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# Internal relative potency factors based on immunotoxicity for the risk assessment of mixtures of per- and polyfluoroalkyl substances (PFAS) in human biomonitoring

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ABSTRACT

Relative potency factors (RPFs) for per- and polyfluoroalkyl substances (PFAS) have previously been derived based on liver effects in rodents for the purpose of performing mixture risk assessment with primary input from biomonitoring studies. However, in 2020, EFSA established a tolerable weekly intake for four PFAS assuming equal toxic potency for immune suppressive effects in humans. In this study we explored the possibility of deriving RPFs for immune suppressive effects using available data in rodents and humans. Lymphoid organ weights, differential blood cell counts, and clinical chemistry from 28-day studies in male rats from the National Toxicology Program (NTP) were combined with modeled serum PFAS concentrations to derive internal RPFs by applying dose–response modelling. Identified functional studies used diverse protocols and were not suitable for derivation of RPFs but were used to support immunotoxicity of PFAS in a qualitative manner. Furthermore, a novel approach was used to estimate internal RPFs based on epidemiological data by dose–response curve fitting optimization, looking at serum antibody concentrations and key cell populations from the National Health and Nutrition Examination Survey (NHANES). Internal RPFs were successfully derived for PFAS based on rat thymus weight, spleen weight, and globulin concentration. The available dose–response information for blood cell counts did not show a significant trend. Immunotoxic potency in serum was determined in the order PFDA > PFNA > PFHxA > PFOS > PFBS > PFOA > PFHxS. The epidemiological data showed inverse associations for the sum of PFOA, PFNA, PFHxS, and PFOS with serum antibody concentrations to mumps and rubella, but the data did not allow for deduction of reliable internal RPF estimates. The internal RPFs for PFAS based on decreased rat lymphoid organ weights are similar to those previously established for increased rat liver weight, strengthening the confidence in the overall applicability of these RPFs.

## 1. Introduction

In recent years, regulatory bodies repeatedly identified the (developing) immune system as a sensitive target of per- and polyfluoroalkyl substances (PFAS)-induced toxicity (EFSA, 2020; ATSDR, 2021; NTP, 2016). There is an overall consistent body of evidence from experimental work as well as epidemiological studies for perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) supporting that immunotoxicity should be included among sensitive human toxicity endpoints (Fenton et al., 2021; DeWitt et al., 2019).

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Several epidemiological studies have examined associations between PFAS exposure and circulating antibody concentrations after vaccination. A study conducted in ~ 500 Faroese children reported an inverse association between serum concentrations of perfluorohexane sulfonic acid (PFHxS), PFOS, PFOA and perfluorononanoic acid (PFNA) at age 5 with antibody concentrations to diphtheria and tetanus at age 5 and 7 after booster vaccination at age 5 (Grandjean et al., 2012). The associations were most pronounced at age 7 and although most consistently observed for PFOS and PFOA, similar trends were observed for PFHxS and PFNA as well. To verify their findings, the authors later conducted a small experiment in Denmark where 12 healthy adults were given a booster vaccination to diphtheria and tetanus (Kielsen et al., 2016). In that study, baseline serum PFOS concentrations were inversely associated with antibody titers at 4 to 10 days after booster vaccination. In most other studies (Granum et al., 2013; Looker et al., 2014; Pilkerton et al., 2018; Shih et al., 2021; Stein et al., 2016a; Timmermann et al., 2020, 2022, Zeng et al., 2019, 2020), but not all (Stein et al., 2016b), lower antibody concentrations were observed with increasing PFAS exposure.

Furthermore, in a cohort of 101 infants from Germany, Abraham et al. (2020) examined associations between serum PFAS concentrations measured in 12-month-old infants with circulating concentrations of vaccine antibodies to diphtheria, tetanus and Haemophilus influenzae type B (Hib) measured at the same age. Most of the infants (~ 80 %) had been breastfed with a mean duration of 7.4 months. Serum PFOA concentrations measured in these infants were inversely associated with antibody concentrations to diphtheria, tetanus, and Hib, while no association was observed for other PFAS. The results from this study were used in the recent opinion by the European Food Safety Authority (EFSA) for derivation of a tolerable weekly intake (TWI) for the sum of PFOA, PFNA, PFHxS and PFOS to protect against possible adverse effects on the immune system in breastfed infants (EFSA, 2020).

Because the immune system is designed to protect host integrity from challenge and potential insult, a gold standard to measure the functioning of the immune system status in animal experiments is to evaluate the host response to challenge by exposing the test animals to an infectious agent or by immunizing them (WHO, 2012). The main body of *T*-cell dependent antibody response (TDAR) studies with PFOA and PFOS demonstrates a robust pattern of findings to support PFOA- and PFOS-associated immunosuppression (Yang et al., 2002; Loveless et al., 2008; Vetvicka and Vetvickova, 2013; DeWitt et al., 2008; DeWitt et al., 2009; DeWitt et al., 2016; Peden-Adams et al., 2008; Zheng et al., 2009; Dong et al., 2009; Dong et al., 2011; Keil et al., 2008), although no change in humoral immune response also has been reported (Loveless et al., 2008; Qazi et al., 2010; Lefebvre et al., 2008; Woodlief et al., 2021). There is more limited data available on other PFAS and replacement products, with immunosuppresive TDAR results for an aqueous film forming foam (AFFF) formulation (McDonough et al., 2020), and hexafluoropropylene oxide-dimer acid (HFPO-DA) (Rushing et al., 2017), and absence of a change in the TDAR for perfluorodecanoic acid (PFDA) (Frawley et al., 2018), PFHxS (Ramhøj, 2018), and different perfluoroether alkyl acids (PFEA) (Woodlief et al., 2021). The *T*-cell independent antibody response (TIDAR) assay follows the same principle, but relies on sensitization to a (non-proteinaceous) *T*-cell independent antigen, such as a polysaccharide- or lipopolysaccharide-enriched structure, to assess the intrinsic ability of B-cells to respond to an antigen (Allman et al., 2019). Several TIDAR studies have been performed with PFOA and PFOS, with two studies (DeWitt et al., 2016; Peden-Adams et al., 2008) illustrating an immunosuppresive response in this assay, whereas in one study (Qazi et al., 2010) the TIDAR was not changed compared to controls.

The resting immune system of a laboratory animal in a controlled, rather pathogen-free environment is a relatively insensitive test system for evaluating chemically induced immune dysfunction (WHO, 2012). Nevertheless, prime routine toxicological studies such as the 28-day and 90-day toxicity studies in rodents (OECD TG 407 (OECD, 2008), OECD TG 408 (OECD, 2018), and NTP TOX series), do include the observation of hematological profiles, lymphoid organ weights, and lymphoid organ histopathology in their protocols. PFAS induced decreases in spleen and thymus weights, alterations in thymic and splenic lymphocyte subpopulations, increased hypocellularity of the bone marrow, atrophy of the thymus, atrophy of the spleen, and atrophy of the lymph nodes in many repeated-dose toxicity studies in rodents (Ehrlich et al., submitted).

In our previous study, we presented internal relative potency factors (RPFs) for PFAS based on liver effects to facilitate mixture risk assessment with primary input from human biomonitoring studies (Bil et al., 2022a). These RPFs were obtained by comparing the hepatotoxic potency of PFAS at blood (serum) concentrations in male rats after subchronic exposure (42–91 days). In mixture risk assessment, these internal RPFs serve to convert PFAS blood concentrations to PFOA equivalents (PEQ) by multiplying with an internal RPF, and these PEQs are then summed to obtain the cumulative PEQ blood concentration per individual. After this conversion, all further risk calculations can be performed as if it considers exposure to PFOA alone. Using the correct set of RPFs is important, because the toxicokinetic differences among PFAS significantly affect their internal and external potency estimates. Several studies showed that recalculation of external intake dose to internal serum concentrations in rats and zebrafish embryos reduced the difference in potency considerably (Bil et al., 2022a; Gomis et al., 2018; Vogs et al., 2019). For interpretation of results from human biomonitoring studies, which commonly present the concentration of PFAS in the human serum or plasma, it is therefore important to use internal RPFs in mixture risk calculations.

A recent experimental study that addressed combination effects of exposure to mixtures of PFOA and PFOS confirmed that maternal and fetal toxicity endpoints, among which hepatotoxicity, were best described by dose-addition and RPFs (Conley et al., 2022). In the current study, we aim to derive internal RPFs based on immunosuppressive effects of PFAS, to further align the RPF methodology with the critical endpoint underlying the EFSA TWI for PFAS (EFSA, 2020). We present the analyses of internal RPFs for eight PFAS [perfluorohexanoic acid (PFHxA), PFOA, PFNA, PFDA, perfluorobutane sulfonic acid (PFBS), PFHxS, PFOS, HFPO-DA] based on lymphoid organ weights, hematological cell counts, and globulin concentration observed in animal toxicity experiments, as well as an exploration of the derivation of internal RPFs based on human epidemiological data. We correlate these findings with the outcomes of functional assays on PFAS, and provide prospects for further research.

## 2. Materials and method

### 2.1. Animal data

#### 2.1.1. Data selection

For derivation of RPFs based on immunotoxicity, functional tests in experimental animals would be most comprehensive (WHO, 2012). Therefore, a literature search was performed to identify functional *in vivo* immunotoxicity tests for PFAS (e.g. TDAR, TIDAR, delayed type hypersensitivity (DTH) response, host resistance to an infectious agent, and natural killer (NK) cell activity). Studies were summarized, tabulated, and assigned a Klimisch score to assess their relevance and reliability (Klimisch et al., 1997; ECHA, 2011). Because the identified studies used diverse protocols with regard to the endpoints assessed, exposure duration, sensitization procedure, sex, species, and strain they were not suitable for derivation of RPFs (Bil et al., 2021). Instead, they were used to support immunotoxicity of PFAS in a qualitative manner.

In order to obtain data suitable for RPF derivation, the literature search was extended to include repeated dose studies in naïve, non-sensitized animals. A database with 42–90 day repeated-dose toxicity studies that was used in our earlier work (Bil et al., 2021, 2022a) was too fragmented with regard to immune parameters measured (Table S1) and

was therefore deemed unsuitable for the purpose of deriving RPFs for immunotoxicity. In contrast, 28-day repeated dose toxicity guideline studies with rats performed by the NTP for PFBS (NTP, 2019a), PFHxS (NTP, 2019a), PFOS (NTP, 2019a), PFHxA (NTP, 2019b), PFOA (NTP, 2019b), PFNA (NTP, 2019b), and PFDA (NTP, 2019b) obtained immunological information (EMA, 2006) according to an identical study protocol and were therefore suitable for RPF derivation. Relevant immune parameters measured are:

- Weight of the spleen and thymus (absolute and relative to body weight);
- Cell counts of leukocytes, neutrophils, lymphocytes, monocytes, basophils, and eosinophils;
- Serum globulin concentration;
- Histopathology of the spleen, thymus and bone marrow.

Besides these NTP studies, 28-day toxicity studies with similar experimental setup were identified for HFPO-DA (Haas et al., 2008) and perfluorobutanoic acid (PFBA) (Butenhoff et al., 2012), equivalent or similar to OECD TG 407 (OECD, 2008).

Data were collected in a database for the substances and effects listed in Table 1. The individual data from the NTP studies (NTP, 2019a; NTP, 2019b) were obtained from the Chemical Effects in Biological Systems (CEBS) database (https://manticore.niehs.nih.gov/cebssearch). Individual data were also obtained for HFPO-DA, extracted from the full study report (Haas et al., 2008) in the HERO database (https://hero.epa.gov/). Summary data were included for PFBA as reported in Butenhoff et al. (2012). When only summary data was available for one substance (i.e. data for PFBA on terminal body weight and liver weight, and data for HFPO-DA on globulin concentration), summary data were also used for the other substances in the RPF analyses. Liver weight was included as a reference.

### 2.1.2. Exclusion criteria

As noted in Bil et al. (2022b) animals in some of the dose-groups of the NTP studies with PFBS, PFNA, and PFDA showed signs of mortality and severe body weight decrements (NTP, 2019a; NTP, 2019b). Observations related to mortality and body weight loss ($\geq$ 20 %) are generally regarded as excessive toxicity/suffering in experimental animals and are therefore defined as humane endpoints (Van Berlo et al., 2022). In contrast, a 10 % reduction in mean terminal body weight compared to the control group is not to be considered a condition of excessive toxicity (Van Berlo et al., 2022; OECD, 2000). In line with the former, McDonough et al. (2020) changed the dosing regime in a subchronic (28-day) oral immunotoxicity study with mice exposed to a PFAS mixture such that the animals did not lose more than 20 % body weight compared to pre-dosing.

Overt toxicity in animal experiments is related to the confounding effect of stress on chemical-induced immunotoxicity, which has received considerable discussion (Pruett et al., 2008; Boverhof et al., 2014). An increase in corticosterone or cortisol release may result in corticosterone-mediated immunosuppression, manifested as increases in circulating neutrophils, decreases in circulating lymphocytes, decreases in thymus weight, decreases in thymic cortical cellularity and associated histopathologic changes, and changes in spleen and lymph node cellularity (WHO, 2012; EMA, 2006). The confounding effect of stress is usually studied in adrenalectomized animals, with supportive evidence from serum corticosteroid levels and characteristic leukograms (WHO, 2012).

In a subchronic (28-day) oral gavage immunotoxicity study with PFOA in male rats, serum corticosterone was measured to study the effect of stress on immune system functioning *in vivo* (Loveless et al., 2008). An increase in serum corticosterone concentration was only reported in 2/10 rats dosed with 10 mg/kg bw/day (with a corresponding mean terminal body weight of −10 % compared to the control group), which did not reach statistical significance. Suppression in a TDAR study with adrenalectomized mice (DeWitt et al., 2009) confirmed that effects of PFOA on the immune system are not the result of stress-related corticosterone production.

