Full length article

Human risk associated with exposure to mixtures of antiandrogenic chemicals evaluated using *in vitro* hazard and human biomonitoring data

Yanying Ma [a], Camilla Taxvig [a], Andrea Rodríguez-Carrillo [b,c], Vicente Mustieles [b,c,d], Lena Reiber [e], Anja Kiesow [e], Nathalie Michelle Löbl [a], Mariana F. Fernández [b,c,d], Tina Vicky Alstrup Hansen [a], Maria João Valente [a], Marike Kolossa-Gehring [e], Madlen David [e], Anne Marie Vinggaard [a,*]

[a] *National Food Institute, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark*
[b] *Center for Biomedical Research (CIBM), University of Granada, Spain*
[c] *Instituto de Investigación Biosanitaria Ibs Granada, Spain*
[d] *Consortium for Biomedical Research in Epidemiology & Public Health (CIBERESP), 18100, Spain*
[e] *German Environment Agency, Berlin, Germany*

ARTICLE INFO

ABSTRACT

Background: Scientific evidence for underestimated toxicity from unintentional exposure to chemical mixtures is mounting. Yet, harmonized approaches on how to assess the actual risk of mixtures is lacking. As part of the European Joint programme 'Human Biomonitoring for Europe' we explored a novel methodology for mixture risk assessment of chemicals affecting male reproductive function.

Methodology: We explored a methodology for chemical mixture risk assessment based on human *in vitro* data combined with human exposure data, thereby circumventing the drawbacks of using hazard data from rodents and estimated exposure intake levels. Human androgen receptor (hAR) antagonism was selected as the most important molecular initiating event linked to adverse outcomes on male reproductive health.

Results: Our work identified 231 chemicals able to interfere with hAR activity. Among these were 61 finally identified as having both reliable hAR antagonist and human biomonitoring data. Calculation of risk quotients indicated that PCBs (118, 138, 157), phthalates (BBP, DBP, DIBP), benzophenone-3, PFOS, methylparaben, triclosan, some pesticides (i.e cypermethrin, β-endosulfan, methylparathion, p,p-DDE), and a PAH metabolite (1-hydroxypyrene) contributed to the mixture effect. The major chemical mixture drivers were PCB 118, BBP, PFOS, DBP, and the UV filter benzophenone-3, together contributing with 75% of the total mixture effect that was primarily driven by high exposure values.

Conclusions: This viable way forward for mixture risk assessment of chemicals has the advantages of (1) being a more comprehensive mixture risk assessment also covering data-poor chemicals, and (2) including human data only. However, the approach is subjected to uncertainties in terms of *in vitro* to *in vivo* extrapolation, it is not ready for decision making, and needs further development. Still, the results indicate a concern for adverse effects on reproductive function in highly exposed boys, especially when considering additional exposure to data-poor chemicals and chemicals acting by other mechanisms of action.

1. Introduction

Humans are exposed to many different chemicals in their everyday life. These stem from multiple sources such as air, water, dust, food, and consumer products (Amato-Lourenço et al., 2020; Domingo and Nadal, 2019; Kampa and Castanas, 2008; Trasande et al., 2018; Wang et al., 2019; Ward et al., 2018). The specific chemical exposure varies based on the behavioural pattern of the individual (Bolon and Haschek, 2020) and exposure is ever-changing with phase-outs and introduction of chemicals to the market (Pimentel et al., 2007). Consequently, humans are exposed to mixtures of chemicals that are dynamic and unique in their compositions.

Multiple component-based mixture studies designed with model compounds have shown that mixtures of chemicals can induce effects greater than those induced by the single chemicals on male reproductive health disorders, *e.g.,* anogenital distance, nipple retention, external sex organ malformations, sex organ weights, and dysgenesis and hypoplasia in male reproductive organs (Christiansen et al., 2009; Hass et al., 2007; Metzdorff et al., 2007). Similar observations have been reported *in vitro* on androgen receptors, (Birkhøj et al., 2004; Ermler et al., 2011; Kjærstad et al., 2010; Orton et al., 2014) estrogen receptors (Rajapakse et al., 2002; Silva et al., 2002), and on sex hormone synthesis (Kjærstad et al., 2010).

Androgen insufficiency in the developing male foetus, which can be a result of chemically induced androgen receptor (AR) antagonism, can lead to shortening of the anogenital distance which is considered a unique, early and non-invasive marker of male reproductive health effects (Scholze et al., 2020). It is plausible that this specific mechanism of action, AR antagonism, constitutes the most important molecular initiating event in the adverse outcome pathway to male reproductive health disorders and therefore is an obvious choice for characterizing hazard in a mixture risk assessment for male reproductive health disorders.

A common way of performing a chemical mixture risk assessment is to base it on the dose addition principle using hazard data retrieved from *in vivo* rodent and human exposure information retrieved from food intake data (Boberg et al., 2021). In some cases, human exposure data from environmental sources is available in addition to the food intake data, as was the case in a previous mixture risk assessment of phthalates (Boberg et al., 2021) or alternatively, human exposure can be predicted by modelling based on chemical data, consumer product data, and population characteristics (Isaacs et al., 2014). Due to inherent uncertainties in these approaches such as lack of *in vivo* data for many chemicals, questionable relevance of hazard data from rodents, and risk of lacking the most relevant exposure sources, there is a need to explore alternative approaches to evaluate risk to chemical mixtures.

In this case study, integrated in the EU Joint programme 'Human Biomonitoring for Europe' we have evaluated the human risk to mixtures of antiandrogenic chemicals based exclusively on human-derived hazard and exposure data. Our hypothesis was that relevant Risk Quotients (RQs) can be defined based on *in vitro* data for human AR (hAR) antagonism and human biomonitoring (HBM) data, reflecting aggregated internal human exposure. Using internal levels in human blood has the advantage that levels represent aggregated levels irrespective of sources and pathway of exposure.

Moreover, we focus on chemicals with antiandrogenic activity that block the hAR. Chemicals with this mode of action are known to be involved in reproductive health disorders observed in boys and men when exposed during foetal life (Schwartz et al., 2019). AR antagonism is the best-known molecular initiating event that is linked to adverse male reproductive health disorders and by default, all chemicals with this specific mode of action belong to the same cumulative assessment group, thus in this case considerations on which grouping criteria needs to be included are redundant.

2. Material and methods

2.1. Retrieving hazard data

A list of compounds previously reported to be hAR antagonists was prepared based on a selection of publications which led to a collection of 231 compounds (Danish Environmental Protection Agency, 2017; Engel et al., 2017; Ermler et al., 2011; Kojima et al., 2019, 2009, 2004; Kortenkamp and Faust, 2010; Orton et al., 2011; Rosenmai et al., 2014; Shen et al., 2009; Vinggaard et al., 2008). The literature search for hazard and exposure data of compounds including inclusion and exclusion criteria followed the procedure described in Fig. 2.

All substances on the bucket list were screened for availability of HBM data by using the approach described later. The HBM data was incorporated into the bucket list leading to a refined list 76 substances with available hazard and exposure data. For all substances on the refined list a comprehensive literature search for additional hazard data was performed using the following search terms in Web of Science:

Search in TOPICS: (Group name(s)/compound name and synonyms and abbreviations (OR between, if more than one)) AND (*vitro*) AND (*androgen*).

If > 500 search results, search in TOPICS: AND (receptor).

If > 500 search results, search in TOPICS: AND ("reporter assay" OR "reporter gene assay").

