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Approaches to mixture risk assessment of PFASs in the European population based on human hazard and biomonitoring data

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ABSTRACT

Per- and polyfluoroalkyl substances (PFASs) are a highly persistent, mobile, and bioaccumulative class of chemicals, of which emissions into the environment result in long-lasting contamination with high probability for causing adverse effects to human health and the environment. Within the European Biomonitoring Initiative HBM4EU, samples and data were collected in a harmonized way from human biomonitoring (HBM) studies in Europe to derive current exposure data across a geographic spread. We performed mixture risk assessments based on recent internal exposure data of PFASs in European teenagers generated in the HBM4EU Aligned Studies (dataset with N = 1957, sampling years 2014–2021). Mixture risk assessments were performed based on three hazard-based approaches: the Hazard Index (HI) approach, the sum value approach as used by the European Food Safety Authority (EFSA) and the Relative Potency Factor (RPF) approach. The HI approach resulted in the highest risk estimates, followed by the RPF approach and the sum value approach. The assessments indicate that PFAS exposure may result in a health risk in a considerable fraction of individuals in the HBM4EU teenager study sample, thereby confirming the conclusion drawn in the recent EFSA scientific opinion. This study underlines that HBM data are of added value in assessing the health risks of aggregate and cumulative exposure to PFASs, as such data are able to reflect exposure from different sources and via different routes.

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1. Introduction

1.1. Mixture exposure to PFASs in human biomonitoring

Per- and polyfluoroalkyl substances (PFASs) are a highly persistent, mobile, and bioaccumulative class of chemicals, of which emissions into the environment are expected to result in long-lasting contamination with high probability for causing adverse effects to human health and the environment (ECHA 2012a; ECHA 2012b; ECHA 2012c; ECHA 2012d; ECHA 2013a; ECHA 2013b; ECHA 2015; ECHA 2017a; ECHA 2017b; ECHA 2019a; ECHA 2019b). Several subclasses among the PFASs, such as perfluoroalkyl carboxylic acids (PFCAs), perfluoroalkyl sulfonic acids (PFSAs) and perfluoroalkyl ether carboxylic acids (PFECAs) comprise substances that induce multi-organ toxicity (ATSDR 2021; EFSA 2020a). Due to their widespread use, persistency, bioaccumulation, and high bioavailability in the environment, humans are exposed to a variety of PFASs via, e.g., contaminated drinking water, food, consumer products, or house dust (Poothong et al., 2020; Colles et al., 2020).

Our current regulatory systems are not designed to adequately address combined exposure to multiple chemicals or aggregate exposure to the same chemical from multiple sources and pathways. Historically, risk assessment is performed for single chemicals and specific applications or uses within confined regulatory domains (Drakvik et al., 2020). For PFASs, as may be the case for many compounds, exposure is underestimated if combined exposure to multiple PFASs and aggregated exposure to the same PFAS from multiple sources and pathways is not included in risk assessment. In addition, considering that several PFASs result in the same adverse health effects, the risk assessment for individual compounds and single exposure sources may lead to an underestimation of the risk (EFSA 2019a). Therefore, mixture toxicity currently receives much attention, to bring forward the importance of assessing combined exposure to substances with similar toxicity profiles (European Commission 2020; Drakvik et al., 2020; EFSA 2019a). In a few exemplary cases, such as for dioxins and related PCBs in food and feed (EFSA 2018a), for phthalates in articles (ECHA 2017c) and food contact materials (EFSA 2019b), and pesticides in food (EFSA 2020b), the cumulative risk resulting from combined exposure to multiple substances has been assessed in a regulatory context.

Human biomonitoring (HBM) is a highly relevant tool to empirically observe aggregated exposure to PFASs in human blood or breast milk, particularly for the long half-life PFASs, and has an important role in screening for exposure to novel PFASs (Kaiser et al., 2021; Kang et al., 2020; Li et al., 2020a; Liu et al., 2019; Miaz et al., 2020). Commonly, studies report highly positive correlation coefficients between PFASs, illustrating that simultaneous exposure to multiple PFASs occurs at the individual level (EFSA 2020a; Kotlarz et al., 2020; Yu et al., 2020; Appendix A, Table A18-A27). This highlights the importance of focussing on combined exposure to multiple PFASs in risk assessment. The HBM4EU Aligned Studies collected samples and data in a harmonized way to derive current internal exposure data for the European population across a geographic spread (Gilles et al., 2021). Blood concentrations were measured in teenagers from nine studies (dataset with N = 1957, sampling period between 2014 and 2021) for 12 different PFASs (Gilles et al., 2021; Govarts et al., submitted; Gilles et al., 2022; Richterova et al., 2023). This dataset served as the basis of internal exposure data for the current paper.

1.2. Aim of this study

We present several ways of assessing the risk of mixtures of PFASs and address the challenges related to risk assessment of combined exposure to several similarly acting substances, when using exposure measurements in the blood plasma/serum of teenagers as primary input.

2. Methods

2.1. Methodologies for calculating mixture risk

2.1.1. The hazard index approach

The hazard index (HI) approach is a generally accepted tool for pragmatic mixture risk assessment that builds on the assumption of dose addition for calculating risk to chemical mixtures (Meek et al., 2011; Boberg et al., 2019). By using this approach, a hazard quotient is calculated for each single compound in the mixture based on the exposure level in the numerator (here human exposure levels, HBM data measured in the HBM4EU project) relative to the effect level (here defined as the effect level observed in human epidemiological studies) (Equation (1)). By summing the hazard quotients (HQs), the HI is calculated and a HI exceeding 1 indicates that a potential risk to human health may exist.

$$Hazard\ Index = \frac{Exp_1}{EL_1} + \frac{Exp_2}{EL_2} + \frac{Exp_3}{EL_3} + \ldots \frac{Exp_i}{EL_i} \quad \text{(Eq. 1)}$$

Exp_i: exposure to compound *i*, expressed as ng/mL blood serum or plasma.

EL_i: the effect level of compound *i,* in ng/mL blood serum or plasma. In this study, we have used human internal exposures (presented as median or geometric mean plasma/serum concentration per study) statistically associated with either a given effect on immunotoxicity or on birth weight reductions.

Hazard Index = the sum of the hazard quotients of each chemical, which is the ratio of the human exposure to the substance relative to the effect level.

2.1.2. The sum value approach

EFSA derived a sum value approach for PFOA, PFNA, PFHxS and PFOS (hereafter called 'EFSA-4') and established a group tolerable weekly intake (TWI) of 4.4 ng/kg bw/week, corresponding to a serum concentration of 6.9 ng/mL in women of reproductive age, based on a serum concentration of 17.5 ng/mL in children of 1-year-old (EFSA 2020a). In their risk assessment, EFSA relied on the assumption that the EFSA-4 are equipotent for immunotoxic effects in humans and can be added without correction for potential differences in toxic potencies.

The cumulative risk, also defined as the risk characterization ratio (RCR), is estimated by summing the serum concentrations of the EFSA-4 in each individual and dividing this by the serum concentration that correspond to the TWI, i.e. the HBM guidance value (GV) (Equation (2)).

$$Cumulative\ risk = \frac{\sum Exp_i}{HBM\ GV_{PFASs}} \quad \text{(Eq. 2)}$$

Exp_i = exposure to compound *i* per individual, whereby compound *i* is PFOA, PFNA, PFHxS or PFOS, in ng/mL blood serum or plasma.

HBM GV PFASs = the PFAS plasma level of 6.9 ng 'EFSA-4'/mL in women of reproductive age that corresponds to a level of 17.5 ng 'EFSA-4'/mL in children.

Cumulative risk = the combined risk from aggregate exposures to multiple PFASs.

