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## A Scoping Literature Review of Toxicological Studies on Per- and Polyfluoroalkyl Substances (PFAS)

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### ABSTRACT

Per- and Polyfluoroalkyl substances (PFAS) encompass a diverse group of industrial chemicals resistant to both environmental degradation and metabolic breakdown due to the strong carbon-fluorine (C-F) bonds dominating their structures and are thus collectively referred to as “forever chemicals”. Within this group, perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) have been extensively studied, and detected in humans, biota and the environment globally. To compile the latest PFAS toxicity data, we conducted a scoping review spanning literature published from January 2019 to December 2023, broadly following the PRISMA guidelines, encompassing epidemiological studies in humans and toxicological studies in experimental animals, *in vitro* assays on human cells, and studies combining experimental and *in silico* approaches. We assessed the available toxicological data and performed an in-depth analysis of studies focusing on potential human health effects, with the aim of leveraging the findings within a cheminformatics framework. Our investigation identified a total of 358 suitable studies. Human exposure studies identified immunotoxic effects as being among the most prevalent endpoints in PFAS toxicity. According to our findings, hepatotoxicity linked to



peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) activation, neurotoxicity, developmental and endocrine effects, and associations with various types of cancer were found amongst the negative health outcomes associated with PFAS exposure. The findings highlight the need for further research to prevent regrettable substitutions and to enable implementation of PFAS-free technologies.

Keywords: PFAS; PFOA; PFOS; Toxicity; Toxicological studies; Human health; Scoping Review

## Environmental Significance Statement

Per- and polyfluoroalkyl substances (PFAS) are highly persistent chemicals used worldwide, resulting in long-term environmental and human exposure. Their widespread presence in water, soil, and biota raises serious concerns due to links with immunotoxicity, hepatotoxicity, endocrine disruption, and developmental effects across species. Despite regulatory actions, emerging “short-chain” and alternative PFAS often exhibit toxicity profiles comparable to legacy compounds, challenging assumptions about their safety. This scoping review synthesizes toxicological data from 358 studies published between 2019 and 2023, comparing legacy and alternative PFAS to provide updated mechanistic insights. These findings highlight the urgent need for integrated risk assessment frameworks and improved regulatory strategies to address the persistence, bioaccumulation, and health hazards of both existing and replacement PFAS

## 1. Introduction

### 1.1 What are PFAS? Definition, Sources, and Legislation

Per- and Polyfluoroalkyl Substances (PFAS) are synthetic industrial chemicals that have been characterized as persistent substances<sup>1</sup> and which are ubiquitous in the environment due to their widespread uses.<sup>2</sup> According to the Organization for Economic Co-operation and Development (OECD), PFAS are defined as fluorinated substances that contain at least one fully fluorinated methyl (-CF<sub>3</sub>) or methylene (-CF<sub>2</sub>-) carbon atom.<sup>3</sup> Due to this carbon-fluorine bond, which is one of the strongest chemical bonds in nature,<sup>4</sup> and their amphipathic properties, PFAS molecules have exceptional stability against chemical<sup>5</sup> and environmental degradation.<sup>6</sup> Thus, PFAS are either very persistent themselves or are precursor compounds that can degrade into persistent and potentially toxic products.<sup>7</sup>

The widespread use of PFAS across various industries, as well as in agriculture –specifically as active ingredients of some pesticides– can be attributed to their unique combination of hydrophilic



and hydrophobic properties, resulting in applications as food packaging materials, aqueous film-forming foams,<sup>8</sup> cosmetics,<sup>9</sup> and even ski waxes,<sup>10</sup> among others. PFAS can be detected not only in the blood of occupationally exposed workers, but also in the general population, especially in those who live near industrial areas that use or produce PFAS. The human population of these regions is exposed to PFAS either via contaminated drinking water,<sup>11</sup> by dietary intake of aquatic products (e.g., marine fish, shellfish, freshwater fish) or through food in general—either directly or indirectly from food packaging, through agricultural practices such as the application of biosolids or even through irrigation with water containing PFAS. Dietary ingestion and consumption of contaminated drinking water have been found to be major exposure pathways to PFAS in the general population.<sup>12</sup> PFAS have also been detected in the atmosphere, with urban regions being the primary sources of perfluoroalkyl acids (PFAAs) to air.<sup>13</sup> Recently, air dispersion modelling for major PFAS manufacturing sites suggests the possibility of soil and water contamination through atmospheric transport.<sup>14</sup> In humans, a common route of PFAS exposure during the prenatal period is through the placenta, where accumulated PFAS can be transferred from pregnant women to foetuses, posing potential health risks to both.<sup>15</sup> Similarly, PFAS have been found in breast milk allowing direct transfer to infants.<sup>9</sup>

Given the ubiquitous exposure, regulatory agencies have made significant efforts to better understand these environmental chemicals, reduce exposure to them, and ultimately protect public health and the environment. Over the past decade, long-chain PFAS—defined as perfluoroalkyl carboxylic acids with carbon chain length  $\geq 7$  or perfluoroalkyl sulfonic acids with  $C \geq 6$ —have been increasingly regulated, as they have been associated with more harmful health effects. A key action of the EU Chemicals Strategy for Sustainability is to phase out PFAS in the EU, allowing their use only if they are proven to be irreplaceable and that their use is essential for society.<sup>16</sup> PFOA, PFHxS, PFOS—and their salts or precursors—are listed in the Stockholm Convention under Annex A - Elimination (PFHxS, PFOA) or Annex B - Restriction (PFOS), so their production and use are banned or restricted, respectively, worldwide. However, the Stockholm Convention waives the ban on PFOA as a component of firefighting foams and allows the production and use of PFOS for multiple purposes. PFOS and PFOA (and their related products) have been restricted or banned by EU Regulation (EU) 2019/1021<sup>17</sup> on Persistent Organic Pollutants (see Figure 1).





**Figure 1.** Timeline of safety restrictions for PFAS in the EU.

Despite stringent regulations and gradual phase-out efforts of legacy PFAS, these substances remain detectable in the environment giving rise to serious health concerns from exposures.<sup>12</sup> Regulatory bodies are primarily focused on setting exposure-based safety thresholds and assessing potential health risks associated with PFAS. However, regulating lesser-known PFAS is challenging due to the limited availability of toxicological data, compounded by the difficulties researchers face in accessing these substances for testing.

### 1.2 Structural Classification and PFAS toxicity

PFAS consist of a carbon chain of 4-14 carbon atoms, where fluorine atoms replace the hydrogen atoms of the carbon chain, and a functional group.<sup>18</sup> Based on their chemical structure, PFAS can be divided into subgroups such as perfluoroalkyl carboxylic acids (PFCAs,  $C_nF_{2n+1}COOH$ ), perfluoroalkane sulfonic acids (PFSAs,  $C_nF_{2n+1}SO_3H$ ), fluorotelomers derivatives (FTOHs, FTSA), ether-containing PFAS (e.g., GenX and ADONA (dodecafluoro-3H-4,8-dioxanonanoate)), and other emerging subgroups like perfluoroether carboxylic or sulphonic acids (PFECAs; PFESAs)<sup>19,20</sup> (see Table 1). These compounds, both legacy and emerging, have been detected in top predators and other biota, demonstrating their persistence, bioaccumulation, and potential to cause ecological and human health impacts.<sup>21</sup>

**Table 1.** PFAS subgroups and their key characteristics.

PFAS Subgroup	Chemical Structure	Example Compound	Key Characteristics
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PFCAs	$C_nF_{2n+1}COOH$	PFOA	Widely detected in the environment and biota; persistent and bioaccumulative when >6C.
PFSA	$C_nF_{2n+1}SO_3H$	PFOS	Known for long environmental persistence and found in top predators and humans when >4C.
PFECAs	Contains oxygen atoms in backbone	GenX	Emerging PFAS; used as a replacement for PFOA, toxic and persistent in the environment.
PFESAs	Contains oxygen atoms in backbone	Nafion by-product 2	Emerging PFAS; used in fuel cells as ionomeric membranes.
Fluorotelomer Alcohols (FTOHs)	$C_nF_{2n+1}CH_2CH_2OH$	6:2 FTOH	Precursor compounds that degrade into other terminal PFAS; detected in various environmental matrices.
Fluorotelomer Sulfonic Acids (FTSAs)	$C_nF_{2n+1}CH_2CH_2SO_3H$	6:2 FTS	Degradation products of FTOHs; commonly found in both aquatic and terrestrial environments.
Perfluoroalkyl Phosphonates (PFPA)	$C_nF_{2n+1}P(O)(OH)_2$	PFPA	Phosphorus-containing PFAS; increasingly detected in the environment.
Perfluoroalkyl Phosphinates (PFPIAs)	$C_nF_{2n+1}P(O)(OH)$	PFPIA	Another phosphorus-containing class; found in water and sediment samples.
Poly-ether PFAS	Contains multiple ether linkages	ADONA	Emerging PFAS; developed as replacements for PTFE synthesis, but still persistent in the environment.

A key feature affecting PFAS behaviour and toxicity is chain length. PFAS are classified into short-chain (PFCAs with  $C \leq 6$  or PFSAs with  $C \leq 5$ ) and long-chain molecules depending on their carbon chain length. The long-chain PFAS, occasionally referred to as legacy PFAS,<sup>22</sup> while shorter derivatives have been developed as industrial alternatives to the legacy ones. However, studies comparing toxicity of long- and short-chain PFAS in human liver cells and zebrafish embryos have shown chain-length-dependent effects, while ether substitutions in emerging PFAS do not consistently reduce toxicity.<sup>23,24</sup>



Among the various PFAS, legacy compounds such as PFOA and PFOS have been most extensively studied and are associated with harmful effects in living organisms and on human health.<sup>25</sup> PFOA was recently upgraded by the International Agency for Research on Cancer (IARC) to Group 1 (carcinogenic to humans) based on evidence from animal studies, mechanistic data, and human epidemiology.<sup>26</sup> Similarly, PFOS has been classified as a Group 2B (possibly carcinogenic) compound.

PFAS exposure has been associated with a broad range of adverse outcomes. Regulatory and health agencies [e.g., Environmental Protection Agency, US (EPA), European Food Safety Authority (EFSA), and the Agency for Toxic Substances and Disease Registry (ASTDR)] have reported evidence of immunotoxicity, hepatotoxicity, endocrine disruption, neurodevelopmental effects, and cancer.<sup>27,28</sup> Experimental and epidemiological studies indicate alterations in the immune system, disruptions in thyroid hormones, lipid metabolism, and early-life development.<sup>8,29,30</sup> Immunotoxicity has emerged as a particularly sensitive endpoint, forming the bases for recent regulatory thresholds in the EU and US.<sup>31,32</sup>

### 1.3 Objective of the Scoping Review

The toxicological profile of the PFAS family is primarily informed by both *in vivo* and *in vitro* studies, particularly for legacy compounds such as PFOA and PFOS. These data are accessible through public databases like the PFAS-Tox Database (<https://pfastoxdatabase.org/>)<sup>33</sup> that provides curated information on biological effects of PFAS up to 2022, while the US EPA's CompTox Chemicals Dashboard<sup>34</sup> compiles molecular and toxicological data across PFAS compounds.

Through analysis of experimental (*in vitro* and *in vivo*) studies, and human epidemiological evidence, this scoping review seeks to map and compare toxicological profiles of legacy and emerging PFAS—defined here as the range, severity, and type of observed biological effects (e.g., hepatotoxicity, immunotoxicity), along with the exposure model and dose-response patterns. This integrative approach is intended to qualitatively evaluate how different PFAS induce toxicity, to enable subsequent cheminformatics analysis of the data, and to identify critical gaps that make it harder to understand their impacts on human health. The review includes studies assessing PFAS toxicological profiles either as single compounds or in mixtures and considers those utilizing Quantitative Structure-Activity Relationship (QSAR) models and cheminformatics approaches, alone or combined with experimental studies to understand the convergence or



divergence of data, supporting advanced predictive modelling of PFAS toxicity in the context of human-health risk assessment.

## 2. Methodology

### 2.1 Protocol and Registration

The aim of the scoping review (ScR) was to gain insights into the toxicological effects of PFAS on human health and how these are determined by the PFAS physicochemical properties, as well as to explore the potential exploitation of these data for machine learning. Thus, the research question underpinning this ScR was: “*What data is available regarding PFAS toxicity to human health and what are the main toxicological impacts and human health endpoints affected by PFAS?*”. The ScR is registered on the OSF database for reviews with the DOI identifier: <https://doi.org/10.17605/OSF.IO/8746M>, and follows the PRISMA<sup>35</sup> guidelines for a scoping review. The completed PRISMA-ScR checklist is presented in Supplementary Table S1.