Compounds belonging to a large chemical class (*e.g.,* polycyclic aromatic hydrocarbons or phthalates) were only searched for using the
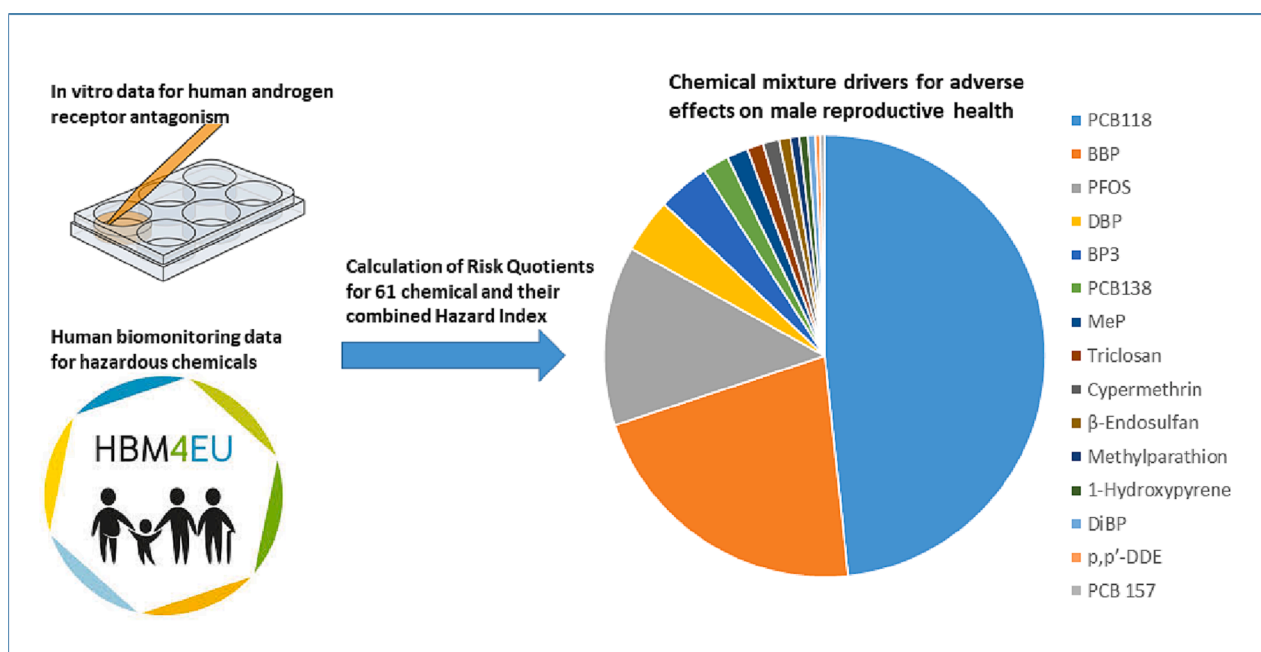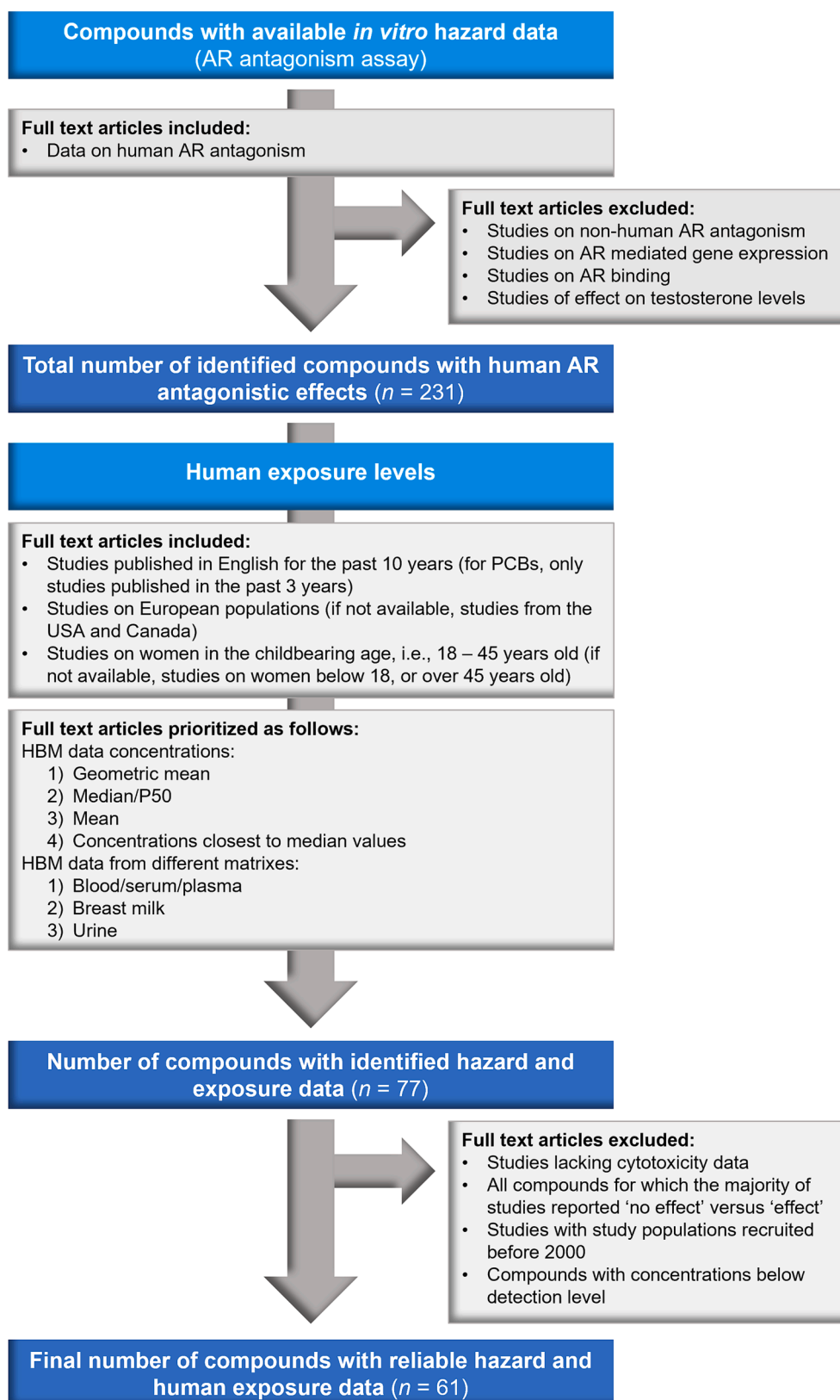

Fig. 1. Graphical abstract illustrating the approach used for identifying the chemical mixture risk drivers for male reproductive health.

Compounds with available *in vitro* hazard data
(AR antagonism assay)

Full text articles included:
- Data on human AR antagonism

Full text articles excluded:
- Studies on non-human AR antagonism
- Studies on AR mediated gene expression
- Studies on AR binding
- Studies of effect on testosterone levels

Total number of identified compounds with human AR antagonistic effects (*n* = 231)

Human exposure levels

Full text articles included:
- Studies published in English for the past 10 years (for PCBs, only studies published in the past 3 years)
- Studies on European populations (if not available, studies from the USA and Canada)
- Studies on women in the childbearing age, i.e., 18 – 45 years old (if not available, studies on women below 18, or over 45 years old)

Full text articles prioritized as follows:
HBM data concentrations:
1) Geometric mean
2) Median/P50
3) Mean
4) Concentrations closest to median values
HBM data from different matrixes:
1) Blood/serum/plasma
2) Breast milk
3) Urine

Number of compounds with identified hazard and exposure data (*n* = 77)

Full text articles excluded:
- Studies lacking cytotoxicity data
- All compounds for which the majority of studies reported 'no effect' versus 'effect'
- Studies with study populations recruited before 2000
- Compounds with concentrations below detection level

Final number of compounds with reliable hazard and human exposure data (*n* = 61)