2.1.3. The relative potency factor approach

The relative potency factor (RPF) approach for mixture risk assessment of PFAS builds on the assumption of dose addition, setting the potency of the index compound PFOA for liver toxicity in rat studies to 1, and expressing the toxicity of the other compounds relative to this as relative potency factors (Bil et al., 2021). For the purpose of evaluating mixtures in blood, RPFs were derived based on (modelled) serum concentrations in the male rat, thus reflecting internal relative potencies (Bil et al., 2022). Internal RPFs were available for nine PFASs (PFBA, PFHxA, PFOA, PFNA, PFDoDA, PFBS, PFHxS, PFOS and HFPO-DA), meaning that for some substances in the HBM4EU survey no internal RPF was available (PFHpS, PFPeA, PFHpA, PFDA, PFUnDA) due to absence of

suitable toxicity and/or toxicokinetic information available, whereas for some substances with an internal RPF (PFBA, HFPO-DA) no measurements were performed in the HBM4EU survey.

PFOA equivalent (PEQ) exposures for each PFAS were calculated by multiplying internal exposure of each individual participant (expressed as concentration in blood serum or plasma) by internal RPFs of the respective PFASs (Equation (3)). Subsequently, PEQ exposures were summed to obtain the sum PEQ for each individual. These may be expressed as percentiles per cohort or aggregated otherwise to reflect population exposure and can be used for risk assessment as if they represented exposure to PFOA solely. The cumulative risk is estimated by summing the PEQ exposure per individual and dividing this by the serum concentrations that correspond to the TWI (Equation (4)).

In combining the RPF approach with the HBM GVs of EFSA, it is assumed that not only the EFSA-4 cause immunotoxicity in humans but also the other PFASs for which RPFs are derived, and that the potency ranking of PFASs based on liver toxicity in animals can be extrapolated to the potency ranking of human toxicities such as immunotoxicity.

$$PFOA\ equivalent\ (PEQ) = Exp_i * RPF_i \quad \text{(Eq. 3)}$$

and

$$Cumulative\ risk = \frac{\sum PEQ_i}{HBM\ GV_{PFASs}} \quad \text{(Eq. 4)}$$

Exp_i = exposure to compound i per individual, whereby compound i is PFBA, PFHxA, PFOA, PFNA, PFDoDA, PFBS, PFHxS, PFOS or HFPO-DA, in ng/mL blood serum or plasma.

RPF_i = internal relative potency factor of compound i, whereby compound i is PFBA, PFHxA, PFOA, PFNA, PFDoDA, PFBS, PFHxS, PFOS or HFPO-DA.

PEQ_i = exposure to compound i per individual expressed in PFOA equivalents, whereby compound i is PFBA, PFHxA, PFOA, PFNA, PFDoDA, PFBS, PFHxS, PFOS or HFPO-DA.

HBM GV PFASs = the PFAS plasma level of 6.9 ng 'EFSA-4'/mL in women of reproductive age that corresponds to a level of 17.5 ng 'EFSA-4'/mL in children.

Cumulative risk = the combined risk from aggregate exposures to multiple PFASs.

2.2. Human biomonitoring data from the HBM4EU aligned studies

HBM4EU aimed to collect exposure data in European countries to sufficiently cover defined geographic regions, with preference given to data on PFAS exposure in teenagers of 12–19 years (Table 1). Serum, plasma or whole blood were the matrices of choice to measure exposure to PFASs (Vorkamp et al., 2021). The 12 PFASs that were included in the HBM4EU survey were PFBS, PFHxS, PFHpS, PFOS, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, and PFDoDA. General information on the number of individuals within each study, sex, age, sampling period, BMI, limit of detection (LOD) and limit of quantification (LOQ) is presented in Appendix A. More information on the data transformation to arrive at harmonized variables may be found in Gilles et al. (2021).

Exposure data are presented as percentiles of exposure per study (Table A6-A17; Appendix A). The 50th (P50) or 95th (P95) percentile values reflect an average or worst-case exposure scenario per study, respectively. These percentiles were used to calculate the HI for the mixture exposure to PFASs per study. When P50 or P95 was <LOD, between LOD-LOQ, or <LOQ, two scenarios were used to calculate the HI. In one scenario, the plasma/serum concentrations below LOQ were set at 0, and hence the HQ of these PFASs were not included in the HI. This scenario is used in the main paper.

In the other scenario, the summary statistics were imputed by LOD/2 (in case of <LOD), (LOD + LOQ)/2 (in case of between LOD and LOQ), or LOQ/2 (in case of <LOQ and LOD not available). This scenario is used in the Supplement (Appendix D). By using the latter approach, the cumulative risk may be driven by the number of substances for which the level is <LOD, between LOD-LOQ, or <LOQ. Moreover, because the LOD and LOQ values differ between studies, the degree by which this imputed value drives the cumulative risk may also differ among studies. The LOD and LOQ values for each study are provided in Appendix A.

In a deterministic approach, such as the HI approach described above, one relies on the assumption that the pairwise correlation among PFAS exposures is 1. However, pairwise correlations among PFAS vary (see Table A18-A27; Appendix A, for Spearman correlations between PFASs per cohort), and the magnitude of the correlations are strongly dependent on exposure source(s) (EFSA 2020a).

For the sum value approach and the RPF approach, we therefore used the mixture exposure at the individual level, using the raw exposure data of the PFAS mixtures for each person separately. This means that for the sum value approach, the serum or plasma concentration for PFOA, PFNA, PFHxS and PFOS were summed per individual, and the percentiles for the EFSA-4 per country were then used to interpret the risk. For the RPF approach, we multiplied the RPF with PFAS exposure concentrations to obtain PEQs per individual, and then presented the percentiles for the sum PEQs per country to interpret the risk.

For the sum value and RPF approach, two scenarios were used for the individuals that had measured PFAS values below LOD/LOQ. In one scenario, all values <LOQ were set at zero, after which the individual PFAS concentrations were summed to obtain sum exposure per individual. In the other scenario, the sum value was calculated in a way that individual PFAS measurements below the LOD/2 (in case of <LOD), LOD + LOQ/2 (in case of between LOD and LOQ), or LOQ/2 (in case of <LOQ) (Govarts et al. submitted). The first scenario is used in the main paper, and the outcome of the latter scenario is provided in the Supplement (Appendix E and F).

Please see Table 2 for an overview of the inclusion of PFASs in the different mixture risk assessments and the data used in these different methods.

Table 1
Human biomonitoring studies aligned within HBM4EU measuring PFASs in teenagers (Gilles et al., 2021).

Study name	Number of participants	Country	HBM4EU region
Riksmaten adolescents[a, c]	300	Sweden	North
Norwegian environmental biobank (NEB) II [b]	177	Norway	North
PCB cohort [a]	292	Slovakia	East
Exposure of children and adolescents to selected chemicals through their habitat environment (SLO CRP) [a]	94	Slovenia	South
Cross-Mediterranean Environment and Health Network (CROME) [a]	52	Greece	South
Biomonitorización en adolescents (BEA) [a]	300	Spain	South
Étude de santé sur l'environnement, la biosurveillance, l'activité physique et la nutrition (ESTEBAN) [a]	299	France	West
German Environmental Survey (GerES-sub) V [b,d]	300	Germany	West
Flemish Environment and Health Studies (FLEHS) IV [a]	143	Belgium	West

[a] Measured in blood serum.
[b] Measured in blood plasma.
[c] Data were obtained in a laboratory that did not participate in the HBM4EU Quality assurance/Quality control (QA/QC) program and consequently was not qualified within HBM4EU. Furthermore, data were initially reported as ng/g. To convert them to ng/mL, the assumption was made that 1 mL blood serum = 1 g blood serum.
[d] This is an unweighted subsample of the German Environmental Survey 2014–2017.

