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An integrated *in vitro* and *in silico* testing strategy applied to PFAS inhibition of antibody production to define a tolerable daily intake

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ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are widely used chemicals known for their persistence, bioaccumulation, and adverse health effects, particularly on the immune system. Epidemiological studies link PFAS exposure to immunosuppression, with increased infection susceptibility and reduced vaccine efficacy. In this paper, we describe the workflow we used to establish an integrated testing strategy (ITS) combining *in vitro* and *in silico* methods to model PFAS inhibition of antibody production and to define a tolerable daily intake. This strategy was based on data generated within an EFSA-sponsored project. Using human peripheral blood mononuclear cells, the effects of PFAS on antibody production were assessed. Mathematical models were then applied to determine PFAS free concentrations *in vitro*, while Physiologically Based Kinetics (PBK) modeling enabled quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) to translate *in vitro* effects into external doses. In addition, the Universal Immune System Simulator was used to predict immune-related outcomes and threshold doses for sensitive populations. Following this strategy, we were able to demonstrate that the oral equivalent effect doses derived through QIVIVE were similar to, or lower than, the tolerable weekly intake established by EFSA for PFAS, indicating that our approach is conservative. We demonstrate the possibility of using alternative methods for studying PFAS toxicity, offering insights into their dynamics and kinetics without animal testing. The strategy provides a promising framework for assessing other chemicals, advancing toxicology toward more human-relevant and ethical practices.

1. Contextualization of the problem: the immune system as critical target of PFAS toxicity and use scenario

Per- and polyfluoroalkyl substances (PFAS) are a group of synthetic chemicals developed starting from the 1940s and valued for their water, oil, stain and heat resistance. These properties have made PFAS widely used in industrial and consumer products, including non-stick cookware, firefighting foams, water-resistant clothing, and food packaging. However, their strong carbon-fluorine bonds make them highly persistent in the environment and resistant to degradation (Habib et al., 2024). Consequently, PFAS are often referred to as "forever chemicals" because they accumulate in ecosystems and human bodies over time (Allen, 2018).

PFAS can enter the environment through various pathways, including emissions from manufacturing facilities, leakage from landfill sites, and discharges from wastewater treatment plants. PFAS contamination is widespread, with traces found in water, soil, air, and even in remote regions. This environmental persistence, coupled with their bioaccumulative nature, poses significant risks to human health (Pan et al., 2024). PFAS contamination in food primarily occurs through bioaccumulation in water sources, aquatic life such as fish and shellfish, plants, and animals. To a lesser extent, these substances can also leach into food from processing equipment and packaging materials, further contributing to their presence in the food supply (EFSA CONTAM

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PANEL, 2020).

Exposure to PFAS is commonly assessed through their plasma or serum concentrations in humans, which reflects both environmental exposure and bioaccumulation. Studies show that PFAS levels in human plasma vary widely depending on factors like geography, diet, and proximity to contaminated sites. The highest concentrations in human blood plasma and serum in adults are detected for perfluorooctanoic acid (PFOA), perfluorooctane sulfonate (PFOS), perfluorononanoic acid (PFNA), and perfluorohexane sulfonic acid (PFHxS). About 90 % of the PFAS concentrations detectable in human blood are represented by these four PFAS (EFSA CONTAM PANEL, 2020). Median serum concentrations for PFOA and PFOS in general populations range between 2 and 10 ng/mL but can be significantly higher, in the mg/mL range, in people living near contaminated sites or in occupational settings (Gebbink and van Leeuwen, 2020; Borghese et al., 2024). These levels correlate with intake from contaminated water, food, and consumer products (Borghese et al., 2024).

Research and epidemiological studies link PFAS exposure to a variety of adverse effects, such as immune system suppression, hormonal disruptions, liver damage, developmental issues in children, and increased risks of cancers like prostate, kidney, and testicular cancer (Fenton et al., 2021; Ehrlich et al., 2023; Garvey et al., 2023; Habib et al., 2024; Martano et al., 2025; Briassoulis et al., 2025). These health impacts vary depending on the type and level of exposure. The European Food Safety Authority (EFSA) has established the safety threshold for the four key PFAS compounds in food, setting the group tolerable weekly intake (TWI) at 4.4 ng/kg of body weight (tolerable daily intake (TDI) is 0.62 ng/kg day). This value is based on the suppression of the immune system's response to vaccination, identified as the most critical health impact associated with PFAS exposure, and linked to an increased risk of immune-related diseases (EFSA CONTAM PANEL, 2020). The TWI value represents the amount of a substance (per kilogram of body weight) ingested weekly over a lifetime, which is not expected to have any adverse effects on health. In a similar manner, the U.S. Environmental Protection Agency (EPA) highlighted that immunotoxicity caused by PFAS, especially in children, is a key element in their risk assessment processes (US EPA, 2022).