Fig. 2. Overview of the inclusion and exclusion criteria for data and papers to be included in the mixture risk assessment. AR – androgen receptor, HBM – human biomonitoring, PCBs – polychlorinated biphenyls.
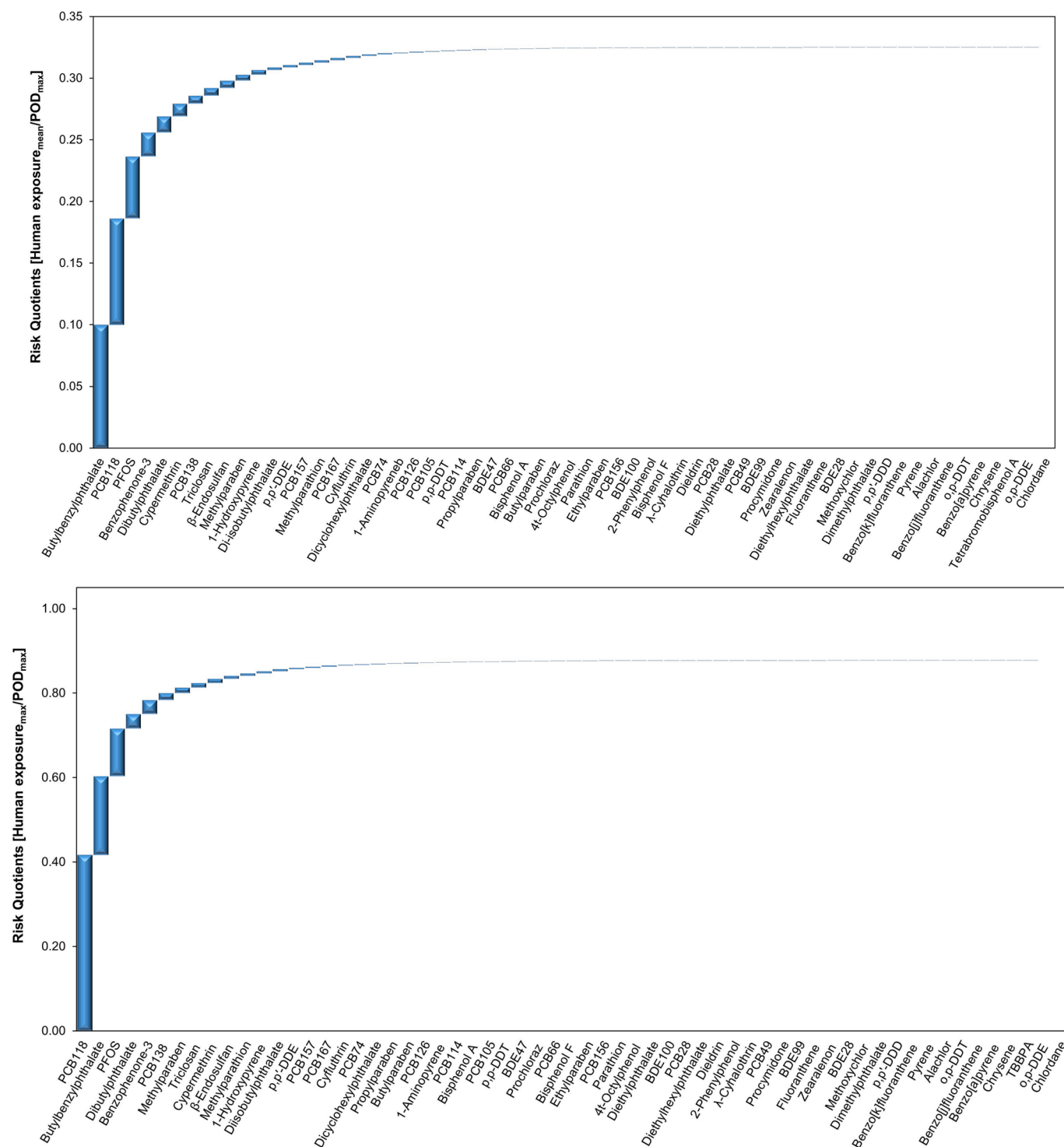
Fig. 3. Identification of risk drivers by ranking risk quotients for antiandrogenic chemicals calculated from human (A) **mean** and (B) **maximum** exposure values divided by POD$_{max}$ for androgen receptor antagonism. BDE – bromodiphenyl ether, DDD – dichlorodiphenyldichloroethane, DDE – dichlorodiphenyldichloro-ethylene, DDT – dichlorodiphenyltrichloroethane, PCB – polychlorinated biphenyl, PFOS – perfluorooctanesulphonate.

group name (*e.g.,* (PAH) OR ("polycyclic aromatic hydrocarbon*" or (phthalate*)).

An inclusion criterion for this search was that the hazard data should stem from hAR antagonism data only, hence, substances for which only animal data existed or *in vitro* effects dealing with *e.g.,* AR mediated proliferation or gene expression, AR binding or effects on testosterone levels were excluded from the list. After thorough evaluation of comprehensive hazard literature on the 77 substances, all studies in

which no information on cytotoxicity was available were omitted, and any response that was obtained at a concentration at which significant cytotoxicity was reported was discarded. For antiandrogenic substances lack of this information is particularly crucial, as a cytotoxic substance could be misclassified as an antiandrogen, as this would lead to a decreased signal as AR blocking would. This curation led to the removal of two substances, 2,2,3,4,4-pentabromodiphenylether and hepta-chloroepoxide, from the list, but also led to a more homogenous hazard

data set, as many values that deviated, typically by being higher than the remaining values within a group, was removed. In addition, all substances for which the majority of the studies reported no effect versus effect were omitted. This curation led to removal of two additional substances, di-isononyl phthalate and bisphenol S from the list. Successively, all studies performed in yeast versus mammalian cells were excluded from the data set.

Hazard values, given as IC_{10} values were used for deriving RQs as a measure of the Point of Departure (POD). In cases where no IC_{10} values were available, the following equation available in GraphPad Prism (ver.8) was used to calculate IC_{10}:

$$IC_x = \left(\frac{F}{100-F}\right)^{\frac{1}{H}} * IC_{50} \Leftrightarrow IC_{50} = \frac{IC_x}{\left(\frac{F}{100-F}\right)^{\frac{1}{H}}} \quad (1)$$

where F is the percent change on the y-axis at IC_x, that is value above 0 and below 100. H is the Hill Slope, which was constrained to −1.

2.2. Exposure data

The 231 compounds were screened for availability of HBM data by reviewing reports published from relevant public organizations such as European Food Safety Authority, European Chemicals Agency, and United States Environmental Protection Agency, followed by a systematic literature search in PubMed. Details of the literature search are showed in the Supplementary Material, Figs. 1-22. All figures detail the specific search terms and boolean terms used for each chemical compound. Studies were selected with the following inclusion criteria: (1) articles published in English during the last 10 years; (2) women within