Based on the above considerations, all dose groups of the 28-day toxicity studies (Butenhoff et al., 2012; Haas et al., 2008; NTP, 2019a; NTP, 2019b) with $\geq$ 20 % reduced mean terminal body weight compared to the control group (not to be confused with mean body weight *loss* (Van Berlo et al., 2022) were excluded from our dataset, meaning that data of the two highest dose groups of PFNA (2.5 and 5 mg/kg bw/day) and PFDA (1.25 and 2.5 mg/kg bw/day) were removed. A lower threshold for effects on body weight, such as a $\geq$ 10 % or $\geq$ 15 % reduction was not deemed necessary, as at these effect magnitudes, no notable increase in serum corticosterone concentration was observed in male rats exposed to PFOA (Loveless et al., 2008). Additionally, the

**Table 1**
Overview of the effect parameters included in the immunotoxicity dataset for per- and polyfluoroalkyl substances (PFAS).

| Parameter | Individual/ summary data used | PFBA | PFHxA | PFOA | PFNA | PFDA | PFBS | PFHxS | PFOS | HFPO-DA |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Butenhoff et al., 2012 | NTP, 2019b | NTP, 2019b | NTP, 2019b | NTP, 2019b | NTP, 2019a | NTP, 2019a | NTP, 2019a | Haas et al., 2008 |
| Terminal body weight | Summary | × | × | × | × | × | × | × | × | × |
| Liver weight [a] | Summary | × | × | × | × | × | × | × | × | × |
| Spleen weight [a] | Individual | – | × | × | × | × | × | × | × | × |
| Thymus weight [a] | Individual | – | × | × | × | × | × | × | × | × |
| Leukocyte count [b] | Individual | – | × | × | × | × | × | × | × | – |
| Neutrophil count [b] | Individual | – | × | × | × | × | × | × | × | × |
| Lymphocyte count [b] | Individual | – | × | × | × | × | × | × | × | × |
| Monocyte count [b] | Individual | – | × | × | × | × | × | × | × | × |
| Basophil count [b] | Individual | – | × | × | × | × | × | × | × | × |
| Eosinophil count [b] | Individual | – | × | × | × | × | × | × | × | × |
| Globulin concentration [c] | Summary | – | × | × | × | × | × | × | × | × |
| Spleen histopathology | Individual | – | × | – | × | – | – | – | × | – |
| Thymus histopathology | Individual | – | – | – | × | × | × | – | – | – |
| Lymph node histopathology | Individual | – | × | – | × | – | – | – | – | – |
| Bone marrow histopathology | Individual | – | – | × | × | × | × | – | × | – |

Note: ×, data available; –, data not available; HFPO-DA, hexafluoropropylene oxide-dimer acid; PFBA, perfluorobutanoic acid; PFBS, perfluorobutane sulfonic acid; PFDA, perfluorodecanoic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid.

[a] Absolute organ weight (in grams) and relative organ to terminal body weight (grams organ weight/grams terminal body weight*1000).

[b] Expressed as thousands per cubic milliliter (K/μL).

[c] Expressed as grams/deciliter (g/dL).

Grubbs outlier test in PROAST was used to detect any other outlying data.

#### 2.1.3. Calculation of internal PFAS serum concentrations for the repeated-dose toxicity studies

Because we were particularly interested in RPFs corresponding to an internal dose (serum levels), the toxicokinetic models presented in Bil et al. (2022a) were used to convert external doses to internal doses. To facilitate this, exposure conditions of the experimental animal studies (Butenhoff et al., 2012; Haas et al., 2008; NTP, 2019a; NTP, 2019b) were implemented in toxicokinetic models to convert the external administered doses of PFAS under these conditions to internal doses, represented as Time-Weighted Average (TWA) serum concentration upon repeated-dose exposure (Table S2). This was confined to the male sex, since toxicokinetic models were obtained for male rats only.

#### 2.1.4. Dose-response analysis and derivation of internal RPFs for PFAS

The RPF approach requires that a) chemicals contribute to a common effect, b) their dose–response curves are (approximately) parallel on log–dose scale, and c) chemicals do not interact (Bosgra et al., 2009; Van Der Ven et al., 2022). Parallelism ensures a constant dose factor between the curves at any effect level, so that RPFs do not depend on a predefined benchmark response (Bil et al., 2022a; Bil et al. 2022b).

An exponential model (Eq. (1)) was fitted to the (continuous) response data plotted against the TWA dose (RIVM, 2021). The fitting procedure included a covariate analysis to ensure that parallel curves were fit to the data by applying the same shape parameters (the maximum response (parameter $c$) and the steepness (parameter $d$)) in the exponential model to all PFAS, but allowing the background (parameter $a$), the potency (parameter $b$) and the residual variance to be different between PFAS. In case the data did not provide sufficient information to estimate the maximum fold change, parameter $c$ was not estimated but fixed to a large value ($10^{18}$ in case of an increasing trend) or a small number ($10^{-18}$ in case of a decreasing trend). This indicates that the response levels-off at an unknown dose level above the applied dose range. A good description of the data of each PFAS by parallel curves was confirmed by visual inspection (Bil et al., 2022a; Slob, 2002; Slob and Setzer, 2014)

$$y = a\left(c^{1-e^{\left(-\left(\frac{x}{b}\right)^{d}\right)}}\right) \quad (1)$$

In addition, the obtained dose–response (including covariates) was evaluated by a trend test, in particular, by comparing the Akaike Information Criterion (AIC) of the exponential models to the AIC of the no-response model ($y = a$). In case a trend was present, the model 'E5-CED in terms of RPF' (model #46 in the PROAST manual) (RIVM, 2021) was used to estimate RPFs. This model is reparametrized such that it directly estimates, based on the potency parameter $b$, the RPFs and their 90 % confidence intervals (Bil et al., 2022a). PFOA was set as the index compound, i.e. receiving an internal RPF of one, and the potency of the other compounds was expressed relative to that of PFOA. We chose PFOA as the index compound because it is one of the best-studied perfluoroalkyl acids. Human biomonitoring guidance values for PFOA are readily available, which enables risk assessment of the summed index compound equivalents.

#### 2.1.5. Correlation plots

To verify our assumption that PFAS would have similar RPFs for different effects in case the underlying mode(s) of action are the same, and thus the RPFs for liver toxicity may be a proxy of the differences in overall toxicity between PFAS (Bil et al., 2021), we compared the internal RPFs for thymus weight to the 42–91 day internal liver RPFs from our previous paper (Bil et al., 2021).

### 2.2. Human data

#### 2.2.1. Data selection

To explore the derivation of internal RPFs based on epidemiological data, we examined the relationship between the sum of PFOA, PFOS, PFNA and PFHxS serum concentrations and immune-specific effect biomarkers measured in NHANES (CDC, 1999; CDC, 2003; CDC, 2005), using the analysis by Stein et al. (2016a) as a starting point. In that study, the authors used NHANES cycles from 1999–2000 and 2003–2004, where both serum PFAS and antibody concentrations for measles, mumps, and rubella had been quantified in 12 to 19 year old teenagers. In their study, Stein et al. (2016a) reported a statistically significant inverse association between serum PFOS and PFOA concentrations with antibody concentrations to mumps and rubella. A statistically significant inverse association was also observed between serum PFHxS and antibody concentrations to rubella. Although inverse trends were also observed between PFHxS and PFNA exposure and mumps antibody concentrations and between PFNA and rubella antibody concentrations, no formal significance was reached. No statistically significant associations were seen between PFAS exposure and measles antibody concentrations.

For the purpose of modeling, we extracted the same NHANES data (cycles 1999–2000 ($n = 551$) and 2003–2004 ($n = 640$), in total $n = 1{,}191$) for the 12 to 19 year old teenagers in the Stein et al. (2016a) publication. This included information on serum PFOA, PFNA, PFHxS, and PFOS concentrations; measured antibody concentrations to measles, mumps, and rubella; as well as information on gender, age, ethnicity, and sampling year. In addition, we also extracted information on blood lymphocyte, monocyte, and neutrophil concentrations determined in the 1999–2000 ($n = 551$), 2003–2004 ($n = 757$), and 2005–2006 ($n = 730$) NHANES cycles. The latter immune parameters were not included in the previous publication by Stein et al. (2016a). When extracting the NHANES data for PFAS serum concentrations and blood cell counts, the age range of the population was kept the same as for the antibody concentrations dataset (12 to 19 year old teenagers).

#### 2.2.2. Exclusion criteria

In line with Stein et al. (2016a), only seropositive individuals were selected (defined as optical density (OD) index $\geq 1.0$ for measles and mumps; OD index converted to international units (IU) $\geq 13$ for rubella). Individuals with antibody concentrations below these thresholds were excluded from our final dataset. Out of the 1,191 individuals in our extracted data, the number of seropositive individuals included were 1,152 for measles, 1,064 for mumps, and 1,112 for rubella.

#### 2.2.3. Dose-response analysis and internal RPF derivation

There are two ways of deriving RPFs, depending on the type of data available. One method is applicable when dose–response data are available for individual compounds, i.e. the experimental unit is exposed to a single compound, not to a mixture. In that case, the RPFs can directly be obtained from (parallel) dose–response curves, as explained above for the animal data. The other method is applicable when dose–response data are available for a mixture, i.e. the experimental unit is exposed to a combination of known compounds. In the latter method, the RPFs are considered as unknown model parameters, which are optimized to the mixture dose–response dataset in order to obtain their values (hereafter referred to as the "Mixture-based method").

In the mixture-based method, the exponential model (Eq. (1)) is applied to the (continuous) response data plotted against the cumulative dose of a mixture, while expressing the cumulative dose as function of each individual compound's ($i$) dose ($x_i$) in the mixture multiplied by its unknown RPF ($RPF_i$) (Eq. (2)).

$$Cumulative\ dose = \sum RPF_i \bullet x_i \quad (2)$$

Hence, RPFs are obtained from mixture dose–response data by

optimizing the fit of the exponential model to the unknown RPF parameters in Eq. (3), whereby the dose ($x$ in Eq. (1)) is substituted by the cumulative dose (Eq. (2)).

$$y = a\left(c^{1-e^{-\left(\frac{\sum RPF_i \bullet x_i}{b}\right)^d}}\right) \quad (3)$$

In fitting this model, the index compound receives an RPF of one. The RPFs of the other compounds are obtained by optimizing the model fit, i.e. the RPFs and model parameters resulting in the best AIC. The AIC integrates the log-likelihood and the number of parameters in one single value. The AIC will decrease when the model approaches the data, while also penalizing for the number of parameters included in the model. Hence, the model with the lowest AIC provides the best fit, and provides the optimal fitted RPF estimates (EFSA, 2017).

Based on Stein et al. (2016a), internal RPFs were derived for PFOA, PFNA, PFHxS, and PFOS by fitting the immune-specific effect data (antibody concentrations or white blood cell counts) to the cumulative dose (cumulative PFAS concentration in serum) using Eq. (3). In this particular case, the cumulative dose was calculated using Eq. (2), whereby substance $i$, represents PFOA, PFNA, PFHxS or PFOS and the dose represents the serum concentration of substance $i$ in ng/mL, and subsequently presenting it as cumulative PEQ dose per individual. PFOA was designated as index compound and received an RPF of one. Because the NHANES data were not expected to provide information to estimate the maximum fold change, parameter $c$ was not estimated but fixed to a large value ($10^{18}$ in case of an increasing trend) or a small number ($10^{-18}$ in case of a decreasing trend). This indicates that the response levels-off at an unknown dose level above the measured dose range.

In addition to this, a covariate analysis was performed. By including a certain factor as a covariate in the dose–response analysis, it can be assessed if dose-responses of subgroups differ from each other based on statistical principles (such as the AIC) (EFSA, 2017). This is particularly relevant in the case of epidemiological data to correct for possible confounders (i.e. factors associated with both exposure and outcome). In the covariate analysis of the NHANES data, it was tested whether the background response (parameter $a$) depended on the subgroups gender, ethnicity, survey year, or a combination thereof, based on the outcomes of the adjusted regression model by Stein et al. (2016a) and the absence of other confounders identified in this study. Covariates were introduced in a stepwise manner. First, the AIC of the model with a particular covariate on parameter $a$ was compared to the AIC of the model without this covariate on parameter $a$. We assumed no different background response among subgroups when the AIC values between the fit with covariate and without covariate was less than five points. When a covariate was observed, combinations of two covariates on the background response were tested and the AICs of these model fits were compared to the AIC of the model fit with the identified covariate on the background response. All other model parameters were assumed the same for all subgroups.

Finally, the obtained dose–response (including covariates) was evaluated by a trend test to check the statistical evidence of a dose-related trend. In this procedure, the AIC of the fitted model was compared to the AIC of the no-response model ($y = a$). We assumed absence of a dose–response trend when the AIC values between the different fits was less than five points. In fitting the no-response model, the RPFs were not estimated, but the cumulative dose was derived by assuming the PFAS are equipotent.

Further background theory to substantiate the mixture-based method, using simulated datasets, is provided in the Supplementary Material "Mixture based RPF derivation method".

### 2.3. Software

All calculations were performed in R (version 4.0.0, R Core Team, https://www.r-project.org/). Specific R packages were used for dose–response analysis and deriving RPFs: PROAST (version 70.3, RIVM, https://www.rivm.nl/en/proast). For downloading and organizing the NHANES data, nhanesA (version 0.6.5.3, Endres, https://CRAN.R-project.org/package = nhanesA) was used.