Within the EFSA project Case Studies NAMS_PFAS Immunotox (Ref. OC/EFSA/SCER/2021/13), we investigated the effects of PFOS, PFOA, PFNA, and PFHxS using human immune cell–based *in vitro* models, assessing several key immunological parameters, including antibody production (Corsini et al., 2024). Mathematical fate and distribution models were used to assess PFAS concentrations within the cell systems, while physiologically based kinetic (PBK) models enabled the quantitative extrapolation from *in vitro* effect concentrations to *in vivo* adverse doses. The 'Universal Immune System Simulator' (UISS), an agent-based modeling platform designed to accurately model and simulate human immune system behavior in physiological or pathological conditions (Pappalardo et al., 2010), was used to extend the ITS framework to evaluate vaccination responses, including in vulnerable populations, through a specialized module, called UISS-TOX, dedicated to predicting immune system perturbations following exposure to chemicals (Pappalardo et al., 2022).

In this paper a twofold objective is presented: on the one hand, we describe the workflow we used to integrate *in vitro* and *in silico* tests developed to establish benchmark doses based on reduced antibody production. On the other hand, through the use of UISS, we advanced integrated modeling approaches capable of representing immune responses, including those elicited by vaccination. The approach is designed to provide a comprehensive framework for assessing chemical immunotoxicological risks and is potentially applicable to a wide range of substances, including pharmaceuticals, environmental contaminants, and industrial chemicals. By leveraging advanced computational models and targeted *in vitro* assays, this strategy enables the prediction of dose-response relationships and supports regulatory decision-making for diverse chemical classes. Overall, findings highlight the utility of ITS in assessing PFAS immunotoxicity and derivation of tolerable daily intake.

2. *In vitro* assessment of PFAS-induced immunosuppression and distribution models

Numerous adverse health effects have been identified in studies of individuals exposed to PFAS through their workplace or environment. Some of these effects are more certain (e.g. immunotoxicity), as they show a strong association, having been consistently observed across different populations and corroborated by evidence from experimental animal models. Staring from the epidemiological evidence of reduced response to vaccination associated with PFAS exposure, we defined a battery of *in vitro* tests to address the effects of PFAS on key immune cells involved in antibody production, including dendritic cells maturation, B and T cells activation as well as immunoglobulin release and primary response to Keyhole limpet hemocyanin using human peripheral blood mononuclear cells (PBMCs) (Corsini et al., 2024). All PFAS were purchased from Sigma-Aldrich (St. Louis, MO, USA), with purities ranging from 95 % to $\geq$ 99 % as specified by the manufacturer, and diluted in dimethyl sulfoxide (DMSO, CAS #67–68–5). The final concentration of DMSO in cell culture was 0.1 %. The initial four PFAS investigated, namely PFOS, PFOA, PFNA, PFHxS, affected all parameters investigated to different extents, with the inhibitory effects on CpG oligodeoxynucleotide 2006 (ODN2006) induced IgG and IgM release showing a clear dose response in both male and female donors (Iulini et al., 2025a). Dose–response relationships and inter-donor variability for these endpoints have been extensively characterized and reported in the original studies (Corsini et al., 2024; Iulini et al., 2025a) and are therefore not reproduced here to avoid duplication. Effects were observed at nominal concentration as low as 1 ng/mL (lowest concentration tested). Subsequently, the effect of three short chain and short half-life PFAS, namely perfluorohexanoic acid (PFHxA), perfluorobutanoic acid (PFBA) and perfluorobutanesulfonic acid (PFBS), on ODN2006-induced IgG and IgM release was also investigated (Iulini et al., 2025b). For these last three PFAS modest effects were found, with a statistically significant reduction only observed for PFHxA at the highest concentration tested (10 mg/mL) (Iulini et al., 2025b).

Under our experimental conditions, adjustments to the nominal applied concentrations that account for sequestration or migration into different compartments of the *in vitro* system (e.g., cells, media constituents, plastic, etc.) were done using the *in vitro* distribution model developed by Armitage et al. (2014; 2021) to translate the nominal concentrations to the free and intracellular concentrations. This allowed to adjust the relevant concentrations at which bioactivity was observed. As set up from the *in vitro* protocol used, the following parameters were used: 48 well plate with a well volume of 500 μL, average cell yield (seeding density) 630.000 cells, cell mass 3.15 ng, 5 % human serum (albumin 42.5 g/L; lipids 1.9 g/L). The results, along with the CAS numbers, molecular weights (MW), and LogPs of the seven PFAS tested, are shown in Table 1. Interestingly, the cellular distribution (MF_{cells}, pink column) was quite different among the different PFAS, with PFOS having the highest value (24.6 %) and PFBS the lowest (0.5 %). PFOS is among the PFAS the chemical mostly taken up by the cells, followed in descending order by PFNA, PFOA, PFHxS, PFHxA, PFBA and PFBS.