### 2.2 Eligibility Criteria

We considered peer-reviewed journal articles published between January 2019 and December 2023, written in English, that reported experimental or observational data on toxicological effects of PFAS. Eligibility was based on first online availability/Epub date where applicable (to account for late indexing/issue assignment). Eligible studies include: (i) *in vivo* studies on laboratory animals (rodent models, zebrafish (*D. rerio*), nematodes (*C. elegans*), and chicken embryos) evaluating health-relevant endpoints, (ii) *in vitro* studies using human cell lines or advanced human-relevant systems (e.g., stem cells, organoids, or organs-on-chip), (iii) studies employing *in silico* approaches such as QSAR models or cheminformatics, and (iv) epidemiological studies of human populations exposed to PFAS. *In vivo* studies using zebrafish and nematodes were considered eligible, as these species are widely accepted models for estimating toxicity and inferring potential health outcomes in humans.<sup>36,37</sup> Their use is also in accordance with the principles of 3Rs (replacement, reduction and refinement) as a partial replacement of rodents.<sup>38</sup>

All studies addressed PFAS either as single compounds or in mixtures. To be eligible, studies had to report findings relevant to the toxicological profile of PFAS, defined here as the range and type of biological effects, including but not limited to hepatotoxicity, immunotoxicity, neurotoxicity, endocrine disruption, and reproductive or developmental toxicity and include all relevant controls. For human epidemiological studies, in addition to the criteria above, the presence of a non-





exposed or lower-exposed control (or reference) group was required to be eligible, to minimize confounding factors.

Papers focusing on the environmental impacts of PFAS toxicity (e.g., ecotoxicity, phytotoxicity) or examining techniques for PFAS detection, determination, degradation, removal, or remediation, were excluded. We also excluded reviews, commentaries, non-English publications, and studies that lacked sufficient detail for toxicity interpretation, machine learning suitability, or data extraction (e.g., concentration-effect relationships, molecular descriptors, gene expression profiles). All inclusion and exclusion criteria are summarized in detail in Table 2.

**Table 2.** Inclusion and exclusion criteria applied in the ScR.

Parameters	Inclusion	Exclusion
<b>Publication date</b>	January 2019 to December 2023	Out of the time period: before 01/2019 and after 12/2023
<b>Language</b>	English	Non written in English
<b>Population</b>	<p><u>Human studies:</u></p> <ul style="list-style-type: none"> <li>a) <i>in vitro</i> human cell lines and</li> <li>b) biomonitoring/epidemiological studies with control groups</li> </ul> <p><u>Animal studies:</u> Only <i>in vivo</i> studies of laboratory animals, specifically: rats, mice, <i>D. rerio</i> (zebrafish) and nematodes (<i>C. elegans</i>), and chicken embryos. <i>In vitro</i> animal studies are accepted only in cases of advanced systems utilizing stem cells, organoids, or organ-on-chip.</p>	<ul style="list-style-type: none"> <li>● Plants</li> <li>● Field studies</li> <li>● Aquatic species, except zebrafish</li> <li>● Other non- laboratory animal species</li> <li>● <i>In vitro</i> studies with animal cell lines</li> <li>● No control or reference groups in human epidemiological studies</li> </ul>
<b>Exposure</b>	<ul style="list-style-type: none"> <li>● Studies focusing on at least one PFAS substance, either legacy or alternative</li> <li>● Individual PFAS or in mixtures</li> </ul>	<ul style="list-style-type: none"> <li>● Not related to PFAS, irrelevant chemicals</li> <li>● Mixtures containing other endocrine disrupting chemicals (EDCs) along with PFAS</li> </ul>
<b>Outcomes</b>	All observed adverse effects on biological systems. Endpoints:	<ul style="list-style-type: none"> <li>● Environmental impacts (soil/water/air pollution)</li> </ul>



- Hepatotoxicity
- Immunotoxicity
- Neurotoxicity
- Endocrine disruption
- Carcinogenicity
- Reproductive or
- Developmental toxicity
- Non-specific toxic effects or effects unrelated to human health
- Toxicokinetics
- Techniques, such as absorption, degradation, remediation, sorption etc.

## Quantitative endpoints:

- LD<sub>50</sub> /LC<sub>50</sub>
- NOAEL/C
- LOAEL/C
- BMD/C

**Study Design**

- *In vivo* studies in rodents, chicken embryos, *D. rerio* (zebrafish) and nematodes (*C. elegans*);
- All human studies, either exposure (epidemiological, cohort studies) or *in vitro* human cell lines;
- *In silico* studies (combined or not with an *in vitro* / *in vivo* experiment)
- Reviews (systematic, critical etc.) combined or not with Meta-analysis
- Case studies and reports
- Editorials, letter to editorial
- Conference papers, research highlights, books

**Data availability**

Either downloadable dataset or retrievable data from the paper. Extractable toxicological data, such as:

- Concentration–response values,
- Molecular descriptors,
- Dose metrics linked to outcomes,
- Gene expression or omics profiles.

On request, or not available data, or not sufficient for utilizing them in a computational framework

Legend: LD = lethal dose; LC = lethal concentration; NOAEL/C = No Observed Adverse Effect Level or Concentration; LOAEL/C = Lowest Observed Adverse Effect Level or Concentration; BMD/C = Benchmark Dose or Concentration



### 2.3 Study Information Sources and Search Terms

The literature search was conducted using two widely recognized databases: PubMed and Scopus. For retrieving studies more specific to chemicals, an additional search was performed in the ACS Publications database. PubMed and Scopus were selected because they contain an abundance of peer-reviewed publications regarding biomedical, environmental, and toxicological literature, while ACS Publications was included for its focus on chemistry-related toxicological studies.

The search strategy was designed to maximise sensitivity and capture the breadth of evidence relevant to the review question, consistent with the exploratory purpose of a ScR. Because PFAS terminology is heterogeneous and inconsistently applied across scientific, regulatory, and technical domains, the strategy prioritised broad, widely recognised terms rather than exhaustive chemical enumeration. Specifically, the term “PFAS” is used as a core search anchor, with PFOA and PFOS as support terms given their widespread usage. The PFAS acronym dominates indexing practices in major bibliographic databases and is routinely used by authors to describe studies involving a wide range of per- and polyfluoroalkyl substances, including those not explicitly named in titles or abstracts. Relying on full chemical names or attempting to list all individual PFAS congeners was deemed neither feasible nor methodologically appropriate, given the large and evolving number of PFAS and the lack of a universally accepted, stable congener list. Additionally, most studies with newer PFAS congeners, designed as alternatives to PFOS/PFOA, include a comparison to the properties and toxicity of one of these two most widely studied congeners, such that we anticipated that use of these terms would further enrich the data corpus generated from the scoping search. Therefore, it was considered acceptable to employ these 3 terms for the search. Using such terminology ensured that the search remained inclusive of studies addressing PFAS as a class, legacy compounds, emerging PFAS, mixtures, and regulatory or environmental assessments that do not specify individual congeners.

Because the objective was to map evidence on PFAS toxicity rather than to systematically catalogue all in silico methodologies, the search strategy intentionally prioritised PFAS-related terminology over method-specific terms. Full search strings for each database, including Boolean operators and filters, are documented in the SI.

The terms that were used to search abstracts, titles, and keywords of papers were thus:



1. ("toxicity" OR "toxicology" OR "cheminformatics" OR "cheminformatics" OR "QSAR" OR "QSPR" OR "machine learning" OR "modeling") AND ("PFAS" OR "PFOA" OR "PFOS")
2. ("toxicity" OR "toxicology") AND ("PFAS" OR "PFOA" OR "PFOS") AND ("cheminformatics" OR "cheminformatics" OR "QSAR" OR "QSPR" OR "machine learning" OR "modeling")

These combinations were utilized to encompass a broad range of studies relevant to PFAS toxicology and computational modelling, aligning with inclusion criteria defined in *Section 2.2*. The initial searches were conducted in March 2024 and updated in May 2025 to ensure inclusion of all eligible studies according to the publication date range defined. The database-specific search criteria are provided in the SI.

#### 2.4 Study Selection and Screening Process

The titles and abstracts of papers obtained using the search terms presented in Section 2.3 yielded an initial total of 3,768 papers. After removing duplicate items, 2,452 unique records remained. Zotero and Excel were used for reference management and duplicate removal. Additionally, screening, organization, and labelling of records were performed using Rayyan software (<https://www.rayyan.ai/>).<sup>39</sup> During the preliminary screening, all titles, abstracts, and keywords were assessed against the eligibility criteria, resulting in 1,244 papers. A more targeted abstract screening was then performed focused on the specific scope of this review, retaining 784 studies. After full-text screening, 351 papers were identified that met all inclusion criteria and presented extractable toxicological data. During this process, 7 additional studies were identified through citation searching and were subsequently added to the final pool of included papers ( $n = 358$ ) due to their relevance and fulfilment of the eligibility criteria. Although many studies provided valuable insights into PFAS toxicity, we chose to include only those that reported structured and interpretable toxicological data (e.g., concentration-response relationships, molecular descriptors, omics readouts) or were accompanied by *in silico* modelling approaches. These criteria ensured the final dataset would be suitable for future machine learning applications.

Study selection and data charting were conducted using a two-stage reviewer model consistent with the flexible methodological expectations of scoping reviews and the PRISMA-ScR reporting framework. One researcher undertook all stages of screening (titles/abstracts and full texts) and data charting to ensure consistency in the application of eligibility criteria and extraction procedures. A second researcher subsequently reviewed the screening decisions and charted data in a complementary validation role. Any uncertainties or discrepancies were resolved through



discussion. This approach maintains transparency and methodological rigour while recognising that scoping reviews, as reflected in PRISMA-ScR, prioritise comprehensive mapping of the evidence base rather than formal risk-of-bias assessment. PRISMA-ScR emphasises clear reporting of the processes used. By documenting each step and incorporating a structured validation layer, this approach supports consistency and transparency while remaining feasible for the scope and resources of the review.

### 2.5 Data Extraction

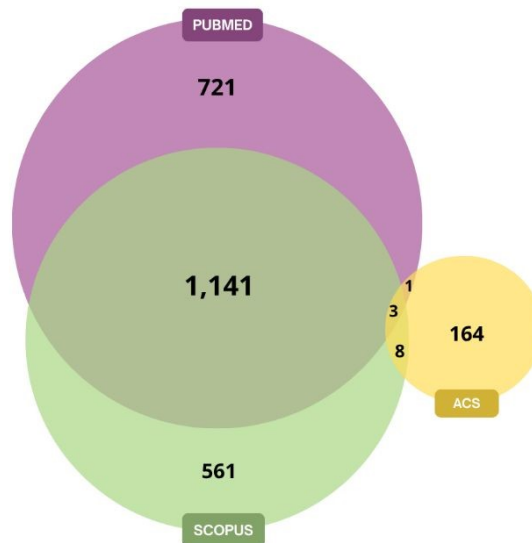
Microsoft Excel was employed to systematically compile and organize information from relevant publications. For each eligible study, data were extracted and categorized according to key study characteristics including the PFAS compound(s) investigated, study design (e.g., case-control human epidemiological studies) or experimental model (model organism or cell type) or *in silico* tools, exposure route, concentration, and reported toxicological outcomes on human health. Author name, publication year, and other relevant details were also documented, supporting the referencing process. By toxicological impacts on human health, we refer to adverse biological effects that compromise normal function or increased disease risk. In our review, these outcomes were identified based on evidence from experimental and human studies and organized into ten outcome categories: hepatotoxicity, immunotoxicity, developmental toxicity, reproductive toxicity, neurotoxicity, endocrine disruption, metabolic disruption, renal toxicity, cardiotoxicity, and carcinogenicity/tumour promotion.