Table 2
Overview of the different mixture risk assessment approaches, the number of PFASs included in these assessments, and the different data that were used in these approaches.

	Hazard index	Sum value	Relative potency factor
Exposure assessment			
PFASs included	PFNA, PFDA, PFUnDA, PFDoDA, PFOS[a] PFOA, PFDA, PFHxS [b] PFOA, PFNA, PFHpS, PFOS [c] PFNA, PFDA, PFDoDA, PFUnDA[d]	PFOA, PFNA, PFHxS, PFOS	PFHxA,[e] PFOA, PFNA, PFDoDA, PFBS, PFHxS, PFOS
Type of data	Summary data, P50 and P95 per substance	Individual data, P50 mixture, P95 mixture	Individual data, P50 mixture, P95 mixture
Hazard assessment			
Type of effect studied in the mixture risk assessment	Birth weight reduction Immunotoxicity	Immunotoxicity	Immunotoxicity
Type of data used to account for differences in toxic potency	Effect levels (ELs) based on immunotoxicity and birth weight reductions in epidemiological studies (Table 3)	None	Internal relative potency factors (RPFs) obtained from rodent liver toxicity data (Appendix C) to convert PFAS serum concentrations to PFOA Equivalents (PEQs)
HBM GV used	None	EFSA sum value for teenagers and their (future) children	EFSA sum value for teenagers and their (future) children

[a] Using the ELs based on Kielsen et al. (2016).
[b] Using the Els based on Grandjean et al. (2012).
[c] Using the ELs based on Meng et al. (2018).
[d] Using the ELs based on Wang et al. (2016).
[e] The Norwegian Environmental Biobank (NEB) II did not contain measurements on PFHxA.

3. Results

3.1. Hazard assessment

3.1.1. Selection of effect levels for critical endpoints in the hazard index approach

EFSA (2020a) considered adverse endpoints on immunotoxicity as the most critical risk factor for exposure to PFASs. Furthermore, it identified decreases in birth weight as potential critical end-point (as well as increase in serum cholesterol and high serum levels of ALT), but noted that the TWI based on immune effects would be protective for the other potential critical end-points. We included impaired vaccination responses as the measure of immunotoxicity as well as decreased birth weight as the critical health effects in the hazard index approach.

All human epidemiological studies mentioned in EFSA (2020a) on immunotoxicity and birth outcomes were reviewed (except the critical study of Abraham et al. (2020)). Studies in which no or only few PFASs showed statistically significant (inverse) associations to immune effects or birth weight were excluded. A total of four studies were selected for further calculations. In these studies, single or multiple linear regression analysis was used to reveal associations between exposure to single PFASs and developmental- or immunological effects. We included the geometric mean or median PFAS exposure as effect level (i.e. point of departure, POD) when the regression coefficients (beta values and 95% confidence intervals) for these PFASs showed a statistically significant association with the exposure. The beta value reflects the magnitude of the effect (including its 95% confidence intervals) that is associated with a doubling of PFAS exposure. Some studies also complemented linear regression with quartile methods, but due to lack of statistical significance in pairwise comparison between quartiles of many of these associations and because not all studies used quartile methods, these outcomes were not used as effect level. A more detailed overview of these studies, including a summary of their conduct and outcomes, is provided in Appendix B. The corresponding PODs can be found in Table 3 and Appendix B.

3.1.2. TWI derivation for the sum value approach as discussed in EFSA (2020a)

Abraham et al. (2020) examined the relation between plasma PFAS concentrations in 1-year-old infants (N = 101) and antibody response against diphtheria, tetanus and Haemophilus influenza type b. EFSA employed benchmark dose (BMD) modelling for exposure to the sum of four PFASs, using a benchmark response of 10% decrease ($BMDL_{10}$) in antibody titres. The tolerable weekly 'EFSA-4' PFASs intake of mothers (of 4.4 ng/kg bw/week), in their life up to pregnancy, was then estimated. The extrapolation of the $BMDL_{10}$ to the TWI consisted of several intermediate steps, of which one was a modelled serum level of 6.9 ng 'EFSA-4' PFASs/mL in mothers at the age of 35 (EFSA 2020a). This value should protect their children from reaching a body burden of 17.5 ng 'EFSA-4' PFASs/mL via breastfeeding.

Thus, the TWI should prevent mothers from reaching PFAS levels in breastmilk at the age of 35 that would lead to a serum/plasma level in their infants that is associated with an impaired immune response. Consequently, high PFAS exposure of breastfed children is considered in derivation of the TWI. Therefore, EFSA (2020a) specifically mentioned that the intake of infants should not be compared to the TWI value. For teenagers we decided to use both a HBM GV value of 17.5 and 6.9 ng/mL for interpreting the risk based on internal exposures. That is, the HBM GV of 6.9 ng/mL should have been lower to be protective for future children of this specific female teenage subgroup, due to further build-up of PFASs in the body up to the age of 35, and therefore slightly underestimates the risk. For exceedance of the HBM GV of 17.5 ng/mL, we assume that teenagers are equally sensitive to immunosuppression by PFASs, however this is an assumption that warrants further study.

3.1.3. Selection of hazard data for RPF derivation

A database for 16 PFASs was previously created based on liver hypertrophy, absolute liver weight increase, and relative to body weight (bw) liver weight increase in the male rat upon oral (gavage) subchronic exposure (42–90 days) (Bil et al., 2021). This database was used for derivation of internal RPFs of nine PFASs, whereby internal, kinetically modelled, time-weighted average serum concentrations in the male rat were expressed against relative (to bw) liver weight increase to obtain RPFs (Bil et al., 2022). The internal RPFs for PFASs are presented in Table 4. The hazard database of the selection of these nine PFASs is to be found in Appendix C. The establishment of toxicokinetic models for many PFASs was hampered by the absence of toxicokinetic information for parametrization, and thus resulted in a lower number of internal RPFs compared to external RPFs.

The RPF approach for PFASs builds on the assumption that the potency ranking based on internal doses giving rise to liver toxicity in the male rat can be applied to humans. Moreover, we assume that the liver RPFs obtained in the male rat also apply to other endpoints, such as immunotoxicity and that all PFASs for which liver RPFs are available will cause an immunotoxic response.

Table 3
Associations between PFASs exposure and either immunotoxic effects or reduced birth weight in new-borns. The beta value represents the magnitude of the effect caused by a doubling of PFAS exposure. Statistically significant associations, of which the median or geometric mean were used as point of departures/effect levels for the hazard quotient calculations, are indicated in bold.