We then examined the relationship between PFAS-induced inhibition of IgG and IgM production (using the concentration of 10 μg/mL) and the PFAS mass fraction distribution across cellular and non-cellular compartments. Statistical analyses were performed using both Spearman's rank and Pearson's correlation coefficients, based on mean responses across donors. A highly significant inverse correlation emerged between PFAS MF_{Cells} and Igs release (Fig. 1). Specifically, higher intracellular PFAS MF_{Cells} were associated with stronger suppression of IgG and IgM secretion (Spearman $r = -0.9286$, $p = 0.0067$; Pearson $r = -0.7628$, $p = 0.0461$). In contrast, no meaningful correlations were observed for PFAS mass fractions in albumin MF_{ALB} or water MF_{WAT} compartments (data not shown). Short-chain PFAS—such as PFHxA,

Table 1
In vitro mass fraction distribution (%) of the different PFAS.

Name	CAS	MW (g/mol)	LogP	MF_{AIR}	$MF_{BULK\ WAT}$	MF_{ALB}	MF_{LIP}	MF_{DOM}	MF_{WAT}	MF_{Cells}	$MF_{Plastic}$
PFOS	1763–23–1	500.1	6.3	0.0	75.3	74.8	0.0	0.0	0.5	**24.6**	0.0
PFNA	355–46–4	464.1	5.9	0.0	84.3	83.4	0.0	0.0	0.8	**15.7**	0.0
PFOA	335–67–1	414.1	5.3	1.1	92.1	90.5	0.0	0.0	1.6	**6.8**	0.0
PFHxS	375–95–1	400.1	5.2	0.0	94.2	92.4	0.0	0.0	1.8	**5.8**	0.0
PFHxA	307–24–4	314.1	4.1	0.0	97.4	86.9	0.0	0.0	10.5	**2.6**	0.0
PFBS	375–73–5	300.1	1.8*	0.0	99.3	3.0	0.0	0.0	96.4	**0.6**	0.0
PFBA	375–22–4	214.0	1.4	0.1	99.3	1.1	0.0	0.0	98.3	**0.6**	0.0

Mass balance (%) was calculated using the model developed by Armitage et al. (2021). MF_{ALB}, fraction bound to albumin; MF_{LIP}, fraction bound to lipids; MF_{DOM}, fraction bound to dissolved organic matter; MF_{WAT}, fraction in water. * estimated (https://pubchem.ncbi.nlm.nih.gov/compound/67815)