childbearing age as target population, and (3) studies on European populations (Fig. 2). When no HBM data fulfilling these criteria was available, articles from other Western populations (USA or Canada) were considered. The following additional steps were applied for selected compounds: For polychlorinated biphenyls (PCBs), only articles published during the last 3 years were searched due to the high number of references found. For compounds without any available information, such as PCB 49, PCB 66, PCB 74, methyl parathion and 4-*tert*-octylphenol, two reports published by National Health and Nutrition Examination Survey (NHANES) were included (CDC, 2009). If available, HBM data concentrations were prioritized in the following order: geometric mean (GM), median/P50, mean, and finally concentrations closest to median values available. If no concentrations above the limit of detection/quantification were measured, the study was excluded from calculation of mean values. Four compounds, namely bitertanol, fenhexamide, bromodiphenyl ether (BDE)85, and 4́OH-BDE17, were omitted from the refined list since none of the identified studies reported concentrations above limit of detection. HBM data was further curated to focus on pregnant women, mothers, or women in the child-bearing age defined as between 18 and 45 yrs. First, if data from these study populations were not available for a substance, the age range was expanded to include younger and elder women. Second, HBM data set was curated to ensure that study populations included recruitment after year 2000. For one substance, heptachlor epoxide, only one study with an older population was available, hence included in the study. In addition, all compounds for which the majority of studies reported 'no effect' versus 'effect' were excluded. For persistent compounds blood/serum/plasma concentrations were used for internal exposure estimates. If not available, alternative matrices were used such as breast milk
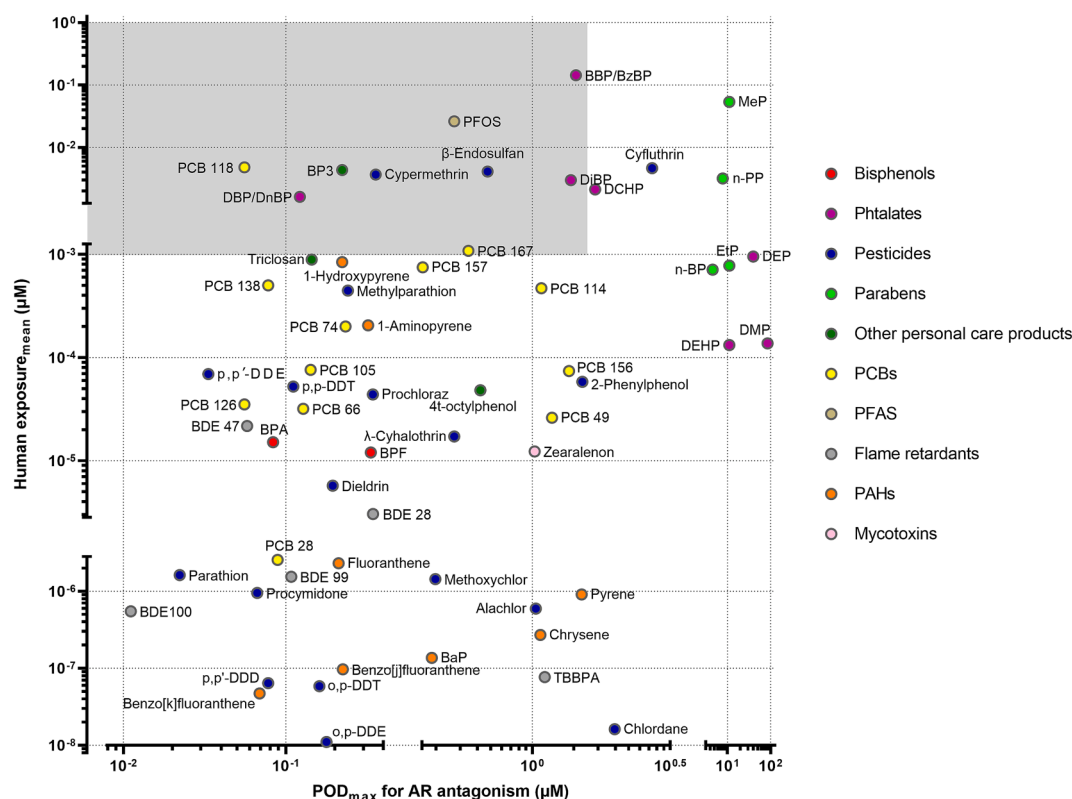
Fig. 4. Human exposure levels expressed as the calculated or measured (A) **mean** and (B) **maximum** blood levels for each of the 61 compounds as a function of their potency *in vitro* (measured as POD_{max} for AR antagonism). Chemicals in the upper left corner (highlighted grey) are the most problematic from a risk perspective as they are the most hazardous chemicals with the highest exposure values. BBP/BzBP – butylbenzylphthalate, BDE – bromodiphenyl ether, BP3 – 2-Hydroxy-4-methoxybenzophenone, BPA – bisphenol A, BPF – bisphenol F, DBP/DnBP – dibutylphthalate, DCHP – dicyclohexylphthalate, DDD – dichlorodiphenyldichloroethane, DDE – dichlorodiphenyldichloroethylene, DDT – dichlorodiphenyltrichloroethane, DEHP – diethylhexylphthalate, DEP – diethylphthalate, DiBP – di-isobutylphthalate, DMP – dimethylphthalate, EtP – ethylparaben, MeP – methylparaben, *n*-BP – butylparaben, *n*-PP – propylparaben, PAHs – polycyclic aromatic hydrocarbons, PCB – polychlorinated biphenyl, PFAS – per- and polyfluoroalkyl substances, PFOS – perfluorooctanesulphonate, TBBPA – tetrabromobisphenol A.
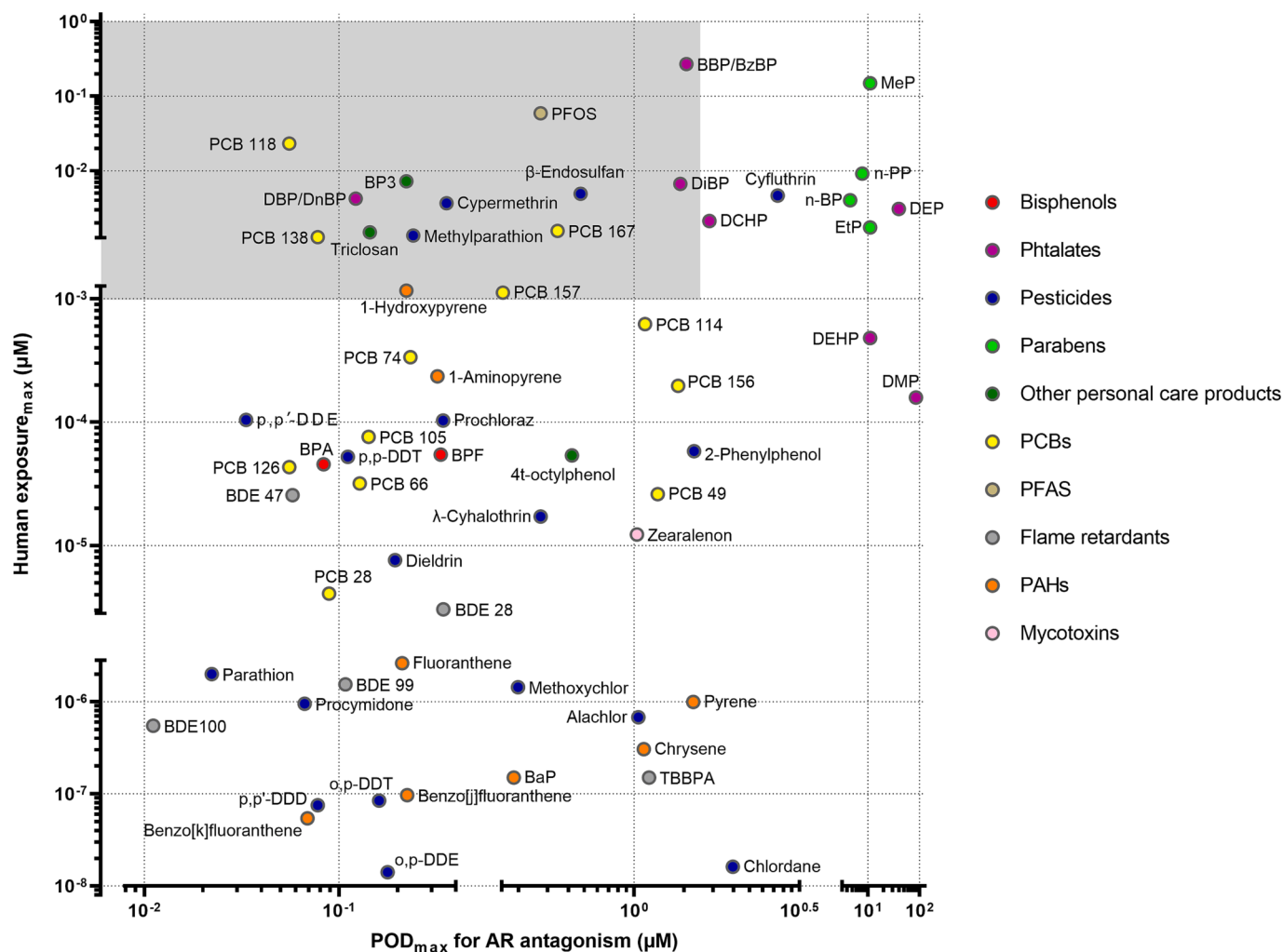
Fig. 4. (*continued*).

levels. For non-persistent compounds urine concentrations were used for calculation of internal exposure estimates if available.

2.2.1. Estimating human blood levels from breast milk levels for lipophilic compounds and from urine levels for fast metabolized compounds

In order to calculate an RQ for each selected chemical, human blood levels of the final 61 compounds were needed (**Suppl. Table 2**). Available blood, serum and plasma levels measured in the lipid fraction were converted to concentrations per L matrix using a factor of 6.19 g lipid/L blood. For the compounds for which the blood levels were not available, blood levels were estimated based on the concentrations in breast milk or urine obtained from additional literature search described below.

For many persistent chemicals, concentrations in breast milk are assumed to mirror concentrations in blood lipids (Aylward et al., 2003). We therefore conducted a literature search in PubMed and Google Scholar to identify studies reporting conversion factors (CFs) between breast milk and the blood matrix for the relevant compounds. For some compounds, CFs were not available, hence, the CFs were chosen as described below (**Suppl. Table 3**).

For the PCBs some studies measured single PCB levels in blood and breast milk (Aylward et al., 2003; DeKoning & Karmaus, 2000; LaKind et al., 2009; Mannetje et al., 2012; Todaka et al., 2010, 2011; Wittsiepe et al., 2007), however, most of the identified studies focused on the sum of PCBs or several of the most common PCBs. Overall, in these studies the CFs fluctuated around 1, thus we used this value for all PCBs. For DDT, levels are often 6–7 times higher in milk than in blood (Wolff,

1983). Mes et al. (Mes et al., 1984) determined the ratio between whole blood and whole milk for chlordane to be 0.2 in 16 women at day 7 postpartum, hence we used a CF of 0.2. According to WHO environmental health criteria (WHO, 1989), the ratio of dieldrin between mother's blood and milk is around 0.5 – 3, hence we set a mean CF of dieldrin to 1.75. For methoxychlor, we only identified data from one *in vivo* study in female rats in which the authors found a CF of 4.05 for the lowest dose level tested (Chapin et al., 1997). We used this CF although human exposure of methoxychlor is anticipated being much lower than for animals under experimental conditions.