## 3. Results

### 3.1 Overview of Studies

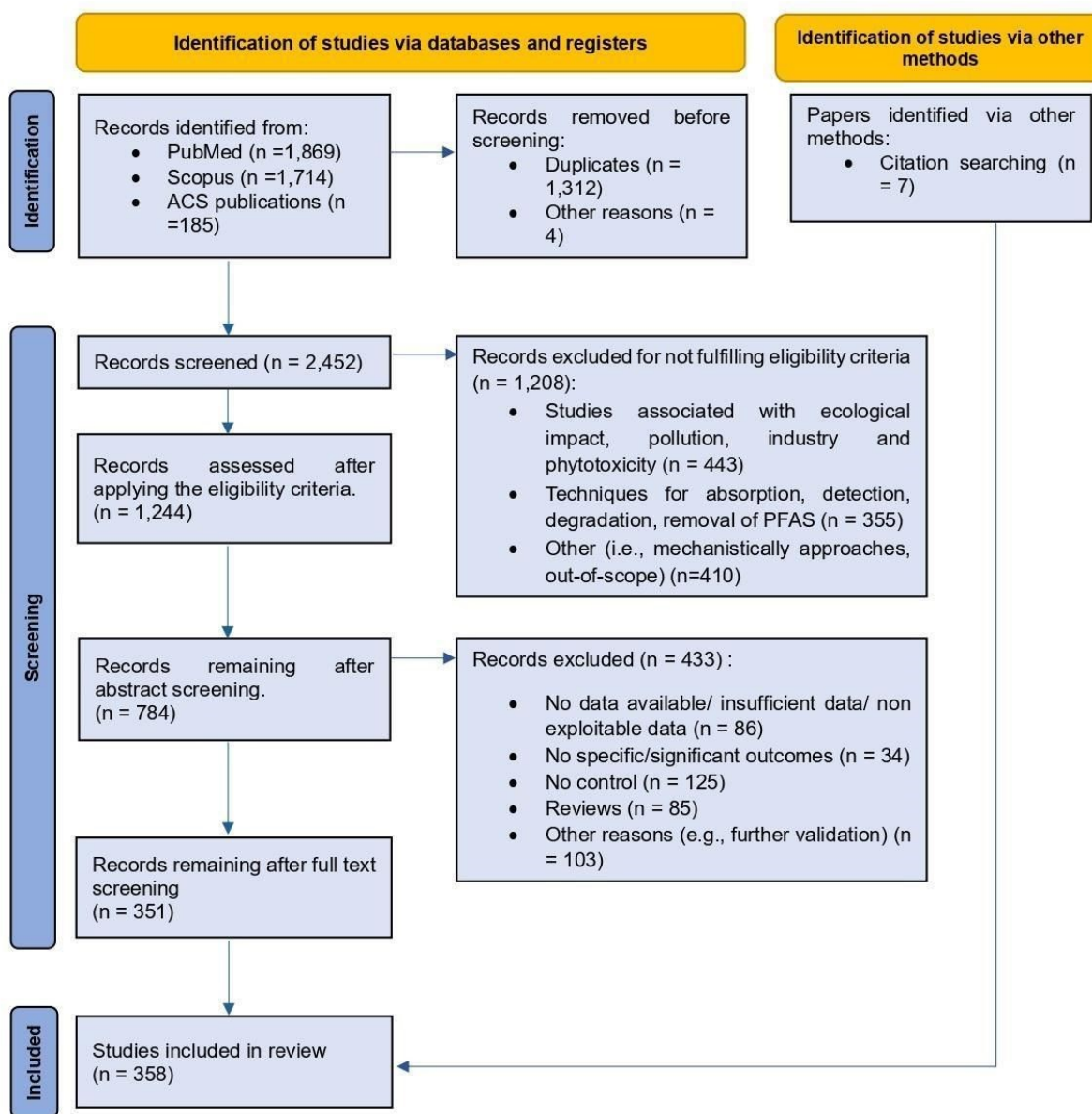
Out of 3,768 records retrieved from PubMed (1,869), Scopus (1,714) and ACS Publications (185), only 358 peer-reviewed papers met the eligibility criteria for inclusion in this scoping review, as outlined in *Section 2.2*. A Venn diagram (see Figure 2) illustrates the overlap of records among the three databases, which helps clarify and evaluate the comprehensiveness of all searches conducted, while the PRISMA flowchart (see Figure 3) outlines the full screening and selection process, as described in detail in *Section 2.4*.





**Figure 2:** Venn diagram showing citation overlap among records retrieved from PubMed (purple), Scopus (green circle), and ACS Publications (yellow circle). Created with BioVenn.<sup>40</sup> Note: BioVenn calculates overlaps based on unique article titles only. Table S2 contains a summary of the most commonly reported PFAS substances identified in the ScR.



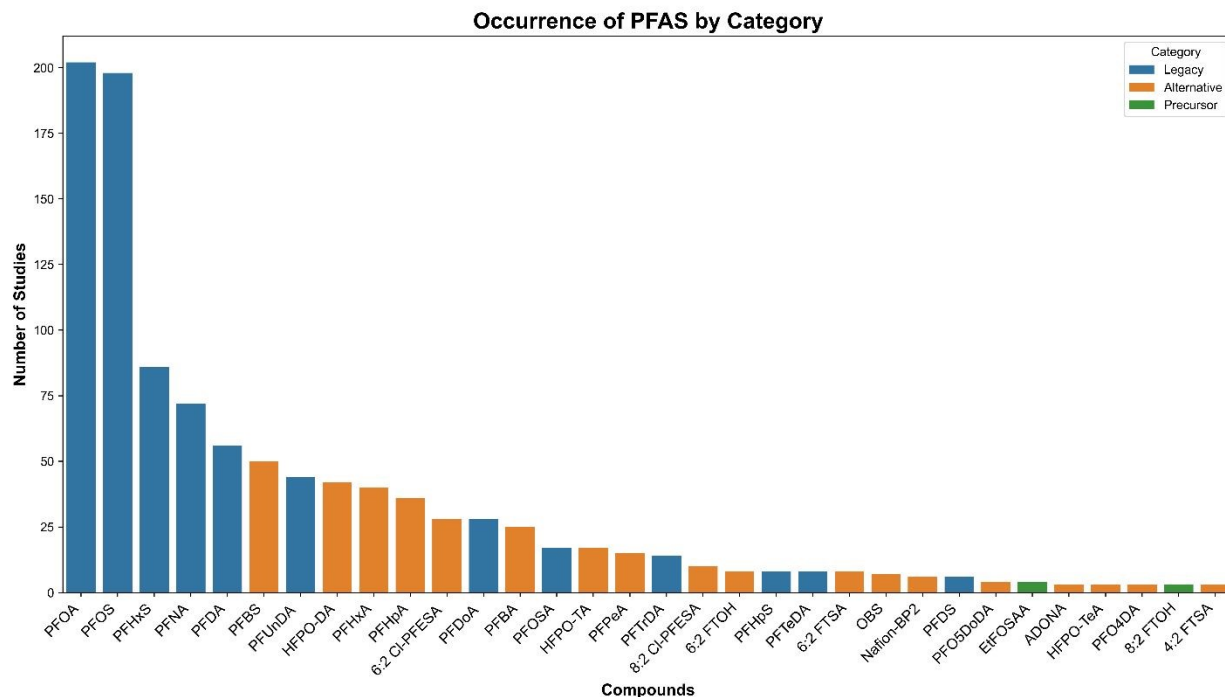


**Figure 3.** A summary of the papers considered at each stage of the scoping review process visualized through the PRISMA flow diagram. 358 studies containing sufficient data for modelling of PFAS toxic effects were identified.

Among the 358 reviewed studies, legacy PFAS compounds —particularly PFOS, PFOA, PFNA, PFHxS, and PFDA— were the most frequently investigated and identified as highly harmful in biological systems. These compounds have received the greatest toxicological attention due to their widespread use and environmental persistence. At the same time, a subset of studies



examined alternative PFAS, either alone, in mixtures or in combination with legacy compounds. Among these, HFPO-DA (or GenX), PFHxA, PFBS, and 6:2 Cl-PFESA (or F-53B) were the most commonly studied alternatives. Notably, several alternatives exhibited toxicity profiles similar to those of their legacy counterparts, while promoted as safer replacements. A breakdown of PFAS occurrence among the reviewed studies—depicting the distinction between legacy and alternative compounds—is provided in Figure 4.



**Figure 4.** Occurrence of PFAS by Category across the included studies. The chart presents the number of studies in which each PFAS was investigated, grouped by category: legacy (blue), alternative (orange), and precursor (green). Only compounds appearing in more than two studies are presented.

To examine the range of data available, the included publications were sorted into five groups based on study design: *in vivo*, *in vitro*, *in silico*, human epidemiological studies, and combined *in silico- in vitro* approaches. The main findings are summarized in Tables 3 to 7, each providing an overview of PFAS-induced effects across different models and methodologies.

### 3.2 *In vivo* studies

This review revealed a wide range of toxicological effects associated with PFAS exposure across model organisms. Here, we highlight representative *in vivo* studies that report recurring or mechanistically informative toxicological endpoints, diversity of PFAS (legacy vs. alternatives),





and cover a range of biological systems including rodents, zebrafish, *C. elegans*, and chicken embryos. Table 3 summarizes the key *in vivo* findings, while the full list of *in vivo* studies, including exposure concentrations, durations, models, and observed outcomes, is available in Supplementary Table S3.

Rodent studies investigated multiple legacy and alternative PFAS and Conley et al.<sup>41–43</sup> revealed both maternal and foetal organ-specific toxicities, such as hepatomegaly, and lipid metabolism disruption, following exposure to PFOS, PFOA, HFPO-DA, and Nafion-BP2 exposure. In another study, intraperitoneal injection of PFOS induced inflammatory bowel disease-like phenotypes in rats<sup>44</sup>. Additionally, skin exposure to PFOA led to immunotoxicity, including reduced thymus and spleen weight, and impaired immune organ development.<sup>45</sup> Complementary findings by Weatherly et al.<sup>46,47</sup> showed that several short-chain alternatives of PFOA—including PFBA, PFPeA, PFHxA, and PFHpA—led to liver toxicity, immunological disruptions, and altered PPAR-regulated gene expression. The aforementioned findings raise concerns about systemic toxicity of short-chain PFAS, which have been designed as safer replacements. Besides, PFOA was found to change circadian rhythm-related gene expression in kidneys through the PPAR signalling pathway, accompanied by oxidative stress.<sup>48</sup> This suggests that PFOA's activation of PPARs may mediate a variety of toxicological outcomes beyond immune or hepatic endpoints. Moreover, neurotoxic effects were observed after PFOA and PFOS exposure, including increased expression of corticotropin-releasing factor, blood brain barrier disruption,<sup>49,50</sup> as well as cognitive-related behavioural changes in mice.<sup>51,52</sup>

Studies in zebrafish models (adults or embryos) also revealed consistent mechanisms and phenotypic outcomes, mainly involving immune and metabolic pathways and extensively investigating developmental effects after PFAS exposure. PFNA was found to impair neurodevelopmental toxicity in zebrafish embryos by leading to abnormal swimming behaviour, after being exposed in environmentally relevant concentrations.<sup>53</sup> Additionally, Zhang et al.<sup>54</sup> demonstrated that 6:2 FTSA—an alternative and precursor analogue—caused immunotoxicity in zebrafish embryos by upregulating pro-inflammatory markers like IL-1 $\beta$ , TNF- $\alpha$ , TLR4, and NF- $\kappa$ B. Similarly, Zhang et al.<sup>55</sup> showed that exposure to PFOA triggered the TLR2/MyD88/NH- $\kappa$ B signalling pathway, causing mitochondrial damage, decreased expression of IL-4, and dysregulation of antibody responses. These results highlight the critical role of NF- $\kappa$ B mediated inflammation in immunological responses triggered by both legacy and alternative PFAS. Another study compared PFOA to its perfluoroalkyl ether alternatives—HFPO-DA and HFPO-TA—reporting shared metabolic toxicity, including high levels of cholesterol and disruption of PPAR $\alpha$ -



regulated genes.<sup>56</sup> The understudied 8:8 perfluoroalkyl phosphinic acid (8:8 PFPIA) was examined in zebrafish embryos by Kim et al.,<sup>57</sup> who showed altered epigenetic regulation and thyroid disturbance.

In *C. elegans*, Lin et al.<sup>58</sup> and Wei et al.<sup>59</sup> showed that legacy PFAS, including PFOA and PFOS, led to metabolic disruption by affecting fatty acid homeostasis. PFBS—an alternative PFOS analogue—was found to decrease reproductive capacity and change offspring's body size, growth rate, and locomotion, in lower internal concentrations than PFOS needed for producing the same effects, showing its greater bioaccumulative potential and potency.

In chicken embryos, Xu et al.<sup>60</sup> showed that HFPO-DA caused hepatotoxicity and developmental cardiotoxicity through PPAR $\alpha$  involvement, while another study<sup>61</sup> further validated the PPAR $\alpha$ -mediated mechanisms for PFOA in both aforementioned toxicities. However, Guo et al.<sup>62</sup> documented PFOA-induced cardiotoxicity via PPAR $\alpha$ -independent pathways.