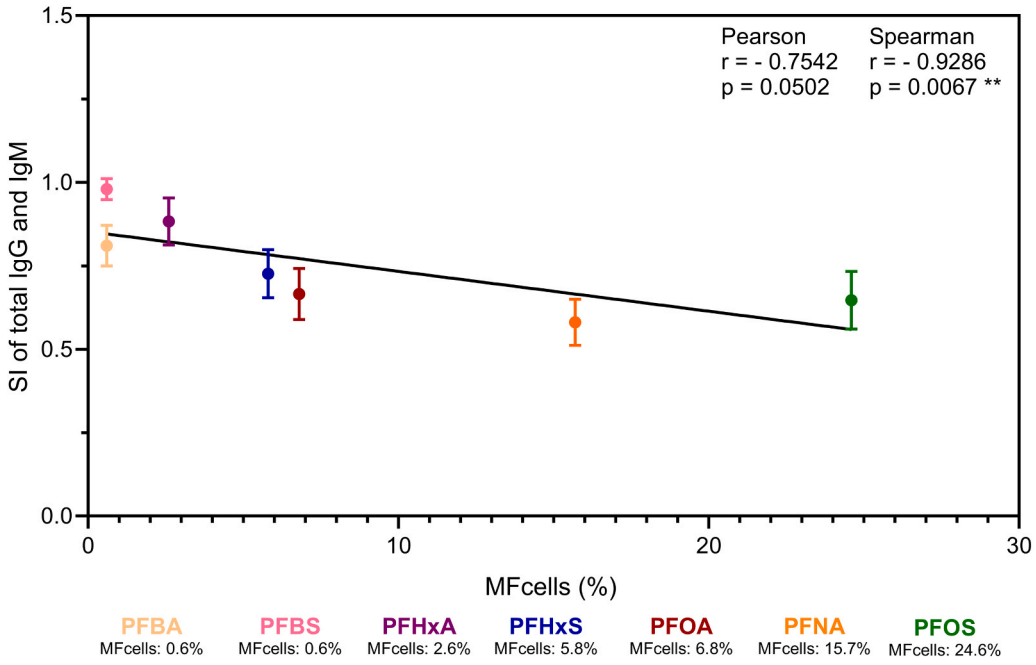

Fig. 1. Higher PFAS intracellular accumulation leads to stronger inhibition of immunoglobulin production. The correlation between the PFAS cell mass fraction distribution (MFcells, %) and the inhibition of total IgG + IgM release following PFAS exposure is shown. The protocol for immunoglobulin production is described in Corsini et al. (2024). Briefly, PBMCs of 5 female and 5 male donors were exposed to PFAS (10 μg/mL) for 24 h and subsequently stimulated for 6 days with 1 μg/mL ODN2006 and 100 IU/mL IL-2 to induce antibody production. The total IgG + IgM values were extracted from Iulini et al. (2025a; 2025b). On the X-axis, the percentages of the different cell mass fraction distributions for each PFAS (also listed in the legend) are shown. On the Y-axis, total IgG and IgM release—normalized to values obtained in vehicle-treated cells (stimulation index, SI)—is reported. Each value represents the mean ± SEM, with n = 10 donors (5 females and 5 males). Spearman and Pearson correlations are reported in the figure.

PFBA, and PFBS—showed minimal cellular accumulation, which was paralleled by markedly lower immunotoxic activity. These findings support the hypothesis that intracellular bioaccumulation is a key mechanistic driver of PFAS-mediated impairment of immunoglobulin production.

Thus, for an accurate interpretation of potency, meaning the ability of a substance to trigger changes in cellular pathways that ultimately lead to adverse outcomes, it is essential to convert nominal concentrations (those added to the culture medium) into actual intracellular concentrations. Only the amount of chemical that enters and accumulates within cells reflects the biologically effective dose capable of driving pathway perturbation and contributing to apical toxic effects. Although a strong inverse correlation was observed between intracellular PFAS accumulation and inhibition of immunoglobulin release, this analysis is based on a limited number of PFAS (n = 7). Therefore, the observed association should be interpreted with caution, as it may partly reflect collinearity with PFAS chain length and related physicochemical properties rather than a fully independent effect of intracellular accumulation *per se*. While the trend is biologically plausible and consistent with known PFAS bioaccumulation behavior, confirmation across a broader and more chemically diverse set of PFAS will be required to strengthen the generalizability of these findings.

3. From *in vitro* to *in vivo*: PBK models and benchmark dose

PBK models are mathematical frameworks used to describe the absorption, distribution, metabolism, and excretion (ADME) of chemicals in the body (Andersen et al., 2003). These models integrate biological and physiological parameters, such as organ sizes, blood flow rates, and enzyme activities, to predict the internal concentrations of chemicals over time (Clewell et al., 2002). PBK models are particularly valuable in toxicology for assessing the risks and effects of chemical exposures in humans. By simulating how chemicals move through different tissues, PBK models help in understanding the potential impacts of various exposure scenarios (Wambaugh et al., 2013). These models are crucial tools in regulatory decision-making, aiding in risk assessments and guiding safe levels of exposure to chemicals in the environment and workplaces. In the present study, the PBK model was based on established EFSA human PBK frameworks developed for PFOA and PFOS and subsequently adapted for PFNA and PFHxS. As lymphatic organs were

not explicitly represented in the model structure, plasma was used as a surrogate compartment for immune system exposure, and plasma area under the curve (AUC) value was selected as the relevant internal dose metric. Model parameters were derived from published kinetic data, and detailed descriptions of model structure and assumptions are reported in Corsini et al. (2024). The PBK model was used to perform quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) or else to translate *in vitro* effective concentrations to respective external doses (Corsini et al., 2024; EFSA CONTAM PANEL, 2020). The QIVIVE process links internal *in vivo* concentrations to external doses by using tools, such as a PBK model, to characterize the relationship between the two. This process can be carried out using two approaches that share the same fundamental principles. In the first approach, an *in vitro* point of departure (PoD) is converted into an *in vivo* internal concentration, which is then used to calculate an external dose (Wetmore et al., 2015). A second approach involves translating *in vitro* concentration-response bioactivity into an external dose-concentration, from which an external PoD is determined (Louisse et al., 2010). However, this assumption may not be appropriate due to several processes influencing the *in vitro* kinetics, such as chemical binding to proteins and lipids in the cell culture medium, evaporation, binding to plastic containers, uptake into the cultured cells, and degradation processes (Groothuis et al., 2015). For those reasons, the *in vitro* bioactive concentration should be adjusted with considerations on medium protein binding and intracellular concentrations (as determined by the distribution model described above). However, as nominal concentrations are easily accessible, using them as the starting point for the extrapolations represents the simplest QIVIVE. This low-tier approach is presented in Fig. 2, and further methodological details are reported in Corsini et al., (2024).

