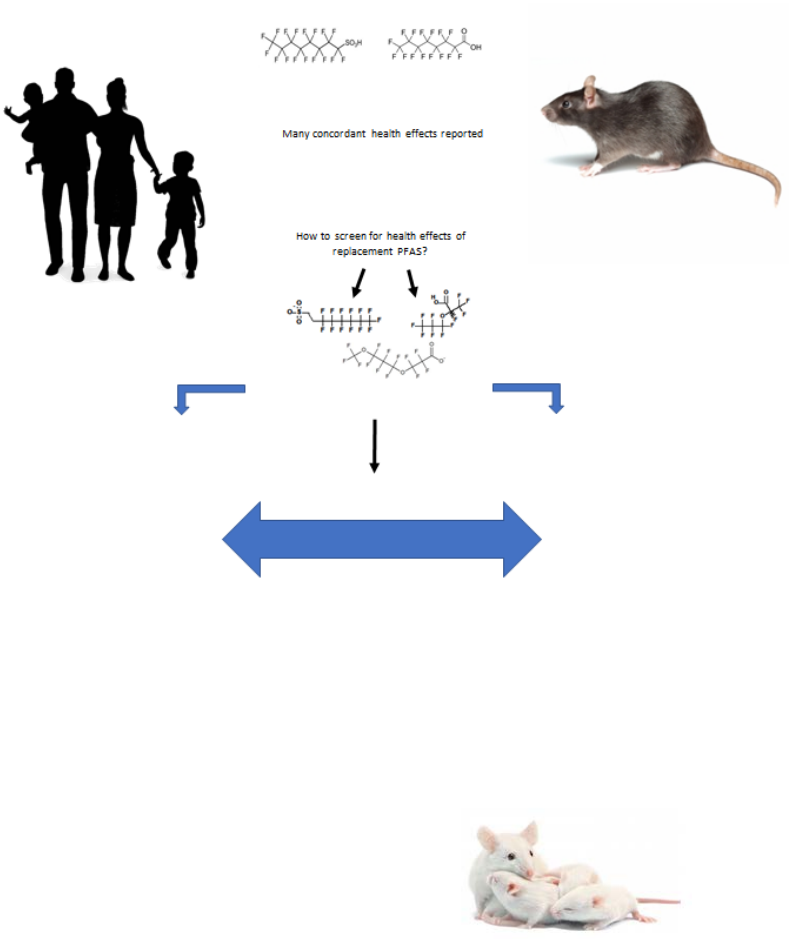
Abstract: Reports of environmental and human health impacts of per- and polyfluoroalkyl substances (PFAS) have greatly increased in the peer-reviewed literature. The goals of this review are to assess the state of the science regarding toxicological effects of PFAS, and to develop strategies for advancing knowledge on the health effects of this large family of chemicals. Currently, much of the toxicity data available for PFAS are for a handful of chemicals, primarily legacy PFAS such as perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). Epidemiological studies have revealed associations between exposure to specific PFAS and a variety of health effects, including altered immune and thyroid function, liver disease, lipid and insulin dysregulation, kidney disease, adverse reproductive and developmental outcomes, and cancer. Concordance with experimental animal data exists for many of these effects. However, information on modes of action and adverse outcome pathways must be expanded, and profound differences in PFAS toxicokinetic properties must be considered in understanding differences in responses between sexes and among species and life stages. With many health effects noted for a relative few example compounds, and hundreds of other PFAS in commerce lacking toxicity data, more contemporary and high throughput approaches such as read across, molecular dynamics, and protein modeling are proposed to accelerate the development of toxicity information on emerging and legacy PFAS, individually and as mixtures. Additionally, an appropriate degree of precaution, given what is already known from the PFAS examples noted here, may be needed to protect human health.

Graphical Abstract

Many health effects have been reported in association with or due to per- and polyfluoroalkyl substances (PFAS) exposures in humans and toxicologic models. Species concordance of effects is evident for a handful of legacy PFAS. With hundreds of PFAS in commerce that lack exposure and health effects data, contemporary and novel methods must be implemented to inform exposed communities, risk assessors and concerned citizens and prioritize those most likely to affect human health.



Keywords: per- and polyfluoroalkyl substances (PFAS); perfluorooctane sulfonate (PFOS); perfluorooctanoic acid (PFOA); persistent compounds, contaminants of emerging concern

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INTRODUCTION

Per- and polyfluoroalkyl substances (PFAS) are ubiquitous in environmental media due to their prolific use in a variety of industrial and consumer products and processes (Jian et al. 2018; Sunderland et al. 2019). Widespread human exposure to PFAS in water, food, and air, coupled with the lengthy environmental persistence and biological half-lives of some PFAS, have led to measurable PFAS in the blood of nearly the entire population in developed countries, with health effects reported globally (Jian et al. 2018; Kato et al. 2011; Khalil et al. 2016; Stubleski et al. 2016). Information needed to evaluate the potential risk of harm from PFAS includes the types of adverse health effects that might occur at environmentally relevant exposures, especially in sensitive life stages. Information is also needed regarding the mode(s) of action for PFAS toxicity, PFAS toxicokinetics (TK) in both humans and laboratory animal models, and dose-response relationships. PFAS risk estimates can be used to inform public health exposure limits that will determine the need for exposure mitigation and environmental cleanup.

There are several challenges in obtaining the information needed to assess human health risk due to the large number of PFAS with a wide range of structures and chemical properties (Buck et al. 2011; Organisation for Economic Co-operation Development 2018; Wang et al. 2017b). Data on the identity, composition, and quantity of PFAS used in products and processes is often treated as confidential business information (CBI) hampering efforts to estimate exposure sources and routes. OECD's chemical inventory

reports over 4,000 substances that contain at least one perfluoroalkyl (–CnF2n–) moiety (Organisation for Economic Co-operation Development 2018), and the U.S. EPA has a curated list of over 8,000 PFAS, included based on structures (U.S. Environmental Protection Agency 2018), on the CompTox Chemicals Dashboard (Williams et al. 2017). The U.S. EPA estimates that more than 600 PFAS are currently in commercial use (U.S. Environmental Protection Agency 2019). Experimental studies of PFAS have been limited by funding and the availability of analytical standards, confounded by the prevalence of background contamination in laboratory materials, and challenged by physicochemical properties such as high surface activity that can interfere with/complicate measurements. Consequently, sufficient information to conduct quantitative risk assessment is currently available for only a relative few PFAS (*Post, 2020*). Further, although typical human exposures involve various combinations of PFAS (Centers for Disease Control and Prevention 2017), only a few efforts address interactions of PFAS mixtures, and a well-founded, scientific basis on which to evaluate their combined toxic potential does not yet exist (Carr et al. 2013; Hoover et al. 2019; U.S. Environmental Protection Agency 2020; Wolf et al. 2014; Zhou et al. 2017).

The Society of Toxicology and Environmental Chemistry (SETAC) held the North America Focused Topic Meeting (FTM) and workshop on *Environmental Risk Assessment of PFAS* on August 12-15, 2019, covering a wide range of topics related to the characterization of health risk posed by PFAS. The overarching purpose of the meeting was to begin a scientific discussion on how best to approach studying, binning, and regulating the large number of PFAS to which people and other species are potentially exposed (for charge questions and other details see Johnson et al., 2020, this

issue). We refer to these PFAS as legacy (those perfluoroalkyl acids for which there is accumulating health data, but may be phased out or decreased in use) and emerging (those which are being used as replacements, often with minimal health effects data). The objectives of the Human Health Toxicity section were to provide an assessment of the state of the science in understanding toxicological effects of PFAS, and to explore and discuss strategies for advancing knowledge on the toxicity of individual and groups of PFAS.

CURRENT KNOWLEDGE OF PFAS TOXICITY IN HUMANS

PFAS, like other chemicals, are potentially capable of producing a wide range of adverse health effects depending upon the circumstances of exposure (magnitude, duration, and route of exposures, etc.) and factors associated with the individuals exposed (e.g., age, sex, ethnicity, health status, and genetic predisposition). Aspects to consider when establishing the health effects of greatest concern are:

1) Effects for which evidence is the strongest. Strength of evidence can come from consistency of effect across studies, strength of effect associations in epidemiological studies, and species concordance, as examples.

2) Effects for which potential impact is greatest. Factors contributing to impact can include severity of effect, functional impairment, persistence, and specific age-groups that are susceptible, as examples.

Brief summaries of candidate PFAS health effects from human and experimental reports are provided in this section (Figure 1).

Immune function

Epidemiological studies have explored relationships between PFAS exposure and laboratory biomarkers of immunomodulation, such as vaccine responses. A doubling of PFOS in maternal serum was associated with a 39% percent ($p<0.001$) reduction in diphtheria antibody concentration in children (age 5), with increased odds of falling below clinically protective values against diphtheria and tetanus at age 7. The authors noted that a "2-fold greater concentration of major PFCs in child serum was associated with a difference of −49% (95% CI, −67% to −23%) in the overall antibody concentration" (Grandjean et al. 2012). Decreased immunological response persisted at age 13 (Grandjean et al. 2017). Adverse associations are also noted for responses to rubella, mumps, and Hemophilus influenza vaccinations in children, and to vaccinations in adults (Abraham et al. 2020; Granum et al. 2013; Looker et al. 2014; Stein et al. 2016). In a single study, modest downregulation of C-reactive protein response, a marker of human systemic inflammation, was also reported to be associated with PFOA blood levels (Genser et al. 2015).

Disease outcomes linked with immunosuppression such as clinician-recorded diagnoses of childhood infections have also been associated with prenatal exposures of PFOS and PFHxS (Goudarzi et al. 2017). A pregnancy cohort study prospectively detected increased risk of airway/throat infections and diarrhea in children through age 10, correlated with cord-blood PFAS measurements (Impinen et al. 2019; Impinen et al. 2018). A recent review concluded that exposure to PFAS in infancy and childhood resulted in an immunosuppressive effect characterized by an increased incidence of atopic dermatitis and lower respiratory tract infections (Kvalem et al. 2020). Some the

immunological effects were sex specific, but the authors cautioned that there were inconsistencies across studies (Kvalem et al. 2020). Overall, available data provide strong evidence that PFAS exposure can suppress human immune response.