**Table 3.** Summary of key toxicological effects associated with PFAS exposure categorized by endpoint.

Toxicological Effect	PFAS Compounds	Number of studies <sup>a</sup>	Model Organisms	Exposure Route	Observed Outcomes
Immunotoxicity	PFOA, PFOS, PFNA, PFHxS, PFBA, PFBS, HFPO-DA (or GenX), HFPO-TA, OBS, 6:2 Cl-PFESA (or F53B), PFMOBA, PFMOPrA, PFOSA, AFFF mixtures	90	Mice, Rats, Zebrafish, <i>C. elegans</i>	Oral (gavage, diet, water) Injection Waterborne	Suppression of IgM antibody response; Altered B/T cell subsets; NF- $\kappa$ B activation; $\uparrow$ TNF- $\alpha$ , $\uparrow$ IL-1 $\beta$ , $\uparrow$ IL-6, $\uparrow$ NLRP3 inflammasome activation; Gut microbiota-driven immune disruption
Hepatotoxicity	PFOA, PFOS, PFHxS, PFNA, GenX, HFPO-TA, Nafion BP2, F-53B, PFMO2HpA, PFMO3NA, PFO4DA, PFO5DoDA, OBS	127	Rats, Mice (wildtype, hPPAR $\alpha$ humanized), Zebrafish, Chicken embryos	Oral (diet, gavage, water) Injection Waterborne	Hepatomegaly, hepatic steatosis, fibrosis; Lipid accumulation; Alterations in PPAR $\alpha$ /PPAR $\gamma$ and AMPK/mTOR signaling; Bile acid metabolism



					disruption; Sex- and diet-dependent outcomes
Developmental toxicity	PFOS, PFOA, PFHxS, PFHxA, PFBA, PFBS, GenX, HFPO-TA, OBS, F-53B, Nafion BP2, PFOSA, PFUnDA, PFPeS	91	Zebrafish, Rats, Mice (including pregnant), Chicken embryos, <i>C. elegans</i>	Oral Waterborne Injection (air-cell)	Embryo/fetal growth restriction; Altered placenta, neonatal mortality; Mitochondrial dysfunction; Cardiac malformations and disrupted circadian rhythm; Delayed hatching; Steroid biosynthesis genes
Neurotoxicity	PFOS, PFOA, PFBS, PFHxS, PFHxA, ADONA, GenX, PFNA, PFHpS, 8:8 PFPiA	60	Mice, Rats, Zebrafish, <i>C. elegans</i>	Oral Waterborne Injection	Cognitive dysfunction; Dopaminergic/astrocytic damage; Microglial activation; Altered neurotransmitters (GABA, glutamate, dopamine); Seizures; Behavioural hypo-/hyperactivity
Endocrine disruption	PFOS, PFOA, PFHxS, PFHxA, PFBA, PFBS, GenX, F-53B, 6:2 FTCA, HFPO-TeA 6:2 FTSA, 8:8 PFPiA	109	Mice, Rats, Zebrafish	Oral Waterborne	Thyroid hormone imbalance ( $\downarrow$ T3/T4); Estrogen/androgen disruption; Altered kisspeptin and LH signalling; Insulin resistance; Disrupted steroidogenesis; Epigenetic regulation via DNA methylation changes
Metabolic disruption	PFOS, PFOA, PFHxS, PFHxA, PFBA, F-53B,	118	Mice, Zebrafish, <i>C. elegans</i>	Oral Waterborne Injection	Lipid/glucose metabolism dysregulation;



	GenX, HFPO-TA, HFPO-TeA, PFO5DoDA, PFO4DA, PFO3OA				Obesity-like phenotypes; Alterations in AMPK, PPAR, and mTOR pathways; Oxidative stress; Diet- and sex-dependent metabolic outcomes
Reproductive toxicity	PFOA, PFOS, PFHxS, GenX, HFPO-TA, HFPO-TeA, PFBS, OBS	67	Rats, Mice, Zebrafish, <i>C. elegans</i>	Oral Waterborne Injection	Reduced fertility; Abnormal gametogenesis, Changes in ovarian/ testicular histology; Hormonal disruption, impaired; Oocyte maturation; Abnormal embryonic implantation; Transgenerational effects
Carcinogenicity and tumour promotion	PFOS, PFOA, GenX	53	Mice, Zebrafish (including transgenic lines in both species)	Oral Waterborne	PFOS promoted liver cancer progression in Kras transgenic zebrafish; Oxidative stress and metabolic reprogramming; Gene expression associated with tumorigenesis
Renal toxicity	PFOS, PFOA	23	Rats, Mice	Oral Intraperitoneal injection	Histopathological renal damage; Tubular epithelial apoptosis; Nephrotoxicity: ↑ serum urea nitrogen, ↑ Cx43 expression



Cardiotoxicity	PFOA, PFOS, GenX	32	Mice, Chicken embryos	Oral, air cell injection	Myocardial injury and cardiac fibrosis; Arrhythmias; Mitochondrial dysfunction; Embryonic cardiotoxicity in chicken and zebrafish models
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<sup>a</sup>Many studies report multiple endpoints and are included in the count in multiple categories.

### 3.3 *In vitro* studies

We combined human *in vitro* evidence from both conventional cell lines and advanced human-relevant systems to highlight toxicological effects of PFAS. The studies highlighted in the results narrative were selected as representative outcomes across diverse *in vitro* models and PFAS types, reflecting recurring findings. Table 4 illustrates these effects across (human) tissues, PFAS used, and experimental designs; the full list of studies presented in Supplementary Table S4.

HepG2, HepaRG, and primary hepatocytes are most common liver models that frequently exhibit lipid dysregulation, stress signalling, and liver inflammation. These include elevated triglycerides and cholesterologenic gene suppression,<sup>63</sup> disruption of bile acid metabolism,<sup>64</sup> unfolded-protein response with steatosis/fibrosis features,<sup>65</sup> and transcriptional programs involving nuclear receptors.<sup>66–68</sup> Higher potency for longer-chain PFCAs in liver spheroids is indicated by mixture experiments, which also show oxidative-stress/ Nrf2 activation along with additive effects in transcriptomic points.<sup>69–71</sup>

Many PFAS (including PFOS, PFHxS, PFNA, PFDA, PUnDA, PFOS precursors, novel sulfonic PFAS) inhibited the human sodium-iodide symporter in engineered HEK293 cells,<sup>72</sup> while structure-dependent uptake has been demonstrated through CD36 in kidney-derived cells.<sup>73</sup> Both legacy (e.g. PFOS, PFOA, PFNA) and alternative PFAS (e.g. 6:2 FTS, GenX) reduced migration/ invasion in placental trophoblast models or changed stress signalling and gene expression.<sup>74–77</sup> In primary lymphocytes, the reduction of T-cell/ NK cells activation at very low PFAS doses<sup>78</sup> and the disruption of cytokines<sup>79</sup> are among the notable immune endpoints.

Pro-carcinogenic and epigenetic alterations (e.g., DNA methylation or histone modification) were observed in breast models, including MCF-10A and MCF-7 cell lines.<sup>80–82</sup> Human sperm and



granulosa, epithelial ovarian, and endometrial cells exhibited additional endocrine and reproductive effects.<sup>83–86</sup> However, one study assessing the effects of the new-generation PFAS C6O4 (perfluoro{acetic acid, 2-[(5-methoxy-1,3-dioxolan-4-yl)oxy]}, ammonium salt) on normal human thyroid cells did not report any adverse outcomes under the tested conditions<sup>87</sup>. Pancreatic  $\beta$ -cells showed ROS-mediated apoptosis with reduced secretion, which was mitigated by antioxidant/ PKA-activating treatments.<sup>88</sup> Neuronal and airway models also reported apoptosis associated with oxidative-stress, barrier dysfunction, and inflammatory signalling.<sup>89–93</sup>

Advanced human-relevant systems such as liver organoids, liver-on-a-chip systems, and animal primary liver spheroids showed pathway-level response to short-chain (e.g., PFBS, HPFO-DA) PFAS at low concentrations (1nM to 1 $\mu$ M),<sup>94</sup> bile-acid disruption, and chain-length dependent potency.<sup>71,95,96</sup> Mechanistic insights were also provided by other specialized models such as neuronal co-cultures (primary rat cells with hiPSCs), where PFOS and PFOA changed GABA<sub>A</sub> receptor activity<sup>97</sup> and mouse embryonic stem cell-derived cardiomyocytes that revealed PFOS-induced disruption of autophagy-lysosome function.<sup>98</sup>

**Table 4.** Representative human *in vitro* of PFAS exposure presenting recurring effects across tissues, PFAS diverse types, and platforms (cell lines, primary cells, and organoids).

Cell Line(s)	Human Tissue Type	PFAS used	Concentration and Time of Exposure	Main Effects	Study
HepaRG and HepG2 (reporter)	Hepatocellular Carcinoma	PFOA, PFOS	PFOA: $\leq 500 \mu\text{M}$ (24 h), $\leq 250 \mu\text{M}$ (48 h); PFOS: $\leq 100 \mu\text{M}$ (24 h), $\leq 50 \mu\text{M}$ (48 h)	Down-regulation of cholesterol/bile-acid genes; Strong CYP7A1 suppression; At 24 h: PFOS (100 $\mu\text{M}$ ) $\uparrow$ cholesterol in supernatant; PFOA $\downarrow$ intracellular cholesterol	Behr et al. <sup>64</sup>
HepG2	Hepatocellular Carcinoma	PFOA	0 to 1000 ng/mL (0–2.4 $\mu\text{M}$ ) for 24h	Insulin-stimulated glycogen synthesis reduced even at 0.1 ng/mL PFOA; glucose uptake blunted $\geq 10$ ng/mL	De Toni et al. <sup>99</sup>
HepaRG and HepG2	Hepatocellular Carcinoma	PFOA, PFBA, PFTA	10–1000 nM for 48h	Increase in ROS production and in expression of TNF $\alpha$ and IL-6; Upregulation of steatosis and fibrosis by activation of UPR pathway	Qi et al. <sup>65</sup>



HepaRG and HEK293	Hepatocellular Carcinoma and Embryonic Kidney	10 PFAS and PFAS mixtures (PFOS, PFOA, PFNA, PFHxS)	5mM for 72h, except PFOS, PFOA, PFNA, PFDA (up to 250µM)	Increased triglyceride accumulation after individual PFAS and PFAS mixtures exposure; steatosis; PPARα activation and up- or downregulation of PPARα target genes (e.g., <i>PLINK2</i> , <i>ADH4</i> , respectively)	Sadrabadi et al. <sup>100</sup>
Huh-7 and HEK293	Hepatocellular Carcinoma and Embryonic Kidney	6:2 CI-PFESA	3–10 µM for 72h and for 24h (luciferase reporter assays)	Dose-dependent lipid accumulation and increased triglycerides; Upregulation of ACOX1 expression and peroxisome fatty acid β-oxidation; Suppression of hsa-miR-532-3p	C. Li et al. <sup>101</sup>
MCF-7 and HepG2	Breast Tumor and Hepatocellular Carcinoma	PFOA	100 and 200 µM 200 and 400 µM	DNA methylation alterations in heterochromatin organization (H3K9me3-post translational modification, target)	W. Liu & Irudayaraj <sup>80</sup>
MCF-10A	Breast Epithelium	PFHxS PFHxA GenX PFO2OA HFBA PFBS	500 pM to 500 µM for 72h	PFHxS induced epithelial cell proliferation, and promoted the migration and invasion potential, by altering cell-cycle regulators, adhesion proteins and histone modifications; PFHxA, HFBA, PFBS and PFO2OA all seem to be safer alternatives	Pierozan et al. <sup>102</sup>
		Binary PFOS and PFOA mixture	100 pM–100 µM for 72h	Cell proliferation mediated by PXR activation, an increase in cyclin proteins levels, decrease in p21 and p53 levels; Alterations in histone modifications; Exposure to higher concentrations of the mixture caused cell death	Pierozan et al. <sup>82</sup>
DU145 and MCF-7	Prostate and Breast Tumors	PFOA	dilutions from 10 <sup>-6</sup> to 10 <sup>-12</sup> M	Increase proliferation of cancer cells; PFOA possible carcinogen	Charazac et al. <sup>103</sup>