The workflow in Fig. 2 illustrates the QIVIVE strategy applied in this study, following the approach described by Wetmore et al. (2015). Briefly, concentration–response data derived from *in vitro* T-independent antibody production assays in human PBMCs were used to identify an *in vitro* PoD. The PoD was determined by providing the benchmark dose (BMD) and its confidence bounds. The resulting *in vitro* PoD was then translated into an equivalent external oral dose by means of PBK modelling. The PBK model was used in a reverse dosimetry framework to estimate the external dose that would lead, under chronic exposure conditions, to an internal concentration (AUC) in the surrogate target compartment (plasma) equivalent to that associated with the *in vitro* effect. This approach enables the integration of mechanistic *in vitro* data with human toxicokinetics to support risk-relevant dose estimation.

The *in vitro* PoD for QIVIVE were determined using the online application Bayesian BMD (https://r4eu.efsa.europa.eu/), which performed Bayesian Benchmark Dose Modeling analyses via the R-package BMABMDR version 0.0.0.9060. This software enables the estimation of the dose corresponding to the benchmark response (BM) of interest. The BMD was reported alongside its lower (BMDL) and upper (BMDU) confidence bounds. A BMR of 0.25, corresponding to a 25 % change in the mean response relative to controls, was selected. This choice is consistent with guidance from regulatory and scientific bodies indicating that, for continuous data derived from *in vitro* or experimental toxicology studies, BMR values in the range of 20–30 % are commonly applied to define biologically relevant effects (U.S. EPA, 2012). Such thresholds are considered appropriate to ensure that the selected point of departure reflects a response magnitude exceeding normal experimental variability and measurement uncertainty inherent to cell-based assays. In contrast, lower BMRs (e.g., 5–10 %), frequently used in epidemiological studies, are intended to capture subtle population-level changes and are generally less suitable for *in vitro* systems, where smaller effect sizes may fall within background noise. Therefore, a 25 % BMR was considered a conservative and biologically meaningful threshold for the present *in vitro* dataset. Nevertheless, the influence of alternative BMR choices on PoD estimation is acknowledged and discussed as a source of uncertainty.

As higher tiers the binding of PFAS to protein, and PFAS distribution into different compartments of the *in vitro* system were used in place on the nominal concentration. In the first case, corrections for albumin binding were applied considering the differences in protein content in the medium versus human plasma. In the second case, results were used as derived from the Armitage model, which facilitated the estimation of PFAS-intracellular concentrations. Here, effective concentrations were used as applied in the *in vitro* experiments. Concentrations (expressed in μM) and responses (expressed in ng/mL) were analyzed with the Bayesian BMD application to obtain a BMC with the required BMR set at 25 %. This concentration was then transformed into μg/L and converted into 7 days (168 h) AUC168h values, considering the 7-day exposure period in the *in vitro* system. Subsequently, the application of PBK modelling was employed to estimate the oral equivalent effect dose that, under chronic exposure conditions, would result in the same AUC in the target system, in this case the immune system. A 5-year exposure timeframe was selected to ensure consistency with the derivation of the TWI for PFAS established by the EFSA CONTAM Panel, which assumes continuous exposure over a 5-year period (EFSA CONTAM Panel, 2020). This assumption is further substantiated by the protracted human elimination half-lives of the PFAS under investigation (PFOA, PFOS, PFNA, and PFHxS), which range from several years, resulting in substantial bioaccumulation and a gradual approach to steady state under repeated exposure conditions. Consequently, a multi-year timeframe is necessary to adequately capture internal dose metrics relevant for chronic immune effects. While the extrapolation from a 7-day *in vitro* exposure to a 5-year chronic *in vivo* exposure introduces uncertainty but serves as a foundational step for risk assessment, and this approach is consistent with current QIVIVE practices aiming to translate short-term *in vitro* effects into chronic human exposure scenarios. All the results obtained are reported in Corsini et al. (2024) and are summarized below in Table 2, categorized by gender. The values expressed in ng/kg day (referring to TDI), represent the combined total of the four individual PFAS (as outlined in the TWI established by EFSA).