## 3. Results

### 3.1. Animal data

#### 3.1.1. Evaluation of functional immunotoxicity studies

A total of 27 functional *in vivo* immunotoxicity studies were identified in the public literature, with the majority of studies focussing on immune effects and exposure to PFOA or PFOS (Table 2). However, recently, also studies became available for substances considered PFAS substitutes, such as HFPO-DA (Rushing et al., 2017), perfluoro-2-methoxyacetic acid (PFMOAA) (Woodlief et al., 2021), perfluoro-2-methoxypropanoic acid (PFMOPrA) (Woodlief et al., 2021), and perfluoro-4-methoxybutanioc acid (PFMOBA) (Woodlief et al., 2021). Most studies focussed on the TDAR to several antigens such as horse red blood cells, sheep red blood cells, keyhole limpet hemocyanin, and ovalbumin (17 in total), of which three also included assessment of the TIDAR to the antigens 2,4-dinitrophenyl-lipopolysaccharide or 2,4,6-trinitrophenyl-lipopolysaccharide (DeWitt et al., 2016; Peden-Adams et al., 2008; Qazi et al., 2010), four studies looked into host resistance to infection after exposure to different mouse influenca virus strains or *Citrobacter rodentium* bacterium (Frawley et al., 2018; Torres et al., 2021; Suo et al., 2017; Guruge et al., 2009), five studies observed the DTH response (DeWitt et al., 2008; Dong et al., 2011; Lefebvre et al., 2008; Frawley et al., 2018; Ramhøj, 2018), six studies considered (*ex vivo*) effects on lymphoproliferation upon exposure to lipopolysaccharide or Concavalin A (Zheng et al., 2009; Dong et al., 2009; Rockwell et al., 2013; Rockwell et al., 2017; Qazi et al., 2009; Mollenhauer et al., 2011), and five studies looked into NK cell activity (Zheng et al., 2009; Dong et al., 2009; Keil et al., 2008; Woodlief et al., 2021; Frawley et al., 2018).

Most studies were assigned a Klimisch score of 2 ('reliable with restrictions') (Klimisch et al., 1997; ECHA, 2011). The main reason for assigning a Klimisch score of 2 was that the investigations could not be subsummed under a testing guideline and differed somewhat in their protocols, but the methods were generally well documented. Some studies however received a Klimisch score of 3 ('not reliable') (Klimisch et al., 1997; ECHA, 2011). Reasons for assigning a Klimisch score of 3 were an unclear and deviating study protocol (Lefebvre et al., 2008), low dosing in combination with a small group size jeopardising the power of the study (Qazi et al., 2010; Ramhøj, 2018; Torres et al., 2021), a less physiologically relevant exposure route (Rockwell et al., 2013; Rockwell et al., 2017), severely impaired food intake and/or body weight decrements (Zheng et al., 2009; Qazi et al., 2009), a changed dosing regimen during the study (Woodlief et al., 2021), and a negative response of the positive control (Woodlief et al., 2021).

Giving most weight to the studies that received a Klimisch score of 2, the majority of studies provide evidence that PFOA (Yang et al., 2002; Loveless et al., 2008; Vetvicka and Vetvickova, 2013; DeWitt et al., 2008; DeWitt et al., 2009; DeWitt et al., 2016; McDonough et al., 2020; Ramhøj, 2018; De Guise and Levin, 2021), PFOS (Vetvicka and Vetvickova, 2013; Peden-Adams et al., 2008; Zheng et al., 2009; Dong et al., 2009; Dong et al., 2011; Keil et al., 2008; Lefebvre et al., 2008), HFPO-DA (Rushing et al., 2017), and a commercial aqueous film forming foam (AFFF) formulation containing C5-C10 PFSA, PFOA, Cl-PFOS or precursors thereof (McDonough et al., 2020), resulted in a suppressed TDAR. Moreover, studies provide indications that exposure to PFOA (DeWitt et al., 2016), and PFOS (Peden-Adams et al., 2008), resulted in a suppressed TIDAR. In line with this, studies observe exposure to PFOS resulted in a decreased host resistance to infection (Suo et al., 2017; Guruge et al., 2009), whereas exposure to PFDA did not result in a

**Table 2**
Functional immunotoxicity studies with per- and polyfluoroalkyl substances (PFAS) in rodents.

| Study | Substance | Species, strain, sex, and group size | Dose, exposure duration, and exposure route | Experimental design and study protocol | Response and effects observed | Klimisch score |
|---|---|---|---|---|---|---|
| Yang et al. (2002) | PFOA | Mouse (C57BL/6, 4–6 M per group) | 0 and 24 mg/kg bw/day for 10 days (diet) | **TDAR study**. Immunization to HRBC (i.v. injection with 200 μL of 5–10 × $10^7$ HRBC/mL in EBSS) on day 5, 6 days before sacrifice. One group continued PFOA treatment for 6 days after immunization (i.e. total of 16 days exposure; others normal chow). Measurement of HRBC-specific IgM levels with PFC assay and ELISA. | **TDAR study**. ↓ plaque formation by HRBC-specific IgM and IgG, ↓ HRBC-specific IgM and IgGs in serum, both at 24 mg/kg bw/day. | **2** |
| Vetvicka and Vetvickova (2013) | PFOA | Mouse (Balb/c, 5 F per group) | 0 and 20 mg/kg bw/day for 21 days (oral gavage) | **TDAR study**. Immunization to OVA twice (2 weeks apart, i.p. injections of 0.1 mg/kg with 100 μL OVA) on days 8 and 15. Second injection was 7 days before sacrifice. Measurement of OVA-specific IgM levels with ELISA.<br>**Other observations.** Splenic and thymic cellularity. | **TDAR study.** ↓ OVA-specific IgM at 20 mg/kg bw/day PFOA. | **2** |
| Loveless et al. (2008) | PFOA | Mouse (Crl:CD-1(ICR)BR, 20 M per group)<br>Rats (Crl:CD(SD)IGS BR, 10 M per group) | 0.3, 1, 10, and 30 mg/kg bw/day for 29 days (oral gavage) | **TDAR study**. Immunization to SRBC (i.v. injection with 0.5 mL of 4 × $10^8$ SRBC/mL (rat) or 0.2 mL of 1 × $10^9$ SRBC/mL (mouse)) on day 23 (rat) or 24 (mouse), seven (rats) or six (mouse) days before sacrifice. Measurement of SRBC-specific IgM levels with ELISA.<br>**Other observations.** Body weight, organ weight and histopathology, hematology, clinical chemistry, corticosterone measurement, spleen and thymus cellularity. | Mouse:<br>**TDAR study.** ↓ SRBC-specific IgM ≥ 10 mg/kg bw/day.<br><br>**Other observations.** ↑ liver weight and liver focal necrosis ≥ 1 mg/kg bw/day. ↓ body weight, spleen and thymus weight, atrophy of lymphoid tissue, eosinophils, total number of thymocytes and splenocytes ↑ neutrophils and monocytes, corticosterone serum levels) ≥ 10 mg/kg bw/day. ↓ lymphocytes at 30 mg/kg bw/day.<br>Rat:<br>**TDAR study.** PFOA had no effect on production of anti-SRBC IgM.<br>**Other observations**. ↑ liver weight, minimal focal liver necrosis and corticosterone serum levels ≥ 10 mg/kg bw/day. | **2** |
| DeWitt et al. (2008) | PFOA | Mouse (C57BL/6N, 8 F per group) | 0 and 30 mg/kg for 15 days (constant group) or 10 days (recovery group); dose response study I with 0, 3.75, 7.5, 17, 30 mg/kg bw/day for 15 days (drinking water); and dose response study II 0, 0.94, 1.88, 3.75, 7.5 mg/kg bw/day<br>for 15 days (drinking water) | **TDAR study**. Immunization to SRBC once (i.v. injection with 4.0 × $10^7$ SRBC in 0.2 mL saline), on day 11, or immunization to SRBC twice (two weeks apart, i.v. injection with 4.0 × $10^7$ SRBC in 0.2 mL saline), on day 11 and day 25. Animals were sacrificed five days after. Measurement of SRBC-specific IgM and IgG levels with ELISA.<br>**DTH study.** On day 11, animals were sensitized with a subcutaneous injection of BSA-CFA (0.05 mL of 2 mg/mL BSA-CFA). At day 18, animals were challenged with an injection of 0.05 mL of heat-aggregated BSA into the right footpad. Footpad thickness was measured 24 h post-challenge.<br>**Other observations.** Body weight, lymphoid organ weights (spleen, thymus). | **TDAR study.** ↓ SRBC-specific IgM both in constant and recovery groups, and ↓ SRBC-specific IgM ≥ 3.75 mg/kg bw/day (dose–response studies I and II). SRBC-specific IgG titers not clearly affected in a dose-dependent manner (↑ at 3.75 and 7.5 mg/kw bw/day only).<br>**DTH study.** DTH responses were not statistically altered by exposure to the tested doses of PFOA.<br>**Other observations**. ↓ body weight at 30 mg/kg bw/day (study II). ↓ spleen and thymus weight ≥ 15 mg/kg bw/day (study I) and ↓ spleen weight ≥ 3.75 mg/kg bw/day (study II). | **2** |

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**Table 2** (*continued*)

| Study | Substance | Species, strain, sex, and group size | Dose, exposure duration, and exposure route | Experimental design and study protocol | Response and effects observed | Klimisch score |
|---|---|---|---|---|---|---|
| Qazi et al. (2009) | PFOA | Mouse (C57BL/6 (H-$2^b$), 4 M per group) | 0, 2 or 40 mg/kg bw/day PFOA for 10 days (diet) | **Lymphoproliferative response.** Immunization to LPS on day 10 (i.v. injection in tail vein with 0.1 mL of 300 μg LPS in saline). Two hours after administration of LPS, blood was collected to quantify the TNFα and IL-6 concentration by ELISA.<br>**Other observations.** The liver, epididymal fat, spleen, and thymus were collected and weighed, total and differential white blood cell counts were determined, and immunofluorescent staining and flow cytometric analysis was performed on peritoneal, bone marrow, and spleen macrophages. | **Lymphoproliferative response.** ↑ *ex vivo* TNFα and IL-6 responses to LPS in the peritoneal cavity and the bone marrow (but not the spleen),<br>**Other observations.** ↓ bw, thymus, spleen and epididymal fat weights, ↓ total circulating WBC, lymphocytes, neutrophils, ↑ macrophages ($CD11^+$) in the bone marrow. Responses changed at 40 mg/kg bw/day compared to controls, but not at 2 mg/kg bw/day.<br>**Severely reduced food consumption in the high-dose group (-75 %).** | **3** |
| DeWitt et al. (2009) | PFOA | Mouse (adrenalectomized or sham-operated C57BL/6N, 6 F per group) | 0, 3.75, 7.5, or 15 mg /kg bw/day for 10 days (drinking water) | **TDAR study.** Immunization to SRBC (i.v. injection with $7.5 \times 10^7$ SRBC in 0.2 mL saline) on day 11, five days before sacrifice. Measurement of SRBC-specific IgM levels with ELISA.<br>**Other observations.** Corticosterone serum levels. | **TDAR study.** ↓ IgM ≥ 7.5 mg/kg bw/day in adx and ↓ IgM at 15 mg/kg bw/day in sham mice.<br><br>**Other observations.** Increase in corticosterone only at 15 mg/kg bw/day in sham mice. Hence, suppression of SRBC-specific IgM was not the result of corticosterone production. | **2** |
| DeWitt et al. (2016) | PFOA | Mouse (PPARα-KO; B6.129S4-Ppartm1GonzN12 and WT C57BL/6-Tac, 4–6 F per group)<br>TIDAR: mouse (C57BL/6N WT, 8 F per group) | PPARα-KO mice compared to WT, exposed to 0, 7.5 or 30 mg/kg bw/day for 15 days (TDAR) or 0, 0.94, 1.88, 3.75, and 7.5 mg/kg bw/day for 15 days (TIDAR) (drinking water) | **TDAR study.** Immunization to SRBC (i.v. injection with $7.5 \times 10^7$ SRBC in 0.2 mL saline) on day 11, five days before sacrifice. Measurement of SRBC-specific IgM levels with ELISA.<br>**TIDAR study.** Immunization to DNP-LPS (i.v. injection with 1 μg DNP-LPS in 0.2 mL saline) on day 11, seven days before sacrifice. Measurement of DNP-LPS-specific IgM levels with ELISA.<br>**Other observations.** Body weights, lymphoid organ weights, splenic lymphocyte phenotypes (the latter in non-immunized PFOA-treated mice). | **TDAR study.** ↓ SRBC-specific IgM at 30 mg/kg bw/day in both PPARα KO (no decline in bw, spleen-, or thymus weight observed) and WT mice (decline in bw, spleen- and thymus weight).<br>**TIDAR study.** ↓ DNP-LPS-specific IgM ≥ 1.88 mg/kg bw/day. | **2** |
| De Guise and Levin (2021) | PFOA | Mouse (B6C3F1, 12–16 F per group) | 0, 1.88 and 7.5 mg/kg bw/day for 28 days (drinking water) | **TDAR study.** Immunization to KLH (i.p. injection with 300 mg KLH/mice in a total volume of 0.5 mL) on day 24, five days before sacrifice. Measurement of KLH-specific IgM levels with ELISA.<br>**Other observations.** Body weight, serum cytokines, serum corticosterone. | **TDAR study.** ↓ KLH-specific IgM ≥ 1.88 mg/kg bw/day.<br>**Other observations.** Serum corticosterone was not significantly correlated with TDAR or measured cytokines. At 5 mg/kg bw/day, ↓ Th2, mixed response for Th1 cytokines (overall favoring a Th1 balance). At both treated groups, ↓ pro-inflammatory cytokines. | **2** |
| Rockwell et al. (2013) | PFNA | Mouse (C57BL/6, 5 per sex/group). | 0 or 0.46 mg/kg bw once (i.p. injection) | **Lymphoproliferative response.** Immunization to LPS on day 14 (i.p. injection with 1 mg/kg LPS or saline). 1.5 h after administration of LPS, blood was collected to quantify the TNFα concentration by ELISA.<br>**Other observations.** Spleen, thymus, kidney and liver were collected on day 14. Splenocyte and thymocyte immunophenotyping was performed by flow cytometry analysis. | **Lymphoproliferative response.** ↑ TNFα<br>**Other observations.** Spleen atrophy, ↓ spleen weight, spleen leukocyte count, spleen red blood cell count, ↑ $CD4^+$, $CD8^+$ in the spleen, ↓ $CD4^+/CD8^+$ cells in the thymus, ↑ $CD4^+$, $CD8^+$, $CD4^-/CD8^-$ cells in the thymus. ↓ $CD14^+$ cells and $CD19^+$ cells in the spleen.<br>**Animals were exposed once via i.p. injection.** | **3** |