Study	Study population	N	Sample matrix	Sampling period	Median/geometric mean exposure and range in ng/mL[a]		Association between PFASs and health effects (beta ± 95% confidence interval)[b], per doubling of exposure		
Meng et al. (2018)	Mother-child pairs	3535 (PFOS and PFOA)	Blood plasma of mothers	First trimester, 1996–2002	PFOS	**30.1** (22.9–39.0)	Birth weight in boys + girls (gr)	PFOS	**−45.2** (−76.8, −13.6)
					PFOA	**4.6** (3.3–6.0)		PFOA	**−35.6** (−66.3, −5.0)
					PFHxS	1.0 (0.7–1.3)		PFHxS	1.2 (−28.3, 30.7)
		2120 (other PFASs)			PFNA	**0.5** (0.4–0.6)		PFNA	**−36.3** (−70.6, −2.0)
					PFHpS	**0.4** (0.3–0.5)		PFHpS	**−38.9** (−72.6, −5.1)
					PFDA	0.2 (0.1–0.2)		PFDA	−9.0 (−43.2, 25.2)
Wang et al. (2016)	Mother-child pairs	106[c]	Blood serum of mothers	Third trimester, 2000–2001	PFOA	1.98 (1.69–2.32)	Birth weight in girls (gr)	PFOA	−80 (−180, 10)
					PFNA	**1.44** (1.19–1.74)		PFNA	**−80** (−160, 0)
					PFDA	**0.37** (0.32–0.42)		PFDA	**−140** (−260, −20)
					PFUnDA	**2.89** (2.12–3.94)		PFUnDA	**−60** (−110, −10)
					PFDoDA	**0.30** (0.25–0.35)		PFDoDA	**−120** (−210, −20)
Grandjean et al. (2012)	Children followed from birth to year 7	440	Blood serum of mothers and children	At age of 5 (children), 2002–2005	PFOS	16.7 (13.5–21.1)	Percentage change in specific antibody response to tetanus vaccine at 7 yrs of age, 2 yrs post-vaccination	PFOS	−23.8 (−44.3, 4.2)
					PFOA	**4.06** (3.33–4.96)		PFOA	**−35.8** (−51.9, −14.2)
					PFHxS	**0.63** (0.45–0.88)		PFHxS	**−19.7** (−31.6, −5.7)
					PFNA	1.00 (0.76–1.24)		PFNA	−17.4 (−34.1, 3.6)
					PFDA	**0.28** (0.21–0.38)		PFDA	**−22.3** (−35.8, −5.8)
Kielsen et al. (2016)	Adults	12	Blood serum	Adults (average age 37 years), NA	PFHxS	0.37 (0.27–0.70)	Percentage change in specific antibody response to diphtheria vaccine 4–10 days post-vaccination	PFHxS	−13.31 (−25.07, 0.29)
					PFHpA	0.12 (0.094–0.14)		PFHpA	6.52 (−28.04, 57.7)
					PFOS	**9.52** (5.38–14.3)		PFOS	**−11.90** (−21.92, −0.33)
					PFOA	1.69 (1.30–2.79)		PFOA	−8.22 (−20.85, 6.44)
					PFNA	**0.66** (0.46–0.80)		PFNA	**−17.90** (−27.99, −6.39)

(*continued on next page*)

Table 3 (*continued*)

Study	Study population	N	Sample matrix	Sampling period	Median/geometric mean exposure and range in ng/mL[a]		Association between PFASs and health effects (beta ± 95% confidence interval)[b], per doubling of exposure	
					PFDA	**0.30** (0.20–0.32)	PFDA	**−18.18** (−29.52, −5.00)
					PFUnDA	**0.21** (0.18–0.27)	PFUnDA	**−12.11** (−22.06, −0.90)
					PFDoDA	**0.039** (0.035–0.048)	PFDoDA	**−15.64** (−28.14, −0.98)

[a] For Meng et al. (2018), median exposure and interquartile range (Q1-Q3) are presented. For Wang et al. (2016), geometric mean and 95% confidence interval are presented. For Grandjean et al. (2012), geometric mean and interquartile range (Q1-Q3 are presented. For Kielsen et al. (2016), median and interquartile range (Q1-Q3) are presented.

[b] For Meng et al. (2018), birth weight was adjusted for infant sex, infant birth year, gestational week of blood draw, maternal age, parity, socio-occupational status, pre-pregnancy body mass index (BMI), smoking and alcohol intake during pregnancy. For Wang et al. (2016), birth weight in girls was adjusted for family annual income, maternal age at delivery, maternal education, maternal previous live children, and maternal pre-pregnancy BMI. Grandjean et al. (2012), antibody response to tetanus vaccination was adjusted for age, sex, and booster type. Kielsen et al. (2016) antibody response was adjusted for sex and age. P-value in all studies was 0.05.

[c] A total of 223 mother-child couples were included in the study, but only statistically significant effects were seen for girls.

Table 4
Internal relative potencies for PFASs for liver toxicity in rodents compared to PFOA that was selected as the reference compound (Bil et al., 2022).

Compound	Internal RPF	Measured in HBM4EU
PFBS	0.2	Yes
PFHxS	0.6	Yes
PFOS	3	Yes
PFBA	2	No
PFHxA	10	Yes
PFOA	1	Yes
PFNA	5	Yes
PFDoDA	10	Yes
HFPO-DA	9	No

3.2. PFAS mixture risk assessments

3.2.1. Calculation of the cumulative risk based on the hazard index approach

Table 5 reports the final HIs calculated for teenagers based on the median exposure (P50). The outcome of this exercise illustrates that for immune effects, the HI is exceeded by taking the average exposure in all study populations except the Slovakian, German, and Spanish study populations, whereas for the Flanders study population the results are equivocal. For decreased birth weight, the HI is exceeded in the French study population, but not in that of Germany, Slovakia, Slovenia, Spain and Flanders. The results are equivocal for the Norwegian, Greek and Swedish study populations. Note that in Table 5, the LOD or LOQ was set at zero in cases where the substance could not be quantified in human blood.

For a more conservative illustration of exposure, the P95 may be used. For individuals with a relatively high exposure to PFASs, there is a risk for a compromised immune response in all cohorts from the various European study populations based on the results of Kielsen et al. (2016) and Grandjean et al. (2012). Moreover, a risk for decreased birth weight caused by PFAS exposure is indicated in all study populations based on the studies by Meng et al. (2018) and Wang et al. (2016) (Table 6).

Calculations that include setting values at half the LOD or LOQ are presented in Appendix D (Table D1 and Table D2). Especially for the studies with a high LOD and/or LOQ, the risk was significantly higher when taking this approach. This indicates that for these study populations, estimation of the risk is highly uncertain since it is mainly driven by the LOD and/or LOQ value.

3.2.2. Calculation of the cumulative risk based on the sum value approach of EFSA (2020a)

In Table 7 (and Appendix E, Table E2), the percentiles of exposure to the EFSA-4 are provided. Exposure to the EFSA-4 resulted in exceedance of the HBM GV in 1.3–24% of individuals per study population when the value of 6.9 ng/mL was used, and when non-detects for the separate compounds were treated as null exposure when calculating the sum. Risk Characterization Ratios (RCRs) were higher than one only in the highly exposed individuals (P95 scenario).

0–1.7% of the study populations exceeded the value of 17.5 ng/mL. The P50 and P95 did not exceed this HBM GV in any of the study populations, and consequently all RCRs remained below one (Table 7). When values below LOD/LOQ were attributed a value of half LOD/LOQ, there was only a marginal difference (Appendix E, Table E1). This is explained by the fact that only a low number of exposure values of the EFSA-4 are below LOD/LOQ (Appendix A).

Another important observation to note is that PFOS exposure is highest among all PFAS congeners, whereby exposure to this substance alone already resulted in exceedance of the HBM GV of 6.9 ng/mL at the higher percentiles of some of the study populations (Appendix A, Table A9).

3.2.3. Calculation of the cumulative risk based on internal RPFs

In Table 8 (and Appendix F, Table F2), the percentiles of exposure to either six (as PFHxA was not analysed in the Norwegian study) or seven PFASs is provided, expressed as PEQs.

Exposure to the sum PEQ resulted in exceedance of the HBM GV of 6.9 ng/mL in 41–96% of the study populations, when non-detects were treated as nulls. 1.7–23% of the study population exceeded the HBM GV of 17.5 ng/mL. RCRs were higher than one for the highly exposed individuals (P95) in all study populations, and below one for the median exposed individuals (P50). Some compounds contributed more to the cumulative risk because they had a high internal RPF (e.g. PFOS, PFNA).

In the scenario where values below LOD/LOQ were attributed a value of half LOD/LOQ, these attributed values were multiplied with their respective RPF. For substances with low detection frequency and for which the LOD/LOQ values were relatively high (e.g. for PFBS, PFHxA, PFDoDA in the French, Flemish and German study), these values contributed significantly to the risk estimate. Interpretation of the risk based on studies with such high LOD/LOQ is uncertain.