Population studies of immune hyperreactive diseases have resulted in mixed findings. Studies on childhood allergy and asthma outcomes have shown no association with PFAS (Impinen et al. 2019; Impinen et al. 2018), while others find substantial effects, including provocative evidence that subgroups of individuals not adequately immunized may be at an increased risk for disease *a priori* (Qin et al. 2017; Timmermann et al. 2017a). For example, a case-control study of Taiwanese children compared the first and fourth quartiles of serum measurements for 11 PFAS with asthma and other immune markers and reported confidence intervals well above 1.0 for PFOA and others (Qin et al. 2017). However, review articles concerning PFAS and childhood allergy and asthma offer nuanced, age- and sex-specific interpretations and advise against firm conclusions (Kvalem et al. 2020).

Chronic autoimmune outcomes, including thyroid disease (see "Thyroid function") and inflammatory bowel disease (IBD), have also been considered. A study in contaminated communities (n=32,254) detected an association between both prevalence and incidence of ulcerative colitis (UC) and PFOA exposure (linear trend p=0.0001; (Steenland et al. 2013)). A worker study (n=3713) found a higher prevalence (p=0.01) and incidence ($p<0.05$) of UC with increasing log PFOA serum concentrations (Steenland et al. 2015). A case-control study of children/young adults from a background exposure community in Atlanta, GA also found higher serum PFOA levels in patients with UC (Steenland et al. 2018b). In contrast to PFOA-related associations in US populations, a

study of a contaminated community in Sweden (n=63,074) did not show a consistent association of IBD with any PFAS exposure (Xu et al. 2020b).

Recent, thorough reviews (DeWitt et al. 2019; National Toxicology Program 2016; Pachkowski et al. 2019) emphasize some key concepts: 1) There is concordance between animal studies and human epidemiological observations that PFAS modifies the human immune response, and 2) There are noted complexities in assuming dose-response continuums, including possible differences in life-stage vulnerability. Authors of these reviews note uncertainty about which outcome will be of most importance but agree that immunotoxicity should be included among sensitive human PFAS toxicity endpoints.

Thyroid function

The C8 Science Panelists concluded a "probable link" of PFOA exposure to thyroid disease, with sex-specific outcomes in women (for hyperthyroid disease) vs. males (hypothyroid disease) (C8 Science Panel 2012). Subsequent reviews drew attention to hypothyroid outcomes in women and children, and to the possibility that populations with *a priori* circulating antithyroid peroxidase antibodies may be at additional risk (Coperchini et al. 2017). A broad childhood disease review noted "some evidence" that PFAS cause childhood hypothyroidism and characterized the number of studies as "limited" for childhood disease conclusions (Rappazzo et al. 2017). A meta-analysis of 12 child and adult studies that excluded populations with higher exposures noted that PFAS exposure is negatively associated with serum total T4 levels, and that "PFAS could induce thyroid dysfunction and disease" (Lee and Choi 2017).

Human thyroid disease is mostly the result of an autoimmune response and is 5-10 times more prevalent in women than men (Tadic et al. 2018). Concerning PFAS and clinically diagnosed outcomes, women in the highest quartile of PFOA exposure (>5.7 ng/mL) reported clinical hypothyroid disease (O.R. 2.2; 95% C.I. 1.4-3.7) over three cycles of NHANES data (1999-2006, n= 3974 adults), with similar findings in men (Melzer et al. 2010). The C8 Science Panel studies (median serum PFOA 26.1 ng/mL) found thyroid disease hazard ratios of 1.00, 1.24, 1.27, 1.36, and 1.37 across cumulative exposure quintiles in women (log-linear trend p = 0.03; (Winquist and Steenland 2014b)), with parallel hypothyroid findings in children aged 1-17 (Lopez-Espinosa et al. 2012). The Ronneby, Sweden, population experienced excess risk of thyroid disease in a discrete time period (1984-2005) among women (Hazard ratio 1.29; 96% C.I. 1.05-1.57) that did not persist over time despite higher cumulative PFAS exposure (Andersson et al. 2019). The authors did not link exposure to hypothyroid outcome, noting a nonmonotonic dose-response relationship (Andersson et al. 2019).

Human population studies augment experimental data that PFAS interact with thyroid hormone binding proteins (Berg et al. 2015; Ren et al. 2016; Zhang et al. 2016a), one of several mechanisms by which PFAS can perturb feedback relationships between free thyroid hormone and the hypothalamic-pituitary-thyroid axis**.** PFAS exposures also interfere with thyroid peroxidase (TPO) enzyme activity in vitro (Song et al. 2012). Several PFAS studies have pursued this putative mechanism, finding that maternal and neonatal thyroid hormone outcomes were more readily detected in those with *a priori* abnormally high circulating anti-TPO antibodies (Webster et al. 2016; Webster et al. 2014). One case-control study investigated congenital hypothyroidism, a rare condition.

Serum concentrations of PFOA (5.40 vs 2.12 ng/mL; p< 0.01), PFNA (1.93 vs 0.63 ng/mL; p< 0.001), PFDA (0.52 vs 0.30 ng/mL; p< 0.005) and PFUnDA (0.98 vs 0.44 ng/mL; p < 0.005) were higher in the diagnosed newborns, and several PFAS, including PFOA and PFHxS, were correlated with thyroid autoantibodies (Kim et al. 2016).

Thyroid disease is not the only concern. Clinicians are concerned about sub-clinically elevated TSH in early pregnancy because it may be associated with several possible adverse maternal-fetal outcomes (Forhead and Fowden 2014). This general concern has prompted numerous PFAS-exposure evaluations of corresponding TSH in maternal serum, cord blood, or newborns. A review of maternal and child biomarkers with PFAS exposure noted that higher TSH has been reported in four second-trimester studies (Ballesteros et al. 2017), but there are also conflicting findings. Studies measuring PFAS in the first trimester have also found associations between PFAS exposure and altered TSH levels in newborns, including non-monotonic patterns of dose response that mirror the marked alterations of thyroid hormone levels during pregnancy (Inoue et al. 2019).

PFAS definitively alter human thyroid hormones, and potentially contribute to thyroid autoimmunity, but do not so far appear to be a cause of thyroid cancer in available studies (Barry et al. 2013; Vieira et al. 2013). Also, thyroid cancer is usually survived, thus morbidity rather than mortality studies are useful.

Liver disease and cancer

Liver is a primary target organ for long-chain PFAS storage, and accompanying experimental evidence of toxicity includes hepatocyte fat infiltration, specific cytochrome

P450 (CYP) pathway induction, apoptosis, hepatocellular adenomas and carcinomas, and disrupted fatty acid trafficking that can be peroxisome proliferator activated receptor alpha (PPARα)-dependent or -independent and present across species (Cui et al. 2009; Filgo et al. 2015; Guillette et al. 2020; Huang et al. 2013; Hui et al. 2017; Li et al. 2017a; Maestri et al. 2006; National Toxicology Program 2020a; Perez et al. 2013; Wan et al. 2012; Xu et al. 2016; Xu et al. 2020a; Yao et al. 2016; Zhang et al. 2019; Zhang et al. 2016b).

Population studies demonstrate significant associations of long-chain PFAS (>6 fluorinated carbons) exposure to higher liver enzymes, such as alanine aminotransferase (ALT) in adults and adolescents (Attanasio 2019; Gallo et al. 2012; Gleason et al. 2015; Nian et al. 2019; Sakr et al. 2007a; Yamaguchi et al. 2013), including in longitudinal studies (Darrow et al. 2016; Sakr et al. 2007b). Following low dose exposures, these associations may be more evident in obese participants (Gallo et al. 2012; Jain and Ducatman 2019e; Lin et al. 2010).

Based on experimental data (Das et al. 2017; Martin et al. 2007; Wan et al. 2012; Wang et al. 2013), non-alcoholic fatty liver disease (NAFLD) has been investigated as a clinical outcome of PFAS exposure mediating consistent population PFAS-altered liver enzyme findings. Studies with NAFLD cytokeratin C18 biomarkers have provided supportive evidence for PFAS inducing steatosis (Bassler et al. 2019). Metabolomic study has been directed at potentially explanatory human glycerophosphocholine and fatty acid profiles (Kingsley et al. 2019; Salihovic et al. 2019; Wahlang et al. 2019). Processes which favor steatosis promote advanced liver disease including liver cancer in humans (Massoud and Charlton 2018; National Toxicology Program 2020a).

Associations of PFAS with advanced human liver disease and liver cancer are technically hard to study for reasons including (and not limited to) lethality, selection of comparison populations, and alterations of excretion mechanics associated with disease states. In a clinic-based study, mostly obese (85%) children aged 7-19 with biopsy-proven NAFLD had more advanced disease associated with PFOS and PFHxS exposure as well as associations with lipid and amino acid pathways linked to NAFLD pathogenesis (Jin et al. 2020). However, an adult study reported that serum PFHxS was inversely associated with hepatic lobular inflammation in morbidly obese bariatric surgery patients (Rantakokko et al. 2015). A study of heavily exposed workers (n=462, geometric mean serum PFOA of 4048 ng/mL) detected significantly increased incident mortality for cirrhosis (RR:3.87, 95% C.I. 1.18-12.7) and liver cancer (RR: 6.69; 95% C.I. 1.71-26.2), compared to a regional population (Girardi and Merler 2019), while no PFAS association to cancer or advanced liver disease was reported in a 3M worker cohort or in the C8 Health study population (Barry et al. 2013; Lundin et al. 2009; Vieira et al. 2013).

Emerging animal toxicology/histology and human population data provide mechanistic clues that PFAS disrupt hepatic metabolism, leading to increased bile acid reuptake and lipid accumulation in liver (Salihovic et al. 2020; Schlezinger et al. 2020). A review of NAFLD and toxicant exposure concluded that PFAS are associated with early steatosis (“fatty liver”), the pre-clinical stage of NAFLD (Armstrong and Guo 2019).