The primary difference between Wetmore and Louisse approaches lies in their methodologies: Wetmore employed a single-point extrapolation, whereas Louisse considered the entire dose-response curve and incorporated different scenarios for PFAS plasma concentrations. The predicted oral equivalent doses for a 25 % reduction in antibody release,

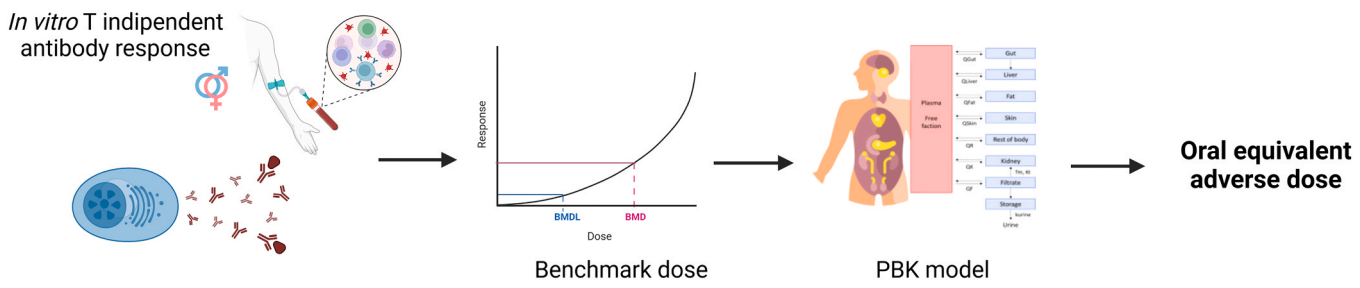

Fig. 2. Schematic representation of the approach used. In line with Wetmore et al. (2015), starting from the *in vitro* effect of PFAS on antibody production, *in vitro* PoD was calculated using the online application Bayesian BMD, which was subsequently translated to the external dose using PBK modelling.

Table 2
Summary of the oral equivalent effect doses resulting from the different QIVIVE approaches analyzed.

		Female (n = 5)	Male (n = 5)
A	Wetmore approach (2015) – nominal concentration	0.039 ng/kg day	0.043 ng/kg day
B	Louisse approach (2010) – nominal concentration	0.039 ng/kg day	0.043 ng/kg day
C	Louisse approach (2010) – correction for protein binding	0.561 ng/kg day	0.616 ng/kg day
D	Louisse approach (2010) – intracellular concentration derived with the Armitage model	0.020 ng/kg day	0.023 ng/kg day

as reported in Table 2, were compared to the EFSA-derived TDI (0.628 ng/kg day) (EFSA CONTAM Panel, 2020). In most scenarios, these doses were significantly lower than the TDI, suggesting that the selected ITS is generally more conservative (Table 2 A, B and C). Among the scenarios within the Louisse approach, the intracellular concentration scenario derived from the Armitage model (Table 2D) proved to be the most conservative (25-fold lower than the TDI), followed by the use of nominal concentrations (Table 2B) (15-fold lower). In contrast, the scenario that corrected for protein binding (Table 2 C) was the least conservative, with results closely aligned with the TDI. These findings underscore the critical importance of selecting appropriate *in vitro* metrics for QIVIVE. Metrics such as nominal concentrations, free chemical concentrations, and protein binding corrections significantly influenced the extrapolated doses. Notably, the use of PFAS intracellular concentrations provided the most cautious and conservative predictions.

4. Universal immune system simulator (UISS) to estimate the immunotoxicity risk

The PBK model was also used to inform the UISS, a mechanistic computational platform designed to simulate and model the immune system. UISS uses an agent-based modeling approach to represent immune responses in various conditions, including the effects of environmental toxins such as PFAS. This platform is capable of estimating immunotoxicity risks, such as reduced responses to vaccination by simulating the complex interactions between immune cells and other biological entities. The UISS can simulate the human immune system and offers the possibility to investigate effects on vulnerable populations.

The UISS platform simulates immune responses within a two-dimensional environment, where biological entities — such as pathogens, immune cells, or cancer cells— are represented as independent agents. Each agent has specific properties, including type, state, location, and behavior. Chemical interactions between agents occur stochastically, driven by factors such as proximity, concentration, and stoichiometric rules. Agents interact according to preestablished parameters, move in response to chemical gradients, and can appear or disappear depending on their interactions with the surrounding environment (Pappalardo et al., 2022).

UISS simulations are statistical, with results varying between runs. However, consistent patterns emerge at the population level though repeated iterations. Each agent occupies a position within a lattice structure and exhibits traits, such as energy levels or lifespan, that influence its movement and interactions. This allows the system to capture complex biological behaviors and can be expanded to include additional details when needed. The platform is built in ANSI C-99, is cross-platform, and tracks biological components either as individual agents or in terms of concentration levels. The model incorporates fundamental immune mechanisms, including clonal selection, thymic education, and immune memory, with interactions simulated as Bernoulli events. Processes like receptor-ligand interactions are modeled based on affinity-based calculations to capture adaptive immune responses. UISS's layered architecture accounts for different aspects of immune responses, disease progression, and therapeutic interventions. The UISS-TOX extension builds upon the general UISS framework to specifically model the immunomodulatory effects of PFAS. In the model, agent behaviors and interaction rules were calibrated based on mechanistic knowledge and experimental evidence of PFAS-induced immunotoxicity derived from the literature, including effects on cytokine production, B- and T-cell activation, and antibody responses. In particular, the model was previously calibrated and evaluated using independent case studies, including allergic contact dermatitis and PFAS-related immunotoxicity (Russo et al., 2022; Iulini et al., 2024). The UISS-TOX translates findings from literature into a conceptual framework of immune interactions, enabling the estimation of how PFAS exposure impacts immune function. UISS-TOX provides a robust tool for exploring immunotoxic responses, including those in vulnerable populations (Russo et al., 2022). A formal sensitivity analysis of individual agent-based parameters was not performed in the present study; however, model robustness was evaluated by simulating multiple exposure scenarios, age groups, and immune challenges, as well as by repeated stochastic simulations to ensure consistency of emergent population-level behaviors. A comprehensive sensitivity and uncertainty analysis of UISS-TOX parameters has been reported in Iulini et al. (2024) and will be further expanded in future applications.