HCT116	Colon Colorectal Carcinoma	PFOS	0.001–10 $\mu\text{M}$ for 48h	Increased proliferation and migration of CRC cells via PI3K/Akt-NF- $\kappa$ B activation and EMT; Upregulation of VEGF, IL-8	F. Li et al. <sup>104</sup>
JEG-3	Placenta Choriocarcinoma	42 PFAS	50– 500 $\mu\text{M}$ the duration of exposure depending on the endpoint evaluated	The placenta may be a direct target of PFAS exposure, with trophoblast cell gene expression and function being disrupted at PFAS levels significantly lower than the calculated cytotoxicity threshold ( $\text{EC}_{50}$ )	Blake et al. <sup>75</sup>
BEAS-2B	Bronchial Epithelium	PFOS, PFOA mixture of PFOA, PFOS, PFBA, PFBS, and GenX	1–500 $\mu\text{M}$ for 24h 1–1000 $\mu\text{M}$ for 24h	PFOA and PFOS alone or in a mixture activate the NLRP3 inflammasome; Combined with <i>in vivo</i> study in mice: inflammation- and immune-related genes differentially expressed; Asthma/airway hyper-responsiveness	Dragon et al. <sup>89</sup>
hBMSCs	Bone marrow	HFPO-DA, HFPO-TA	0.1–10 $\mu\text{M}$ for 7–21 days	HFPO-TA promoted abnormal proliferation at 1 $\mu\text{M}$ , while higher doses downregulated CDK and CASP expression; Both HFPOs repressed MSC stemness markers, and impaired osteogenic differentiation; Carcinogenesis-related signalling disruption	Pan et al. <sup>105</sup>
Primary Human Lymphocytes	Peripheral Blood Lymphocytes	PFOS	50 $\mu\text{M}$ for 72h	Interleukins (IL-1, IL-4, IL-6 and IL-8) induction after PFOS treatment; Alterations in lymphocyte functions and lymphocytic system through cell signalling; Changes in differentiation and migration of antigen presenting cells	R. Li et al. <sup>79</sup>



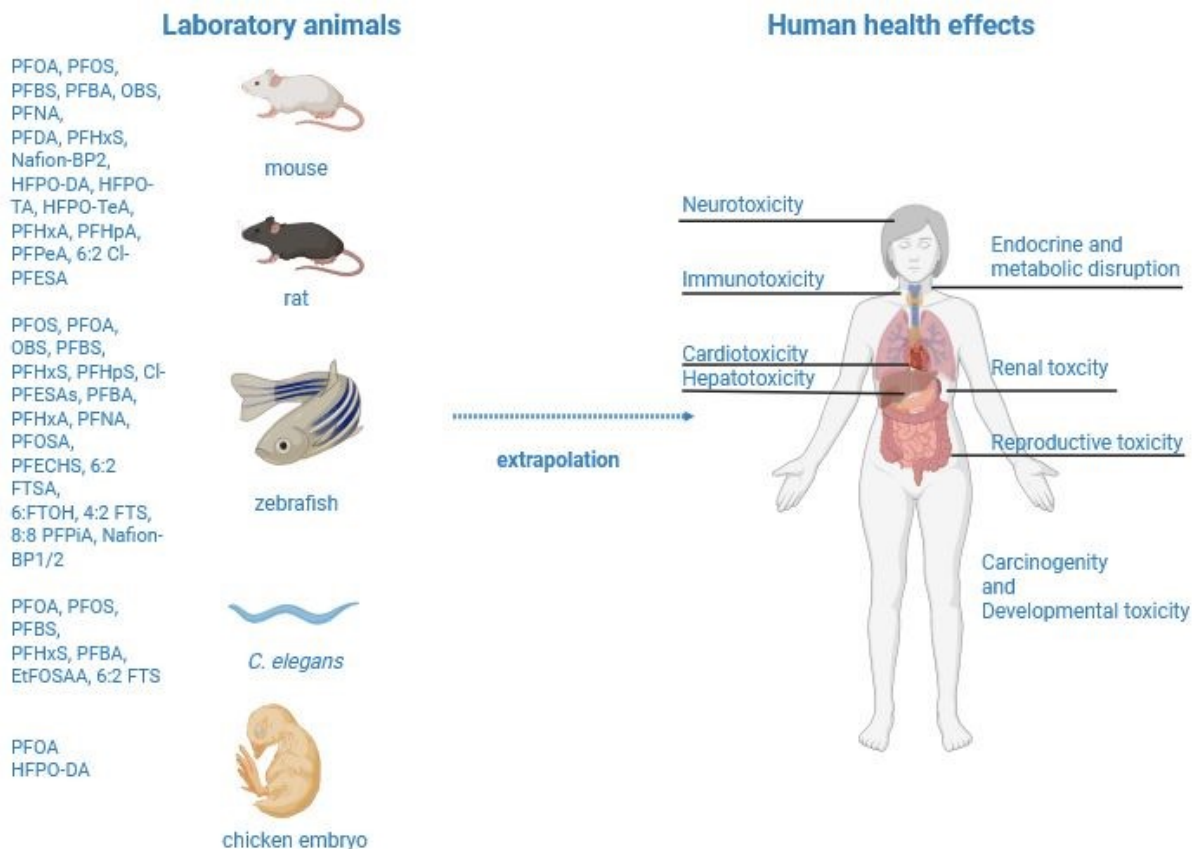


Astroglia SVG p12 cells	Foetal Brain	PFOA	10-200 $\mu\text{M}$ for 24, 48 or 72h	10 $\mu\text{M}$ PFOA: elevated glutamate levels, while 80 $\mu\text{M}$ PFOA: $\uparrow$ expression of S100B, tachykinin, and CYP1A1 $\rightarrow$ neurotoxicity	Osemwegie et al. <sup>106</sup>
RTCs	Renal Tubular Epithelium	PFOS	100 $\mu\text{M}$ for 24h	Increased cytosolic ROS and PFOS- mediated apoptosis oxidative stress and renal toxicity	L.-L. Wen et al. <sup>107</sup>
Liver-on-a-chip (HepaRG + HMEC-1 + THP-1)	Liver (hepatocytes, endothelial, macrophage co-culture)	PFBS, PFHxA, HFPO-DA, PFHxS, 6:2 FTOH	1 nM and 1 $\mu\text{M}$ for 48 h	Viability largely unaffected (except $\downarrow$ at 1 $\mu\text{M}$ 6:2 FTOH); Gene expression changes: $\uparrow$ CYP1A1, $\downarrow$ CYP1A2; PFAS-induced oxidative stress and AHR/CAR pathway activation	Solan et al. <sup>94</sup>

**Legend:** AHR/CAR: aryl hydrocarbon receptor/ constitutive androstane receptor; CASP: caspase genes; CDK: cyclin-dependent protein kinase EMT: epithelial-mesenchymal transition; HEK293: cell line isolated from kidney of a human embryo; HepaRG: derived from a female patient with hepatocellular carcinoma, but they exhibit characteristics of both hepatocytes and biliary epithelial cells; MCF-7: human breast cancer cell line with estrogen, progesterone and glucocorticoid receptors; MCF-10A: a non-tumorigenic breast epithelial cell line with stem cell properties; Primary human lymphocytes were isolated from 30 human donors; male: female= 1:1; RTCs: renal tubular cells; S100B: astrocytes biomarker; Tachykinin: inflammatory marker; TNF: tumour necrosis factor; UPR: unfolded protein response; VEGF: vascular endothelial growth factor.

After cross-referencing the data collected from both *in vivo* and *in vitro* studies, the main toxic effects that were supported by several research studies and are potential health outcomes in humans, are presented in Figure 5.





**Figure 5.** A summary of human health effects of PFAS derived from laboratory animals' studies and supported by *in vitro* assessments on human cell lines. The main model species (left) and the highlighted adverse outcomes (right) are depicted. Created with "BioRender.com".

### 3.4 *In silico* studies

Most of the *in silico* studies meeting the inclusion criteria were either coupled with *in vitro* assays within the same research article or formed part of the authors' ongoing research. Machine learning and deep learning approaches, high-throughput screening methods, molecular dynamics simulations and development of QSAR models are the main *in silico* methods found in PFAS toxicity papers. Several studies utilizing *in silico* approaches combined them with *in vitro* methods either for data acquisition or validation purposes. The latter are summarized in Table 5; the papers where only *in silico* methods were applied are presented in Table 6.



**Table 5.** *In silico* approaches combined with *in vitro* studies for PFAS toxicity prediction.

Study	Dataset	<i>In silico</i> tools and combined <i>in vitro</i> methods	Endpoints
Hoover et al. <sup>108</sup>	PFOS, PFOA, PFHxS, PFHxA (testing their cytotoxicity) External dataset of 24 single PFAS and 1,380 binary mixtures	MTT assay for cell survival (in vitro toxicity test) QSAR modelling: 195 1D and 2D descriptors 3 division techniques with MLR and GA utilized to improve the precision of QSAR models	IC <sub>50</sub> of single-chemicals and of the mixtures converted into a negative logarithmic molar scale ( <i>pIC</i> <sub>50</sub> )
J. Li et al. <sup>109</sup>	PFBA, PFBS, PFHxA, PFHxS, PFOA, PFOS, PFNA, PFDA, PFPeA, PFPPA	MVLN cell assay for defining estrogen receptor (ER) agonists/ antagonists; EADock2 docking calculation and MD simulation; QSAR model (-log EC <sub>50</sub> of luciferase activity) based on molecular connectivity indices (MCIs) and solvation connectivity indices of the tested compounds	MCF-7 cells (human breast cancer cell line); Binding affinity of PFAS in the ER; Binding stability of PFOS; Antiestrogenic activity of PFAS
Massarsky et al. <sup>110</sup>	Cytotoxicity data for human liver HepG2 cells for PFOA and PFOS from ToxCast's Attagene, Apredica, and Tox21/NCGC datasets	<u>ToxCast</u> - <u>Reactome</u> (Pathway analysis) framework corresponded to potential human or animal hazards; Method evaluation (score ratios, scores and the relationship between them)	Dose-response and cell viability; (AC <sub>50</sub> values and 'active'/ 'inactive'); Genes potentially affected upon exposure to PFOA or PFOS; Biological pathways
C. Zhao et al. <sup>111</sup>	17 PFAS with various chain- lengths	Inhibitory potency ( <i>in vitro</i> assay), Docking similarity analysis and correlation analysis of molecular structures (calculated by absolute value after subtracting the molecular length of cortisol) of PFAS with IC <sub>50</sub> values	Human placental and rat renal 11β- HSD2 (11β-hydroxysteroid dehydrogenase 2); IC <sub>50</sub> of PFAS





Enyoh et al. <sup>112</sup>	PFNA, PFHxA, PFDA, PFOS, PFBS, PFOA	Docking on selected binding pockets of secretory Immunoglobulin A (SIgA) of human breast milk; Fractional factorial design for evaluation of toxic effects	Binding affinity to SIgA; Inhibitory constant (K <sub>i</sub> ); Relative toxicity factor (RT <sub>f</sub> ) and index (RT <sub>I</sub> )
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**Legend:** EC<sub>50</sub>: concentration causing 50% cell death; GA: genetic algorithms; IC<sub>50</sub>: half-maximal inhibitory concentration and for cytotoxicity IC<sub>50</sub> is the concentration causing 50% cell death; MLR: multiple linear regression; MLVN cell assay: based on the human breast cancer cell line MCF-7 BUS stably transfected with the luciferase reporter gene and estrogen-responsive element derived from the Xenopus vitellogenin A2 gene; MTT: measurement of activity of mitochondria as a means of assessing the number of living cells; QSAR: quantitative structure-activity relationship model.