Lipid and Insulin Dysregulation

Cross-sectional and longitudinal investigations indicate that PFAS increase serum total and LDL cholesterol in adults and children (Dong et al. 2019; Eriksen et al. 2013; Fisher et al. 2013; Fitz-Simon et al. 2013; Frisbee et al. 2010; Fu et al. 2014; Geiger et al. 2013; He et al. 2018; Koshy et al. 2017; Li et al. 2020; Lin et al. 2019; Liu et al. 2020a; Nelson et al. 2010; Seo et al. 2018; Skuladottir et al. 2015; Starling et al. 2014; Steenland et al. 2009; Winquist and Steenland 2014a; Zeng et al. 2015), including clinically defined high cholesterol (Lin et al. 2019; Steenland et al. 2009; Winquist and Steenland 2014a). Studies of large populations, featuring wide exposure ranges, demonstrate that serum lipids rapidly increase beginning at background (1-10 ng/mL) serum concentration and then are followed by attenuating ("plateaued") cholesterol measurements as exposures (log-transformed) to long-chain PFAS increase (Frisbee et al. 2010; Li et al. 2020; Steenland et al. 2009). These findings suggest partially saturable mechanisms; thus, cholesterol dose-response at pharmacologic or acutely toxic doses should be viewed with caution; associations can be missed or may be misleading when an environmental range of exposure is absent. At background exposure levels, residual associations may be more detectable in obese participants (Jain and Ducatman 2019d; Timmermann et al. 2014), a finding congruent with experimental PFAS outcomes in rodents fed "western" or high fat diets (Quist et al. 2015; Rebholz et al. 2016; Tan et al. 2013). Human gene expression pathways provide support for an interaction of obesity and PFAS exposures and suggest possible sex differences (Fletcher et al. 2013). A pharmacokinetic model predicts that about half of the PFOS-exposed population would experience a > 20% rise in serum cholesterol (Chou and Lin 2020). Risk-assessment implications for low-PFAS dose increases in cholesterol have been noted (Li et al. 2020; New Jersey Drinking Water

Quality Institute Health Effects Subcommittee 2017) and a review of population and toxicity data concluded that dyslipidemia is the strongest metabolic outcome of PFAS exposure (Sunderland et al. 2019).

Human PFAS lipid findings may be related to experimental findings of induced adipogenesis, impaired bile acid metabolism/synthesis, strongly decreased CYP7A1 enzyme activity, altered fatty acid transport, and intracellular lipid accumulation with steatosis, including in PPAR-α-null or PPAR-α-humanized animals (Behr et al. 2020b; Bijland et al. 2011; Bjork et al. 2011; Das et al. 2017; Filgo et al. 2015; Guruge et al. 2006; Lau et al. 2007; Liu et al. 2020b; Salihovic et al. 2019; Schlezinger et al. 2020; Wang et al. 2014; Zhang et al. 2019). Independent of PFAS exposure, similar alterations in metabolic pathways have been related to disrupted fatty acid beta-oxidation and increased free cholesterol in toxicology studies. (Perla et al. 2017).

Cross-sectional studies of diabetes outcomes can be misleading for reasons discussed in the renal section (see “Kidney disease, uric acid, and kidney cancer”). Emerging longitudinal and diabetes clinical trial data indicate that PFAS may increase human insulin resistance, associated with dysregulated lipogenesis activity (Alderete et al. 2019; Lin et al. 2019). Longitudinal studies of clinically diagnosed diabetes patients have sometimes associated PFAS exposures with diabetes (Sun et al. 2018) or with small changes in glycemic markers (Cardenas et al. 2017); however, diabetes associations to date are not consistent (Cardenas et al. 2017; Donat-Vargas et al. 2019; Karnes et al. 2014). Future studies should consider whether PFAS may instigate autoimmune diabetic outcomes in humans, as shown in experimental studies (Bodin et al. 2016). Experimental data reveal that PFAS activate G protein coupled receptor 40 (GPR40), a free fatty acid

regulated membrane receptor on islet ß cells, stimulating insulin secretion (Qin et al. 2020; Zhang et al. 2020a).

Kidney disease, uric acid, and kidney cancer

Extended human half-lives of long-chain PFAS are attributed to active renal tubular reabsorption. Of concern, legacy PFAS such as PFOA and PFOS are concentrated in renal tissues, and histopathologic, molecular, oxidative stress and epigenetic studies provide evidence of potential nephrotoxicity (Rashid et al. 2020; Sakuma et al. 2019; Stanifer et al. 2018; Wen et al. 2016). Additionally, the strong influence of kidney reabsorption on the extended half-lives of long chain PFAS is consistent with both human protein binding and experimental PFAS excretion data.

Human studies have associated legacy PFAS exposure to diminished glomerular filtration and/or defined chronic kidney disease in adults and children (Blake et al. 2018; Kataria et al. 2015; Shankar et al. 2011; Watkins et al. 2013). However, this outcome may be due to reverse causation (Dhingra et al. 2017; Watkins et al. 2013). Some reviews of the available epidemiologic and toxicologic evidence suggest causative links between PFAS and diminished kidney function and chronic kidney disease (Ferrari et al. 2019; Stanifer et al. 2018); these authors also note several knowledge gaps and uncertainty about which proposed mechanisms of action are most important. A propensity score approach to NHANES data (Jain and Ducatman 2019c; Zhao et al. 2020) and a study with repeated PFAS and health measures over an 18-year period (Blake et al. 2018) recently concluded that PFAS exposure likely causes diminished renal glomerular filtration.

Uric acid, a biomarker of increased risk for renal disease (Obermayr et al. 2008), is also consistently associated with PFAS exposure in adults and children (Geiger et al. 2013; Gleason et al. 2015; Kataria et al. 2015; Qin et al. 2016; Steenland et al. 2010; Zeng et al. 2019), including a visible dose response curve that begins at or near historic background levels in human populations (Steenland et al. 2010; Zeng et al. 2019). Serum PFAS concentrations exhibit an inverted U-shaped pattern related to glomerular filtration, initially exhibiting a modest accumulation as glomerular filtration begins to decrease, and then decreasing in advancing renal disease, likely due to failure of normal strong reabsorption mechanisms in moderate to severe kidney disease (Jain and Ducatman 2019c). This finding is more dramatic across stages of glomerular filtration when there is also albuminuria (Jain and Ducatman 2019b). Studies suggest the association of PFAS to uric acid is not due to reverse causation and is underestimated because the failing kidney excretes long-chain PFAS but retains uric acid. An implication is that population outcomes that occur in the presence of either albuminuria or moderate to severe renal disease such as hypertension (Jain 2020) and uric acid (a biomarker of renal disease) (Jain and Ducatman 2019a; Zeng et al. 2019) can be underestimated in cross-sectional studies; in other words, the link between these health outcomes and PFAS exposure is obscured in these studies due to enhanced PFAS excretion patterns in the presence of either albuminuria or moderate to severe kidney disease. Furthermore, the strong influence of renal reabsorption on the long half-lives of long chain PFAS, is consistent with both human protein binding of PFAS and experimental PFAS excretion rates in high dose rodent studies (Cheng and Ng 2017).

Kidney cancer diagnoses have been increasing since 1975, a finding that is partially independent of improved detection, with 5-year cancer-specific survival of around 80% (Gandaglia et al. 2014). The C8 health studies noted longitudinal (n= 32254) increases of kidney cancer (Hazard Ratio: 1.10; 95% C.I. 0.98-1.24) and kidney cancer mortality (Barry et al. 2013; Steenland and Woskie 2012; Vieira et al. 2013). A review of six published studies found long-chain PFAS exposure associated with kidney cancer or kidney cancer mortality, with risks ranging from 1.07-12.8 (Stanifer et al. 2018). Subsequent preliminary data from the heavily exposed Veneto, Italy, population also suggest a significant increase in kidney cancer mortality with PFAS exposure (Mastrantonio et al. 2018). Evidence is accumulating for PFAS as a cause of chronic disease and kidney cancer. Study designs must consider the peculiar PFAS excretion mechanics involved in and associated with kidney disease.

Reproductive and Developmental Outcomes

PFOA impairs human sperm motility and sperm penetration into viscous media (Sabovic et al. 2020; Yuan et al. 2020) and is longitudinally associated with lower sperm concentration/count and higher adjusted levels of luteinizing and follicle stimulating hormones in young men (Joensen et al. 2009; Song et al. 2018; Vested et al. 2013). PFAS serum concentrations are also cross-sectionally associated with deleterious markers of semen quality (Louis et al. 2015; Pan et al. 2019).

Legacy and emerging PFAS have been found in follicular fluid (Kang et al. 2020). PFAS appear to alter endometrial regulation such as progesterone activity in young women (Di Nisio et al. 2020b), and possibly menstrual cycle length (Lum et al.

2017). PFAS associations to menarche and menopause may be substantially due to reverse causation since menstruation is a route by which women eliminate PFAS (Dhingra et al. 2017), partially explaining why men have higher PFAS levels than women in the same communities. Women on birth control and who do not menstruate or with poor cyclicity due to age, activity level, or disease (PCOS) may have elevated PFAS levels in comparison to menstruating women. PFAS exposure has been associated with endometriosis in the US and in China (Campbell et al. 2016; Louis et al. 2012; Wang et al. 2017a), but the specific PFAS associated with this effect vary among studies.

Time-to-pregnancy (fecundity) studies provide indirect evidence of changes in fertility. Methodologic considerations include maternal and paternal age, parity (which in turn affects serum PFAS), and health status. Among 1,240 women in the Danish National Birth Cohort, PFOS exposure was associated with decreased fecundity (median serum PFOS 35.5ng/mL; (Fei et al. 2009)). Reverse causation may explain this finding, as it is duplicated in parous but not among non-parous women (Bach et al. 2015; Whitworth et al. 2012). Prospective odds of actual infertility in the Maternal-Infant Research on Environmental Chemicals (MIREC) cohort (n=1743) at low dose exposures were associated with PFOA (geometric mean 1.66 ng/mL; OR=1.31; 95% C.I. 1.11-1.53) and PFHxS (OR=1.27; 95% C.I. 1.09-1.48) (Velez et al. 2015). The reported fertility rate improved following water filtration in a PFAS-contaminated community (incidence rate ratio 0.73, 95% C.I. 0.69-0.77 prior to filtration) along with measures of birthweight (Waterfield et al. 2020).

PFAS are reliably moved cross the placenta and enter breast milk (Gyllenhammar et al. 2018; VanNoy et al. 2018); serum PFAS in young children generally exceeds

maternal serum concentrations (Eryasa et al. 2019; Fromme et al. 2010; Papadopoulou et al. 2016). Population studies provide evidence that breast-feeding duration and milk quantity is adversely affected by PFAS exposure (Romano et al. 2016; Rosen et al. 2018; Timmermann et al. 2017b).