In the selected ITS, UISS-TOX was used to investigate the sensitivity of children and elderly population to PFAS and to predict the threshold dose for immunotoxic effects, such as a 10 % decrease in the response to vaccination. The combination of PBK models and UISS enabled the investigation of potential immunotoxicity based on plasma concentrations profiles generated through PBK modelling. To facilitate this integration, plasma concentration data were recorded every 8 h, formatted into Excel spreadsheets, and input into the UISS-TOX module. Using the PKB model, UISS-TOX was provided with three distinct PFAS concentrations across different age groups (children, young adults, and the elderly) to predict immune responses under two scenarios: a general bacterial challenge and a response to two widely used vaccines, anti-H1N1 and anti-diphtheria. The collected results and the efficacy of the integrated models were published by Iulini et al. (2024). Briefly, three cohorts were considered: children (0–4 years), young adults (25–26 years), and elderly individuals (65–66 years). The focus was on the impact of PFAS on cytokine levels, immunoglobulins (IgM and IgA), and B- and T-cell dynamics, capturing both cellular and humoral immune responses. The simulations revealed that higher PFAS exposure led to reduced cytokine production, immunoglobulin levels, and B- and T-cell activity. Notably, the young adult cohort (25–26 years) was the most affected, showing significant declines in cytokine levels and B- and CD4 + cell dynamics, particularly in conditions that mimicked the oral exposure of individuals living in contaminated areas. UISS also modeled the impact of PFAS on vaccine responses (Iulini et al., 2024; Corsini et al., 2024). In a cohort of 100 young adults, the same exposure condition caused a substantial reduction in the immune response to an anti-H1N1 vaccine. Similarly, in a cohort of 100 children (0–10 years), a marked decrease in vaccine response was observed after the second anti-diphtheria vaccine challenge under the same exposure scenario (Iulini et al., 2024; Corsini et al., 2024). The most important results obtained are summarized in Table 3 reported below. The higher sensitivity predicted for young adults does not reflect an increased intrinsic vulnerability, but rather a stronger functional immune response at baseline, which results in a larger measurable decline upon PFAS exposure. This pattern has been consistently observed in UISS-TOX simulations and is in line with known age-dependent immune dynamics reported in the literature (Iulini et al., 2024; Corsini et al., 2024). It is also important to highlight that the scenario simulating the TDI set by EFSA in 2020 for the exposure to PFOA, PFOS, PFNA and PFHxS, did not result in a significant effect on the selected endpoints. This indicates that the TDI, established as a safety level by EFSA, does not impact on

Table 3
Summary of the UISS-TOX results for young-adults cohort (25–26 years) reported in Iulini et al. (2024).

	Endpoint	Not exposed	Individuals living contaminated areas
After generic bacterial challenge	IL-2	24000 pg/mL	14000 pg/mL
	IL-6	90000 pg/mL	65000 pg/mL
	TNF-α	47000 pg/mL	34000 pg/mL
	IL-17	900000 pg/mL	450000 pg/mL
	IgM	70 entities/μL	18 entities/μL
	IgA	35000 entities/μL	3800 entities/μL
After anti-H1N1 vaccination	IgG	600 antibody titer (GMT)	200 antibody titer (GMT)
After anti-diphteria vaccination	IgG	7500 GM antibody titer (GMT)	1500 antibody titer (GMT)

Cytokine concentrations are reported as pg/mL. Immunoglobulin values reported as assay-derived units (entities/μL). Antibody responses after vaccinations are reported as geometric mean titers (GMT; reciprocal dilution).

our systems either. Consequently, this reinforces our confidence in ITS itself.