3.2.4. Comparison between approaches

PFAS exposure may pose a health risk in the teenagers' HBM4EU study population where risk estimates exceed an RCR/HI of one (Figs. 1 and 2). The HI approach resulted in the highest risk estimates based on

Table 5
PFAS mixture risk assessment based on the Hazard Index (HI) approach. HIs for PFASs were calculated for nine European teenage populations based on P50 values, where non-detects are treated as zeros. Hazard quotients are summed up to calculate the HI. A HI >1 indicates a potential risk.

	EL (ng/mL)		Norway	Sweden	Slovakia	Slovenia	Greece	Spain	Belgium	France	Germany
Immunotoxicity											
Kielsen et al. (2016)											
Adults											
PFOS	9.52	HQ	0.29	0.28	0.14	0.17	0.22	0.14	0.23	0.21	0.27
PFNA	0.66		0.67	0.57	0.26	0.38	0.63	0.43	0.48	0.82	ND
PFDA	0.3		0.43	0.49	0.17	0.47	0.57	ND	ND	0.72	ND
PFUnDA	0.21		0.41	ND	ND	0.29	0.17	ND	ND	0.52	ND
PFDoDA	0.039		ND	ND	ND	ND	ND	ND	ND	0.00	ND
		HI	**1.81**	**1.34**	**0.57**	**1.30**	**1.58**	**0.57**	**0.72**	**2.27**	**0.27**
Grandjean et al. (2012)											
Children											
PFOA	4.06	HQ	0.32	0.28	0.17	0.21	0.22	0.16	0.27	0.36	0.31
PFHxS	0.63		0.75	0.62	0.46	0.37	0.44	ND	0.78	1.09	0.62
PFDA	0.28		0.46	0.53	0.18	0.50	0.61	ND	ND	0.77	ND
		HI	**1.53**	**1.43**	**0.81**	**1.08**	**1.27**	**0.16**	**1.05**	**2.22**	**0.93**
Birth weight reduction											
Meng et al. (2018)											
Mothers											
PFOA	4.6	HQ	0.28	0.25	0.15	0.19	0.19	0.14	0.24	0.32	0.27
PFOS	30.1		0.09	0.09	0.05	0.05	0.07	0.04	0.07	0.07	0.09
PFNA	0.5		0.89	0.75	0.34	0.50	0.83	0.56	0.64	1.08	ND
PFHpS	0.4		ND	NA	0.08	0.08	0.13	ND	ND	ND	NA
		HI	**1.26**	**1.09**	**0.61**	**0.82**	**1.22**	**0.75**	**0.95**	**1.47**	**0.36**
Wang et al. (2016)											
Mothers											
PFNA	1.44	HQ	0.31	0.26	0.12	0.17	0.29	0.20	0.22	0.38	ND
PFDA	0.37		0.35	0.40	0.14	0.38	0.46	ND	ND	0.59	ND
PFDoDA	2.89		ND	ND	ND	ND	ND	ND	ND	ND	ND
PFUnDA	0.3		0.29	ND	ND	0.20	0.12	ND	ND	0.36	ND
		HI	**0.95**	**0.66**	**0.25**	**0.75**	**0.86**	**0.20**	**0.22**	**1.33**	**0**

EL = effect level.
HI = hazard index.
HQ = hazard quotient.
NA = not available.
ND = not detected (values below LOD or LOQ were set at zero).

P95 exposure, up to an HI of 6.2 for immune effects seen in the French study population. This is followed by the RPF approach, for which the highest RCR was 4.3 in the Swedish study population based on P95 exposures. By using the sum value approach, the highest RCR based on P95 exposure was 1.8, observed for the Swedish study population.

4. Discussion

The current work explores the use of HBM data in risk assessment, and the outcomes of each risk assessment should be interpreted in light of the uncertainties of the approaches considered. In order to perform these mixture risk assessment exercises, we have used approaches that have their advantages as well as some built-in assumptions and limitations, as we explain below.

4.1. Internal exposure data

In the mixture risk assessments, we have included recent PFASs exposure data from 2014 or later from teenagers in the European population. The HBM4EU Aligned Studies collected samples and data in a harmonized way and analysed them in laboratories that were qualified in the HBM4EU QA/QC program, but also built further on existing capacity. For this reason, there is still some heterogeneity in the data, e.g. the age of the study participants and sampling years and geographical representativity for Europe (Appendix A, Table A1). Furthermore, most studies quantified PFASs in serum, but two studies quantified PFASs in plasma (NEB II and GerES V). Consequently, differences due to this different exposure matrix cannot be excluded, although it is anticipated that the concentrations quantified in serum or plasma only differ slightly (Poothong et al., 2017). Lastly, not all studies measured total PFASs levels, being the sum of branched and linear forms, but only considered the linear form (i.e. the ESTEBAN and FLEHS IV studies). It may be that serum/plasma levels may have been higher by including both branched and linear forms.

In the HBM4EU Aligned Studies, short as well as long chain PFASs were measured in blood samples, but the detection frequencies of substances such as PFBS, PFPeA, and PFHxA were overall low. Apart from the high LOD/LOQ in some studies that could explain this, it may also have to do with the exposure matrix in which the substances were measured. In general, for short-chain PFASs, the half-life in humans is quantified in the range of days to months compared to years for the long-chain PFASs (EFSA, 2020a). For this reason, urine may also be a relevant matrix to consider to assess the exposure, complementary to blood samples. In a recent HBM study among the US population, paired urine and blood samples were obtained, which showed that urine may also be a relevant exposure matrix for PFASs with a short elimination half-live

Table 6
PFAS mixture risk assessment based on the Hazard Index (HI) approach. HIs for PFASs were calculated for nine European teenage populations based on P95 values, whereby non-detects are set at zero. Hazard quotients are summed up to calculate the hazard index. A HI >1 indicates a potential risk.

	EL (ng/mL)		Norway	Sweden	Slovakia	Slovenia	Greece	Spain	Belgium	France	Germany
Immunotoxicity											
Kielsen et al. (2016)											
Adults											
PFOS	9.52	HQ	0.74	0.86	0.65	0.61	0.55	0.32	0.77	0.65	0.62
PFNA	0.66		1.52	1.30	0.70	0.77	1.27	0.91	1.12	2.09	1.11
PFDA	0.3		1.01	1.43	0.57	0.94	1.33	0.92	1.67	1.69	1.24
PFUnDA	0.21		1.28	1.67	0.38	0.59	0.81	1.12	1.14	1.37	ND
PFDoDA	0.039		ND	ND	ND	0.77	0.63	ND	ND	ND	ND
		HI	**4.55**	**5.27**	**2.30**	**3.68**	**4.58**	**3.28**	**4.70**	**5.80**	**2.96**
Grandjean et al. (2012)											
Children											
PFOA	4.06	HQ	0.51	0.58	0.34	0.36	0.54	0.25	0.44	0.65	0.77
PFHxS	0.63		1.89	1.75	1.58	0.67	1.41	1.21	2.22	3.69	1.56
PFDA	0.28		1.08	1.54	0.61	1.00	1.42	0.99	1.79	1.81	1.32
		HI	**3.48**	**3.87**	**2.53**	**2.03**	**3.37**	**2.46**	**4.45**	**6.15**	**3.65**
Birth weight reduction											
Meng et al. (2018)											
Mothers											
PFOA	4.6	HQ	0.45	0.51	0.30	0.31	0.48	0.22	0.39	0.57	0.68
PFOS	30.1		0.23	0.27	0.20	0.19	0.17	0.10	0.24	0.20	0.20
PFNA	0.5		2.01	1.72	0.93	1.02	1.68	1.20	1.48	2.76	1.46
PFHpS	0.4		0.25	NA	0.49	0.28	0.36	0.63	ND	ND	NA
		HI	**2.95**	**2.51**	**1.92**	**1.81**	**2.69**	**2.16**	**2.11**	**3.54**	**2.34**
Wang et al. (2016)											
Mothers											
PFNA	1.44	HQ	0.70	0.60	0.32	0.35	0.58	0.42	0.51	0.96	0.51
PFDA	0.37		0.82	1.16	0.46	0.76	1.08	0.75	1.35	1.37	1.00
PFDoDA	2.89		ND	ND	ND	0.01	0.01	ND	ND	ND	ND
PFUnDA	0.3		0.89	1.17	0.27	0.41	0.57	0.79	0.80	0.96	ND
		HI	**2.41**	**2.93**	**1.05**	**1.53**	**2.23**	**1.95**	**2.67**	**3.29**	**1.51**

EL = effect level.
HI = hazard index.
HQ = hazard quotient.
NA = not available.
ND = not detected (values below LOD or LOQ were set at zero).