We identified only few studies reporting CFs of pesticides between human milk and blood levels (**Suppl. Table 3**). For procymidone no CF was identified, thus we estimated it to be 1, assuming equilibrium between blood and breast milk (Marchitti et al., 2017). Polycyclic aromatic hydrocarbons (PAHs) were measured in both maternal serum and breast milk of 20 volunteers (Tsang et al., 2011). We used CFs from this study, where 0.24, 0.86 and 0.82 for benzo[k]fluoranthene, chrysene and pyrene were set, respectively. As we did not identify any specific CFs for the remaining PAHs on the list, we used the CF for the total PAHs i.e 0.74 determined in this study. Finally, we identified one study (Cariou et al., 2008), comparing the concentrations of tetrabromobisphenol A (TBBPA) in maternal serum and breast milk from 93 volunteers. In this study the authors found the CF between these matrixes to be approximately 1.6, hence we used this CF. For conversion of levels in breast milk fat to levels in breast milk a factor of 0.3 g lipid/L milk was used.

2.2.2. Estimating human blood levels from urine levels for fast metabolized compounds

Compounds that have a relatively short biological half-life mostly have HBM data measured in urine. First, we normalized urinary exposure concentrations to creatinine-standardized concentrations using 1.30 g/L as the mean urine creatinine concentration across all ethnic groups of females between 20 and 39 years from the NHANES cohort 1988–1994 (Barr et al., 2005).

We used available urine concentrations for compounds given in **Suppl. Table 4** to estimate the concentrations in the blood. Blood levels were estimated using equation 2 (Koch et al., 2007) and 3 given below.

$$DI[mol/kg_{bw} \cdot day] = \frac{\left(\dfrac{conc_{metabolite}\left[\frac{g}{L}\right]}{MW_{metabolite}\left[\frac{g}{mol}\right]}\right)}{Creatinine\left[\frac{g_{crt}}{L}\right]} \cdot \frac{CE\left[\frac{g_{crt}}{kg_{bw}}/day\right]}{F_{UE}} = \frac{UE[mol/g_{crt}] \cdot CE\left[\frac{g_{crt}}{kg_{bw}}/day\right]}{F_{UE}} \quad (2)$$

First, the daily intake (DI) of a substance was estimated by using equation 2, where UE is the molar urinary excretion of the respective metabolite(s), CE is the creatinine excretion rate normalized by body weight, which was set to 0.023 g/kg/day for a pregnant woman (Lioy et al., 2015), F_{UE} (%) is the molar ratio between the amount of metabolite(s) excreted in the urine and the amount of parent compound absorbed. The molecular weight of creatinine 113.12 g/mol was applied.

$$C_p[mol/l] = [DI^{mol}/kg_{bw}] \cdot day \frac{t^{1/2}/day}{0,693} \frac{1}{V_d[L/kg_{BW}]} \quad (1)$$

Secondly, the blood concentrations were calculated by applying a simplified one-compartment toxicokinetic model, where C_p is the blood plasma concentration after exposure to dose X, $T_{1/2}$ is the biological half-life of the substance and V_d is the apparent volume of distribution. This model assumes total bioavailability, intestinal absorption and that the compound reaches a steady state level (Fromme et al., 2007).

For two substances (i.e., prochloraz and alachlor) we used the F_{UE} obtained from animal studies due to lack of human data. No studies reported F_{UE} and $T_{1/2}$ for bisphenol F, but as previous studies have reported bisphenol F and bisphenol A to have similar properties (Punt et al., 2019; Rochester and Bolden, 2015) we assumed these to be similar.

For some phthalates, data was not available (i.e., dicyclohexylphthalate, diethylphthalate and dimethylphthalate). Hence, for dicyclohexylphthalate we used the values for diethylhexyl- phthalate (Koo et al., 2002), whereas for the latter two, we used the values for dibutylphthalate (monobutylphthalate) reported by (Gennings et al., 2014).

For 4-*tert*-octylphenol we did not find any data and estimated the F_{UE} to be 0.9 based on expert judgement.

For the two metabolites of cyfluthrin, *cis*- and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA), we assumed equal excretion. In a study by Hays et al., 2009 a total of 0.33 mg *cis*- and *trans*-DCCA was excreted in urine after oral intake of 2.6 mg cyfluthrin. The mass excretion fraction (F_{UE} (mass) and its subsequently conversion into F_{UE} (molar) was calculated using eq. 4 given (Aylward et al., 2018).

$$F_{UE}(molar) = F_{UE}(mass) * (MW_{parent}/MW_{metabolite}) \quad (4)$$

For several compounds $T_{1/2}$ and V_d values could not be found. In these cases we used computationally predicted values from the CompTox Chemicals Dashboard (U.S. Environmental Protection Agency, 2020), except for parathion which was not available. For 1-aminopyrene we used V_d for its parent compound 1-nitropyrene due to lack of data. All estimated blood levels of compounds with HBM data from urine together with the parameters used for the conversions are given in **Suppl. Table 4**.

2.3. Calculating the risk quotient for each individual compound and the Point of Departure Index to illustrate the mixture risk

The following equation was used to calculate the RQ for each chemical:

$$Risk\,Quotient(RQ) = Human\,blood\,level(\mu M)/POD\,for\,hAR\,antagonism(\mu M) \quad (5)$$

where aggregated HBM data, defined as the mean or the maximum human blood levels or estimated human blood levels, were used as exposure data, and the POD values for hAR antagonism *in vitro* were used to define the hazard level.

The Point of Departure Index (PODI), which is a rough measure of the cumulative risk to the chemical mixture, was calculated by summing up the RQs for each compound of the mixture:

$$PODI = \sum \left(RQ_{comp1} + RQ_{comp2} + RQ_{comp3} + RQ_{comp4} + \ldots\ldots RQ_{compN}\right) \quad (6)$$

3. Results

3.1. Identification of compounds with available hazard data

In total, we identified 231 compounds for which hazard values for human AR antagonism was available, but many compounds had to be excluded due to lack of exposure data, cytotoxicity data or other issues as illustrated in Fig. 2. For the 61 remaining compounds, the hazard values were reported either as mean POD (POD_mean) values across all identified studies or as the maximum (=lowest) POD (POD_max) value identified for each compound. In several cases, the IC_{10} values that formed the basis for the POD values were converted from other ICx values (such as IC_{50}) as reported in **Suppl. Table 1** for each compound with the corresponding references.

We compared the dataset on the observed POD values obtained from the literature for the 61 chemicals with the Integrated Chemical Environment (ICE) Curve Surfer tool dataset for hAR antagonism, given as Activity Concentration at Cut-off (ACC) values. We identified an overlap of 30 chemicals for which data were available in both datasets (**Suppl. Table 5**). ACC is defined as the concentration at which a concentration–response curve first reaches a user-defined cut-off, being this commonly set at 15 to 20 % of effect (Escher et al., 2021). Although the ACC values retrieved for these 30 compounds ranged from 1.3- to over 200-fold higher than our POD values, a strong positive correlation was found between ACC and both POD_mean ($r_S = 0.752$; $p < 0.001$) and POD_max ($r_S = 0.695$; $p < 0.001$), giving us confidence in the validity of our dataset.

Mixture effects of AR antagonists have previously been measured at IC_{10} and IC_{20} (Orton et al., 2014), and as we know that even a non-detectable activity of single compounds can contribute to the overall mixture effect as shown previously *in vitro* (Silva et al., 2002) and *in vivo* (Hass et al., 2007; van der Ven et al., 2022), we decided to use the IC_{10} as a reliable POD for AR antagonism.

3.2. Identification of compounds with available hazard and exposure data

Combining the results from the hazard data and HBM data search, resulted in a final list of 61 compounds for which reliable hazard and exposure data were available (Table 1). Of these 61 compounds, 15 had HBM data obtained in plasma/serum/blood/cord blood, 19 had levels in breast milk, and 27 had levels obtained in urine. **Suppl. Table 2** summarises the HBM mean and maximum levels for each substance in plasma/serum/whole blood/cord blood, breast milk and urine, respectively, together with the corresponding references.