A systematic review reported that PFOA exposure was associated with a small decrease in infant birthweight; the meta-analysis estimated a 1 ng/mL increase in PFOA was associated with an ~19 gram reduction (95% C.I. -29.8 to -7.9 g) in birth weight (Lam et al. 2014). The authors noted similarities in experimental studies (Johnson et al. 2014; Koustas et al. 2014) and concluded that there was "sufficient" human and corroborative toxicology evidence of a detrimental effect of PFOA on birthweight (Johnson et al. 2014; Koustas et al. 2014; Lam et al. 2014). However, another meta-subpopulation analysis focused on early pregnancy or the time shortly before conception, detecting only a small and nonsignificant association, which was less subject to bias (Steenland et al. 2018a). Different approaches to the possible confounding role of shifting glomerular filtration rates in pregnancy can affect interpretations; evidence suggests this consideration can, at most, only partially explain associations of PFAS exposure to decreased birthweight (Interstate Technology and Regulatory Council 2020; Wikstrom et al. 2020). A recent review of mostly prospective cohort studies (n=24 studies) noted PFAS associated with altered fetal/postnatal growth measures, such as lower birthweight. Many (22) of the relevant studies suggest developmental and childhood immunomodulatory effects, while 21 studies concerning neurodevelopment were inconclusive (Liew et al. 2018). The authors of the review noted methodologic challenges of developmental and newborn epidemiology, including consideration of critical

exposure windows for developmental effects, the effects of breast-feeding and parity on maternal PFAS levels, and the variety of possible mechanistic explanations for growth outcomes, such as disruption of glucocorticoid and thyroid hormone metabolism *in utero* (Liew et al. 2018). Recent Faroe Island studies report that prenatal PFAS effects on thyroid hormone status do not support a causal relationship (Xiao et al. 2020).

Review articles suggest that prenatal exposure to PFOA may increase risk of subsequent childhood adiposity, noting that steroid hormones, retinoid X receptor (RXR), and other pathways may be contributing to this effect (Hall and Greco 2019; Halldorsson et al. 2012). Prospective evidence supports this relationship in adults with a high risk of diabetes (Cardenas et al. 2017). However, some well performed community studies do not support this outcome in adults or children (Barry et al. 2014; Martinsson et al. 2020).

Based on several preliminary findings, supported by longitudinal follow up studies (Avanasi et al. 2016a; Avanasi et al. 2016b; Darrow et al. 2013; Savitz et al. 2012; Stein et al. 2009), the C8 Science Panel concluded that PFOA is probably linked to pregnancy-induced hypertension or pre-eclampsia. Population-level evidence implicating additional PFAS having this effect has included studies with longitudinal designs(Borghese et al. 2020; Huang et al. 2019; Wikstrom et al. 2019). Experimental support includes PFAS effects on human trophoblast migration in vitro (Szilagyi et al. 2020) and recent evidence of PFOA and GenX (or hexafluoropropylene oxide dimer acid, HFPO–DA) effects on mouse placenta, as well as excessive gestational weight gain (Blake et al. 2020). However, a recent longitudinal study did not find an association of PFAS with pregnancy-associated hypertension (Huo et al. 2020).

The possibility that circulating PFAS may reduce bone mineral density has been investigated. Cross sectional and practical trial associations have been found in adults (Di Nisio et al. 2020a; Hu et al. 2019; Lin et al. 2014), and there is emerging longitudinal evidence from a mother-child pair study indicating that children may also be affected (Cluett et al. 2019).

Testicular cancer diagnoses are increasing steadily, a trend unrelated to improved detection (Cheng et al. 2018; Park et al. 2018). Most patients diagnosed (>90%) will be cured and die of other causes; mortality studies therefore provide little help understanding disease risk factors. The C8 Science Panel detected longitudinal evidence for increased testicular cancer risk (1.35; 95% C.I. 1.00-1.79) for cumulative PFOA exposure (Barry et al. 2013). There is ample supportive data of testicular damage following PFAS exposure, including strong evidence of endocrine disruption, but the cell-specific associations are different in humans (germ cell) than the outcomes in rodents (stromal).

PFAS have deleterious effects on conception, pregnancy, and infant development. The underlying birthweight data are mostly supportive, while subsequent growth, and adiposity literature is mixed. The most sensitive reproductive/developmental outcomes are a topic of ongoing discussion.

Outcomes replicated across populations such as perfluorocarboxylate (PFCA) and perfluorosulfonate (PFSA) exposures associate with down-regulation of immune response; increases in cholesterol, liver enzymes, and uric acid; alterations in thyroid hormone binding proteins; growth deficits; and effects on breast milk and lactation indicate priority areas for understanding mechanisms and health implications.

CURRENT KNOWLEDGE OF PFAS TOXICITY IN EXPERIMENTAL MODELS

Animal studies have focused most intensely on PFOA and PFOS, using laboratory rodents and, more recently, zebrafish as models. Perfluoroalkyl acids (PFAAs) of varied carbon-chain lengths as well as a few replacement chemicals with ether linkages in the carbon backbone (such as GenX and ADONA) have also been examined, with outcome profiles thus far generally consistent with legacy chemicals. The varying extent of responses is likely related to toxicokinetic disposition (excretion or half-life) and relative potency/affinity of the individual chemical for binding to receptor proteins. Some PFAS (i.e., PFHxS, PFOA, and PFNA) have longer half-lives in mice than rats, and typically much longer half-lives in humans (Table 1). These differences in elimination kinetics complicate the cross-species evaluation of toxicity. Additionally, some PFAS (such as PFOA and PFNA), exhibit a profound sex difference in the rate of chemical elimination and bioaccumulation in the rat: females eliminate them much faster than males (Table 1). Sex differences in half-lives, although important, are much smaller in humans and have a different explanation. The mouse also typically has more limited sex-based PFAS elimination differences, making this species more amenable for extrapolation to humans, especially for mechanistic and toxicity evaluations.

In general, human health effects associated with PFOA and PFOS exposure (described in the section “Current Knowledge of PFAS Toxicity in Humans”) have also been reported in animal models: hepatic/lipid metabolic toxicity, developmental toxicity, immune suppression, tumor induction, endocrine disruption, and obesity. These findings are often derived from well-controlled laboratory experiments in more than one species using wide

dose ranges that are often orders of magnitude higher than typical human exposure to account for differences in half-life across species. Some of the phenotypic findings are supported by in vitro mechanistic investigation and/or molecular queries on target tissues. Our understanding of the toxicologic properties of PFAS other than PFOA and PFOS is notably less advanced and, in the case of emerging manufactured replacements and byproducts, completely unexplored.

Hepatic and metabolic toxicity

In rodent studies, dose-dependent increases in liver weight, hepatocellular hypertrophy associated with vacuole formation, and with/without increased peroxisome proliferation have been observed with a significant body burden of PFAS, especially for the most persistent and potent long-chain homologues. Hepatocyte proliferation and necrosis/apoptosis are outcomes occurring at relatively low doses. This is also true for a new replacement chemical, GenX, which altered liver histopathology and function, and increased apoptosis in mice and fish (Blake et al. 2020; Guillette et al. 2020). Correspondingly, transcriptional activation of mouse and, to a lesser extent, human PPARα-related genes in liver were detected in adult-exposed models; activation of other nuclear receptors such as PPARγ, constitutive androstane receptor (CAR), and pregnane X-receptor (PXR) has also been reported. These nuclear receptors, metabolic sensors that regulate lipid and glucose metabolism and transport, and inflammation, tend to be more responsive in tissues of rodents than in humans (Rosen et al. 2017; Wolf et al. 2012). Recent work using developmental models report that mitochondrial dysfunction is associated with hepatocellular hypertrophy in young adult mice, and other fatty acid metabolism pathways are activated (Jones et al. 2003; Shabalina et al. 2016). Steatosis is

also a common feature of PFAS chronic exposure in rodents. PFAS exposure in rodent models typically decreases serum cholesterol, whereas elevations of circulating cholesterol levels have been reported in humans. The mode of action concerning serum cholesterol is debatable. For example, PFOA exposure increased liver weight, increased liver enzymes, and led to persistent histopathological changes (particularly damage to the bile duct) in livers of wild type and PPARα-null rodent strains [reviewed in (Division of Science and Research New Jersey Department of Environmental Protection 2019)]. Many of these effects are reversible upon cessation of PFAS exposure and this observation has been interpreted by some as "adaptive" responses to exposure. However, this reversibility is irrelevant to ongoing environmental PFAS exposure (for instance, from drinking water), because exposure will persist until contamination is remediated. In summary, there is strong confluence of animal toxicology/histology and human population data that PFAS disrupt hepatic metabolism and lead to lipid accumulation in liver, although mechanism(s) are unclear. Effects on bile acid metabolism, mitochondrial perturbation and cholestatic mechanisms deserve further investigation at human relevant exposures.

Reproductive and developmental toxicity

Only a few reproductive toxicity studies of males and females are available, primarily focusing on long-chain PFAS. Profound developmental toxicity has been described following gestational and lactational exposure to PFOS, PFOA and PFNA in mice (Das et al. 2015; Lau et al. 2006; Thibodeaux et al. 2003) and in mice and rats gestationally exposed to GenX (Blake et al. 2020; Conley et al. 2019). Neonatal morbidity and mortality were seen with exposure to high doses of legacy PFAS; growth deficits and developmental delays were noted in offspring exposed to lower doses.

Evidence of lactation impairment was seen in mice at doses of 5 mg PFOA/kg body weight (White et al. 2007), leading to increased offspring mortality (Lau et al. 2006); recent studies have implicated a role of placental dysfunction in these adverse developmental outcomes (Blake et al. 2020). Deficits of mammary gland development were also observed in mice exposed to PFOA (doses of 1 mg PFOA/kg body weight and lower) during gestation, which persisted into adulthood, although these exposure levels did not alter body weight, lactational function or neonatal growth of offspring (F1 or F2 mice) (Macon et al. 2011; Tucker et al. 2015; White et al. 2011b). Systematic reviews support a relationship between *in utero* exposure to PFOA and PFOS and fetal growth in animals and humans, and the relationship between PFOA and reduced fetal growth in mice was recently validated (Blake et al. 2020; Koustas et al. 2014). PFAS are also reported to have reproductive effects such as ovulation failure in mice (Zhang et al. 2020b).

Immunotoxicity

A few long-chain PFAS (PFOS, PFOA, PFNA and PFDA) have been shown to alter immune status in rodents and non-human primates. Effects are predominantly immunosuppressive and include reductions in thymus and spleen weights and associated immune cell populations, in numbers of circulating immune cells, in certain aspects of innate immunity (i.e., natural killer cell cytotoxicity), in infectious disease resistance, and in antibodies produced in response to an antigen (i.e., an analog to the vaccine response in humans). In their 2018 draft Toxicological Profile for Perfluoroalkyls, the US Agency for Toxic Substances and Disease Registry (ATSDR), noted changes to these aforementioned immune parameters observed in experimental rodents exposed to PFOA,

PFOS, PFNA, PFHxS, PFDeA, PFBS, or PFBA (Agency for Toxic Substances and Disease Registry 2018). The U.S. National Toxicology Program (NTP) conducted a systematic review of the immunotoxicological literature for PFOA and PFOS and concluded that PFOA and PFOS were presumed to be immune hazards to humans based on a high level of evidence for suppression of antibody responses in experimental animals and a moderate level of evidence for suppression of antibody responses in humans (National Toxicology Program 2016). The ATSDR (Agency for Toxic Substances and Disease Registry 2018) also included a decreased antibody response to vaccines (PFOA, PFOS, PFHxS, and PFDeA) and increased risk of asthma diagnosis (PFOA) among the list of adverse health effects in PFAS-exposed humans. Reduction in the antibody response to a vaccine, an adaptive immune function, is a well-accepted measure of immunotoxicity, is consistent with the mode of action (MOA) for the effects of fatty acids on immune system function (Fritsche 2006), and is compelling evidence that the immune system is a sensitive target of PFAS.