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**Table 2** (*continued*)

| Study | Substance | Species, strain, sex, and group size | Dose, exposure duration, and exposure route | Experimental design and study protocol | Response and effects observed | Klimisch score |
|---|---|---|---|---|---|---|
| Rockwell et al. (2017) | PFNA | Mouse (C57BL/6 J, 5 per sex/group). | 0 or 0.46 mg/kg bw once (i.p. injection) | **Lymphoproliferative response.** Immunization to LPS on day 28 (i.p. injection with 1 mg/kg LPS or saline). 1.5 h after administration of LPS, blood was collected to quantify the TNFα concentration by ELISA.<br>**Other observations:** Spleen, thymus and liver were collected on day 28. Splenocyte and thymocyte immunophenotyping was performed by flow cytometry analysis. | **Lymphoproliferative response**. ↑ TNFα<br>**Other observations.** ↑ $CD14^+$ cells and ↓ $CD19^+$ cells in the spleen. Spleen and thymus atrophy, ↓ spleen and thymus weights, ↑ $CD4^+$, $CD8^+$ in the spleen, ↓ $CD4^+/CD8^+$ cells in the thymus.<br>**Animals were exposed once via i.p. injection.** | **3** |
| Frawley et al. (2018) | PFDA | Mouse (B6C3F1/N, 8 F)<br>Rat (SD, 8 F) | Mouse: once each week (days 1, 8, 15, and 22) at doses of 0, 0.3125, 0.625, 1.25, 2.5, and 5 mg/kg bw/week (oral gavage)<br>Rat: 0, 0.125, 0.25, 0.5, 1, and 2 mg/kg bw/day for 28 days (oral gavage) | **TDAR study.** Immunization to SRBC and KLH (i.v. injection with 2 mg KLH and an unknown quantity of SRBC respectively) on day 23 (in rat and mouse), and measurement of anti-SRBC and anti-KLH with ELISA. In a separate assay, immunization to SRBC on day 25 and measurement of spleen IgM with APC response.<br>**NK cell activity.** Splenocytes and $^{51}$Cr-labelled YAC-1 cells were prepared in different ratios (200:1, 100:1, 50:1, 25:1, 12.5:1, 6.25:1). Then, 100 μL supernatant was counted using a γ-counter.<br><br>**DTH study.** On days 21 and 29, animals were challenged with a subcutaneous injection of C. albicans ($2 \times 10^7$ organisms for rats, $1 \times 10^7$ for mice) in the right footpad. Footpad thickness was measured right before the second challenge, and 24 h post-challenge.<br><br>**Mononuclear phagocyte system (MPS) activity.** Uptake and vascular clearance of $^{51}$Cr-labelled SRBC by fixed macrophages in the liver, spleen, thymus, lung and kidney. Intravenous injection with $^{51}$Cr-labelled SRBC on day 29. SRBC serum half-life and relative organ uptake was determined using a γ-counter 30–60 min. post-injection.<br><br>**Host resistance to infection study.** Mice (treated for 28 days as described above) were infected intranasally with *Influenza A*/Hong Kong/*8/86* (H3N2) virus at three challenge levels (1:2420, 1:440, and 1:80 dilutions). Mice were observed twice daily for 21d for changes in appearance, locomotion, and respiration.<br><br>**Other observations.** Body weight, organ histopathology, total and differential white blood cell counts, spleen cell immunophenotyping, bone marrow DNA synthesis, colony formation, and differentials. | **TDAR study (mouse and rat).** No PFDA exposure-related effects were observed on the AFC response to SRBC in rats or mice, or in the serum IgM levels to SRBC or KLH in rats.<br>**Host resistance to infection (mouse).** Treatment with PFDA did not decrease survival compared to the control during the 21 day post-challenge observation period.<br>**DTH (mouse and rat).** No increased footpad swelling was observed in treated groups compared to the control group.<br><br>**MPS activity (rat).** Altered functional activity of mononuclear phagocytic system in liver and thymus ≥ 0.25 mg/kg bw/day.<br><br>**Other observations (mouse).** ↓ spleen weight, splenic atrophy (20 %), ↓ total spleen cells, Ig + and NK + cells at 5.0 mg/kg bw/week PFDA, and ↓ CD3+, CD4+, CD8+, and Mac3 + cells in the spleen ≥ 1.25 mg/kg bw/week PFDA.<br><br>**Other observations (rat).** No change from controls on lymphoid organ weights, leukocyte subpopulations, and bone marrow cellularity. | **2** |

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**Table 2** (*continued*)

| Study | Substance | Species, strain, sex, and group size | Dose, exposure duration, and exposure route | Experimental design and study protocol | Response and effects observed | Klimisch score |
|---|---|---|---|---|---|---|
| McDonough et al. (2020) | AFFF formulation (containing C5-C10 PFSA, PFOA, Cl-PFOS or precursors thereof) | Mouse (C57BL/6, 6 per sex/group) | AFFF formulation (based on 0, 1.88, 3.75, 7.5, or 10 mg/kg bw/day PFOS + PFOA measured in the formulation) for 10 days followed by 6 days of depuration (oral gavage) | **TDAR study.** Immunization to SRBC (i.v. injection with $7.5 \times 10^7$ SRBC in 0.2 mL saline) on day 11, five days before sacrifice. Measurement of SRBC-specific IgM levels with ELISA.<br><br>**Other observations.** Body weight, liver weight, lymphoid organ weights, spleen cellularity, splenic lymphocyte subpopulations. | **TDAR study.** ↓ SRBC-specific IgM in F and M ≥ 7.5 mg/kg bw/day.<br><br>**Other observations.** ↓ rel. spleen weight in M at 10 mg/kg bw/day and ↓ body weight, rel. thymus weight in F and M ≥ 7.5 mg/kg bw/day. ↑ liver weight in all treated dose groups in M and F compared to the control groups. | **2** |
| | PFOA | Mouse (C57BL/6, 6 per sex/group) | 0 and 7.5 mg/kg bw/day PFOA) for 10 days followed by 6 days of depuration (oral gavage) | **TDAR study.** Immunization to SRBC (i.v. injection with $7.5 \times 10^7$ SRBC in 0.2 mL saline) on day 11, five days before sacrifice. Measurement of SRBC-specific IgM levels with ELISA. | **TDAR study.** ↓ SRBC-specific IgM in F and M at 7.5 mg/kg bw/day. | |
| Ramhøj (2018) | PFHxS | Rat (HanTac: WH, dams, 8 or 20 litters per group in two separate experiments) | Dams were exposed to 0, 25, or 45 mg/kg bw/day and 0, 0.5, 5 and 25 mg/kg bw/day PFHxS in two separate experiments during GD7-PND22 (oral gavage) | **TDAR study.** Immunization to KLH in weaned offspring twice (14 days apart, 200 µL of 1.5 mg/ml KLH via i.p. injection). At PND28 and PND37 in experiment 1 and at PND34 and PND43 in experiment 2. Measurement of KLH-specific IgM and IgG with ELISA.<br><br>**DTH study.** The day before sacrifice, animals were challenged intradermally with an injection of 20 µL of 5 mg/ml KLH or 20 µL saline in the left and right ear respectively. Right after sacrifice, the thickness of the ear was measured and weighted.<br><br>**Other observations.** Lymphoid organ weights. | **TDAR study.** No effects on IgM and IgG responses up to the highest dose tested compared to the control group.<br><br>**DHT study.** No increased ear swelling was observed in treated groups compared to the control group.<br><br>**Other observations.** No effects on lymphoid organs in M or F at any dose apart from ↓ in lymph node wt in M at PND 16 in 25 and 45 mg/kg bw/day dose groups in experiment 1.<br><br>**Challenging regimen and timepoint of measuring KLH-specific IgM and IgG differs from other TDAR studies. Also the positive control cyclophosphamide was negative.** | **3** |
| Peden-Adams et al. (2008) | PFOS | Mouse (B6C3F1, 5 per sex/group for TDAR study, 10 F per group for TIDAR study) | 0, 0.166, 1.66, 3.31, 16.6, 33.1, and 166 µg/kg bw/day for 28 days (oral gavage) (TDAR study)<br><br>0 and 334 µg/kg bw/day for 21 days (oral) (TIDAR study) | **TDAR study.** Immunization to SRBC (i.p. injection, 0.1 mL of 25 % SRBC in PBS) on day 23, five days before sacrifice. Measurement of SRBC-specific IgM with PFC assay.<br><br>**TIDAR study.** Immunization to TNP-LPS (i.v. injection in tail vein, 100 µL of 1 µg TNP-LPS/µL) on day 14, seven days before sacrifice. Measurement of TNP-LPS-specific IgM with ELISA.<br><br>**Other observations.** Splenic and thymic CD4/CD8 subpopulations. | **TDAR study.** ↓ SRBC-specific IgM ≥ 1.66 µg/kg bw/day in M and ≥ 16.6 µg/kg bw/day in F.<br><br>**TIDAR study.** ↓ TNP-specific IgM at 334 µg/kg bw/day in F.<br><br>**Other observations.** Alteration of splenic (but not thymic) CD4/CD8 *T*-cells ≥ 3.31 µg/kg bw/day in M, and alteration of splenic and thymic CD4/CD8 *T*-cells ≥ 3.31 µg/kg bw/day in F. | **2** |

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**Table 2** (*continued*)

| Study | Substance | Species, strain, sex, and group size | Dose, exposure duration, and exposure route | Experimental design and study protocol | Response and effects observed | Klimisch score |
|---|---|---|---|---|---|---|
| Zheng et al. (2009) | PFOS | Mouse (C57BL/6, 12 M per group) | 0, 5, 20 and 40 mg/kg bw/day for 7 days (oral gavage) | **TDAR study.** Immunization to SRBC (i.p. injection with 0.1 mL of a 25 % SRBC suspension in PBS) on day three, five days before sacrifice. Measurement of SRBC-specific IgM with PFC assay.<br><br>**NK cell activity.** Splenocytes and Yac-1 cells were prepared in the ratio 10:1. Then, the amount of LDH released from lysed Yac-1 cells was determined to observe NK cell activity.<br><br>**Lymphoproliferative response.** Isolated splenocytes were exposed to either 10 μg/mL ConA or LPS. After that, proliferation was determined using the MTT assay.<br><br>**Other observations.** Body weight, organ weights (liver, kidney, spleen, and thymus), thymus and spleen cellularity, serum corticosterone, thymic and splenic CD4/CD8 subpopulations. | **TDAR study.** ↓ SRBC-specific IgM ≥ 5 mg/kg bw/day.<br><br>**NK cell activity.** ↓ NK cell activity ≥ 20 mg/kg bw/day<br><br>**Lymphoproliferative response.** ↓ splenic leukocyte proliferation in response to ConA and LPS ≥ 5 mg/kg bw/day.<br><br>**Other observations.** ↓ splenic and thymic cellularity, ↑ liver weight ≥ 5 mg/kg bw/day. ↓ body weight, thymus and spleen weight, ↓ splenic and thymic lymphocyte subpopulations, ↑ serum corticosterone ≥ 20 mg/kg bw/day.<br><br>**Severe impairment of terminal body weight and food intake ≥ 20 mg/kg bw/day. Mean terminal body weight decreased with approximately 15 % and 25 % at 20 and 40 mg/kg bw/day respectively.** | **3** |
| Dong et al. (2009) | PFOS | Mouse (C57BL/6, 10 M per group) | 0, 0.008, 0.08, 0.42, 0.83, and 2.1 mg/kg bw/day<br>for 60 days (oral gavage) | **TDAR study.** Immunization to SRBC (i.p. injection with 0.1 mL of a 25 % SRBC suspension in PBS) on day 57, four days before sacrifice. Measurement of SRBC-specific IgM with PFC assay.<br><br>**NK cell activity.** Splenocytes and Yac-1 cells were prepared in the ratio 10:1. Then, the amount of LDH released from lysed Yac-1 cells was determined to observe NK cell activity.<br><br>**Lymphoproliferative response.** Isolated splenocytes were exposed to either 10 μg/mL ConA or LPS. After that, proliferation was determined using the MTT assay.<br><br>**Other observations.** Body weight, organ weights (liver, spleen, and thymus), thymus and spleen cellularity, serum corticosterone, thymic and splenic CD4/CD8 subpopulations. | **TDAR study.** ↓ SRBC-specific IgM ≥ 0.08 mg/kg bw/day.<br><br>**NK cell activity.** ↓ NK cell activity ≥ 0.83 mg/kg bw/day.<br><br>**Lymphoproliferative response.** ↓ splenic leukocyte proliferation in response to ConA and LPS ≥ 0.83 mg/kg bw/day.<br><br>**Other observations.** ↓ body weight, spleen and thymus weights, spleen and thymus cellularity, splenic and thymic *T*-cell subpopulations ≥ 0.42 mg/kg bw/day. ↑ serum corticosterone level ≥ 0.83 mg/kg bw/day. | **2** |
| Dong et al. (2011) | PFOS | Mouse (C57BL/6, 6 M per group) | 0, 0.0083, 0.017, 0.083, 0.42 and 0.83 mg/kg bw/day<br>for 60 days (oral gavage) | **TDAR and DTH study.** Immunization to SRBC (i.v. injection with $4.0 \times 10^7$ SRBC in 0.2 mL saline) on day 54 in 12 animals per group, seven days before sacrifice. On day 60, 6 animals per group received a SRBC booster immunization (footpad injection with $4.0 \times 10^7$ SRBC in 0.2 mL saline) for assessment of a DTH response (footpad swelling) and different immunoglobulin determinations (IgGs, IgE). Measurement of SRBC-specific immunoglobulins with ELISA. | **TDAR study.** ↓ SRBC-specific IgM ≥ 0.083 mg/kg bw/day.<br><br>**DTH study.** No increased footpad swelling was observed in treated groups compared to the control group.<br><br>**Other observations.** ↓ bw change, food consumption, spleen and thymus weights ≥ 0.833 mg/kg bw/day. ↑ SRBC-specific IgGs and SRBC-specific IgE at 0.83 mg/kg bw/day. ↑ IL-4 ≥ 0.083 | **2** |