Although our aim was to obtain approximate blood levels of all compounds for the mixture risk assessment, for 19 compounds, most reliable data were available in human milk. Hence, we performed a comprehensive literature search to identify available CFs between milk

Table 1
The identified chemicals with hazard data for androgen receptor antagonism, corresponding human exposure levels in blood as well as the calculated risk quotient for each chemical. The total Point of Departure Index is given at the bottom of the table.

Chemicals			Hazard data				Exposure data		Mixture risk assessment	
Compound	Abbreviation	CAS no.	*In vitro* AR antagonism data				Human biomonitoring data		RQs (mean exposure)	RQs (max exposure
			IC$_{50}$ mean (µM)	IC$_{10}$ mean (µM)	IC$_{50}$ max (µM)	IC$_{10}$ max (µM)	Mean (µM)	Max (µM)	HBM mean/ IC$_{10}$ max	HBM max /IC$_{10}$ max
Bisphenol A	BPA	80–05-7	2.7	3.02E-01	0.75	8.33E-02	1.51E-05	4.55E-05	1.81E-04	5.46E-04
Bisphenol F	BPF	620–92-8	4.9	5.48E-01	3.0	3.33E-01	1.20E-05	5.46E-05	3.59E-05	1.64E-04
Butylbenzylphthalate	BBP/BzBP	85–68-7	387.3	4.30E + 01	13	1.44E + 00	1.44E-01	2.69E-01	9.99E-02	1.87E-01
Dibutylphthalate	DBP/DnBP	84–74-2	175.3	1.95E + 01	1.1	1.22E-01	1.61E-03	4.22E-03	1.32E-02	3.45E-02
Dicyclohexylphthalate	DCHP	84–61-7	15.2	1.69E + 00		1.69E + 00	2.12E-03	2.12E-03	1.25E-03	1.25E-03
Diethylphthalate	DEP	84–66-2	436.5	4.85E + 01	358	3.98E + 01	9.50E-04	3.08E-03	2.39E-05	7.74E-05
Diethylhexylphthalate	DEHP	117–81-7	275	3.06E + 01	>100	1.11E + 01	1.32E-04	4.79E-04	1.19E-05	4.31E-05
Di-isobutylphthalate	DiBP	84–69-5	18.6	2.07E + 00	12.4	1.38E + 00	2.98E-04	6.67E-03	2.16E-03	4.84E-03
Dimethylphthalate	DMP	131–11-3	769	8.54E + 01		8.54E + 01	1.37E-04	1.58E-04	1.60E-06	1.85E-06
Mono-*n*-butylphthalate[a]	MBP	131–70-4	0.12							
2,2′,3,4,4′,5′-hexachlorobiphenyl	PCB 138	35065–28-2	3.99	4.43E-01	0.7	7.78E-02	5.00E-04	1.29E-03	6.43E-03	1.65E-02
2,2′,4,5′-tetrachlorobiphenyl	PCB 49	41464–40-8	10.64	1.18E + 00		1.18E + 00	2.61E-05	2.61E-05	2.21E-05	2.21E-05
2,3,3′,4,4′,5′-hexachlorobiphenyl	PCB 157	69782–90-7	3.61	4.01E-01		4.01E-01	7.49E-04	1.12E-03	1.87E-03	2.78E-03
2,3,3′,4,4′,5-hexachlorobiphenyl	PCB 156	38380–08-4	12.28	1.36E + 00		1.36E + 00	7.39E-05	1.97E-04	5.42E-05	1.45E-04
2,3,3′,4,4′-pentachlorobiphenyl	PCB 105	32598–14-4	1.28	1.42E-01		1.42E-01	7.59E-05	7.59E-05	5.34E-04	5.34E-04
2,3,4,4′,5-pentachlorobiphenyl	PCB 114	74472–37-0	9.72	1.08E + 00		1.08E + 00	4.69E-04	6.21E-04	4.34E-04	5.75E-04
2,3,4,4′,5,5′-hexachlorobiphenyl	PCB 167	52663–72-6	5.28	5.87E-01		5.87E-01	1.08E-03	1.56E-03	1.84E-03	2.66E-03
2,3′,4,4′,5-pentachlorobiphenyl	PCB 118	31508–00-6	2.88	3.20E-01	0.5	5.56E-02	4.80E-03	2.31E-02	8.64E-02	4.17E-01
2,3′,4,4′-tetrachlorobiphenyl	PCB 66	32598–10-0	1.15	1.28E-01		1.28E-01	3.18E-05	3.18E-05	2.48E-04	2.48E-04
2,4,4′,5-tetrachlorobiphenyl	PCB 74	32690–93-0	6.45	7.17E-01	2.1	2.33E-01	2.00E-04	3.35E-04	8.57E-04	1.44E-03
2,4,4′-trichlorobiphenyl	PCB 28	7012–37-5	4.97	5.52E-01	0.8	8.89E-02	2.56E-06	4.08E-06	2.88E-05	4.59E-05
2,4,6- trichlorobiphenyl[a]	PCB 30	35693–92-6	7.79							
2,4′-dichlorobiphenyl[a]	PCB 8	34883–43-7			0.9					
3,3′,4,4′,5-pentachlorobiphenyl	PCB 126	57465–28-8	1.51	1.67E-01	0.5	5.56E-02	3.51E-05	4.32E-05	6.32E-04	7.77E-04
Methylparaben	MeP	99–76-3	154	1.71E + 01	>100	1.11E + 01	5.37E-02	1.49E-01	4.84E-03	1.34E-02
Ethylparaben	EtP	120–47-8	106	1.18E + 01	>100	1.11E + 01	7.76E-04	1.73E-03	6.99E-05	1.56E-04
Propylparaben	*n*-PP	94–13-3	78	8.67E + 00	70	7.78E + 00	3.17E-03	9.14E-03	4.07E-04	1.17E-03
Butylparaben	*n*-BP	94–26-8	56	6.22E + 00	41.0	4.56E + 00	7.10E-04	4.02E-03	1.56E-04	8.81E-04
Triclosan		3380–34-5	3.5	3.90E-01	1.3	1.44E-01	8.88E-04	1.49E-03	6.15E-03	1.03E-02
Perfluorooctanesulphonate	PFOS	111873–33-7/ 1763–23-1	13.35	1.48E + 00	4.7	5.22E-01	2.62E-02	5.90E-02	5.02E-02	1.13E-01
1-Aminopyrene		1606–67-3	2.89	3.21E-01		3.21E-01	2.05E-04	2.35E-04	6.37E-04	7.33E-04
1-Hydroxypyrene		5315–79-7	3.51	3.90E-01	2.0	2.22E-01	8.39E-04	1.16E-03	3.78E-03	5.22E-03
Benzo[*a*]pyrene	BaP	50–32-8	4.74	5.27E-01	3.9	4.33E-01	1.36E-07	1.50E-07	3.15E-07	3.45E-07
Benzo[*j*]fluoranthene		205–82-3	2.0	2.24E-01		2.24E-01	9.68E-08	9.68E-08	4.32E-07	4.32E-07
Benzo[*k*]fluoranthene		207–08-9	0.62	6.89E-02		6.89E-02	4.71E-08	5.42E-08	6.83E-07	7.87E-07
Chrysene		218–01-9	9.97	1.11E + 00	9.63	1.07E + 00	2.71E-07	3.05E-07	2.53E-07	2.85E-07
Fluoranthene		206–44-0	1.97	2.19E-01	1.9	2.11E-01	2.31E-06	2.62E-06	1.09E-05	1.24E-05
Pyrene		129–00-0	13.58	1.51E + 00		1.51E + 00	9.06E-07	9.97E-07	6.01E-07	6.61E-07
2,4-Dimethoxy-1-nitro-benzene[a]		4920–84-7	10.18							
2-Hydroxy-4-methoxybenzophenone	BP3	131–57-7	11.28	1.25E + 00	2.0	2.22E-01	4.33E-03	7.24E-03	1.95E-02	3.26E-02
2,2́,4,4́-Tetrahydroxybenzophenone[a]	BP2	131–55-5	3.1		1.3					
Zearalenon		17924–92-4	9.19	1.02E + 00		1.02E + 00	1.23E-05	1.23E-05	1.20E-05	1.20E-05
p,p′-DDE		72–55-9	3.54	3.94E-01	0.3	3.33E-02	6.92E-05	1.04E-04	2.08E-03	3.11E-03
o,p-DDT		789–02-6	2.01	2.24E-01	1.45	1.61E-01	5.83E-08	8.46E-08	3.62E-07	5.25E-07
o,p-DDE		3424–82-6	2.9	3.26E-01	1.6	1.78E-01	1.10E-08	1.41E-08	6.19E-08	7.96E-08
p,p'-DDD		72–54-8	0.7	7.78E-02		7.78E-02	6.41E-08	7.50E-08	8.24E-07	9.64E-07