**Table 6.** *In silico* approaches investigating PFAS toxicity.

Study	Dataset	In silico tools	Endpoints
Feinstein et al. <sup>113</sup>	8,163 EPA-listed PFAS compounds, including fluorinated chemicals with two or more C-F bonds	Random forests, Deep Neural Networks (DNN), Graph Convolutional Networks (GCN), and Gaussian processes; Molecular descriptors (Mordred, ECFP, GCN)	Acute oral toxicity — LD <sub>50</sub> of PFAS compounds (predicted); Model confidence
Singh & Hsieh <sup>25</sup>	23 major PFAS with sufficient ToxCast data	Toxicological prioritization of selected chemicals with visual analytics tool, ToxPi. A numerical chemical-assay matrix generated and inputted into ToxPi for holistic visualization of activity.	Oxidative stress indicators, inflammatory markers, receptors (i.e., AR, PPARα) and cell cycle markers
Lai et al. <sup>114</sup>	EPA PFAS Master List (7,751 molecules)	Molecular generation to expand EPA Master List with JTVAE model; 36 molecules used for small descriptor correlation set, (GenX as reference substance); 1,826 descriptors generated for each molecule; Molecular	268,558 PFAS molecules generated; Binding affinity to hPXR and PPARγ and complex stability; Free energies; CMC

L. Li et al. <sup>115</sup>	165 suspected PFAS, 19 novel PFAS identified	docking and molecular dynamics simulations for the complex PFAS-hPXR and PFAS-PPAR $\gamma$ Tox21-GCN model (Graph Convolutional Neural Network, DeepChem platform); GenRA model (EPA); OECD bioactivity model	Nuclear receptor responses (ER, PPAR $\gamma$ ); Stress responses; Predicted hepatotoxicity, chronic and developmental toxicity
Azhagiya Singam et al. <sup>116</sup>	5,206 PFAS	Identification of 14 PFAS that are predicted to interact strongly with vitamin D receptor (VDR) by using molecular docking, molecular dynamics, and free energy binding calculations	Binding affinity to VD; Potentially producing harmful effects on the immune, endocrine, and bone homeostasis
Dawson et al. <sup>117</sup>	11 PFAS using published data experimentally collected from 4 species	Prediction of descriptors of $t_{1/2}$ using OPERA modelling platform; <sup>118</sup> Model development using R caret package; <sup>119</sup> Characterization of predicted chemicals	Half-lives; Renal elimination; Whole-body clearance; Steady-state concentration
Dharpure et al. <sup>120</sup>	15 short-chain and 16 long-chain PFAS retrieved from PubChem and literature	Molecular docking (AutoDock Vina); Binding energy analysis; Amino acid substitution modelling; Sequence alignment	Binding affinity to transthyretin (TTR); Thyroid hormone disruption potential; Interspecies toxicity prediction
Gallagher et al. <sup>121</sup>	24 PFAS	Classification-based and regression-based QSAR models; Generalized Read-Across (GenRA) to predict toxic outcomes	EC <sub>50</sub> for binding affinity to human serum albumin (HSA); Chronic to sub-chronic toxicity to HSA

Note: AR: androgen receptor; CMC: critical micelle concentration, defined as the surfactant concentration at which micelle formation is first seen in solution;<sup>122</sup> JTVAE: junction tree variational autoencoder; QSAR: quantitative structure- activity relationships; ToxPi: toxicological prioritization index.



### 3.5 Epidemiological studies

We incorporated 69 epidemiological studies encompassing a diverse range of age groups, geographical areas, and exposure contexts, including only four that focused on occupational environments.<sup>123–126</sup> This wide inclusion illustrates the prevalence of PFAS exposure worldwide and supports the increasing human environmental impact. A notable portion of these studies are derived from regions with high exposure, such as the Veneto Region in Northern Italy—one of the most heavily PFAS-polluted areas worldwide.<sup>83</sup> Chinese populations are also considered highly exposed as reported in the Isomers of C8 Health Project<sup>127</sup> and Shanghai Birth Cohort.<sup>128</sup> Moreover, considerable PFAS levels were detected in general population-based cohorts such as NHANES national (U.S.) survey, or the CHARGE study, highlighting the severity of the problem globally.

Four studies of “Isomers of C8 Health Project in China” are included, establishing the connection between PFOA and PFOS exposure and human health effects, such as nephrotoxicity<sup>129</sup> and hepatocellular dysfunction,<sup>130</sup> highlighting the already discussed hepatotoxic effects of PFAS. These studies were also focused on CI- PFESAs alternatives (6:2 CI-PFESA and 8:2 CI-PFESA) that are mainly produced in China, indicating thyroid hormones alterations<sup>131</sup> and associations with metabolic syndrome.<sup>132</sup> However, longitudinal studies have been suggested in most cases as necessary for further validation and assessment.

Human studies also emphasize critical life stages, including the prenatal and neonatal periods. Adverse birth outcomes, such as low birth weight or preterm births,<sup>133–135</sup> neurodevelopmental issues,<sup>136,137</sup> and implications in inflammatory markers, adiposity or related biochemical pathways<sup>138,139</sup> were associated with exposure during pregnancy and infancy—through maternal serum, or placental transfer. Immunotoxic effects after early-life PFAS exposure were confirmed in children by decreased vaccine efficacy,<sup>140</sup> and immunomodulating effects on allergies and infectious diseases.<sup>141</sup> Another study involving children and adolescents corroborated the immunotoxicity of PFAS, revealing that the mixture of PFNA and PFHxS or PFOS alone were associated with higher risk of common cold.<sup>142</sup> These effects are similar to the developmental pathways and immunotoxicity observed in animal models and *in vitro* studies. Additionally, a follow-up research in a pregnancy cohort reported that maternal PFAS levels were associated with increased risk of breast cancer in daughters, particularly when high levels of EtFOSAA—a PFOS precursor—coincided with high cholesterol, indicating that maternal PFAS exposure may



affect long-term cancer susceptibility.<sup>143</sup> Nevertheless, a study, in adolescents and adults, showed no association between serum PFAS concentrations and antibody response after COVID-19 vaccination.<sup>144</sup>

In adults, PFAS have been associated with a range of systemic effects and hormone-related health outcomes. Several studies have reported high risks of cancer, including breast,<sup>145,146</sup> ovarian,<sup>145</sup> and kidney cancers,<sup>147,148</sup> linked to specific PFAS, such as PFOA, PFHxS, and PFNA. Nephrotoxicity has also been supported by correlations between PFAS exposure and kidney biomarkers, suggesting also that urine may serve as a useful matrix for assessing such exposures.<sup>126</sup> Reproductive toxicity was also found to be associated with PFAS exposure both in males and females: in young men, long-chain PFAS (e.g., PFTTrDA, PFDoA) were inversely associated with Leyding cells' function, while PFBS was linked to increased levels of reproductive hormones;<sup>149</sup> in women, exposure to perfluorododecanoic acid (PFDoA) was correlated with an elevated risk of infertility due to polycystic ovarian syndrome.<sup>150</sup> Additionally, PFAS have been linked with metabolic disturbances, as Donat-Vargas et al.<sup>151</sup> identified that long-term exposure can lead to reduced insulin resistance, whereas Schillemans et al.<sup>152</sup> reported opposing associations between PFAS and two different glycerolipids regarding the risk of type 2 diabetes. Similarly, another study revealed mixed associations: while most PFAS were inversely associated with risk of type 2 diabetes, PFOA was positively associated with fasting glucose and several other PFAS (e.g., PFNA, PFUnDA, PFOS, and 6:2 Cl-PFESA) were linked to high cholesterol levels, suggesting PFAS exposure as potential diabetogenic factor.<sup>153</sup> Increased cholesterol levels were found even at lower concentrations.<sup>154</sup> Finally, the potential inverse correlation between PFAS and Crohn's disease,<sup>155</sup> highlights the complexity of PFAS toxicodynamics and the need for further research. Table 7 summarizes representative epidemiological studies that highlight the extensive distribution of PFAS and related compounds, as well as their health impacts. Detailed information on all the epidemiological studies included is presented in Supporting Information Table S6.

**Table 7.** Representative epidemiological studies, their population context, PFAS focus, and health endpoints and associations.

Study	Study Design and Population	PFAS detected	Outcomes <sup>a, b</sup>	Key Findings/ Association
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Jain & Ducatman <sup>156</sup>	C-S, U.S general adult population (NHANES 2011-2014), <i>n</i> = 2883 (1801 nonobese, 1082 obese, BMI ≥ 30)	PFOA, PFOS, PFNA, PFHxS, PFHpA, PFDA,	Liver function biomarkers: alanine aminotransferase (ALT), aspartate aminotransferase (AST), $\gamma$ -glutamyl transferase (GGT)	Positive associations of PFOA, PFHxS, PFNA with ALT and GGT, but only in obese participants. PFOS showed no association. Link between PFAS and hepatic steatosis
Shin et al. <sup>157</sup>	Population-based C-C mother-infant pairs ( <i>n</i> =453; 239 ASD cases, 214 typical development, controls)	PFOS, PFOA, PFHxS, PFNA	Lowest and highest quartile of modelled prenatal maternal PFAS; ORs with 95% CI of child diagnosis of ASD	Prenatal maternal PFHxS and PFOS were associated with increased risk of child diagnosis of autism spectrum disorder (ASD)
Tsai et al. <sup>146</sup>	C-C, 120 breast cancer cases and 119 controls (25-80 years old women) in Taiwan	PFHxS, PFOA, PFOS, PFNA, PFDA, PFUnDA	Breast cancer with stratification by age and estrogen receptor (ER) status; ORs with 95% CIs risk of ER <sup>+</sup> or ER <sup>-</sup> tumours	PFOS was associated with increased breast cancer risk in women ≤50 years; PFHxS and PFOS were associated with high risk of ER <sup>+</sup> tumours in women ≤50 years; PFNA and PFDA were inversely associated with ER-tumours in young women
Skogheim et al. <sup>158</sup>	Nested C-C, children from MoBa cohort ( <i>n</i> = 821 ADHD cases, <i>n</i> = 400 ASD, <i>n</i> = 980 controls)	PFOA, PFNA, PFDA, PFUnDA, PFHxS, PFOS	ADHD and ASD diagnosis from health registry; Adjusted ORs across quartiles, spline models, and PFAS mixtures.	Prenatal exposure to PFOA, associated with high risk of ADHD and ASD; Inverse U-shaped association for PFOA with ASD; Mixture analysis showed inverse associations between PFAS mixture and ASD.
Ku et al. <sup>159</sup>	P birth cohort ( Taiwan Birth Panel Study II), <i>n</i> = 486 pregnant women (2004-2005); The lowest quartile served as the	PFOS, PFOA, PFNA, PFUA	Methylation levels at 5 CpG sites in MEST promoter (cord blood), infant birth weight	Higher prenatal PFOS levels were associated with lower methylation of the MEST gene Lower methylation was in turn associated with reduced birth





	control/reference group			weight. MEST methylation partially mediated the PFOS- birth weight association; Stronger associations in girls
Qu et al. <sup>160</sup>	C-C, 156 rheumatoid arthritis (RA) cases and 156 matched healthy controls	PFOA, PFOS, PFNA, PFDA, PFUnA, PFDoA	RA diagnosis based on 2010 ACR criteria; Rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibody (ACPA) levels.	PFOA associated with increased RA risk and positively correlated with RF and ACPA levels; No associations for other PFAS
H. Li et al. <sup>161</sup>	C-C, 355 newly diagnosed acute coronary syndrome (ACS) cases and 355 age- and sex-matched controls in Shijiazhuang Hebei, China	PFOA, PFOS, PFNA, PFHxS, PFUnDA, PFDA, PFBA	ORs with 95% CI for associations between PFAS and ACS risk (single and multi-PFAS models); PFAS mixtures and ACS (qgcomp analysis); Platelet-related parameters	PFOA and PFOS were associated with increased ACS risk; PFUnDA also showed positive association. PFHxS and PFDA were inversely associated. No overall effect from PFAS mixtures; Associations were significant mainly in males
Siwakoti et al. <sup>162</sup>	Nested C-C, pregnant women ( <i>n</i> = 501; 128 preterm cases, 373 term controls) from LIFECODES cohort	PFOA, PFNA, PFDA, PFUA	Preterm birth (overall, spontaneous, placental), BW Z-score, small for gestational age (SGA), large for gestational age (LGA)	PFNA, PFDA, PFUA associated with higher odds of placental preterm birth, especially in males; PFDA and PFOA associated with higher odds of LGA in females
Tian et al. <sup>135</sup>	C-C, women ( <i>n</i> = 251) with preeclampsia ( <i>n</i> = 82) and healthy ( <i>n</i> = 169) participants from Hangzhou, China	15 PFAS	Maternal serum PFAS concentrations (OR, 95% CI); Systolic and diastolic blood pressures (mmHg). Risk of low birth weight (LBW) and SGA in women with preeclampsia	PFOA and 6:2 Cl-PFESA were positively associated with preeclampsia and foetus development (LBW/SGA)



van Gerwen et al. <sup>163</sup>	Nested C-C, 88 patients with thyroid cancer (cases) and 88 healthy controls in the BioMe population	8 PFAS	ORs and 95% CIs of log <sub>2</sub> -PFAS; ORs per increase in IQR; Logistic Regression (Wald Test), p- values for association with thyroid cancer diagnosis	PFOS associated with increased rate of thyroid cancer
Y. Chen et al. <sup>164</sup>	Population-based C-C Children born in California aged ≤5 years at diagnosis; 501 retinoblastoma (cases) and 899 controls	PFOS, PFOA, PFNA	Means, SDs, medians, IQRs for detected PFAS in dried blood spots. ORs and 95% CI for neonatal PFAS exposure and retinoblastoma	PFOS and PFOA might contribute to retinoblastoma risk in children born in California

**Legend:** 95% CI: 95% confidence interval; ADHD: attention-deficit/hyperactivity disorder; ASD: autism spectrum disorder; BioMe: a medical record-linked biobank at the Icahn School of Medicine at Mount Sinai in New York; BW: birthweight; CHARGE: CHildhood Autism Risk from Genetics and Environment; IQR: interquartile range; MoBa: Norwegian Mother, Father and Child Cohort Study; OR: Odds ratio; SD: standard deviation.