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**Table 2** (*continued*)

| Study | Substance | Species, strain, sex, and group size | Dose, exposure duration, and exposure route | Experimental design and study protocol | Response and effects observed | Klimisch score |
|---|---|---|---|---|---|---|
| | | | | | mg/kg bw/day, ↑ IL-10 at 0.83 mg/kg bw/day, ↓ IL-2 and INF-γ at 0.83 mg/kg bw/day. | |
| | | | | **Other observations.** Body weight, organ weights (liver, spleen, and thymus), serum corticosterone, measurement of cytokines. | | |
| Keil et al. (2008) | PFOS | Mouse (B6C3F1, 10–12 dams per group) | 0.1, 1, and 5 mg/kg bw/day on GD 1–17 (oral gavage) | **TDAR study.** Immunization to SRBC (i.v. injection with 7.5 × $10^7$ SRBC in 0.2 mL saline) in eight-week-old F1 pups (6 sex/dose), four days before sacrifice. Measurement of SRBC-specific IgM with PFC assay.<br><br>**NK cell activity.** Splenocytes and Yac-1 cells were prepared in different ratios (200:1, 100:1, 50:1, 25:1, 12.5:1, 6.25:1). Then, lysis was determined by lysing $^{51}$Cr-labeled Yac-1 cells with 0.1 % Triton X in complete media.<br><br>**Other observations.** Body weight, organ weight (liver, thymus, spleen, uterus), spleen and thymus cellularity, splenic and thymic CD4/CD8 subpopulations, nitrite production by peritoneal macrophages. | **TDAR study.** ↓ of SRBC-specific IgM in M at 5 mg/kg bw/day at week 8.<br><br>**NK cell activity.** ↓ NK cell activity in M ≥ 1 mg/kg bw/day and in F at 5 mg/kg bw/day at week 8.<br><br>**Other observations.** At week 4, ↑ liver weight in M at 5 mg/kg bw/day. ↓ $CD3^+$ and $CD4^+$ thymocytes in M at 5 mg/kg bw/day. In maternal animals ↓ CD4:CD8 ratio at 5 mg/kg bw/day. | **2** |
| Lefebvre et al. (2008) | PFOS | Rat (SD, 10–15 per sex/group) | 0.14, 1.33, 3.21, 6.34 (M) and 0.15, 1.43, 3.73, 7.58 (F) mg/kg bw/day for 28 days (diet) | **TDAR and DTH study.** Immunization to KLH twice (i.p. injection with 1 mg KLH) on day 14 and day 21, 14 and 7 days before sacrifice. On day 28, rats were injected with 2 mg heat-inactivated KLH or saline in the left and right footpad respectively, to measure swelling after 24 h. Right after measuring footpad swelling, blood serum was sampled for measuring KLH-specific IgG with ELISA.<br><br>**Other observations.** Body weight, organ weights (liver, thymus, spleen), organ histopathology (liver, thymus, spleen, mesenteric lymph nodes), total serum immunoglobulin (IgM, IgA, IgG, unchallenged), peripheral blood lymphocyte phenotyping, splenocyte proliferation. | **TDAR study.** ↑ KLH-specific IgG in M at the highest dose in M, but not in F.<br><br>**DTH study.** There was no statistically significant change in footpad swelling.<br><br>**Other observations**. ↓ body weight and ↑ liver weight at the two highest dose groups (M, F), and ↓ spleen (M) and thymus (M, F) weight at the highest dose. ↑ total IgM (unchallenged) in F at the highest dose. No effect on peripheral blood lymphocyte phenotype, or splenic leukocyte proliferation in response to ConA and LPS.<br><br>**Challenging regimen and timepoint of measuring KLH-specific IgG differs from other TDAR studies.** | **3** |
| Qazi et al. (2010) | PFOS | Mouse (B6C3F1, 5 M per group) | 0 and 250 μg/kg bw/day for 28 days (diet) | **TDAR study.** Immunization to SRBC (i.p. injection with 0.1 mL of a 1:10 SRBC suspension in PBS) on day 23, five days before sacrifice. Measurement of SRBC-specific IgM and IgG with ELISA and IgM with PFC assay.<br><br>**TIDAR study.** Immunization TNP-LPS (i.v. injection with 0.1 mL of 100 μL/mL TNP-LPS in 0.9 % NaCl) on day 23, five days before sacrifice. Measurement of TNP-LPS-specific IgM with ELISA.<br><br>**Other observations.** Organ and tissue weights (liver, epididymal fat, spleen, and thymus), serum corticosterone, immunophenotyping of the thymus and the spleen. | **TDAR, TIDAR, and other observations.** No change in the TDAR or TIDAR at 250 μg/kg bw/day compared to the control group. Serum levels of SRBC-specific IgM and IgG or levels of TNP-LPS-specific IgM were not altered by PFOS treatment. ↑ weight of the liver at 250 μg/kg bw/day. Cellular compositions of the thymus and spleen were not altered. No effect on corticosterone levels observed compared to the control group.<br><br>**Study was performed with one low dose and had small group sizes.** | **3** |

(*continued on next page*)

**Table 2** (*continued*)

| Study | Substance | Species, strain, sex, and group size | Dose, exposure duration, and exposure route | Experimental design and study protocol | Response and effects observed | Klimisch score |
|---|---|---|---|---|---|---|
| Guruge et al. (2009) | PFOS | Mouse (B6C3F1, 30 F per group) | 0, 5 and 25 μg/kg bw/day for 21 days (oral gavage) | **Host resistance to infection study.** Challenge with mouse influenza virus strain A/PR/8/34 (H1N1) intranasally (100 pfu/mouse in 30 μL PBS) at day 21. After treatment animals were observed for morbidity and mortality for another 20 days. | **Host resistance to infection study.** Reduced survival of infected animals (↓ body weight 10 days post challenge, ↑ mortality) at 25 μg/kg bw/day. Organ weights (liver, kidney, lung, spleen, thymus) of the treated groups, were not statistically significantly different from the control group. | **2** |
| Suo et al. (2017) | PFOS | Mouse (C57BL/6, sex not specified, 4 per group) | 0 and 2 mg/kg bw/day for 18 days (oral gavage) | **Host resistance to infection study**. Challenge with mouse *Citrobacter rodentium* bacterium strain DBS100 ($10^{10}$ cfu) in 200 μL PBS at day 7 via oral gavage. PFOS exposure was continued until the end of the experiment. Intestinal lamina propria lymphocytes were harvested during exposure and infection. | **Host resistance to infection study**. At 2 mg/kg bw/day, PFOS inhibited the outgrowth of the pathogen (↑ of IL-22 from ILC3 cells). In the later phase ↑ bacterial counts and ↑ of inflammatory cytokines, (↑ of IL-22 and IL-17 from ILC3 cells, ↑ IFN-γ from $CD3^-$ cells, ↑ of IL-22 and IL-17 from Th17 cells), reduced mucus production, dysbiosis, ↑ levels of *E.coli*. | **2** |
| Torres et al. (2021) | PFOS | Mouse (C57BL/6, 4–5 per sex/group (exp. 1) and 8–10 per sex/group (exp. 2)) | 0 and 1.5 μg/kg bw/day for 28 days (oral gavage) | **Host resistance to infection study.** **Experiment 1:** Challenge with mouse influenza virus strain A/WSN/33 (H1N1) intranasally ($1e^6$ pfu/mouse) at day 28. Body weights were monitored. After 11 days, BALF was collected from the lungs, and blood, lungs, liver and spleen were collected.<br><br>**Experiment 2:** Challenge with mouse influenza virus strains A/WSn/33(H1N1) (WSN-$OVA_1$) and A/WSN/33(H1N1) (WSN-$OVA_{11}$) intranasally ($2e^4$ pfu/mouse) at day 28. Body weights and tissue collections were as described above. | **Host resistance to infection study.** **Experiment 1.** No effect on virus-induced weight loss, on the number of inflammatory cells, T cells, and granulocytes in the lung. ↓ $CD4^+$ and ↑ $CD8^+$ T cells in BALF and ↑ $CD4^+CD44^{Hi}$ in the lung at 1.5 μg/kg bw/day.<br><br>**Experiment 2.** No effect on virus-induced weight loss, no effect on inflammatory cells in BALF, no difference in the percentage of antigen-specific $CD4^+$ or $CD8^+$ T cells in the spleen. ↑ number of antigen-specific $CD4^+$ T cells in the spleen at 1.5 μg/kg bw/day.<br><br>**Study was performed with one low dose and had small group sizes.** | **3** |
| Qazi et al. (2009) | PFOS | Mouse (C57BL/6 (H-$2^b$), 4 M per group) | 0, 2 and 40 mg/kg bw/day PFOS for 10 days (diet) | **Lymphoproliferative response.** Immunization with LPS on day 10 (i.v. injection in tail vein with 0.1 mL of 300 μg LPS in saline). 2 h after administration of LPS, blood was collected to quantify the TNFα and IL-6 concentration by ELISA.<br><br>**Other observations.** The liver, epididymal fat, spleen, and thymus were collected and weighed, total and differential white blood cell counts were determined, and immunofluorescent staining and flow cytometric analysis was performed on peritoneal, bone marrow, and spleen macrophages. | **Lymphoproliferative response.** ↑ in the *ex vivo* TNFα and IL-6 responses to LPS in the peritoneal cavity and the bone marrow (but not the spleen), ↓ food consumption, bw, and thymus, spleen and epididymal fat weights, ↓ total circulating WBC, lymphocytes, ↑ macrophages ($CD11^+$) in the bone marrow. Responses were observed at 40 mg/kg bw/day, but not at 2 mg/kg bw/day.<br><br>**Severely reduced food consumption in the high-dose group (-65 %).** | **3** |
| Mollenhauer et al. (2011) | PFOS | Mouse (B6C3F1, 5 F per group) | 0, 0.0331, 0.0993 or 9.93 mg/kg bw/day for 28 days (oral gavage). | **Lymphoproliferative response.** Immunization with LPS on day 10 (i.p. injection with 0.1 mL of 25 μg/mL LPS solution). 1 h after administration of LPS, blood was collected to quantify the TNFα and IL-6 concentration by ELISA. Peritoneal lavage fluid cytokine levels were determined using ELISA.<br><br>**Other observations.** Body weight, spleen mass, and spleen cellularity were determined. | **Lymphoproliferative response.** No clear dose–response in TNFα and IL-6 serum concentrations up to the highest dose. ↑ *ex vivo* IL-6 production by peritoneal macrophages at 9.93 mg/kg bw/day in F challenged *in vivo* with LPS but no effect on TNFα.<br><br>**Other observations.** ↓ bw and spleen weight at 9.93 mg/kg bw/day, spleen cellularity not affected. | **2** |

(*continued on next page*)