Table 1 (*continued*)

Chemicals			Hazard data				Exposure data		Mixture risk assessment	
Compound	Abbreviation	CAS no.	*In vitro* AR antagonism data				Human biomonitoring data		RQs (mean exposure)	RQs (max exposure
			IC$_{50}$ mean (µM)	IC$_{10}$ mean (µM)	IC$_{50}$ max (µM)	IC$_{10}$ max (µM)	Mean (µM)	Max (µM)	HBM mean/ IC$_{10}$ max	HBM max /IC$_{10}$ max
p,p-DDT		50–29-3	3.06	3.40E-01	1	1.11E-01	5.24E-05	5.24E-05	4.72E-04	4.72E-04
Tetrabromobisphenol A	TBBPA	79–94-7	10	1.11E + 00		1.11E + 00	7.65E-08	1.50E-07	6.88E-08	1.35E-07
2,2′,4,4′,5-Pentabromodiphenyl ether	BDE 99	60348–60-9	4.39	4.87E-01	0.97	1.08E-01	1.54E-06	1.54E-06	1.43E-05	1.42E-05
2,2′,4,4′-Tetrabromodiphenyl ether	BDE 47	5436–43-1	1.77	1.97E-01	0.52	5.78E-02	2.17E-05	2.56E-05	3.76E-04	4.43E-04
2,2′,4-Tribromodiphenyl ether	BDE 28	41318–75-6	3.1	3.44E-01		3.44E-01	3.04E-06	3.04E-06	8.83E-06	8.84E-06
2,2,4,4,6-Pentabromodiphenyl ether	BDE100	189084–64-8/32534–81-9	2.06	2.29E-01	0.1	1.11E-02	5.48E-07	5.48E-07	4.93E-05	4.93E-05
4 t-Octylphenol		27193–28-8	16.37	1.82E + 00	5.85	6.49E-01	4.82E-05	5.37E-05	7.42E-05	8.27E-05
Procymidone		32809–16-8	0.9	1.01E-01	0.6	6.67E-02	9.50E-07	9.50E-07	1.43E-05	1.43E-05
Prochloraz		67747–09-5	7.7	8.60E-01	3.1	3.43E-01	4.38E-05	1.03E-04	1.28E-04	2.99E-04
Pyrimethanil[a]		53112–28-0	126.00							
Fenhexamide[a]		126833–17-8	8.10							
Chlorpropham[a]		101–21-3	38.70		30.6					
Pirimiphos methyl[a]		29232–93-7	25.40		22					
Methylparathion		298–00-0	3.22	3.57E-01	2.17	2.41E-01	4.45E-04	1.35E-03	1.84E-03	5.58E-03
Methoxychlor		72–43-5	4.26	4.73E-01	4.01	4.46E-01	1.44E-06	1.44E-06	3.23E-06	3.23E-06
β-Endosulfan		33213–65-9	8.61	9.57E-01	6.20	6.89E-01	4.10E-03	4.91E-03	5.95E-03	7.14E-03
Chlordane		57–74-9	17.88	1.99E + 00		1.99E + 00	1.61E-08	1.61E-08	8.11E-09	8.11E-09
Cypermethrin		52315–07-8	28.36	3.15E + 00	3.22	3.57E-01	3.66E-03	3.66E-03	1.02E-02	1.02E-02
Dieldrin		60–57-1	4.50	5.00E-01	1.75	1.94E-01	5.73E-06	7.62E-06	2.95E-05	3.92E-05
Cyfluthrin		68359–37-5	24.44	2.72E + 00		2.72E + 00	4.63E-03	4.63E-03	1.70E-03	1.70E-03
λ-Cyhalothrin		91465–08-6	48.60	5.40E + 00	4.7	5.22E-01	1.72E-05	1.72E-05	3.29E-05	3.29E-05
Bitertanol[a]		55179–31-2	2.70							
Alachlor		15972–60-8	9.26	1.03E + 00		1.03E + 00	5.94E-07	6.79E-07	5.78E-07	6.60E-07
Fenitrothion[a]		122–14-5	0.205							
Parathion		56–38-2	0.20	2.22E-02		2.22E-02	1.62E-06	1.99E-06	7.27E-05	8.97E-05
2-Phenylphenol		90–43-7	13.70	1.52E + 00		1.52E + 00	5.82E-05	5.82E-05	3.82E-05	3.82E-05
Point of Departure Index =									**0.32**	**0.88**

[a] compounds for which no HBM data was found. AR – androgen receptor, RQs – risk quotients.

and blood and subsequently estimated the blood level of these compounds. A summary of the CFs for each compound with corresponding estimated blood levels in breast milk is given in **Suppl. Table 3**, together with the obtained mean and maximum blood levels of each compound.

3.3. Mixture risk assessment of antiandrogenic compounds

Using the required hazard and HBM data, we calculated the RQs for each compound based on both the mean and the maximum exposure levels, respectively. The POD values for hAR antagonism were selected to reflect the lowest observed effect levels, as even low, non-detectable levels of chemicals have been shown to contribute to the final mixture effect (Silva et al., 2002; Hass et al. 2007; van der Ven et al., 2022). Table 1 summarises the calculated RQs together with the corresponding hazard and HBM levels. The cumulative mixture risk – called the PODI – was calculated as the sum of the RQs of all compounds based on either mean exposure levels (Fig. 3a) or maximum exposure levels (Fig. 3b). No compound had an individual RQ above 1. The PODI for the mixture of all 61 AR antagonistic compounds was calculated being 0.88 based on maximum exposure levels. The 15 compounds that mainly contributed to the RQ and therefore can be considered the mixture risk drivers were the PCBs (PCB118, 138, 157), the phthalates (butylbenzylphthalate (BBP), dibutylphthalate (DBP), di-isobutylphthalate), the UV filter benzophenone-3, perfluorooctanesulphonate (PFOS), methylparaben, triclosan, some pesticides (i.e. cypermethrin, β-endosulfan, methylparathion, and p,p-dichlorodiphenyldichloroethylen (DDE)), and a PAH metabolite (1-hydroxypyrene) (Fig. 3b). Notably, in this study-five compounds: PCB118, BBP, PFOS, DBP and benzophenone-3 were identified as the major mixture risk drivers, constituting approximately 75 % of the total risk. The remaining 56 compounds contributed with a minor factor to the PODI. When the RQs were calculated from the mean HBM levels, corresponding to a less stringent exposure scenario, the PODI was found to be 0.32 (Fig. 3a). The top 15 compounds were the same as when the mixture risk calculation was based on the maximum exposure levels.

When using the Hazard Index for mixture risk assessment, uncertainty factors are usually included in the hazard metrics (*e.g.,* in Acceptable Daily Intakes = ADI) to account for interspecies differences (different kinetics in rats versus humans) and inter-individual differences (different sensitivities among humans). With our approach there is no need for an uncertainty factor to account for species differences, but an uncertainty factor of 10 could have been included to account for sensitivity differences in humans and in that case the PODIs would increase by a factor of 10. However, as we have also calculated the PODI based on maximum human exposure levels and the lowest reported POD, we consider this a worst-case scenario and find no need to include a generic, default uncertainty factor as well.

Another way of illustrating the chemicals that drive or do not drive a given mixture effect or human risk is shown in Fig. 4. All chemicals in the upper left corner belong to the most potent antiandrogenic compounds, to which humans are relatively more exposed and may be considered chemicals of concern. The top 5 chemicals (PCB118, BBP, PFOS, DBP and benzophenone-3) are placed horizontally in the top of the upper, left quadrant, indicating that it is the exposure levels rather than the hazard levels that drive the risk to these chemicals. This is valid when considering both mean (Fig. 4a) and maximum human exposure levels (Fig. 4b). Totally, thirteen out of the top 15 chemicals are located in the upper left quadrant (Fig. 4b), but two chemicals were located outside the quadrant, methylparaben for which exposure levels are estimated to be high and p,p-DDE that is among the most potent chemicals for AR antagonism. In contrast, chemicals in the lower right corner are the least potent compounds to which human are not markedly exposed and are therefore chemicals of less concern for risk assessors and risk managers.