Table 7
PFAS mixture risk assessment based on the sum value approach. Percentiles of the distribution for the sum of EFSA-4 (PFOA, PFNA, PFOS, PFHxS) calculated for nine European teenage populations, where non-detects were set at zero. Risk Characterization Ratios (RCR) were calculated for each population (for median and high exposures, respectively) as well as the percentage of participants exceeding the HBM GV.

	Participants	Percentiles of the sum EFSA-4 in serum or plasma (ng/mL)		Participants exceeding the HBM GV of 6.9 ng/mL		RCR based on the P50 and the HBM GV of 6.9 ng/mL	RCR based on the P95 and the HBM GV of 6.9 ng/mL	Participants exceeding the HBM GV of 17.5 ng/mL		RCR based on the P50 and the HBM GV of 17.5 ng/mL	RCR based on the P95 and the HBM GV of 17.5 ng/mL
	N	P50	P95	N	%	RCR P50	RCR P95	N	%	RCR P50	RCR P95
Norway	177	5.15	11.0	31	17.5	0.75	1.59	0	0	0.29	0.63
Sweden	300	4.82	12.4	69	23.0	0.70	1.80	5	1.7	0.28	0.71
Slovakia	292	2.59	9.24	22	7.5	0.38	1.34	4	1.4	0.15	0.53
Slovenia	94	2.99	8.15	7	7.5	0.43	1.18	0	0	0.17	0.47
Greece	52	3.83	8.91	7	13.5	0.56	1.29	0	0	0.22	0.51
Spain	299	2.38	5.18	4	1.3	0.34	0.75	1	0.3	0.14	0.30
Belgium	300	4.18	10.2	51	17.0	0.61	1.48	1	0.3	0.24	0.58
France	143	4.91	11.3	34	23.8	0.71	1.64	2	1.4	0.28	0.65
Germany	300	4.35	9.78	49	16.3	0.63	1.42	3	1	0.25	0.56

(Calafat et al., 2019). The CONTAM panel recommended to conduct additional human biomonitoring studies on paired samples (blood--urine), to identify the relevant matrices for biomonitoring of various PFASs (EFSA 2020a). This would be a useful follow-up in additional HBM studies on PFASs under the PARC initiative.

Organofluorine mass balance analysis measures the total extractable

Table 8
PFAS mixture risk assessment based on the Relative Potency Factor (RPF) approach. Percentiles of the distribution for the sum of six or seven PFASs calculated for nine European teenage populations, where non-detects were set at zero. The exposure levels were corrected relative to the potency of PFOA, and all values were expressed as ng PFOA equivalents (PEQ)/mL serum or plasma. Risk Characterization Ratios (RCR) were calculated for each population (for median and high exposures, respectively), and the percentage of participants exceeding the HBM GV for the sum of four PFASs.

	Substances in sum PEQ	Participants	Percentiles of the sum of PFASs in serum or plasma expressed as PEQ ng/mL		Participants exceeding the HBM GV of 6.9 ng/mL		RCR based on the P50 and the HBM GV of 6.9 ng/mL	RCR based on the P95 and the HBM GV of 6.9 ng/mL	Participants exceeding the HBM GV of 17.5 ng/mL		RCR based on the P50 and the HBM GV of 17.5 ng/mL	RCR based on the P95 and the HBM GV of 17.5 ng/mL
	#	N	P50	P95	N	%	RCR P50	RCR P95	N	%	RCR P50	RCR P95
Norway	6[b]	177	12.4	28.5	169	95.5	1.80	4.13	32	18	0.71	1.63
Sweden	7[a]	300	11.8	29.9	266	88.7	1.71	4.33	68	23	0.67	1.71
Slovenia	7[a]	94	7.24	21.9	51	54.3	1.05	3.17	7	7.5	0.41	1.25
Slovakia	7[a]	292	6.88	26.2	145	49.7	1.00	3.80	27	9.3	0.39	1.50
Greece	7[a]	52	11.1	25.8	49	94.2	1.61	3.74	9	17	0.63	1.47
Spain	7[a]	299	6.26	13.7	123	41.1	0.91	1.99	5	1.7	0.36	0.78
Belgium	7[a]	300	9.86	27.6	230	76.7	1.43	4.00	48	16	0.56	1.58
France	7[a]	143	10.7	27.4	130	90.9	1.55	3.97	25	17	0.61	1.57
Germany	7[a]	300	9.63	23.9	232	77.3	1.40	3.46	30	10	0.55	1.37

[a] PFBS, PFHxS, PFOS, PFHxA, PFOA, PFNA, PFDoDA.
[b] PFBS, PFHxS, PFOS, PFOA, PFNA, PFDoDA.

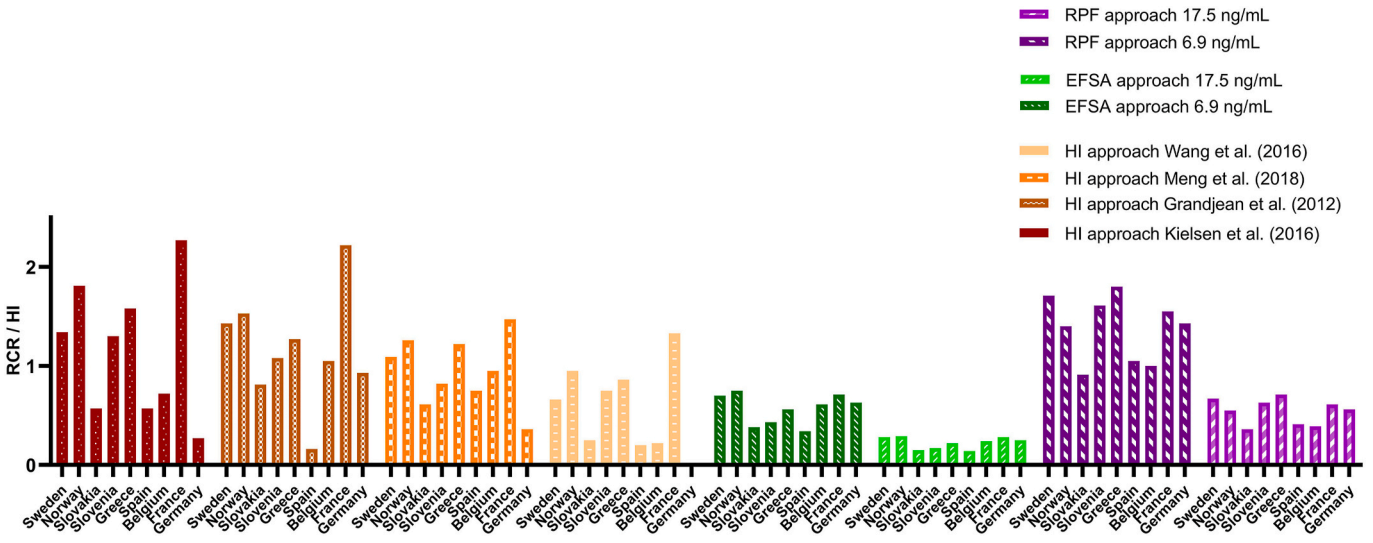

Fig. 1. Risk characterization ratio (RCR)/hazard index (HI) for simultaneous exposure to multiple PFASs based on P50 sum exposure in nine European teenage populations. Exposure values below limit of quantification (LOQ) were treated as nulls. Information on the number of individuals within the human biomonitoring study, sex, age, sampling period, LOD and LOQ is presented in Appendix A.