**Table 2** (*continued*)

| Study | Substance | Species, strain, sex, and group size | Dose, exposure duration, and exposure route | Experimental design and study protocol | Response and effects observed | Klimisch score |
|---|---|---|---|---|---|---|
| Vetvicka and Vetvickova (2013) | PFOS | Mouse (Balb/c, 5 F per group) | 0 and 20 mg/kg bw/dayfor 21 days (oral gavage) | **TDAR study**. Immunization to OVA twice (2 weeks apart, i.p. injections of 0.1 mg/kg with 100 μL OVA) on days 8 and 15. Second injection was 7 days before sacrifice. Measurement of OVA-specific IgM levels with ELISA. **Other observations.** Splenic and thymic cellularity. | **TDAR study.** ↓ OVA-specific IgM at 20 mg/kg bw/day PFOS. | |
| Rushing et al. (2017) | HFPO-DA | Mouse (C57BL/6, 6 per sex/group) | 0, 1, 10, or 100 mg/kg bw/day for 28 days (oral gavage) | **TDAR study.** Immunization to SRBC (i.v. injection with $4 \times 10^7$ SRBC in 0.2 mL saline) on day 24, five days before sacrifice. Measurement of IgM by ELISA.<br><br>**Other observations.** Body weight, organ weights (liver, thymus, spleen), immunophenotyping of spleen. | **TDAR study.** ↓ SRBC-specific IgM in F, but not in M, at 100 mg/kg bw/day.<br><br>**Other observations.** ↑ $CD8^+$ and $CD4^-/CD8^-$ T cells in M at 100 mg/kg bw/day. ↓ relative spleen weight in F at 100 mg/kg bw/day. | **2** |
| Woodlief et al. (2021) | PFMOAA | Mouse (C57BL/6, 4–6 per sex/group) | 0, 0.00025, 0.0.025 and 2.5 mg/kg bw/day for 30 days (drinking water) | **TDAR study.** Immunization to SRBC (i.v. injection with $4 \times 10^7$ SRBC in 0.2 mL saline) on day 26, five days before sacrifice. Measurement of IgM with ELISA.<br><br>**Other observations.** Body weight, organ weights (liver, thymus, spleen), immunophenotyping of thymus and spleen. | **TDAR study.** No statistical differences were detected in the TDAR in M or F animals given PFMOAA compared to the control group.<br><br>**Other observations**. No statistically significant effect was observed on body weight, lymphoid organ weight, and thymus and spleen immunophenotyping, up to the highest dose tested. | **3** |
| | PFMOPrA | Mouse (C57BL/6, 4–6 per sex/group) | 0, 0.5, 5, and 50 mg/kg bw/day for 30 days (drinking water) | **TDAR study.** Immunization to SRBC (i.v. injection with $4 \times 10^7$ SRBC in 0.2 mL saline) on day 26, five days before sacrifice. Measurement of IgM with ELISA.<br><br>**NK cell activity.** YAK-1 cells were prepared 5 days before the NK cell assay. Spleens were processed and lymphocytes were isolated. Lymphocyte and YAK-1 cells were prepared in three ratios (5:1, 10:1, and 30:1). After that, the percent specific lysis was determined by flow analysis.<br><br>**Other observations.** Body weight, organ weights (liver, thymus, spleen), immunophenotyping of thymus and spleen. | **TDAR study.** No statistical differences were detected in the TDAR in M or F animals given PFMOPrA compared to the control groups.<br><br>**NK cell activity.** There was no statistically significant change in NK cell activity.<br><br>**Other observations.** ↓ in spleen weight in F at 0.5 and 50 mg/kg bw/day, ↑ in spleen weight in F at 5 mg/kg bw/day.<br><br>**Dosing regimen of M animals was increased (2-fold) after week one of the experiment.** | |
| | PFMOBA | Mouse (C57BL/6, 4–6 per sex/group) | 0, 0.5, 5, and 50 mg/kg bw/day for 30 days (drinking water) | **TDAR study.** Immunization to SRBC (i.v. injection with $4 \times 10^7$ SRBC in 0.2 mL saline) on day 26, five days before sacrifice. Measurement of IgM with ELISA.<br><br>**NK cell activity.** YAK-1 cells were prepared 5 days before the NK cell assay. Spleens were processed and lymphocytes were isolated. Target cells (500 μL) were added to effector cells in three E:T ratios (5:1, 10:1, and 30:1). After that, the percent specific lysis was determined by flow analysis. | **TDAR study.** No statistical differences were detected in the TDAR in M or F animals given PFMOBA compared to the control group.<br><br>**NK cell activity**. There was no statistically significant change in NK cell activity.<br><br>**Other observations**. ↑ B cells and NK cells in the spleen in M at all doses compared to the control group, and ↓ B cells and NK cells in the spleen at 50 mg/kg bw/day in F compared to the control group. | |

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**Table 2** (*continued*)

| Study | Substance | Species, strain, sex, and group size | Dose, exposure duration, and exposure route | Experimental design and study protocol | Response and effects observed | Klimisch score |
|---|---|---|---|---|---|---|
| | | | | **Other observations.** Body weight, organ weights (liver, thymus, spleen), immunophenotyping of thymus and spleen, natural killer cell activity. | | |
| | PFOA | Mouse (C57BL/6, 4–6 per sex/group) | 0, and 7.5 mg/kg bw/day for 30 days (drinking water) | **TDAR study.** Immunization to SRBC (i.v. injection with $4 \times 10^7$ SRBC in 0.2 mL saline) on day 26, five days before sacrifice. Measurement of IgM with ELISA. | **TDAR study.** No statistical differences were detected in the TDAR in M or F animals given PFOA compared to the control group. | |
| | | | | **Other observations.** Body weight, organ weights (liver, thymus, spleen), immunophenotyping of thymus and spleen. | **Other observations.** ↓ relative spleen weight at 7.5 mg/kg bw/day in F.<br>**PFOA, serving as the positive control, was also negative in the TDAR study.** | |

Note: Relevance and reliability of the study was determined using the Klimisch score as described in Klimisch et al. (1997) and ECHA Guidance R.4 (ECHA, 2011). AFC, antibody forming cell; BALF, bronchoalveolar lavage fluid; BSA-CFA, bovine serum albumin in complete Freund's adjuvant; ConA, Concanavalin A; DNP, 2,4-dinitrophenyl; DTH, delayed-type hypersensitivity; EBSS, Earle's balanced solution; F, female; HFPO-DA, hexafluoropropylene oxide-dimer acid; HRBC, horse red blood cells; i.p., intraperitoneal; i.v., intravenous; KLH, keyhole limpet hemocyanin; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; M, male; NK, natural killer; OVA, ovalbumin; PBS, phosphate-buffered saline; PFBA, perfluorobutanoic acid; PFBS, perfluorobutane sulfonic acid; PFC, plaque forming cell; PFDA, perfluorodecanoic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFMOAA, perfluoro-2-methoxyacetic acid; PFMOPrA, perfluoro-2-methoxypropanoic acid; PFMOBA, perfluoro-4-methoxybutanioc acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; PND, postnatal day; SRBC, sheep red blood cells; TDAR, *t*-cell dependent antibody response; TIDAR, *t*-cell independent antibody response; TNP, 2,4,6-trinitrophenyl.

change in host resistance to infection nor a reduced TDAR compared to consecutive controls (Frawley et al., 2018). The majority of studies looking into the DTH response provide evidence that PFAS do not affect DTH, as exposure to PFOA (DeWitt et al., 2008), and PFDA (Frawley et al., 2018), did not result in changed DTH responses compared to the respective control groups. With regard to the innate immune system, studies provide evidence that PFAS exposure results in decreased activity of NK cells and/or a decreased number of NK cells upon exposure to PFDA (Frawley et al., 2018), and PFOS (Zheng et al., 2009; Dong et al., 2009; Keil et al., 2008).

### 3.1.2. Dose-response analyses

Analyses were performed for absolute and relative organ weights (liver, spleen, thymus), white blood cell (sub)populations (counts of total leukocytes, neutrophils, lymphocytes, monocytes, basophils, and eosinophils), and globulin concentration, fitting the no-response and exponential (Eq. (1)) models. Histopathological observations in the spleen, thymus, bone marrow and lymph nodes were not considered for dose–response analysis because only a selection of PFAS caused changes in these organs (Table 1). Likewise, basophil and eosinophil counts were not considered for dose–response analysis because the variation in their counts was only minimal (min–max of basophil counts in the dataset was 0–0.06 K/μL and min–max of eosinophils counts was 0–0.33 K/μL). We show the dose–response modelling procedure for relative thymus weight below for illustrative purposes. The dose–response analyses for the other effects are provided in Figs. S1-S5.

For the relative thymus weight dataset, the exponential model with a fixed value for parameter *c* provided the best fit (Fig. 1a). Visual inspection of the individual dose–response curves of the substances indicated a good description of the data by parallel curves (Fig. 1b).

### 3.1.3. Internal RPFs for immunotoxicity

Internal RPFs were successfully derived for absolute thymus weight, relative thymus weight, absolute spleen weight, and globulin concentration. The dose–response analyses for relative spleen weight, total leukocytes, neutrophils, lymphocytes, and monocytes did not show a significant trend and hence no internal RPFs could be derived based on these datasets.

Based on the data of thymus weight decrease, PFDA, PFHxA, and PFNA are most potent at the serum level, followed by PFOS, PFBS, PFOA and PFHxS (Fig. 1 and Table 3). Internal potency estimates range from 7 to 0.5. Whereas an internal RPF could be obtained for PFDA based on absolute thymus weight decrease, there was no significant trend observed in the relative thymus weight data, indicating less confidence in the internal RPF of 6 for this PFAS based on the absolute thymus weight decrease. HFPO-DA induced no change in thymus weight compared to the control group, and hence no internal RPFs were obtained for this substance.

Internal RPFs could also be derived based on the dataset for absolute spleen weight (Fig. S2 and Table 3). PFDA and PFNA were most potent at the serum level, followed by PFOS, PFOA, PFBS, and PFHxS. Potency estimates range from 12 to 0.3. The dose–response analysis for PFHxA and HFPO-DA did not show a significant trend in the data (Fig. S2), and hence no internal RPFs were obtained. The dose–response analysis for relative spleen weight did not provide a significant trend at all, indicating an overall lower confidence in the internal RPFs for PFAS based on absolute spleen weight (Fig. S5). The internal RPFs of globulin concentration were generally lower compared to the internal RPFs of lymphoid organ weight (Fig. 2 and Table 3). The 90 % confidence intervals of the internal RPFs are provided in Table S3.

### 3.1.4. Internal RPFs for hepatotoxicity

Internal RPFs were also successfully derived based on the dataset for relative liver weight (Fig. S4 and Table 3). Potency estimates ranged from 0.6 to 25. HFPO-DA was most potent, followed by PFDA, PFHxA, PFOS, PFNA, PFBS, PFBA, PFOA, and PFHxS. In an earlier analysis, we

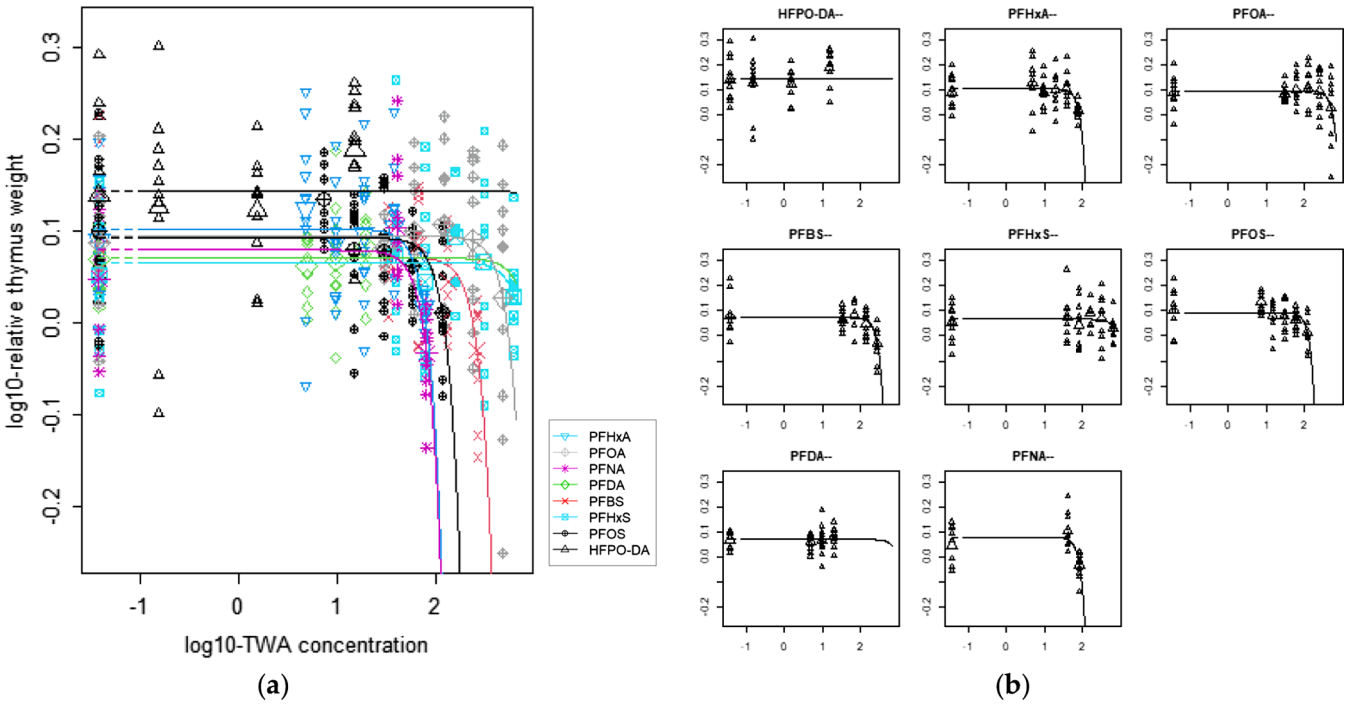


**Fig. 1.** Dose-response analysis for time-weighted average (TWA) serum concentration (μg/mL) of perfluorobutane sulfonic acid (PFBS), perfluorohexane sulfonic acid (PFHxS), perfluorooctane sulfonic acid (PFOS), perfluorohexanoic acid (PFHxA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), and hexafluoropropylene oxide-dimer acid (HFPO-DA) expressed against relative thymus weight (grams organ weight/grams body weight*1000) from the 28-day toxicity studies (Butenhoff et al., 2012; Haas et al., 2008; NTP, 2019a; NTP, 2019b) (a) Dose-response curves for eight PFAS on a $\log_{10}$-scale obtained by simultaneously fitting the exponential model with a study specific background response. Note: Larger symbols represent the geometric mean of each dose group, smaller symbols represent individual data. (b) Individual dose–response curves for the same relative thymus weight data to confirm parallel curve fitting is applicable to all PFAS. Note: Larger symbols represent the geometric mean of each dose group, smaller symbols represent individual data.

derived internal RPFs for relative liver weight based on oral 42–91 day toxicity data (Bil et al., 2022a). Interestingly, the order of potency was quite similar, with PFHxA and perfluorododecanoic acid (PFDoDA) (both internal RPF of 10) being most potent, followed by HFPO-DA, PFNA, PFOS, PFBA, PFOA, PFHxS and PFBS. Earlier, we confirmed that the confidence intervals of the external RPFs based on these two hepatotoxicity datasets overlapped, apart from PFBS (Bil et al. 2022b).