Tumor induction

PFAS are not known to be directly mutagenic; PFOA, PFOS, and other tested PFAS, show little or no evidence for induction of gene mutation, clastogenicity or aneuploidy in vitro or in vivo by a direct mode of action (see (EFSA Panel on Contaminants in the Food Chain 2020), for details). There is evidence that PFAS can induce DNA damage, such as strand breaks, and other genotoxic effects, secondary to oxidative stress ((EFSA Panel on Contaminants in the Food Chain 2020)). This occurs at concentrations/doses that are high relative to human environmental exposures to PFAS

and the mechanism is such that their dose-response will be sub-linear. Hence, PFAS are unlikely to be of mutagenic concern in exposed populations.

PFOA and PFOS have been shown to induce tumors in adult-exposed rodents and fish. Liver adenomas, pancreatic acinar cell tumors and testicular Leydig cell adenomas have been detected in rats treated chronically with PFOA (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2017a), as well as its replacement, GenX (Caverly Rae et al. 2015). Following gestational and chronic exposure to PFOA, 58% of male rats demonstrated pancreatic tumors at the lowest dose administered (National Toxicology Program 2020b). This finding has spurred MN and CA policy makers to consider cancer as an endpoint in risk assessment, whereas the European Food Safety Authority (EFSA Panel on Contaminants in the Food Chain 2020) has the opinion that there is not adequate evidence for a link between exposure to PFAS and cancer risk in humans. This "tumor triad" profile has been associated with the PPARα-mediated molecular signaling pathway in rats exposed to high doses of PFAS. Consequently, liver tumors involving this MOA are not considered relevant to humans at equivalent PFAS exposures (Post et al. 2017). The human relevance of PPARα-mediated pancreatic tumors in rodents remains to be determined. Liver lesions evident in PPARα-null mice exposed to PFOA during pregnancy and lactation (Filgo et al. 2015), suggest a non-PPARα mediated liver response. Induction of liver tumors mediated by estrogen receptor activation has also been reported in fish (Tilton et al. 2008) and several non-PPARα mediated hypotheses, including increased reactive oxygen species (ROS) formation, oxidative stress, and mitochondrial dysfunction, decreased tumor cell surveillance by the immune system, and diminished gap junction cellular communication are documented

(IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2017b; New Jersey Drinking Water Quality Institute Health Effects Subcommittee 2017).

Endocrine disruption

The primary evidence of endocrine disrupting potential of PFAS involves induction of hypothyroxinemia and reduction of serum testosterone in rats. An early review of PFAS endocrine disrupting properties in humans concluded “thyroid may be one axis significantly affected by PFOA exposure while the animal toxicology literature is less certain due to technical issues.” (White et al. 2011a).

PFAS effects on thyroid hormone status detected in animal studies differs from classical hypothyroidism, in that reduction of circulating total thyroxine (T4) is not accompanied by a compensatory increase of thyroid-stimulating hormone (TSH). A possible mechanism for these effects may be related to the propensity of protein binding of legacy PFAS, which could lead to displaced T4 binding to its carrier proteins (transthyretin and thyroxine-binding globulin). Human population studies augment animal data that show PFAS interacts with thyroid hormone binding proteins (Berg et al. 2015; Ren et al. 2016; Zhang et al. 2016a), one of several mechanisms by which PFAS can perturb feedback relationships between free thyroid hormone available to cells (free T4) and the hypothalamic-pituitary axis. Some estrogenic effects of PFAS have also been illustrated by in vitro studies, although there is no evidence of direct transactivation of estrogen, androgen, or glucocorticoid receptors (Behr et al. 2018; Behr et al. 2020a)

The evidence for PFAS affecting estrogen receptors in humans and animals is mixed. While studies have identified some PFAS as being without estrogenic activity

(Behr et al. 2018; Borghoff et al. 2018; Gogola et al. 2019), others suggest an ability of PFAS to modulate or even activate estrogen receptor-mediated effects (Benninghoff et al. 2010; Bjerregaard-Olesen et al. 2019; Kjeldsen and Bonefeld-Jørgensen 2013; Qiu et al. 2020; Wang et al. 2018), with some effects only observed in aquatic organisms (Chen et al. 2016; Chen et al. 2018; Wei et al. 2009). Microarray analyses of human primary hepatocytes confirmed that PFOA activated the ER pathway (Buhrke et al. 2015).

Neurotoxicity

Potential adverse effects of PFAS on the nervous system and functions have not been widely investigated. A few studies reported neurotoxicity of PFOS, PFHxS and PFOA in cell culture systems (Slotkin et al. 2008), as well as altered behavioral responses (Goulding et al. 2017) and deficits in learning and memory ability in rodents (Viberg et al. 2013). In contrast, no significant developmental neurotoxic effects were seen from prenatal exposure to PFOS in U.S. EPA guideline-based studies with rats (Butenhoff et al. 2009).

Obesity

Numerous cell-based assays in human and mouse pre-adipocytes and animal studies with and without high fat diets have consistently shown that some PFAS have the potential to increase lipid production by adipocytes/fat pads (van Esterik et al. 2016). Exposure of pregnant mice to low doses of PFOA produced obesity in young adult female offspring (Hines et al. 2009; van Esterik et al. 2016), a finding that was recapitulated in Danish women compared by i*n utero* PFOA exposures (Halldorsson et al. 2012). PFOA and GenX also increased weight gain of pregnant mice (Blake et al. 2020),

an effect also seen in women during pregnancy (Ashley-Martin et al. 2016), although discordant results have been reported in other studies (Barry et al. 2014; Ngo et al. 2014). These apparently disparate findings in experimental models may be associated with differences among mouse strains examined, exposure periods, statistical methodology, and/or the rodent diets used.

There are specific differences in human and rodent health outcomes that deserve further investigation – 1) cholesterol metabolism, 2) thyroid effects, 3) mode of action (MOA) for liver effects (different or same), and 4) kidney transporter or other MOA leading to large differences in half-life. However, species concordance in the six human health effects sections discussed herein, support a weight of evidence for the handful of extensively studied PFAS.

Human health advisory and guidance values for a few PFAS have been issued to date by U.S. EPA, ATSDR, several individual state environmental agencies or health departments, as well as regulatory agencies in Canada and Europe that are largely (but not exclusively) based on toxicological findings in animal models. However, risk assessment scientists have not reached consensus in selecting a singular apical endpoint as a point of departure for assessments. Three toxicological features of PFAS that have been commonly highlighted, based on their sensitivity (low dose effect), strength of evidence (robust corroborating studies with mechanistic support for human relevance), and corresponding findings noted in epidemiological investigation, include hepatotoxicity (and alterations in lipid metabolism), developmental toxicity and immunotoxicity. It should be noted that apical endpoints that drive risk assessments often differ among

individual PFAS, perhaps highlighting the complexity of these chemicals and the family of PFAS, in general.

IMPORTANCE OF TK IN UNDERSTANDING PFAS TOXICITY

Species and sex differences

Few of the substantial number of structurally diverse PFAS have been tested for toxicological effects. Some available toxicological information has come from studies in animals, where marked species and (in rat) sex differences in half-life for some PFAS (Table 1) have been observed and relevance to humans is uncertain. These differences are due to TK and toxicodynamic factors. There are also differences in mean PFAS serum levels between men and women in the same communities. Children may have elevated serum levels compared to parents, even with the same exposures (Daly et al. 2018; Emmett et al. 2006; Graber et al. 2019), for reasons relating to transplacental transfer, breast feeding, and body mass (Blake et al. 2020; Daly et al. 2018; Emmett et al. 2006; Graber et al. 2019). Transplacental transfer of PFAS confers a substantial burden to the newborn infant. Because the infant has a smaller overall mass and blood volume, PFAS are concentrated, increasing PFAS/volume (Koponen et al. 2018). Additionally, transfer of PFAS is common through lactation, and the longer a child breast-feeds, the higher the burden (Gyllenhammar et al. 2018; VanNoy et al. 2018).

Effects of co-morbidity on PFAS TK

Factors affecting renal function can influence PFAS TK. As previously discussed herein, opposing types of causation should be considered. Human TK appear to vary

bidirectionally with changing renal function, leading to non-monotonic dose-response relationships and, depending on the study goal, possibly to errors in estimating disease associations. As progress is made in the field of PFAS TK, new chemistries may have different clearance factors and nuances that vary by PFAS group or structures, and that will need to be investigated to accurately model half-lives in different exposure subgroups.

Sources of information on TK in humans; strengths and limitations of studies

Some PFAS half-life data in humans were obtained from retired industry workers, particularly those who worked with PFOS, PFOA and PFHxS (Olsen et al. 2007). Since then, these estimates have been modified slightly or confirmed with longitudinal data and modeling from contaminated communities once uncontaminated water options were provided (Bartell et al. 2010; Li et al. 2018). Other contemporary PFAS estimates are derived from biomonitoring studies of production workers, blood donors, study participants, and/or occupationally exposed cohorts (Olsen et al. 2009; Olsen et al. 2017; Russell et al. 2013; Zhang et al. 2013). Some caution must be taken in using these data as variables affecting PFAS clearance may not be taken into consideration (age, sex, menstruation, disease, and medication status), and may contribute to confounding.

The challenge in determining a reliable human half-life in these types of studies is that exposure does not end with a clean water source or retirement/change of job and continued exposures vary over potential depuration periods. Model components may also vary in subclasses. Children (small blood volumes and large fraction of exposures comes from drinking), pregnant women (large increase in blood volume and water intake),

parous women (transfer to fetus and breast milk), and athletes (water intake elevated) are examples of subpopulations with expected variation in half-life compared to adult men (Post et al. 2017). There will be more human estimates of PFAS forthcoming that involve variations in half-life (Post et al 2017). Realistic computational modeling can help, so long as it clearly characterizes exposures and applicable populations. The continued goal should be to provide predictive values for those PFAS lacking actual measurements, based on chemical structures and trusted physiological parameters.