organofluorine (EOF) in the blood, and provides an indication of what fraction of the EOF can be attributed to particular PFASs, when performed together with a targeted PFAS analysis (Aro et al., 2021). Several studies have shown that the percentage of EOF accounted for by the sum of identified legacy PFASs in the human blood decreased in recent years, meaning that the fraction of unknown PFASs in human blood increased during this period (Aro et al., 2021; Miaz et al., 2020; Yeung and Mabury 2015). This stipulates the importance of organofluorine mass balance analysis and non-targeted- and suspect screening to gain insight into the exact PFAS composition in human blood, thereby enabling the identification of new and emerging PFASs. Currently the total PFASs mixture exposure is likely underestimated by focussing mainly on known PFCAs and PFSAs.

4.2. Hazard data

In both the HI approach and the sum value approach, we fully relied on human epidemiological data in risk assessment to estimate mixture risk, which is a novelty in risk assessment. However, the human epidemiological data we have used in the HI approach have limitations that one should be aware of. A clear distinction between cross-sectional studies, prospective studies, and case-control studies has not been made in the hazard assessment. Furthermore, the models used in these epidemiological studies were based on single or multiple linear regression analysis, and did not consider model averaging to address model uncertainty. Another inherent issue to the use of hazard data obtained from human epidemiological studies is the lack of a background response. This means that it is generally unknown how the response observed relates to the response if very low or no exposure occurs. This problem may be overcome by the use of an interpercentile range, whereby the data are divided over a sufficient number of ventiles/deciles/quartiles so that the lower percentiles(s) may serve as a background response (Li et al., 2020b).

The PODs we took for the HI approach are the average PFAS serum

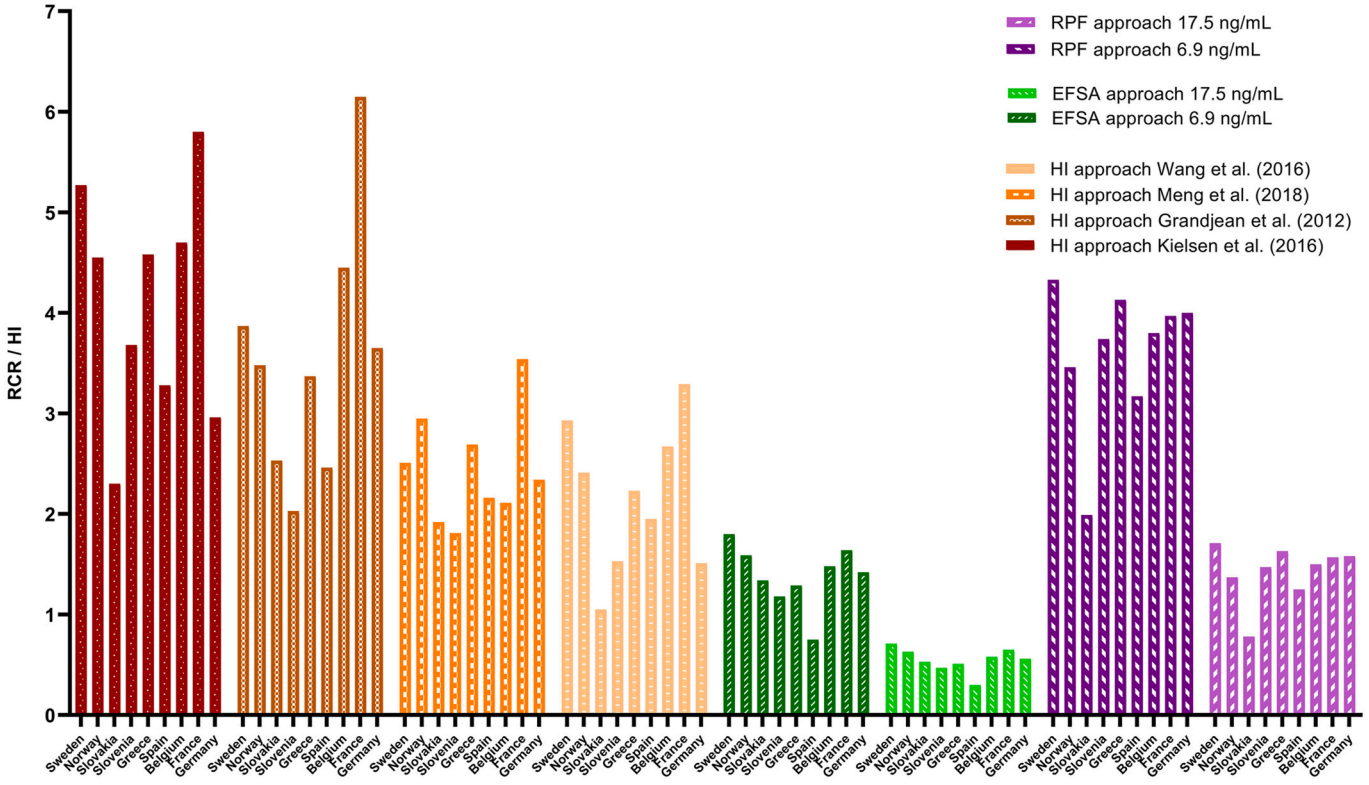

Fig. 2. Risk characterization ratio (RCR)/hazard index (HI) for simultaneous exposure to multiple PFASs based on P95 sum exposure in nine European teenage populations. Exposure values below limit of quantification (LOQ) were treated as nulls. Information on the number of individuals within the human biomonitoring study, sex, age, sampling period, LOD and LOQ is presented in Appendix A.

levels in a given study statistically significantly associated with the health outcome, which depends on the population studied and the exposure to PFASs in these populations. Furthermore, the serum concentration of one PFAS may be highly correlated with the serum concentration of another PFASs (as indicated in Appendix D) and therefore, it is impossible to determine which PFAS(s) drive(s) the association. If one of the components would be completely responsible for the adverse effect observed in the study, the others would not be expected to have any impact. A specific consequence of this is that PFASs with low concentrations (e.g. PFNA or PFHxS) may be statistically correlated with the health outcome in the study using a single pollutant model, but this may (at least partly) be due to confounding with the higher concentration PFASs (e.g. PFOS) with which it is correlated. In such cases, the POD of the low concentration PFASs would be overprotective. Discussion on the issues of using epidemiological data for risk assessment took place in 2018, when EFSA presented its scientific opinion on PFOS and PFOA (EFSA 2018b; EFSA 2018c). EFSA later performed BMD modelling for the sum of four PFASs in the recent opinion, to derive a $BMDL_{10}$ assuming equipotency of the four PFASs and absence of other PFASs/-substances or stressors which could also (partly) contribute to the effect.