**Table 3**
Internal relative potency factors (RPFs) for absolute and relative thymus weight, absolute spleen weight, globulin concentration and relative liver weight based on time-weighted average (TWA) serum concentrations after 28-day repeated-dose exposure to eight per- and polyfluoroalkyl substances (PFAS) in male rats (Butenhoff et al., 2012; Haas et al., 2008; NTP, 2019a; NTP, 2019b).

| Substance | Abs. thymus | Rel. thymus | Abs. spleen | Globulin | Rel. liver |
|---|---|---|---|---|---|
| PFBA | – | – | – | – | 1 |
| PFHxA | 6 | 7 | NR | 0.9 | 6 |
| PFOA | 1 | 1 | 1 | 1 | 1 |
| PFNA | 6 | 7 | 6 | 1 | 4 |
| PFDA | 6 | NR | 12 | 2 | 7 |
| PFBS | 2 | 2 | 0.7 | 0.1 | 2 |
| PFHxS | 0.5 | 0.7 | 0.3 | 0.03 | 0.6 |
| PFOS | 4 | 4 | 2 | 0.2 | 5 |
| HFPO-DA | NR | NR | NR | 1 | 25 |

Note: –, data not available; HFPO-DA, hexafluoropropylene oxide-dimer acid; NR, no significant trend in the dose–response analysis; PFBA, perfluorobutanoic acid; PFBS, perfluorobutane sulfonic acid; PFDA, perfluorodecanoic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid. PFOA was set as the index compound, and was therefore assigned an internal RPF value of 1.

### 3.1.5. *Correlation plots*

In order to evaluate if the previously derived internal RPFs for relative liver weight increase, based on oral 42–91 day toxicity data (Bil et al., 2022a) would be in line with the immunotoxicity dataset currently used, the correlation between the internal RPFs for hepatotoxicity

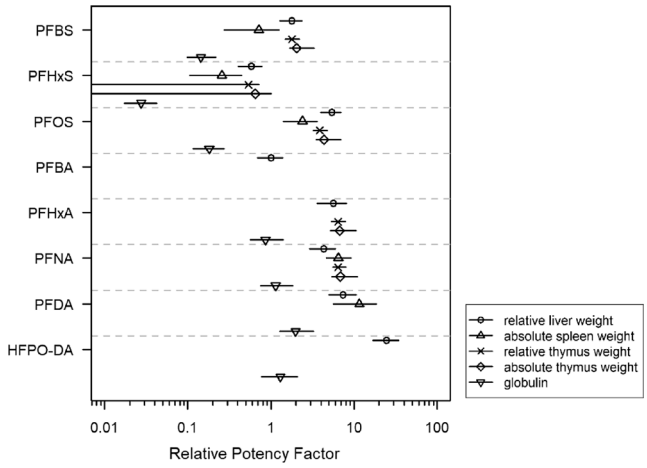


**Fig. 2.** Internal relative potency factors (RPFs) and their 90% confidence intervals for eight per- and polyfluoroalkyl substances (PFAS) based on thymus weight, spleen weight, liver weight, and globulin concentration. Note: lines indicate the 90% confidence intervals of the internal RPFs. HFPO-DA, hexafluoropropylene oxide-dimer acid; PFBA, perfluorobutanoic acid; PFBS, perfluorobutane sulfonic acid; PFDA, perfluorodecanoic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid.

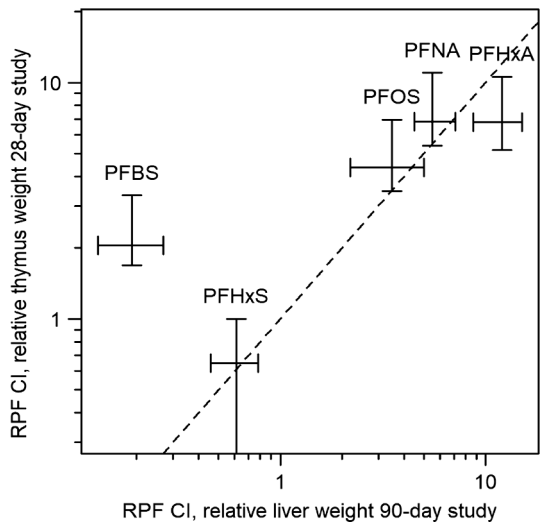


**Fig. 3.** Correlation between the internal relative potency factor (RPF) 90% confidence intervals (CIs) for relative thymus weight from the 28-day dataset used in the current paper (Butenhoff et al., 2012; Haas et al., 2008; NTP, 2019a; NTP, 2019b) and the internal RFF 90% CIs for relative liver weight increase in Bil et al. (2022a). Note: The dashed line represents the unity line. Because in Bil et al. (2022a) no internal RPF for PFDA could be derived, and in the current paper no internal RPFs could be derived for HFPO-DA and PFBA for relative thymus weight, the total number of substances included in this correlation plot is five and not eight. CI, 90% confidence interval; PFBS, perfluorobutane sulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOS, perfluorooctane sulfonic acid; RPF, relative potency factor.

reported in Bil et al. (2022a) and the internal RPFs for relative thymus weight of the 28 day toxicity dataset (Butenhoff et al., 2012; Haas et al., 2008; NTP, 2019a; NTP, 2019b) was examined. Generally, the confidence intervals of the internal RPFs for relative liver weight and relative thymus weight overlap, except for those of PFBS (Fig. 3), indicating that the internal RPFs between these datasets are overall in line.

### 3.2. Human data

#### 3.2.1. Covariate analyses for measles, mumps, and rubella antibody concentrations

The exponential model (Eq. (3)) was fitted to the measles, mumps, and rubella antibody concentration data plotted against the cumulative PEQ dose using gender, sampling year, ethnicity, or a combination thereof as covariate on the background response (parameter $a$) (Table 4). Taking the dose–response fitting procedure of rubella antibodies as an example, fitting the exponential model with gender as covariate on parameter $a$ resulted in an AIC of 2107.58, sampling year in an AIC of 2108.00, and ethnicity in an AIC of 2087.42. In comparing these AICs to the fit of the model without applying any covariate on parameter $a$ (AIC of 2106.38), only the model fit with ethnicity as covariate differed with five points or more from the fit without applying any covariates (-18.96), indicating a difference in rubella antibody levels among ethnic groups. For measles, mumps and rubella antibodies, lowest AICs were obtained using ethnicity as a covariate compared to applying no covariate on parameter $a$. For mumps, comparison of the AIC also provided evidence for gender as a covariate on the background response. However, application of both gender and ethnicity as a covariate on the background response did not improve the fit of the model sufficiently compared to the AIC of the model fit by only applying ethnicity as covariate on the background response.

#### 3.2.2. Trend analyses for measles, mumps, and rubella antibody concentrations

The best fit of the exponential model (including covariates) was compared to the no-response model (including covariates) to check the statistical evidence of a dose-related trend (Table 4). Using again the fitting procedure for rubella antibodies as an example, the exponential model fit with ethnicity as a covariate on parameter $a$ (AIC of 2087.42) compared to the no-response model fit with ethnicity as a covariate on parameter $a$ (AIC of 2111.66) resulted in an increased AIC by 24.24 points, indicating statistical evidence of a trend in the data. When comparing the AIC of the fitted model to the no-response model, the data did not show a trend for measles, but for rubella and mumps it did (bold fit in Table 4, Fig. 4).

#### 3.2.3. Internal RPFs based on measles, mumps, and rubella antibody concentrations

Resulting internal RPFs for PFOA, PFNA, PFHxS, and PFOS based on mumps and rubella antibody concentrations were large ($> 10^2$ for mumps and $> 10^4$ for rubella) and confidence intervals ranged from zero to infinity in most cases. The lower 5 % confidence bound of the internal RPF derived for PFOS based on the rubella data and the lower 5 % confidence bound of the internal RPF derived for PFOS and PFNA based on the mumps data were 0.5, 0.08 and 1.2 respectively (Fig. 5). The upper 5 % confidence bounds of all internal RPFs approached infinity.

Similar analyses were performed for the white blood cell counts. No trend in the data was observed. The results are provided in Table S4 and Fig. S6.

## 4. Discussion

We successfully derived internal RPFs for eight PFAS based on thymus weight decrease, spleen weight decrease, and reductions in serum globulin concentration in the male rat. The results of our study fit to the overall picture that PFAS cause immunosuppression and degeneration of lymphoid tissues. There is ample evidence from animal toxicity experiments illustrating that exposure to PFAS causes atrophy of the spleen, thymus, and lymph nodes, results in bone marrow cell depletion, decreases the TDAR and the TIDAR, reduces the host resistance to infections, leads to decreased NK cell activity, and reduces clonal production of leukocytes. The results of our study indicate that, for all PFAS evaluated in this manuscript, internal RPFs based on lymphoid organs are overall similar to those previously derived based on liver weight (Bil et al., 2022a). This increases the confidence to apply our internal RPFs to PFAS blood concentrations, and to compare the resulting cumulative PEQ blood concentration with the blood equivalent of the PFAS TWI established by EFSA, which is based on immune suppression in children. Furthermore, this finding may justify the use of the larger set of external liver weight RPFs (of 23 substances) in combination with the EFSA TWI in risk assessment of external intake mixtures.

The only PFAS that had a clearly distinct internal RPF based on effects on liver weight and lymphoid organ weights was HFPO-DA. For this substance, no internal RPFs for changes in weights of the lymphoid organs could be obtained because, in the HFPO-DA applied dose-range, the thymus and spleen weights of male rats did not show a dose–response trend. Haas et al. (2008) however used a top-dose of 30 mg/kg bw/day for males in their 28-day toxicity study with HFPO-DA, while a 90-day toxicity study illustrates male rats can tolerate a dose of 100 mg/kg bw/day (Haas et al., 2009), indicating the 28-day study was dosed too low. Hence, the absence of internal RPFs for thymus and spleen weight for HFPO-DA may be explained by a lack of suitable data from studies using higher doses rather than a lack of response. This is supported by the observation that oral exposure to HFPO-DA for 28 days in male mice caused an increase in the number of $CD8^+$, $CD4^+/CD8^+$, and $CD4^-/CD8^-$ T cells in the spleen at a dose of 100 mg/kg bw/day compared to the control group (Rushing et al., 2017).

Whereas the internal RPFs derived using data on thymus, spleen, and

**Table 4**
Akaike Information Criterion (AIC) of different combinations of model fits and covariates for measles, mumps, and rubella antibody concentration (OD index or IU respectively) plotted against the cumulative dose of perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorohexane sulfonic acid (PFHxS), and perfluorooctane sulfonic acid (PFOS) as PFOA Equivalents (PEQ) in blood of teenagers (age 12–19) in the National Health and Nutrition Examination Survey (NHANES) cycles of 1999–2000 and 2003–2004 for measles ($n$ = 1,153), mumps ($n$ = 1,064), and rubella ($n$ = 1,112).

| Substance | Endpoint | Model | Covariate on parameter $a$ | AIC[a] | Change in AIC | |
|---|---|---|---|---|---|---|
| | | | | | Covariate analysis | Trend analysis |
| Sum PEQ[b] | Anti-measles | Equation (1); | None | 2622.64 | | |
| | | | Gender | 2621.96 | −0.68 | |
| | | $c$ fixed at $10^{-18}$ | Sampling year | 2620.94 | −1.70 | |
| | | | Ethnicity | 2552.54 | −70.10 | |
| | | No-response | Ethnicity | **2554.60** | | **+2.06** |
| Sum PEQ[b] | Anti-mumps | Equation (1); | None | 1235.96 | | |
| | | | Gender | 1229.78 | −6.18 | |
| | | $c$ fixed at $10^{-18}$ | Sampling year | 1237.40 | +1.44 | |
| | | | Ethnicity | **1217.80** | **−18.16** | |
| | | | Ethnicity and gender | 1214.70 | −3.10 | |
| | | No-response | Ethnicity | 1227.04 | | +9.24 |
| Sum PEQ[b] | Anti-rubella | Equation (1); | None | 2106.38 | | |
| | | | Gender | 2107.58 | +1.20 | |
| | | $c$ fixed at $10^{-18}$ | Sampling year | 2108.00 | +1.62 | |
| | | | Ethnicity | **2087.42** | **−18.96** | |
| | | No-response | Ethnicity | 2111.66 | | +24.24 |

Note: the best model fit is indicated in bold.
[a] An improvement of the AIC by 5 points or more indicated statistical evidence of a better fit of the model.
[b] The total PFOA Equivalent (PEQ) dose was calculated by applying the optimized RPFs for PFOA (RPF = 1), PFNA, PFHxS, and PFOS when fitting the exponential model. In fitting the no-response model, PFAS were assumed to be equipotent.

liver weight are overall similar for each substance, the internal RPFs derived based on serum globulin deviate substantially. The maximum internal RPF of 2 (for PFDA) based on serum globulin concentration was much lower than that of the maximum internal RPF of 12 (also for PFDA) based on spleen weight, and a similar trend is observed for the other substances. Although the globulin concentration in serum is suggested to be linked to immunosuppression (EMA, 2006), it is a rather nonspecific indicator of immunotoxicity. The globulin fraction of serum total protein consists of α-, β-, and γ-globulins (O'Connell et al., 2005). Alpha- and β-globulins are produced by hepatocytes, and the γ-globulins (immunoglobulins) by B-lymphocytes. Thus, while a decrease in serum globulin concentration could indicate the γ-globulin fraction is decreased, it could also indicate a decrease in α-globulin and/or β-globulin fractions. Some studies measured the α-, β-, and γ-globulin fractions in male rats, noting the $\alpha_2$-globulin fraction was decreased after exposure to PFDoDA (Kato et al., 2015), and the $\alpha_1$-globulin and γ-globulin fractions were decreased and increased after exposure to perfluorooctadecanoic acid (PFODA) respectively (Hirata-Koizumi et al., 2012). Furthermore, because these total globulin measurements were performed in unchallenged animals, their meaning with regard to immune suppression is considered limited. The antigen-specific IgM and/or IgG serum concentrations measured in the TDAR and TIDAR