PBPK/TK modeling in different aged populations

PFAS TK are influenced by their interactions with proteins (Andersen et al. 2006; Katakura et al. 2007; Nakagawa et al. 2008; Weaver et al. 2009) in the blood and other tissues (Figure 2). Certain TK features are saturable, and thus dosing in TK studies is of profound importance. Studies of renal reabsorption mechanisms in mammals show that reduced activity of transporters such as Oatp1a1, through inactivation (e.g., genetic manipulation, castration, treatment with estrogen) or by saturation at increasing doses, leads to substantial reductions in half-lives of PFOA and PFOS (Andersen et al. 2006; Nakagawa et al. 2008; Weaver et al. 2009; Yang et al. 2009).

These protein-associated TK processes were recently incorporated into a model for PFOA in the male Sprague-Dawley rat (Cheng and Ng 2017), which provides a useful platform to explore how changes in protein interactions might affect estimates of PFAS half-life (Figure 3). At high doses, it is typical to see clear bi-phasic behavior with rapid initial clearance, during which the serum half-life appears to be shorter, followed by a much longer tail once processes are saturated (e.g., renal reabsorption; Figure 3A). In a

similar fashion, the magnitude of internal dose and rate of serum clearance can be profoundly influenced by proteins known to bind PFAS, such as serum albumin (Figure 3B). Increasing and decreasing the extent of reabsorption in the kidney increases and decreases the serum half-life, respectively (Figure 3C). Finally, the effect of saturating reabsorption is magnified when the half-life is longer due to increased serum binding (Figure 3D). In this, case taking an initial slope to calculate the serum half-life at high doses would lead to a profound underestimation.

Differences in protein expression, circulating levels, and even protein type across populations, sex, and species could lead to important species and sex differences in PFAS biological half-lives (Han et al. 2012); such differences should be investigated and taken into account in the extrapolation to human equivalent doses. Because expression of proteins may change at different life stages, clearance factors and TK may also change.

Given the large number of species-, sex-, and age-specific differences that have been observed, coupled with the lack of data for many PFAS, the parameterization of complex physiologically-based TK models remains a persistent challenge. Therefore, lower-resolution models (e.g., 1-compartment or few-compartment models) may be more appropriate for species and settings where insufficient data are available for reasonably accurate parameterization. Alternatively, in silico and in vitro methods are under development that could aid in parameterization in the absence of in vivo data, as discussed in the section “New approaches for developing PFAS toxicity information.”.

SO MANY PFAS, SO LITTLE TIME: ACCELERATING THE PACE OF DISCOVERY

Importance of determining mode-of-action and AOP

Information on MOAs and/or adverse outcome pathways (AOPs) is invaluable in 1) establishing human relevance of experimental evidence, 2) assessing causality in epidemiological studies, 3) applying "read across" to PFAS for which there is little toxicological information, 4) assessing risks from mixtures, 5) guiding development and interpretation of new approach methodologies (NAMs), 6) informing the development of biomarkers in epidemiologic investigation, and 7) identifying potentially vulnerable sub-populations and life-stage specific effects (LaLone et al. 2017; Meek et al. 2014). Verified MOAs and AOPs can inform risk assessment based on intermediate effects and enable development of NAMs-based approaches to assess PFAS safety (Meek et al. 2014).

Postulated MOAs/AOPs for PFAS

Mechanistic studies have been performed on only a few PFAS. These have been shown to activate a range of putative molecular initiating targets, among which are the nuclear receptors PPARα, PPARγ, PPARβ/δ, CAR, PXR, LXRα and ERα (Bijland et al. 2011; Bjork et al. 2011; Li et al. 2019; Rosen et al. 2017). However, MOAs verified by agreed procedures (World Health Organization 2020) have been established for few reported effects of PFAS and those that have been interrogated involve activation of PPARα, and at higher doses CAR, as molecular initiating events (MIEs) (Klaunig et al. 2012; Rosen et al. 2017). Several AOPs involving these molecular targets are in various stages of development (Organisation for Economic Co-operation Development 2020) but few have yet to be endorsed by the OECD following their agreed procedures

(Organisation for Economic Co-operation Development 2017). Demonstration of receptor activation alone is insufficient to establish involvement of a MOA or AOP in an observed effect, for which an overall weight of evidence approach is necessary (World Health Organization 2020).

(Andersen et al. 2007) provides a useful, albeit dated, review of possible PFAS MOAs. Established MOAs are restricted largely to liver and include species-specific hepatic hyperplasia and liver tumors (Butenhoff et al. 2012; Corton et al. 2018; Elcombe et al. 2012). Available studies on PFBS, PFHxS, PFHxA, PFNA and PFDA suggest that they share molecular targets with similar consequences, albeit with differences in potency, in part due to differences in their excretion and protein-interaction kinetics (Zeilmaker et al. 2018). However, studies in vitro have established intrinsic differences in potency among PFAS analogues. Potency in activating PPARα showed some relationship with PFAS chain length (Wolf et al. 2008). A MOA or AOP provides a causal chain of key events between chemical exposure and outcome. The established MOAs for PFOS and PFOA provide a causal explanation for development of liver tumors observed in rodents on exposure to these compounds, through activation of PPARα, and the possible relevance to humans. However, this does not mean that other effects of PFAS are due to activation of PPARα, or that other pathways might not lead to liver tumors in humans, such as secondary to the primary effect of steatosis.

Until recently, there has been little study of MOAs/AOPs for effects of PFAS other than hepatic outcomes in rodents, particularly for critical effects, such as immunosuppression and developmental toxicity, and from PFAS other than PFOS and PFOA (Temkin et al. 2020); EFSA, 2020). The ability of various PFAS to interact with

and modify lipid metabolism is, however, an intriguing hypothesis (Andersen et al. 2007; Jones et al. 2003; Pouwer et al. 2019; Tan et al. 2013; Xu et al. 1999). Other putative molecular initiating/key events for PFAS, in addition to nuclear receptor activation, include gap junctional inhibition to disrupt cell-cell communication, mitochondrial dysfunction, interference of protein binding, partitioning into lipid bilayers, oxidative stress, altered calcium homeostasis, and inappropriate activation of molecular signals controlling cell functions. Many of these effects are consistent with a non-specific action of PFAS on the cellular lipid membrane (Bourre et al. 1989; Casares et al. 2019; Dodes Traian et al. 2012; Spector and Yorek 1985). However, these alternative events lack robust evidence to support a specific pathophysiological role in the multi-faceted effects of PFAS. A better characterization of the MOAs/AOPs for PFAS toxicities remains an important area of future investigation, necessary to improve our understanding of PFAS impacts on human health.

At present there is insufficient evidence to determine which, and to what extent, these molecular interactions play a pathophysiological role in observed adverse outcomes of PFAS (Michigan PFAS Science Advisory Panel 2018). Hence, there is a need to integrate such mechanistic information into a weight of evidence framework, first by establishing the MOA or AOP linking a proposed chain of key events to an adverse outcome and then by demonstrating that at human exposure levels of PFAS the established AOP or MOA is causal in the adverse outcome observed. The substantial advantage offered by such an approach is the ability to read across from representative members of appropriate PFAS groupings, based on quantitative information from NAMs and exposure estimates. Hence, better characterization of the MOAs/AOPs for PFAS

toxicities remains a critical area of future investigation and will allow us to understand which adverse PFAS-modified pathways must be interrogated prior to new chemicals joining this class. Predicting PFAS activity in the body should be the goal prior to approving novel PFAS for use.

New approaches for developing PFAS toxicity information

When it comes to determining which PFAS should be prioritized for further testing, there are too many chemicals, even in one subclass, for traditional approaches. Numerous creative and high throughput methodologies are being developed and tested to provide valuable data on PFAS with no toxicity data.

1. Collaborative Approaches

Problem formulation and approach must be guided by available equipment, funds, and technical staff, and important principles:

a. What biological activity and toxicology information can be generated in a *responsive timeframe*?
b. Can this information be used to make public health decisions?
c. What are appropriate tools to bring to this problem (platforms, species/sex of cells used, metabolic competency of the model system, and data analysis)?
d. How do we organize and what are best mechanisms to report useful biological activity/toxicological information?

Developing “how” to evaluate potential health effects of new PFAS requires some thought to PFAS heterogeneity. Although sub-class names have been suggested by

several investigators (Buck et al. 2011; Sha et al. 2019; Wang et al. 2017b), there is still disagreement on those groupings. Additionally, half-lives and persistence are not predictable based on structure, and exposure routes may be complex. Given that traditional approaches to generate toxicity information are resource intensive, NAMs, which may include in vitro high throughput toxicity (HTT) screening and TK testing, will be needed to inform further (in vivo) testing of PFAS.

One example of how agencies/institutes are collaborating to prioritize a list of PFAS needing further study is the REACT Program (Responsive Evaluation and Assessment of Chemical Toxicity). Scientists from the U.S. EPA and NIEHS NTP have joined forces to determine if read across approaches would work. Essentially, they will use existing data for a data-rich substance (the source; e.g., PFOA or PFOS) as an anchor for a data-poor substance (the target; a novel PFAS), which is considered similar enough to the source substance to use the same data as a basis for the safety assessment. For example, the NTP 28-day PFAS or chronic PFOA data sets (National Toxicology Program 2020c) could be used as an anchor. The goal is to bin PFAS by biological activities and then use in vitro to in vivo extrapolation (IVIVE) data and models to estimate oral equivalent exposures for PFAS. For example, multiple biological endpoints (Table 2) were chosen to generate data on 150 PFAS (Patlewicz et al. 2019), representing several structural sub-classes for use in read across.

Selecting assays shown in Table 2 based on PFOA and PFOS health effects covers a broad range of biology. However, due to the structural diversity of PFAS, biological activity of subclasses of PFAS may be missed, but can be addressed in two ways. First, using transcriptomics as a screen, similar and unique pathways altered by

different PFAS can be identified. Second, structure-activity relationships (SARs) may predict potentially missing biological activities. As an example, Leadscope model predictions conducted at NIEHS predicted biology that was covered in assays already chosen for evaluation, which increased confidence in approaches chosen. Because model predictions are only as robust as data sets from which they are generated, these outputs should be used to identify assays for screening efforts, and not as synonymous with toxicities induced by PFAS. Ultimately, the REACT program aims to prioritize PFAS for additional targeted testing and follow up with in vivo studies as needed.