Type of study design: C: Cohort; C-C: Case-Control; C-S: Cross-Sectional; RCT: Randomized Control Trial.

<sup>a</sup> Studies during pregnancy and mother-infant pairs studies were adjusted for: newborn sex, child's birth year, parity, maternal age, pre-pregnancy body mass index (BMI), smoking during pregnancy, socioeconomic status, maternal ethnicity, birthplace and schooling, and breastfeeding duration.

<sup>b</sup> Studies in adult populations were adjusted for: sex, age, race, BMI, smoking, alcohol drinking, education, and socioeconomic status.

### 3.6 Relative Concentrations and Comparison among Study Types

*In vivo* studies frequently use PFAS concentrations that are higher than the environmentally relevant levels. For example, drinking water exposures closer to environmental levels (1-100 ng/L for PFOA, PFOS, PFBA, and PFBS) resulted in more subtle endocrine changes,<sup>165</sup> while oral PFOA exposures of 1-20 mg/kg/day in mice (≈25-500 μM serum equivalents) caused liver damage, lipid dysregulation, inflammation, and reproductive toxicity.<sup>166-168</sup>



Concentrations in *in vitro* systems varied widely, from nanomolar to hundreds of micromolar. In line with *in vivo* liver outcomes, HepaRG cells treated to 20  $\mu\text{M}$  PFOA or PFOA showed impaired cholestatic potential and bile acid metabolism<sup>64</sup>. In contrast, HepG2 cells exposed to quantiles closer to human serum levels (0-1000 ng/L;  $\sim$  0-2.4  $\mu\text{M}$  PFOA) showed reduced glucose uptake and glycogen synthesis.<sup>99</sup> Evans et al.<sup>169</sup> revealed receptor activity in the low micromolar range, with PPAR $\alpha/\gamma$  activation being reported at 1-10  $\mu\text{M}$  for PFOA, PFNA, PFOS, HFPO-DA, and NBP2. Pro-inflammatory signalling alterations were reported at 10-1000 nM (PFOA and HFBA) in a more recent study with hepatocytes,<sup>65</sup> indicating that certain mechanistic changes occur well within the range observed in exposed human populations.

Human internal serum concentrations are generally lower, though they may differ by exposure environment. Chinese chemical workers had mean serum concentrations of 5.43  $\mu\text{g/mL}$  ( $\sim$ 10.8  $\mu\text{M}$ ) and urinary levels of 201 ng/mL ( $\sim$ 0.48  $\mu\text{M}$ ) for 23 PFAS congeners, while local residents' urine had only 6.18 ng/mL ( $\sim$ 0.015  $\mu\text{M}$ ).<sup>126</sup> Notably, Australian firefighters joining the service after AFFF (PFOS-based) replacement had serum PFOS levels similar to general population, while firefighters who had previously exposed to AFFF had increased serum PFOS (27 ng/mL;  $\sim$ 54nM) and PFHxS (14 ng/mL;  $\sim$ 27nM), with half-lives of 5–7.8 years.<sup>170</sup> The highest levels were found in occupational cohorts from Veneto PFAS production facilities, with maximum PFOA concentrations above 13,000 ng/mL ( $\sim$ 30  $\mu\text{M}$ ) and median concentrations about 80 ng/mL ( $\sim$ 0.19  $\mu\text{M}$ ).<sup>123</sup> A mortality study in the same facilities documented similar exposure levels, with a geometric mean of 4048 ng/mL ( $\sim$ 9.7  $\mu\text{M}$ ) PFOA, which was associated with diabetes, cirrhosis and liver cancer.<sup>125</sup> However, the general population in the Veneto region reported comparable levels, with young adults having 2  $\mu\text{M}$  serum PFOA, significantly associated with lipid alterations.<sup>154</sup>

NHANES-based analyses, including general population outside of the occupational settings, frequently reported widespread but lower background exposure. According to Bulka et al.<sup>171</sup> the median levels of PFOA and PFOA in American adults were around 1.6 ng/mL ( $\sim$ 4 nM) and 4.5 ng/mL ( $\sim$  9 nM), respectively. Similarly, Omoike et al.<sup>145</sup> showed median PFHxS concentrations of 1.1 ng/mL ( $\sim$ 4 nM), while Jain and Ducatman<sup>156</sup> showed that PFAS were detectable in almost all participants, confirming persistence.

The broad range of PFAS concentrations—from nanomolar (nM) levels in the general population to micromolar ( $\mu\text{M}$ ) levels in occupational environments—is highlighted by these data collectively,



underscoring the challenge of aligning toxicological studies with real-world human exposures. In addition to these biomonitoring comparisons, Supplementary Table S5 consolidates toxicological results for key alternative PFAS congeners compared to legacy compounds (PFOA, PFOS), highlighting similarities in hepatotoxicity, immunotoxicity, and developmental effects despite variations in bioaccumulation capacity.

## 4. Discussion

### 4.1 Interpretation of Key Findings, Species Differences, and Context

The present scoping review, which combines experimental and epidemiological data, identified toxicological effects of PFAS, particularly legacy ones, PFOS and PFOA. Immunotoxicity and hepatotoxicity were the most frequently reported endpoints across all study types. However, responses vary by species and conditions, though. Specifically, rodent studies indicate that PFOA induces hepatotoxicity and lipid dysregulation through PPAR $\alpha$  activation,<sup>172</sup> whereas in humanized PPAR $\alpha$  mice the lipid outcomes are less pronounced,<sup>173</sup> reflecting interspecies toxicokinetic differences. These differences between rodents and humans, including faster renal clearance and shorter PFOA half-life, resulted into inconsistent lipid outcomes, and emphasizes how important the human-relevant models (e.g., humanized PPAR $\alpha$  mice) are for more accurate assessment of lipid and cholesterol alterations.<sup>174–176</sup> Peroxisome proliferation and liver hypertrophy are specific PPAR $\alpha$ -mediated effects mostly observed in rodents and are less applicable to humans, due to humans less susceptibility to this pathway according to the study of Corton et al.<sup>177</sup> Additionally, PFOS and PFOA can activate other nuclear receptors including PPAR $\gamma$ , CAR, and PXR, suggesting that several pathways may influence hepatic outcomes,<sup>178,179</sup> while the implication of various PPAR isoforms ( $\alpha$ ,  $\delta$ , and  $\gamma$ ) in PFAS-induced liver damage is further confirmed by short-chain alternatives of PFOA (e.g., PFHpA, PFHxA, and PFPeA).<sup>47</sup> An *in vitro* receptor-binding study by Evans et al.<sup>169</sup> supports that many PFAS—including HFPO-DA, NBP2, and other compounds—can activate both human and rat PPAR $\alpha$  and PPAR $\gamma$  isoforms, while another study in human mesenchymal stem cells reports promotion of adipogenesis via PPAR $\gamma$ .<sup>180</sup> These findings highlight that receptor binding is consistent, but the systemic outcomes differ by PFAS type and model species.

Another recurring endpoint was immune disruption, characterized among others by decreased lymphocyte responses, cytokine modulation, and NF- $\kappa$ B activation. Suppressed vaccine antibody production—a recognized biomarker of adaptive immune dysfunction—was frequently reported



in both human and animal studies.<sup>140,142,181</sup> Human epidemiological evidence has repeatedly indicated that higher PFAS serum levels are associated with decreased responses to routine vaccinations in children.<sup>140,182,183</sup> Two regulatory actions, EFSA's 2020 group tolerable weekly intake (TWI) and the NASEM 2022 clinical guidelines<sup>31,184</sup> are supported by this endpoint. Beyond antibody suppression, exposure to PFAS has led to alterations in T-cell proliferation, NK cell activity, and cytokine balance, suggesting a broader impact of adaptive and innate immunotoxic mechanisms.<sup>78,79</sup> These immune alterations can be considered molecular initiating events and key events (e.g., NF- $\kappa$ B activation, reduced antibody response), but they do not yet constitute fully validated AOPs in the [OECD AOP-Wiki](#). Accordingly, they should be interpreted as mechanistic hypotheses rather than established adverse outcome pathways.<sup>185</sup> Collectively, these results highlight immunotoxicity as one of the most recurring and conserved across species outcomes.

An important observation, based on *in vitro* (human liver cell lines) and *in vivo* models, is that the short-chained PFAS present similar toxicity profiles as their long-chain counterparts. This highlights an important risk, since short chain PFAS, including PFBS, 6:2 Cl-PFESA, and HFPO-DA (GenX), have shown hepatotoxic, endocrine, developmental, and immunological effects,<sup>42,101,186,187</sup> were developed as potentially safer alternatives to the legacy compounds. The developmental toxicity of these short-chained compounds was mainly studied in animal models (e.g., rodents and zebrafish) and the studies highlighted the multi-faceted effects at the organism level. These observations further strengthen the concerns raised about the systemic toxicity of short-chained PFAS. New cell culture techniques, like liver organoids and spheroids, or liver-on-a-chip, have provided a more robust approach to the study of PFAS toxicity and their potential mechanisms of action.

However, the direct correlation between the results obtained from the *in vivo* models and human biology can be misleading. The variability in protein expression levels between tissues and various cell types may impact the effects of PFAS. Moreover, the metabolic rates and consequently the exposure to PFAS may vary across individuals and is highly dependent on various factors, such as environment and social behaviour. Based on the analysis of the *in vivo* studies, PFAS effects on hepatotoxicity are the most studied endpoints with endocrine and metabolic disruptions following (Table 3). On the other hand, there are extensive gaps regarding the impact of PFAS on heart and renal function (Table 3) as the least studied effects. This observation correlates with the trends observed in the literature, where the most commonly reported effects involve the hepatic function and metabolic assessment. Moreover, the identified



data gaps in renal toxicity and cardiotoxicity are in agreement with a recent study by <sup>188</sup>, who reported that QSAR/ML-based PFAS toxicity models have focused mainly on hepatotoxicity and endocrine disruption endpoints. This suggests that other endpoints need to be addressed and thus expand our understanding of PFAS-related health effects.

The epidemiological studies, across various environments, have highlighted the extent of PFAS prevalence. However, most studies have focused on areas with high exposure levels, as the nature of epidemiological cohort studies is that they are often designed to assess impacts of a known exposure source or occupational activity.<sup>189</sup> Another reason is that high exposure may have more pronounced effects in the populations being observed and thus be more easily documented, making it easier to correlate toxicological outcomes (establish probable cause) with specific PFAS congeners (e.g. PFOA).<sup>190</sup> Although there has been extensive study of PFAS in both *in vitro* and *in vivo* models over the last decade, epidemiological studies have been focusing on the impact of PFAS in embryo development and their long-term exposure effects in children. These studies have highlighted the immunotoxicity of PFAS especially during early-life exposure and the various developmental effects.

#### 4.2 Limitations and Data Gaps

Existing evidence-maps, such as the PFAS- Tox Database and the EPA/EHP PFAS-150 project, have documented many studies, but without integrating findings across study types or harmonizing dose metrics.<sup>33,191</sup> By contrast, this ScR provides updated mechanistic background by limiting studies to those published between 2019-2023, reporting exposure concentrations (*Sections 3.3 and 3.6*) and explicitly comparing legacy with alternatives PFAS. However, several limitations were detected across the reviewed literature.