As a default assumption, EFSA assumed in their risk assessment equipotency of PFOA, PFNA, PFOS and PFHxS (the 'EFSA-4') at POD level (immune effect in the child). In human epidemiological studies it is difficult to determine relative potencies and EFSA noted that the stronger association for PFOA compared to PFOS as indicated by the human epidemiological data, conflicted with the higher potency that is seen for PFOS compared to PFOA in various animal studies. Studying relative potencies in epidemiological studies is difficult in practice, as the serum concentration of one PFAS may be highly correlated with the serum concentration of other PFASs. Therefore, data from experimental animal studies can be used to explore if such an assumption on equipotency is likely, even if this approach suffers from the general issue of species differences. Our exercise on the derivation of internal relative potencies leads to question the assumption of equipotency between PFOA, PFOS, PFHxS and PFNA, because differences in potency between PFAS were observed at the serum level.

Concerning the limitations of the RPF approach, it is assumed that the PFASs for which an RPF is available exert the same toxicodynamic features as PFOA during similar exposure durations. Nevertheless, this extrapolation step needs some critical reflection. For other data-rich substances included in the assessment, such as PFOS, it would be valuable to evaluate whether the use of the RPF under- or overestimates the risk when substance-specific exposure and hazard data are used. Furthermore, it must be noted that the derivation of the internal RPFs is based on a limited dataset, namely liver effects in the male rat. The relevance of rodent data for use in risk assessment of PFASs has been questioned due to uncertainties in species concordance for certain endpoints, such as lipid perturbations and liver carcinogenicity, and differences in toxicokinetics and bioaccumulation potential among species (Corton et al., 2018; Pizzurro et al., 2019; Fenton et al., 2021). Such apparent discrepancies led to the conclusion that more information is needed regarding the mode(s) of action and adverse outcome pathways for PFAS toxicity, PFAS toxicokinetics in humans and experimental animals, and dose-response relationships among sexes, species and life-stages, whereas for many effects concordance between human epidemiological findings and experimental animal data exists (Fenton et al., 2021).

By using the RPFs in combination with the EFSA TWI, it is (provisionally) assumed that (1) other PFASs apart from 'EFSA-4' exert an adverse effect on the immune system and (2) the potency ranking of PFAS for liver toxicity in the rodent is the same as the potency ranking of PFAS for immunotoxicity in the human. A thorough comparison of PFASs relative potencies in relation to other effects, sex, life-stages, and species, and in particular for critical effect such as immune effects and mammary gland development, would be of added value.

4.2.1. Risk characterization

With regard to the different approaches:

- The mixture risk assessment using the HI approach demonstrated PFAS exposure may result in a health risk in the HBM4EU study population, when considering both P50 and P95 values. In this assessment, more than four PFASs were included, which is an advantage over the sum value approach. The basis of this mixture risk assessment was human data only, which is an advantage compared to the RPF methodology. However, due to the positive correlation between different PFASs in epidemiological studies this approach is likely to result in overestimation of risk. Finally, the HI approach used as such is more conservative to the other two approaches caused by the fact that combined exposure has not been assessed at the individual level but only at the population level.
- The sum value methodology is the most straight forward PFAS mixture risk assessment performed here, as the HBM GV of the 'EFSA-4' is directly compared to the recent European mixture exposure of the same compounds. This approach led to the conclusion that exposure to the sum of the EFSA-4 resulted in exceedance of the HBM GVs only when looking at P95 exposure. Since the P50 and P95 values were based on summing exposure to the EFSA-4 per individual, the influence of >1 correlations is avoided. This approach relies on human data, therefore not involving issues regarding interspecies differences. However, only four substances were included in the assessment, whereas exposure to more PFASs is apparent in the HBM studies. Furthermore, this approach assumed equipotency of PFASs at the internal level, which may not be the case.
- The RPF method showed that exposure to six or seven PFASs, expressed as the sum PEQ, resulted in exceedance of the HBM GVs when considering P50 and P95 values. In this assessment, more than four PFASs were included, which is an advantage over the sum value approach. Furthermore, since the P50 and P95 values were based on summing PEQ per individual, the influence of >1 correlations was avoided. Moreover, differential potencies of the different PFASs is taken into account in the mixture risk assessment. However, this includes an uncertainty related to the extrapolation of the RPFs based on liver toxicity in experimental animals to immunotoxicity in humans. Ideally, this approach should include a serum concentration on which the toxicological point of departure is based, expressed in PEQs (i.e. an 'RPF adjusted' HBM GV). However, due to practical reasons it is currently not feasible to adjust the POD underlying the EFSA TWI from EFSA-4 sum exposure to PEQs. This is recommended to take into account later on, should the EFSA TWI be revised in the future.

4.3. Strengths of this study

In the current paper, we performed mixture risk assessments with HBM data as primary input. HBM data are very valuable to calculate the cumulative risk resulting from exposure to PFASs from multiple sources. Since exposure to PFASs stem from a wide variety of sources across different routes, exposure is underestimated if combined exposure to multiple PFASs and aggregated exposure to the same PFAS from multiple sources and pathways is not included in risk assessment. Hence, these mixture risk assessments targeted both combined exposure to multiple chemicals and aggregate exposure to the same chemicals from multiple sources and pathways.

Another strength of our study is the use of individual exposure data. Within HBM4EU, individual data could be shared via a single collaboration agreement, which restricted exchange of data to the information required to answer the research question defined (Gilles et al., 2021). Via this collaboration agreement, we were able to use individual exposure data to calculate summed exposure to PFASs on an individual level, rather than relying on summary statistics (e.g. P50, P95). In this way, sum exposure to multiple PFASs was not overestimated.

Lastly, EFSA considered exposure from dietary intake for specific age groups and expressed this against the TWI of 4.4 ng/kg bw/week. Based on these calculations, EFSA concluded on a risk for both adolescents and adults based on mean and high dietary intake. Overall, the results in the current mixture risk assessments are in line with the risk characterization in the EFSA opinion, which strengthens the overall conclusion that adverse health effects may arise in the European population due to PFAS mixture exposure.

5. Conclusion and perspectives

The mixture risk assessments show that combined exposure to PFASs is too high and may result in a human health risk in a substantial fraction of the HBM4EU study population, thereby confirming the conclusion drawn in the recent EFSA scientific opinion. Long-term exceedance of the HBM GV is undesirable and a reason to reduce human exposure to a level below this threshold. Thus, it is important to map exposure sources to PFASs and target them with effective policy measures so that human exposure to PFASs in general will be reduced.

For future research, the following recommendations aid to refine and improve mixture risk assessment of PFASs using human biomonitoring data:

- In general, we observed that risk assessments, based on HBM data obtained from studies that relied on relatively high LOD and LOQ, are very uncertain. We therefore stress the need for improving the analytical methods for human biomonitoring and for bringing down the LOD and LOQ of the PFAS analyses;
- We recommend to perform human biomonitoring studies that observe paired blood-urine samples to have a better insight into exposure to short-chain PFASs;
- We recommend to perform organofluorine mass balance analysis and non-targeted- and suspect screening to gain insight into the exact PFAS mixture composition in human blood, thereby enabling the identification of new and emerging PFASs;
- We recommend to further explore linkages between PFAS mixture exposure and health effects that would allow point of departure setting for PFAS mixture risk assessment, taking into account the background response of the health effects studied;
- We recommend to derive RPFs for PFASs that currently have none available, and to validate relative potencies of PFASs in relation to other effects, sex, life-stages, and species, and in particular for critical effects such as immune effects;
- We recommend to develop human-based *in vitro/ex vivo* models for immunotoxicity and developmental toxicity that can mimic the human responses. We advise, complementary to this, to investigate mode(s) of action and adverse outcome pathways for PFASs to overcome data gaps and to shed light on species concordance.

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Declaration of competing interest

The authors have no conflicts of interest to declare.

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Supplementary data

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