2. Molecular Dynamics and Protein Interactions

Advances in computational tools, many developed for drug discovery, allow environmental and public health researchers to better anticipate some impacts of emerging contaminants even in the absence of substantial experimental data (Rabinowitz et al. 2008). For example, molecular docking and molecular dynamics to predict strengths of interactions between biomolecules and contaminants can be an in vitro screening tool for assessing legacy and emerging PFAS for bioaccumulation potential, to identify potential sites of toxic action (Cheng and Ng 2018; Li et al. 2019; Ng and Hungerbuehler 2015; Salvalaglio et al. 2010), and to gain insights into toxic mechanisms (Sheng et al. 2018). Relatively strong binding with particular proteins (e.g., serum albumin, liver fatty acid binding protein) has already proven useful in correlating PFAS structure with potential for bioaccumulation (Cheng and Ng 2017; Ng and Hungerbühler 2014). Tools including molecular docking and molecular dynamics can correlate relative binding affinities of emerging PFAS with these target proteins, and subsequently compared with

affinities of legacy chemicals with known bioaccumulation potentials, thus providing a first-tier rapid screening mechanism (Cheng and Ng 2018; Luebker et al. 2002).

The use of fluorinated substances in pharmaceutical products has led to an unexpected data source for discovery of structural features in PFAS associated with various types of bioactivity. These data were recently used to train machine learning models to predict potential bioactivity for thousands of untested PFAS (Cheng and Ng 2019). Classification approaches such as these serve as preliminary screening tools for identifying PFAS as a first step in a tiered assessment when detailed mechanistic information is not available.

3. Addressing Mixtures

PFAS are a mixtures issue based on their potential for complex exposure patterns. Communities with water monitoring programs reporting PFAS concentrations demonstrated that they are exposed to mixtures of PFAS. This mixture may be from one or more point-sources releasing multiple PFAS and/or PFAS byproducts into air and water, such as a Chemours plant in North Carolina, and suggest exposures may be substantial (McCord and Strynar 2019). However, numerous other PFAS sources are known to impact community exposure to PFAS mixtures, such as landfill leachate, biosolids recycling, and aqueous film forming foam (AFFF) contamination of drinking water sources, among others (Solo-Gabriele et al. 2020; Sunderland et al. 2019). AFFF and other mixtures evident in drinking water, food packaging, health and beauty products, and food-based sources are often poorly characterized (Sunderland et al. 2019; Susmann et al. 2019).

Discussions on whether PFAS may be addressed using a relative potency framework or toxic equivalency factor (TEF) approach are on-going. PFAS could be grouped by bioaccumulation and persistence (toxicokinetics), function (biology), molecular initiating events (MIE), potency factors derived from several assays, or by subclass (structural similarity).

SPECIAL CONSIDERATIONS IN FUTURE STUDY DESIGNS

Future epidemiological studies

Future human studies need to characterize immune outcomes including (and not limited to) immune effects from exposure in early pregnancy and possible roles of PFAS in initiating allergic and autoimmune processes, conditions for which dose-response is hard to predict. Interactions of immune pathways with liver and lipid toxicity deserves additional consideration.

Liver and lipid studies have reasonably characterized associations between PFAS and effects and should now address why and what to do about it. Characterization of possible *a priori* susceptibility, such as in the obese, is important. Human and animal lipid data suggest that future experimental studies should focus on mitochondrial toxicity, alterations in bile acid metabolism, cholestasis, and resultant steatosis. These outcomes are already known to be associated with altered serum lipids, liver enzymes, and uric acid in the human population regardless of PFAS exposure (Arguello et al. 2015; Cohen and Fisher 2013; Jensen et al. 2018; Sattar et al. 2014).

Studies of human kidney markers related to PFAS exposures illustrate the importance of understanding physiology to inform study design choices and reasonable

interpretations. PFAS have complex excretion mechanics that vary with dose, state of the healthy and progressively diseased kidney, as well as a potentially additional causative effect on kidney disease outcome(s). Appropriate definition of biological and mechanistic targets and more precise investigation of PFAS subclasses will better inform study designs and research questions. For example, consistent reports of disrupted cholesterol metabolism should prompt mechanistic studies evaluating effects on steroid hormones that may influence cancer, fecundity, lactation, and developmental signals seen in human population data. More attention could be given to effects of PFAS on the hypothalamic-pituitary-gonadal axis, and then reconsidered based on life stages.

The history of long-chain PFAS studies indicates that collaborative team approaches featuring clinical, epidemiologic, computational modeling, and laboratory toxicological expertise are needed. Future population designs and more sensitive analytical methodologies should address replacement chemicals, typically found as mixtures; study designs must account for shorter PFAS half-lives and unpredictable PFAS detection in exposed individuals/communities. Innovative use of biomarkers in specifically designated risk subpopulations (obesity, immune) will likely be important.

Sex differences, nonmonotonic dose responses, sensitive subpopulations

Although serum level differences exist between men and women similarly exposed to individual PFAS, sex-dependent differences in half-life have not been reported in human populations for short- (PFBS, PFBA) or long-chain perfluoroalkyl acids thus far (Li et al. 2017b). Perhaps the half-life differences between sexes is similar to inter-individual variability and cannot be detected above background or studies deriving datasets used to model half-lives were not designed to detect sex differences

(convenience sampling or workers were mostly male, etc). However, sex-specific elimination half-lives are defined (Table 1) for some PFAS in rodent models. Additionally, developmental exposure studies in experimental models have consistently shown effects at lower doses than adult only exposures and should be given priority in testing replacement chemicals. In vitro and alternative models that capture developmental susceptibility are encouraged. In summary, care should be taken in testing replacement PFAS in rodent or alternative (cell-based or zebrafish, for example) models, to consider possibilities of sex-based differences in elimination half-lives, dose range used (to include human relevant exposures), life stage represented in the model system, and variability of the response so that issues highlighted enable the use of data generated for risk assessment.

Future experimental model studies

Experimental rodent studies have been essential in confirming PFAS health effects (liver and thyroid disease, and lipid homeostasis), even when effects were not identical to those in humans; in some cases, novel targets (mammary and immune changes) were identified in animals. Future animal, cell-based, and HTT screening should enhance transparency in reporting to include blinded dose allocation, reporting of all data, adherence to Animal Research Reporting in Vivo Experiments (ARRIVE) guidelines (Kilkenny et al. 2010), and dose ranges that approach human relevancy (adjusted to reflect the differences in elimination between species and potentially chronic exposures), so that they influence systematic reviews that may now be used in chemical regulation.

Model selection for health effects evaluation is critical. An appropriate model should be sensitive, susceptible to the outcome(s) of interest (obesity, immune), and produce outcomes that will inform human health effects. Alternative research models, such as transgenic mice, zebrafish, developmental models for most affected target tissues, and diet-challenged designs in susceptible rodent strains will strengthen our knowledge of PFAS-related health effects. Validation of fish neurobehavior models to inform mammalian, including human, developmental responses is needed.

Finally, advanced human cell-based platforms – that have been validated for relevant outcomes in humans – will facilitate concurrent screening of larger numbers of PFAS but bioavailability of PFAS in the culture system needs to be understood, as binding to media proteins or labware, the instability of some PFAS in some vehicles, and altered metabolism may exist in some cases (Gaballah et al. 2020; Liberatore et al. 2020).

Future alternative approaches

One way to determine toxicity of the large number of PFAS compounds currently used in commerce is to develop quantitative structure activity relationships (QSARs). Such QSARs attempt to define relationships between a PFAS compound structure with a specific biological activity or response that identifies or is a biomarker for toxicity. Few data are available for receptor binding of PFAS, mainly limited to a few PFCAs and PFSAs, and even between carboxylates and sulfonates of similar chain length substantial differences have been observed (Cheng and Ng 2017; 2018). If there are substantial differences between perfluoroalkyl carboxylic and sulfonic acids, which differ only in their acid head group, construction of a successful QSAR for the large and diverse class

of all PFAS will be particularly challenging. Several QSARs may be developed, each predictive of toxicity of a distinct class/sub-class of PFAS, based on a unique functional moiety or other feature. While this brings additional challenges in finding sufficient data for QSAR training and validation, big data approaches, such as the recently developed machine learning models to predict PFAS bioactivity (Cheng and Ng 2019), show promise for advancing these computational approaches at the screening level.

For example, it may be determined by affinity for receptor-specific binding and non-specific interactions with cellular membranes, that the specific toxic effect exhibits a multiphasic dose response reflecting two potential mechanisms of action. In addition, the critical effect may change with levels of PFAS exposure. Add to this that people are typically exposed to PFAS mixtures, each of which may have a different affinity for a binding site and ability to impact cellular membrane fluidity, the potential to predict PFAS toxicity becomes extremely complicated. In the foreseeable future, we may be limited to assessing PFAS toxicity using high throughput assays designed to inform regulators as to the relative toxicity of PFAS mixtures or compounds. Such approaches are suited to the use of artificial intelligence (i.e., machine learning approaches) that synthesize data from multiple sources to identify bioaccumulation potential, relevant pathways triggered, protein binding affinities, and MOAs involved in the development of individual and mixture toxicity of PFAS.

The utility of any future approach to determining PFAS toxicity must consider tissue-specific MOAs. Such an approach may rely on molecular dynamic interactions with specific binding sites on enzymes/storage/transport proteins or the non-specific ability to alter cell membrane fluidity by which membrane bound protein activities are

altered within a particular organ/system. Regardless of the MOA, model, and/or simulation, the predictive result should be biologically plausible and represent dose-effect responses across species.

Conclusions

Future research on the health effects of replacement PFAS and mechanistic studies on legacy PFAS must apply "lessons learned" such as those highlighted here. There are only a handful of PFAS with enough health effects data for use in decision making, as evidenced by state-led standard setting. There are numerous health effects reported for those PFAS tested, which sets this family of chemicals apart from many others and elevates the need for precautionary action. With hundreds of PFAS lacking health effects data, translational research teams using innovative methodologies and carefully-designed studies will be critical to our state of knowledge on PFAS-related health effects and our enhanced strategies for informing risk assessment of this large family of chemicals.

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Figure

Figure 1. Effects of per- and polyfluoroalkyl substances (PFAS) on human health. Used with permission from: (European Environment Agency 2019). Original sources for this figure: US National Toxicology Program 2016; C8 Health Project Reports 2012; WHO IARC 2017; Barry et al. 2013; Fenton et al. 2009; and White et al. 2011.