5. Sources of uncertainty

Several uncertainties are associated with the methodology applied in the present study. In the present study, experiments were conducted using PBMCs from 10 healthy adult donors (5 males and 5 females, aged 18–65 years), allowing assessment of potential sex-related differences in PFAS-induced effects. While this design captures an element of biological variability, the relatively limited number of donors may not fully reflect inter-individual variability in the general population and could be expanded in future studies to further strengthen robustness. Moreover, the results strongly depend on the selected BMR, and hence, the predicted oral equivalent effect doses would vary with different BMR choice. At present, the magnitude of change in *in vitro* immune readouts that can be clearly considered adverse remains uncertain, and the use of a 25 % effect size, while conservative for *in vitro* systems, contributes to this uncertainty. An additional source of uncertainty relates to the extrapolation from a short-term 7-day *in vitro* exposure to a long-term 5-year *in vivo* exposure scenario. This approach assumes equivalence between *in vitro* and *in vivo* AUC metrics and represents a simplification of complex temporal dynamics. While this assumption is supported by the long elimination half-lives of PFAS and the chronic exposure scenario underlying the EFSA TWI (EFSA CONTAM, 2020), further research is needed to better understand time-dependent effects and exposure duration relationships in QIVIVE. Nonetheless, this approach can serve at least as a first tier in human health risk assessment.

Uncertainties are also inherent to the PBK model structure and parametrization. As mentioned before, the current PBK models do not explicitly represent lymphatic or immune organs; therefore, plasma concentrations were used as a surrogate for immune system exposure. Moreover, enterohepatic recirculation of PFAS is not included in the model, although ongoing research aims to address this limitation. For PFNA and PFHxS, kinetic parameterization is less extensively documented than for PFOA and PFOS, adding further uncertainty. In relation to QIVIVE, the selection of the *in vitro* concentration metric as a PoD constitutes a pivotal source of uncertainty. The use of nominal *in vitro* concentrations does not account for *in vitro* kinetic processes such as protein binding, plastic adsorption, cellular uptake, or degradation, which may affect biologically effective concentration. Although alternative metrics accounting for protein binding and *in vitro* distribution were explored, the selection of the most appropriate metric remains chemical and context-dependent.

Finally, both the PBK modelling and the QIVIVE approach applied for the translation of *in vitro* concentrations to external doses are of a deterministic nature. This means that all model parameters are held fixed at a central value and therefore do not account for variability or uncertainty. Understanding and quantifying these elements embedded at each step of hazard and risk assessment is important to increase confidence in the use of new approach methodologies (NAMs) (Judson et al., 2011; Berggeren et al., 2017; Loizou et al., 2021). Overall, variability and uncertainty in parameter values should be considered when applying such models for QIVIVE. Nonetheless, given that the predicted oral equivalent effect doses were generally below or close to human-based TDI, the overall approach can be considered conservative and suitable as a first-tier assessment in human health risk evaluation.

6. Conclusion

The use of *in silico* models enabled the derivation of an acceptable daily intake, which was found to be similar or lower than the actual value established in the 2020 EFSA Opinion depending on different QIVIVE approaches. This result suggests that the present methodology is more conservative. This aligns with numerous studies demonstrating that in vitro bioactivity offers a conservative estimate of the dose required to elicit toxicological responses in traditional animal-based studies (Paul-Friedman et al., 2020; Judson et al., 2024). Our approach also predicted a reduced response to vaccination. This comparison was made by extrapolating the TWI value presented in the 2020 EFSA Opinion (EFSA CONTAM Panel, 2020) to an acceptable daily intake, derived from the QIVIVE data included in this report. Overall, the proposed approach demonstrates the utility of NAMs in providing mechanistic insights to predict PFAS immunotoxicity without relying on animal studies.

Efforts to mitigate PFAS risks involve better detection methods, safer alternatives, and remediation strategies for contaminated environments. Policymakers and researchers are actively exploring ways to address the challenges posed by these chemicals to safeguard both public health and ecosystems (Itumoh et al., 2024). With the example reported here, the application of selected NAMs offers a valuable tool for regulatory risk assessment by providing detailed mechanistic insights, supporting efforts to better understand and mitigate associated risks. The proposed battery of tests is also useful in the hazard identification of immunotoxic compounds, considering that the gold standard in assessing immunotoxicity in animal study is the T cell dependent antibody response, which is recognized *in vivo* as the main relevant target of the interaction of chemical with the immune system (Luster, 1992).

Ethical standards

the manuscript does not contain clinical studies or patient data.

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CRediT authorship contribution statement

Martina Iulini: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Janssen Aafke W. F.:** Writing – review & editing. **Emanuela Corsini:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Alicia Paini:** Writing – review & editing, Investigation, Formal analysis. **Styliani Fragki:** Writing – review & editing, Investigation, Formal analysis. **Francesco Pappalardo:** Writing

– review & editing. **Giulia Russo:** Writing – review & editing, Formal analysis. **Karsten Beekmann:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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