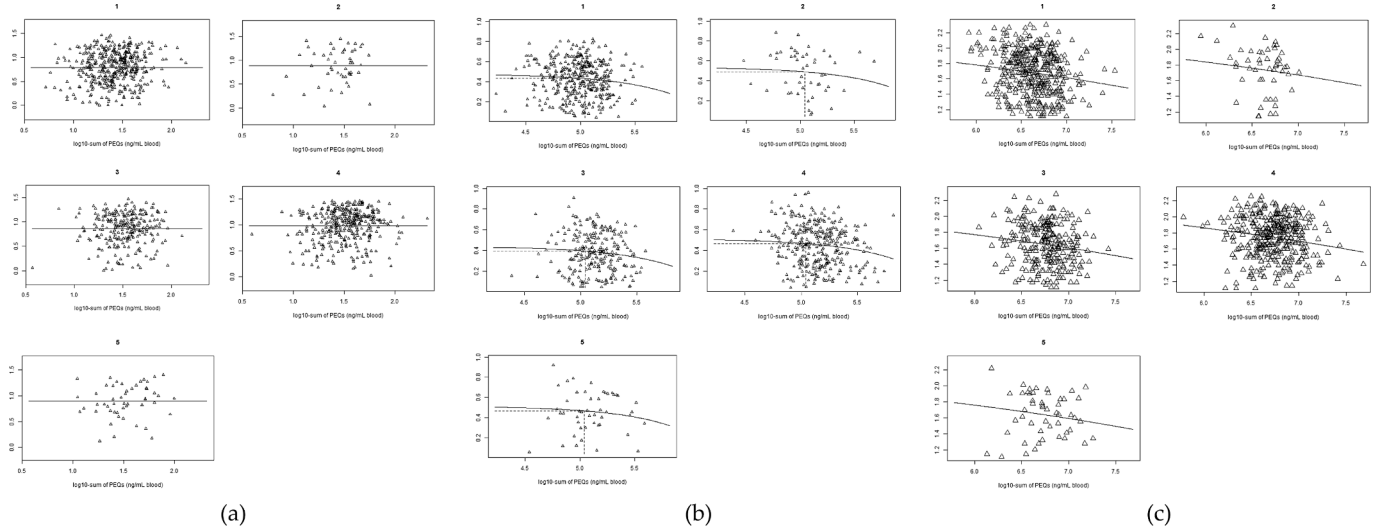


**Fig. 4.** (a) Dose-response data and curves of measles (OD index) against the sum PEQ in ng/mL blood by fitting the no-response model with ethnicity as a covariate on parameter a. In fitting the no-response model, the assumption is made that PFOA, PFNA, PFHxS and PFOS are equipotent. (b) dose–response data and curves of mumps (OD index) against the sum of PEQ in ng/mL blood by fitting the exponential model with ethnicity as a covariate on parameter a. Optimized RPF values are used to calculate the sum PEQ. c) dose–response data and curves of rubella (IU) against the sum PEQ in ng/mL blood by fitting the exponential model with ethnicity as a covariate on parameter a. Optimized RPF values are used to calculate the sum PEQ. Note: both axes are on log10-scale. Headers above the plots indicate ethnicity. Ethnicity was coded according to self-identified categories by NHANES: 1, Mexican American; 2, Other Hispanic; 3, non-Hispanic white; 4, non-Hispanic black; and 5, other non-Hispanic race including non-Hispanic multiracial). IU, international units; OD, optical density; PEQ, PFOA equivalent; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid.

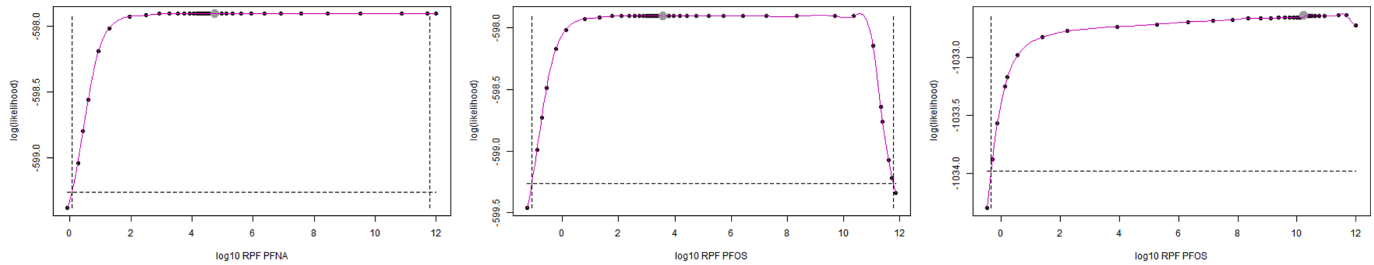


**Fig. 5.** Loglikelihood profiles of optimized RPF values for single PFAS resulting from the exponential model fit of the sum PFOA equivalent dose against antibody concentrations of mumps and rubella respectively, using ethnicity as covariate on the background response. (a) RPF loglikelihood profile of PFNA for mumps with a lower 5 % confidence bound of 1.2 and an upper 5 % confidence bound of $\geq 10^{12}$; (b) RPF loglikelihood profile of PFOS for mumps with a lower 5 % confidence bound of 0.08 and an upper 5 % confidence bound of $\geq 10^{12}$; (c) RPF loglikelihood profile of PFOS for rubella with a lower 5 % confidence bound of 0.5 and an upper 5 % confidence bounds of $\geq 10^{12}$. Note: the pink line indicates the estimated loglikelihood profile, the large grey dot indicates the optimized RPF value. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

assay after antigenic challenge should be treated as a considerably more precise measure of the adaptive immune response compared to total serum globulin concentration in unchallenged animals.

Our attempt to derive RPFs based on cross-sectional NHANES data among 12 to 19 year old US teenagers was unsuccessful. In the previous publication by Stein et al. (2016a), inverse associations were consistently reported for PFOA, PFNA, PFHxS, and PFOS with antibody concentrations to mumps and rubella, although formal significance was not always reached for all four compounds. Similar to those findings, our modeled sum PEQ was significantly (as measured by a change in AIC) associated with a reduction in antibody concentrations to mumps and rubella. However, when attempting to extract information on RPFs from the model, the resulting RPF parameters were highly imprecise as reflected by their wide confidence intervals ranging from approximately zero to infinity. The moderate to higher pairwise correlation between individual PFAS (with $r = 0.23$ to 0.66) combined with a relatively small exposure range for some of the substances (i.e. geometric mean and 95 % CI of PFHxS and PFNA serum concentrations of 2.47 (2.15, 2.85) and 0.765 (0.648, 0.903) ng/mL respectively) (Stein et al., 2016a), as well as a high interindividual variation in the measured antibody concentrations, is likely to have contributed to these inconclusive estimates. In the U.S., children are typically vaccinated with a combined measles, mumps, and rubella (MMR) vaccine at the age of 12–15 months (first dose) and 4–6 years (second dose) (Perrone and Meissner, 2020). The high interindividual variation in the NHANES dataset may be explained by the fact that it is unknown whether the teenagers were treated with the MMR vaccine, and if so, at what age. In addition, in the NHANES dataset there was a long period between (booster) vaccination and sampling. Consequently, by using this specific dataset, it may be challenging to link the exposure (current) to the biological effect (historical), which ideally should be determined at a fixed time interval after vaccination. All of the above considered, the correlated exposure and other methodological challenges associated with observational data did not allow for derivation of RPFs using the NHANES data. We encourage researchers to apply our methodology to other human datasets with lower correlations among PFAS, a larger PFAS exposure range, and, for vaccination studies, datasets with reported vaccination and sample withdrawal preferably relatively soon after immunization, in order to derive internal RPFs for PFAS based on human data.

The mode(s) of action underlying PFAS immunomodulation are largely unknown and may be due to an interplay of different pathway perturbations at various levels. Mechanistic studies provide evidence that PFAS modulate relevant nuclear receptors and transcription factors [peroxisome proliferator-activated receptors (PPARs), nuclear factor kappa-B (NF-κB)], $Ca^{2+}$-signaling, oxidative stress, and cytokine levels that affect various cell types of the innate and adaptive immune system (Ehrlich et al., submitted). Others have suggested PFAS immunotoxicity in rodents is partially the result of PPARα-activation for which interspecies differences are at hand (Antoniou et al., 2022). However, the interplay of different pathway perturbations underlying PFAS immunotoxicity is still to be unraveled, and evidence with PPARα knockout mice suggests PFAS-induced immunotoxicity is multifactorial (DeWitt et al., 2016), illustrating immunotoxicity may be induced through pathways that do not involve PPARα. Besides this, there is debate on the human relevance of PPARα-induced liver tumors observed in rodents, (Corton et al., 2018; Guyton et al., 2009) but not with regard to other effects. Pharmacological literature provides evidence that PPAR activators suppress inflammatory activity in various cell types by inhibiting the expression of proinflammatory genes (Delerive et al., 2001; Christofides et al., 2021). In a study with coronary artery disease patients, treatment with fibrate for four weeks resulted in reduced IL-6, C-reactive protein, and fibrinogen levels, and in patients with hyperlipoproteinaenemia type 11b, treatment with fenofibrate for four weeks resulted in reduced plasma interferon-γ and tumor necrosis factor-α levels (Delerive et al., 2001). Such information provides evidence that compounds targeting PPARs in the human body could, in a non-clinical setting, give undesired effects, whereby anti-inflammatory properties of a substance may manifest as immunosuppression.

An interesting option for further research, considering the effects of PFAS observed on the thymus in the current study, is to screen a series of PFAS for their effect on *ex vivo* (mitogen or antigen stimulated) thymocytes or advanced *in vitro* models recapitulating thymocyte maturation (Asnaghi et al., 2021). Other than that, additional studies are foreseen in new projects on mixture toxicity and risk assessment of chemical contaminants. Mixture effects of PFAS and the use of RPFs in their combined assessment are considered in the EFSA roadmap for action on risk assessment of combined exposure to multiple chemicals (RACEMiC) (De Jong et al., 2022) as well as in the partnership for the assessment of risks from chemicals (PARC; https://www.anses.fr/en/content/european-partnership-assessment-risks-chemicals-parc). In RACEMiC, it is foreseen that RPFs should be derived based on existing *in vivo* data in combination with relevant data generated using new approach methodologies (NAMs). Appropriate NAMs, in combination with quantitative *in vitro-in vivo* extrapolation (QIVIVE) methods, should be used to investigate, group and rank the large number of compounds without the need for respective *in vivo* data. PARC will also contribute to this activity by developing (networks of) relevant adverse outcome pathways (AOPs), associated NAMs, and integrated approaches to testing and assessment (IATAs) for various health effects, including immunotoxicity, and by demonstrating their applicability for regulatory purposes through case-studies.

For practical application of the RPF method for PFAS, we furthermore stimulate scientific debate to work towards agreement of RPFs for PFAS. Several efforts have been made since 2018 on deriving RPFs for PFAS, such as RPFs based on cytotoxicity and intracellular reactive oxygen species formation in human HepG2 cells *in vitro* (Amstutz et al.,

2022), based on gene expression in human HepaRG cells (Louisse et al. in preparation) and in human liver spheroids *in vitro* (Reardon et al., 2020); based on thyroid hormone disruption potential *in vitro* (Behnisch et al., 2021); and based on several systemic toxicity effects *in vivo* (Goodrum et al., 2021; Bil et al. 2022b; Rietjens et al., 2021; Zeilmaker et al., 2018). The RPF analyses performed in these publications do not necessarily adhere to the same methodological approaches and criteria, for instance on ensuring parallel curve fitting (Bil et al. 2022b). It is therefore important to have discussions on correct methods for RPF derivation, establishing criteria for grouping, and eventually setting harmonized values for RPFs in an international context. Such discussions could take place at the WHO level, as has been the case for establishing consensus toxic equivalency factors (TEFs) for dioxins and dioxin-like compounds since 1993 (Ahlborg et al., 1994), or within another established regulatory structure.

All in all, our RPF method holds great promise as an approach for risk assessment of combined exposure to multiple chemicals. Development and refinement of mixture-based risk assessment tools for PFAS will continue to be an important topic in future. Because it is both difficult and time-consuming to enforce the conduct of *in vivo* toxicity studies for emerging and phased-out environmental contaminants in a European regulatory setting, it is urgently needed to move towards reliance on new methodological approaches as the basis for mixture risk assessment.

## 5. Conclusion

Functional tests that characterize the cellular and functional status of the immune system upon antigenic challenging, and routine toxicity studies that observe effects on the resting immune system, show a robust picture of PFAS causing suppression of the immune response. In our study, internal RPFs were successfully derived for PFAS based on rat thymus weight, spleen weight, and globulin concentration. Immunotoxic potency in serum based on lymphoid organs was determined in the order PFDA > PFNA > PFHxA > PFOS > PFBS > PFOA > PFHxS and ranged from 12 to 0.3. The internal RPFs based on lymphoid organ weights are similar to those previously established for liver weight, strengthening the confidence in the general applicability of these RPFs. The epidemiological data showed inverse associations for the sum PEQ with serum antibody concentrations to rubella and mumps, but the data did not allow for deduction of reliable internal RPF estimates. Exploration with (future) mixture dose–response datasets may help to further substantiate the quantitative estimates of internal RPFs for PFAS with the use of human data. Taken together, our RPF method holds great promise for performing risk assessment of combined exposure to multiple chemicals.

## CRediT authorship contribution statement

**Wieneke Bil:** Funding acquisition, Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Veronika Ehrlich:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Guangchao Chen:** Conceptualization, Formal analysis, Software. **Rob Vandebriel:** Conceptualization, Writing – review & editing. **Marco Zeilmaker:** Conceptualization, Writing – review & editing, Supervision. **Mirjam Luijten:** Funding acquisition, Conceptualization, Writing – review & editing. **Maria Uhl:** Conceptualization, Writing – review & editing. **Philip Marx-Stoelting:** Conceptualization, Writing – review & editing. **Thorhallur Ingi Halldorsson:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Bas Bokkers:** Conceptualization, Methodology, Formal analysis, Software, Writing – original draft, Writing – review & editing, Visualization, Supervision.

## 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.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2022.107727.

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