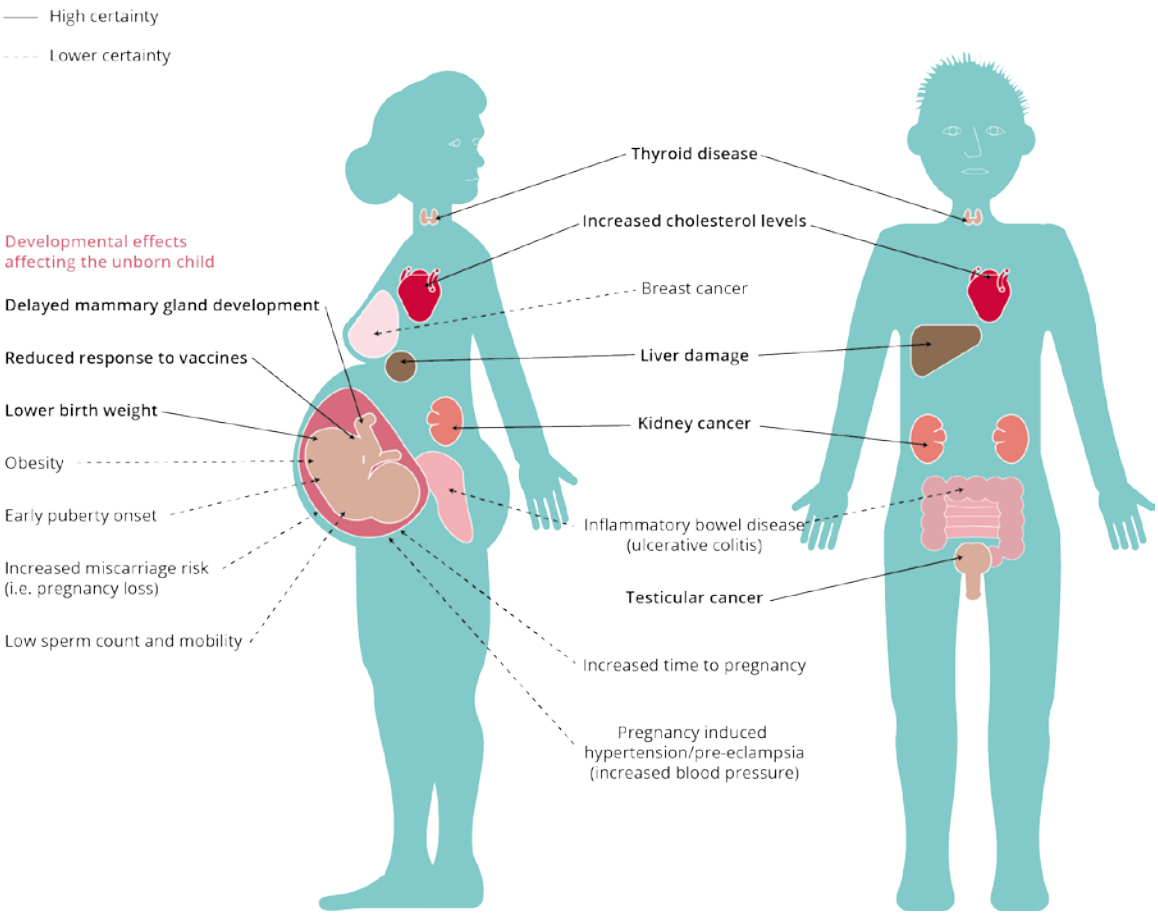

Figure 2. Example of proteins that are known to influence per- and polyfluoroalkyl substances (PFAS) toxicokinetics through binding (which affects tissue distribution and accumulation) and facilitation of membrane transport (which affects clearance and reabsorption). Illustrated for kidney and blood.

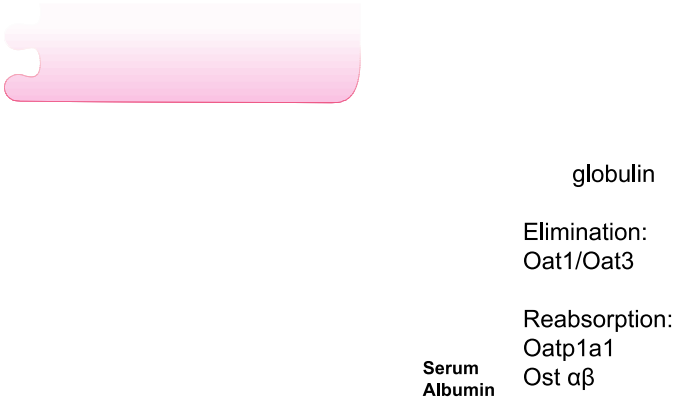

Figure 3: Simulations based on (Cheng and Ng 2017) perfluorooctanoic acid (PFOA) toxicokinetic model for Sprague-Dawley rats. (A) Effect of dose on initial half-life. (B) Effect of higher and lower levels of serum albumin, which binds to PFOA, on serum clearance dynamics. (C) Effect of extent of reabsorption in kidney on serum half-life, based on Oatp1a1 activity. (D) Effect of dose on elimination kinetics when half-life is longer due to higher albumin binding.

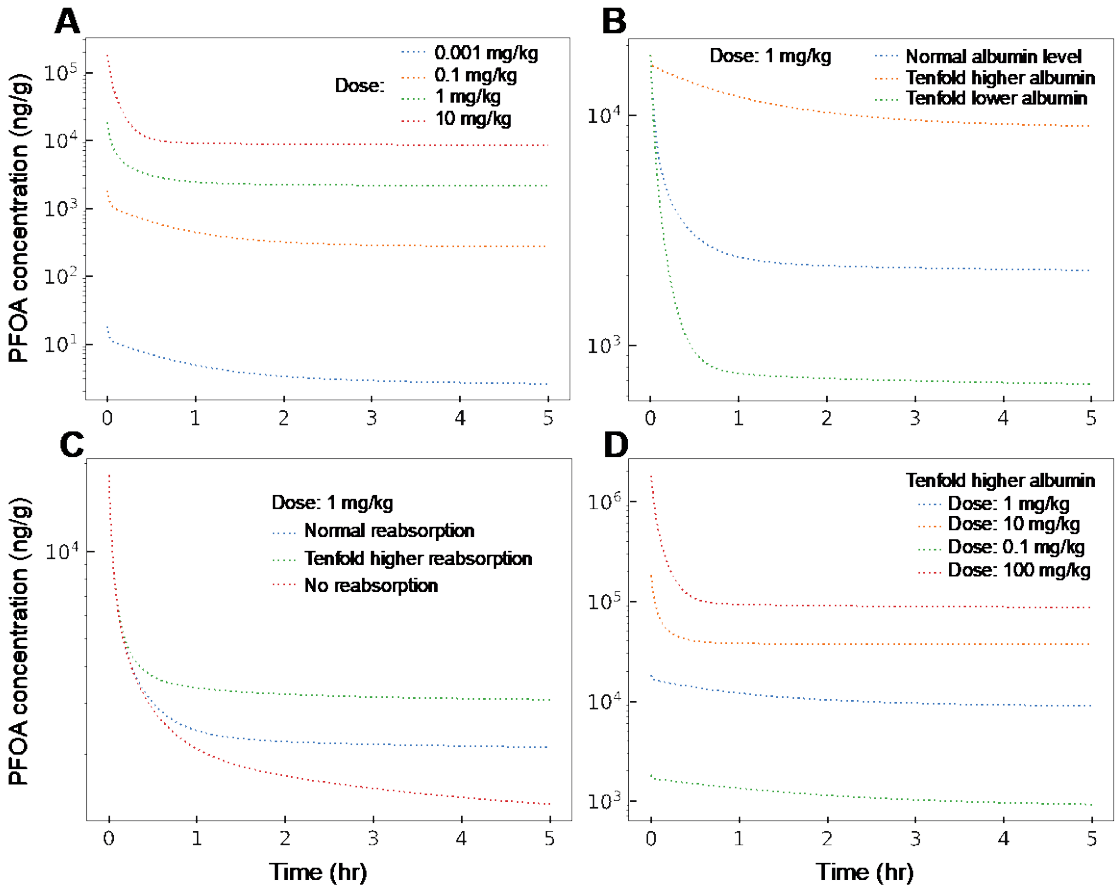

Table 1. PFAS serum half-life estimates in rat, mouse, monkey, and humans

	PFBS (C4)	PFHxS (C6)	PFOS (C8)	PFBA (C4)	PFHxA (C6)	PFHpA (C7)	PFOA (C8)	PFNA (C9)	PFDA (C10	F-53	GenX

																				)	B	
	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M
Rat	0.6-4.0 hours	2.1-4.5 hours	1.8 days	6.8 days	62-71 days	38-41 days	1.0-1.8 hours	6-9 hours	0.4-0.6 hours	1.0-1.7 hours	1.2 hours	2.4 hours	2-4 hours	4-6 days	1.4-6.4 days	31-55 days	59-75 days	40-80 days			8 hours	3 hours
Mouse	4.5 hours	5.8 hours	25-27 days	28-30 days	31-38 days	36-43 days	3 hours	12 hours	~1.2 hours	~1.6 hours			16 days	22 days	26-68 days	34-69 days					18 hours	20 hours
Monkey	3.5 days	4.0 days	87 days	141 days	110 days	132 days	1.7 days		2.4 hours	5.3 hours			30 days	21 days								
Humans	28 days		5.3-8.5 years		3.4-5.0 years		3 days		32 days		1.2-2.5 years		2.1-3.8 years		2.5-4.3 years				15.3 years			

Note: F: Female; M: Male. Modified from (Lau 2012). Blank boxes indicate that reliable data have not been reported to our knowledge. Due to inconsistencies in the methodology by which data sets for individual PFAS were generated for men and women, and the fact that they were generally similar within PFAS, human data are not separated by sex. The values in this table are determined from data in multiple references to indicate the

landscape of vast differences between species and PFAS and should not be considered precise.

Table 2. Fit-for purpose assays proposed in REACT program

Endpoint of Interest	Assay Proposed
High throughput transcriptomics	Metabolically competent human liver cells/MCF-7 (Tempo-Seq®)
Hepatotoxicity	2D HepaRG® cells
Developmental toxicity	Zebrafish embryo assay
Developmental neurotoxicity	Multi-Electrode Array in neonatal cortical cells and neurite outgrowth
Immunotoxicity	Cytokine alterations in human vascular endothelial cells (BioSeek®)
Hepatic Clearance	Metabolic clearance in 50 donor-pooled hepatocyte suspensions
Plasma protein binding	Serum protein binding assay using human serum
Enterohepatic recirculation	Qualyst B-CLEAR® hepatocyte transporter assay
In vitro disposition	In vitro disposition in cell lines under study

REACT = Responsive Evaluation and Assessment of Chemical Toxicity