First, there are still several exposure mismatches. *In vitro* systems are usually tested in 1-100  $\mu\text{M}$  range, while *in vivo* experiments generally use higher oral dosages (e.g., up to 125 mg/kg HFPO-DA in rats.<sup>42</sup> These levels are higher than the average human biomonitoring results (<10  $\mu\text{M}$ , even in highly exposed groups). In epidemiological NHANES studies, the median serum levels of PFOS and PFOA in U.S. adults were approximately 4-5 ng/mL ( $\approx 9$  nM) and 1-2 ng/mL ( $\approx 4$ -5 nM), respectively.<sup>145,171</sup> In contrast, Veneto occupational cohorts had median levels of PFOA of 80 ng/mL ( $\sim 0.19$   $\mu\text{M}$ ), with a maximum of > 13,000 ng/mL ( $\sim 30$   $\mu\text{M}$ ).<sup>123</sup> Exposures in micromolar range (e.g., median serum PFOA at 1-6  $\mu\text{M}$ ) are further confirmed by historical occupational



datasets,<sup>192</sup> while more recent studies<sup>193</sup> reported comparable PFOS and PFOA concentrations (1-2  $\mu\text{M}$ ) in the serum of environmentally exposed population despite phase-out efforts.

Second, mixtures effects remain underexplored despite evidence that PFAS mixtures can produce either synergistic, cumulative or even antagonistic effects, usually distinct when compared to those of single compounds.<sup>194–196,69,71</sup> Even though humans are usually exposed to complex PFAS mixtures rather than single compounds, there is a lack of comprehensive mixture testing. This may be attributed to the limited knowledge of which mixtures should be studied and in which proportional concentrations. It is difficult to determine which compound combinations are most abundant in the various environments, while the issue of chronic low-dose exposure remains insufficiently investigated.

Third, data on alternatives is still inconsistent. While compounds like HFPO-DA (GenX) and 6:2 Cl-PFESA are increasingly investigated, only a small number of experimental datasets support other alternatives such as PFECAs (e.g., PFO5DoDA, PFMO2HpA, and PFMO3NA)<sup>197–200</sup> or PFPiAs (e.g., 8:8 PFPiA)<sup>57</sup> with variability in their reported toxicity. For example, Guo et al.<sup>198</sup> found that PFMO2HpA exhibited reduced hepatotoxicity compared to GenX, while PFMO3NA documented stronger hepatotoxic effects. A zebrafish study showed that of the PFECAs, PFO5DoDA caused greater thyroid and developmental toxicity than PFO4DA or PFO3NA.<sup>200</sup> Collectively, these findings demonstrate that toxicity varies significantly among alternatives, highlighting the necessity for thorough assessment across chemical subclasses—including the understudied ones—instead of presuming consistent safety among the derivatives. This comparative viewpoint (Supplementary Table S5) emphasizes that although certain alternatives, like PFBS or PFHxA, are less bioaccumulative, they still display similar toxicities to PFOS and PFOA, and substances such as 6:2 Cl-PFESA or NBP2 demonstrate emerging toxicological profiles similar to legacy ones.

Finally, variability in reporting still limits comparability. Numerous studies do not provide standardized dose-response data, consistent units (e.g., converted to  $\mu\text{M}$ ), or internal concentration assessments. In addition to the lack of harmonized reporting protocols, there is considerable variation observed in exposure levels across different environments (industrial, urban, and agricultural). These site-specific differences require extensive study to better assess minimal or safe exposure levels and to fully understand PFAS toxicity and their association with specific outcomes. Addressing these challenges will require uniform experimental protocols, consistent reporting metrics and the incorporation of computational toxicology methods like QSAR



modelling, toxicity prioritization indices (e.g., ToxPi), and AI-driven techniques such as ComptoxAI<sup>25,201</sup> to enhance experimental findings and enable systematic comparisons across studies. In this context, it is important to state that our review focuses on studies published between January 2019 and December 2023 (see also Table 2), which poses an important limitation. Moreover, as with many reviews, the study-screening strategy cannot fully preclude selection bias. Accordingly, some toxicity categories (notably carcinogenicity/tumour promotion) may be underrepresented for certain PFAS congeners, because relevant studies fall outside our timeframe and/or may not be captured by the predefined search string.

#### 4.3 Regulatory Implications and Future Perspectives

Regulatory frameworks are increasingly acknowledging the complexity of PFAS. For example, the U.S. EPA has established a Hazard Index MCL of 1 for combinations of two or more PFHxS, PFNA, PFBS, and HFPO-DA, specifically considering cumulative health risks,<sup>202</sup> while European Chemicals Agency (ECHA) is progressing with a detailed PFAS proposal under REACH, reflecting regulatory efforts at grouping PFAS instead of evaluating them separately<sup>7</sup>. This direction supports the idea that regulatory decisions are not based solely on chain length and functional groups (e.g., sulfonic moieties), that have been extensively proved correlated with bioaccumulation and persistence.<sup>19,20</sup> Recent research indicates that PFAS mixtures can produce additive results that differ from those of individual compounds.<sup>195,196</sup> Moreover, as mentioned in Section 4.2, some alternatives—including PFMO3NA and 8:8 PFPiA—continue to be poorly characterized despite indications of varying toxicity.

As noted in the previous section, there are important limitations in the current research body regarding the impact of PFAS in human health. Despite the numerous studies available, this group of chemicals includes a large number of compounds that remain underrepresented (e.g., GenX) or have not been studied extensively. Moreover, the lack of standardised reporting of experimental outcomes and protocols creates challenges for traceability and reproducibility of results.

To address these deficiencies, regulatory agencies are encouraging New Approach Methodologies (NAMs), which integrate *in vitro* tests, high-throughput screening, and computational models to rank chemicals prior to extensive application.<sup>203,204</sup> The methodological inconsistencies and variability highlighted in this review emphasize this necessity: NAMs can assist in bridging differences among study types and offer organized, scalable resources to aid





regulatory assessments. To address these methodological inconsistencies between the various studies, harmonized PFAS panels and mixtures could greatly expand the research field and help elucidate potential synergistic effects and their overall impact. Recent *in silico* developments—such as neural networks for predicting bioactivity<sup>115</sup> and extensive docking of PFAS to endocrine receptors<sup>116</sup>—demonstrate the capability of NAMs to enhance conventional testing. Nonetheless, these methods should currently be considered as supplementary evidence rather than independent proof in regulatory decision-making, with validation and standardization across agencies still needed.<sup>201</sup> Additionally, the lack of data regarding chronic exposure to many PFAS congeners or low-dose exposure further complicates risk assessment. To further enhance the impact of *in silico* tools in PFAS risk assessment, tools like translational PBPK modelling, which aims to translate, or scale, findings from a PBPK model between species (e.g., from rats to humans) or across life stages (e.g., from adults to children) from one cohort to another, may provide significant advantages.<sup>205</sup>

## 5. Conclusion

This scoping review emphasizes the extensive and complex nature of toxicological data regarding PFAS. Recurring results from *in vivo* (177 papers), *in vitro* (91 papers), *in silico* (21 papers), and epidemiological (69 papers) studies indicate hepatotoxicity and immunotoxicity as frequent and sensitive endpoints, alongside with developmental, endocrine, neurotoxic, and carcinogenic effects. Several alternative PFAS, such as HFPO-DA, PFBS, and 6:2 Cl-PFESA, demonstrate toxicological profiles similar to legacy compounds, while other less-studied subclasses are still poorly understood. PFAS, alongside various alternatives, exhibit a range of toxicities, though the evidence is still inconsistent, particularly concerning mixtures and newer compounds. Another major issue that has been highlighted in the text is the lack of any consistent information regarding the impact of the chemicals in low-dose exposure or during chronic exposure. Currently, there are almost no epidemiological studies that focus on the long-term effects of PFAS. This gap may underestimate the effect of the compounds in major organs (e.g. heart) or hide their effects on reproductive health. Moreover, the lack of long-term observation may hide any correlation between PFAS and carcinogenicity. These gaps emphasize the necessity for standardized testing to support regulatory efforts like the EPA's Hazard Index and ECHA's group restrictions. Incorporating NAMs with conventional data will be crucial for progressing PFAS risk health assessments towards PFAS-free technologies.

## Abbreviations



<b>6:2 CI-PFESA (or F-53B)</b>	Perfluoro(2-(6-chlorohexyl) oxy) ethanesulfonic acid)
<b>6:2 FTCA</b>	2-(Perfluorohexyl)ethanoic acid
<b>6:2 FTOH</b>	2-(Perfluorohexyl)ethanol
<b>6:2 FTSA</b>	6:2 Fluorotelomer sulfonic acid
<b>8:2 CI-PFESA</b>	11-chloroeicosafuoro-3-oxaundecane-1-sulfonic acid
<b>8:8 PFPiA</b>	8:8 Perfluoroalkyl phosphonic acid
<b>AMPK</b>	AMP-activated protein kinase
<b>AOPs</b>	Adverse Outcome Pathways
<b>ATSDR</b>	Agency for Toxic Substances and Disease Registry
<b>BMD</b>	Benchmark Dose
<b>BMC</b>	Benchmark Concentration
<b>CAR</b>	Constitutive Androstane Receptor
<b>EC<sub>50</sub></b>	Half -maximal Effective Concentration
<b>ECHA</b>	European Chemicals Agency
<b>EFSA</b>	European Food Safety Authority
<b>EPA</b>	Environmental Protection Agency (U.S.)
<b>GABA</b>	Gamma-aminobutyric acid
<b>HFPO-DA (or GenX):</b>	Hexafluoropropylene oxide dimer acid
<b>HFPO-TA</b>	Hexafluoropropylene oxide trimer acid
<b>HFPO-TeA</b>	Hexafluoropropylene oxide tetramer acid
<b>hiPSCs</b>	Human induced Pluripotent Stem Cells
<b>IC<sub>50</sub></b>	Half -maximal Inhibitory Concentration
<b>IL-</b>	Interleukin-
<b>LD<sub>50</sub></b>	Median Lethal Dose
<b>LH</b>	Luteinizing hormone
<b>Nafion BP2 (or NBP2)</b>	Nafion by-product 2 (or H-PFMO2OSA)
<b>NASEM</b>	National Academies of Sciences, Engineering, and Medicine
<b>NF-κB</b>	Nuclear factor kappa-light-chain-enhancer of activated B cells
<b>NK</b>	Natural killer (cells)
<b>NTP</b>	National Toxicology Program
<b>OECD</b>	Organization for Economic Co-operation and Development
<b>OBS</b>	Sodium p-perfluorous nonenoxybenzene sulfonate
<b>PFAS</b>	Per- and Polyfluoroalkyl Substances
<b>PFBS</b>	Perfluorobutane sulfonic acid
<b>PFCAs</b>	Perfluoroalkyl carboxylic acids
<b>PFDA</b>	Perfluorodecanoic acid
<b>PFECAs</b>	Perfluoroether carboxylic acids
<b>PFECHs</b>	Perfluoroethylcyclohexanesulfonate
<b>PFESAs</b>	Perfluoroether sulfonic acids
<b>PFHpA</b>	Perfluoroheptanoic acid
<b>PFHxA</b>	Perfluorohexanoic acid
<b>PFHxS</b>	Perfluorohexane sulfonic acid
<b>PFMOAA</b>	2-methoxyacetic acid
<b>PFMOBA</b>	Perfluoro4-methoxybutanoic acid



<b>PFMOPrA</b>	Perfluoro-2-methoxypropanoic acid
<b>PFNA</b>	Perfluorononanoic acid
<b>PFO2OA</b>	Perfluoro diEther octananoic acid
<b>PFOA</b>	Perfluorooctanoic acid
<b>PFOS</b>	Perfluorooctane sulfonic acid
<b>PFOSA</b>	Perfluorooctanesulfonamide
<b>PFPeA</b>	Perfluoropentanoic acid
<b>PFPiAs</b>	Perfluoroalkyl Phosphinates
<b>PFSAs</b>	Perfluoroalkyl sulfonic acids
<b>PFTA</b>	Perfluorotetradecanoic acid
<b>PFUnDA</b>	Perfluoroundecanoic acid
<b>PPAR</b>	Peroxisome Proliferator-Activated Receptor
<b>PXR</b>	Pregnane X receptor
<b>ROS</b>	Reactive oxygen species
<b>ScR</b>	Scoping Review
<b>TNF-<math>\alpha</math></b>	Tumor necrosis factor alpha

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### Data Availability

Supplementary Material (Tables S3-S5)

### CRedit author contribution statement

**MP:** Methodology, Formal analysis, Data curation, Visualization, Writing – original draft; **HT:** Methodology, Formal analysis, Writing – review and editing; **KDP:** Methodology, Formal analysis, Writing – review and editing; **FD:** Funding Acquisition, Formal Analysis, Writing – review and editing; **DC:** Writing – review and editing; **IL:** Formal Analysis, Writing – review and editing; **AA:** Supervision, Project Administration, Writing – review and Editing, Conceptualization; **GM:** Supervision, Conceptualization, Writing – review and editing.

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