4. Discussion

All mixture risk assessments have their assumptions, strengths and limitations and for transparency these are highlighted for our case study. Our first assumption was that male reproductive health disorders arise during foetal life and the dominating mechanism of action for these adverse health effects is AR antagonism. Antagonism of the hAR is the best known and documented molecular initiating event for adverse male reproductive effects and provides a clear link to the *in vivo* male reproductive disorders such as cryptorchidism, hypospadias, poor semen quality or testicular cancer (Schwartz et al., 2019). We have previously demonstrated how *in vitro* data on hAR antagonism together with physiologically based kinetic (PBK) modelling can predict *in vivo* reductions in anogenital distance, which is known as a sensitive biomarker for male reproductive disorders (Scholze et al., 2020). This clear link was the rationale for selecting data on this mechanism of action as the hazard data for mixture risk assessment.

We also assumed that a maternal blood chemical level corresponding to a POD for AR antagonism will lead to a minor AR blocking in the male foetus and contribute to the total AR blocking of the mixture, which ultimately may translate into a shortened anogenital distance in the new-born. We know from previous studies that AR antagonists in a mixture will act additively and give rise to a mixture effect also at low chemical levels (Ermler et al., 2011; Hass et al., 2007). Thus, even very low non-detectable levels of AR antagonists will add up to contribute to the mixture effect, a phenomenon popularly termed 'something from nothing'. This has been documented *in vitro* in an AR reporter gene assay (Ermler et al., 2011) and *in vivo* in male rat pups that were exposed to antiandrogenic chemicals during foetal life and for which mixture effects in the form of reduced anogenital distances were documented (Hass et al., 2007). Moreover, we have found that we can predict shortening of anogenital distance by combining *in vitro* data for hAR antagonism with PBK modelling (Scholze et al., 2020). For six antiandrogenic pesticides we showed that by using hAR antagonism data in a 'reverse dosimetry' mode in our PBK models, we could predict oral doses that would cause an adverse effect on anogenital distance in male rat pups. Predicting effects in rats allowed us to validate the approach and results showed that this alternative approach worked well. Based on these studies, it is a realistic assumption that AR antagonistic chemicals will contribute to a combined effect on endpoints for male reproductive health disorders.

Our final assumption was that compounds with AR antagonistic effects belong to the same assessment group, which is indeed the case and therefore can be considered a default assumption.

4.1. Assumptions, strengths and limitations of our approach for hazard evaluation

The advantages of using *in vitro* hazard data for mixture risk assessment are that (1) human *in vitro* data can be used circumventing the uncertainties in using hazard data from rodents, (2) more compounds can be included due to larger availability of *in vitro* compared to *in vivo* hazard data and (3) no grouping considerations are needed as the *in vitro* active compounds by default belong to the same cumulative assessment group.

A disadvantage, however, is that only compounds acting by this specific mechanism of action are included. We know that compounds with other known or unknown modes of action can also induce male reproductive health disorders (*e.g.,* 2,3,7,8-TCDD (EFSA, 2018) and paracetamol (Bauer et al., 2021), and the exclusion of these may probably lead to an underestimation of risk.

Moreover, there are uncertainties in the use of *in vitro* potencies for hazard evaluation that applies to the general use of *in vitro* data. Uncertainties exist in translating *in vitro* potencies to *in vivo* outcomes in the human foetus. This last point is particularly critical, as we do not know the magnitude of the freely-dissolved concentration in cytoplasm of the

chemicals in the hAR assays and therefore do not know the 'true' potencies of the compounds. We are using nominal concentrations of the chemicals added to the assay, which may not always estimate *in vivo* effective concentrations, as the nominal concentration poorly reflects the concentration at the molecular target in cells *in vitro* (*e.g.*, Gilbert et al., 2015; Mundy, 2004; Proença et al., 2021). Chemicals can differentially distribute between *in vitro* assay compartments, including serum constituents in exposure medium, microtiter plastic plates, headspace, and extracellular matrices. The partitioning of test chemicals to these extracellular compartments either enhances or reduces the concentration at the molecular target (Gilbert et al., 2015; Mundy, 2004; Proença et al., 2021). However, the freely-dissolved concentration in cytoplasm is responsible for initiating effects and can be referred to as the biologically effective concentration, and ideally we would like to know this value to relate it to the actual exposure levels at the target. Such estimations have previously been derived for specific compounds (*e.g.*, Gilbert et al., 2015; Mundy, 2004; Proença et al., 2021), but are at present not available for a large number of compounds and may not be so in the near future. Thus, hazard levels of the compounds may be over- or underestimated when using the nominal POD values for *in vitro* hAR antagonism, as the likelihood is high that the 'true' POD values based on intracellular concentrations in the assay will be either higher or lower. Moreover, we would ideally like to know the concentration of each single chemical in the mixture at the target in the testis of the foetus at human relevant exposure levels. However, such measured data do not exist. But it is possible to develop PBK models including a foetal compartment that predicts foetal levels based on knowledge on human biomonitoring levels (in *e.g.*, blood or urine) with a reasonable accuracy. Previously, we have developed a generic PBK model that could predict foetal levels of pesticides in rats within a factor of 5 (Scholze et al., 2020), and such models are available in the US for a large number of compounds, but more development is needed and should be a focus point for future research within this field.

As a short term solution, a measure of the free concentration of the chemical in the cell media that takes protein binding, loss of compound due to non-specific binding, evaporation, etc., into account may be sufficient, relating this level to the human serum concentration as a second best option. Thus, we suggest to routinely measure the free concentration of chemicals in the media of *in vitro* assays in the future in order to obtain more useful data.

4.2. Assumptions, strengths and limitations of our approach for exposure evaluation

Concerning the exposure data, the advantages of using HBM data for mixture risk assessment are that: (1) exposure levels are based on measured biomonitoring data reflecting real-life exposure in humans, and (2) data are aggregated and cover all exposure sources and routes. The disadvantages are that: (1) aggregated HBM values from general populations are used, which do not represent individual exposure levels, (2) for many compounds urinary or breast milk levels are measured, thereby causing the need of identifying or estimating toxicokinetic parameters for each substance to convert the urine or milk levels to blood levels. This conversion of such HBM data is pragmatic and encumbered with uncertainty. For the 19 compounds that were measured in human breast milk, the conversion can be made with relatively high certainty as reliable CFs were available. For the 27 compounds with reported urine concentrations, uncertainties were introduced in the estimations as several parameters were predicted values obtained from the CompTox Chemicals Dashboard and a relatively simple first order kinetic model was used.

We used internal exposure levels of pregnant women as a proxy of exposure levels of the male fetus. This will give rise to uncertainty as some chemicals will appear with lower and others with higher concentration in the fetus compared to the mother. In this paragraph we will discuss this issue for the major chemical mixture drivers identified. For

per- and polyfluoroalkyl substances (PFAS), a direct comparison of maternal blood levels and levels in human fetal organs have been measured (Mamsen et al., 2017). For all 5 PFAS, the levels were lower in the fetal organs compared to the blood of the mother (7–26 % of the mother's levels), with a PFOS level that was 7 % of maternal levels. However, when comparing PFOS levels in cord blood with maternal blood, the cord blood levels were found to be 30 % of maternal levels (Manzano-Salgado et al., 2015).

In a study on organochlorine (OC) levels in human maternal and cord blood (Junqué et al., 2020), it was found that OC levels including PCBs were correlated between the mother and the cord blood and that the cord blood levels were approximately 30–50 % of maternal levels at delivery, although exceptions were found. For one of the other mixture drivers, benzophenone-3, fetal and cord blood levels were found to be approximately 10 % of those in maternal serum (Krause et al., 2018). In a study on endocrine disruptors, concentrations of the Mono(2-ethylhexyl) phthalate, octylphenol, and 4-nonylphenol in cord blood samples were reported to be approximately 80 % of levels in maternal blood. In contrast, polybrominated diphenyl ethers (PBDEs) levels in cord blood samples were significantly higher than those in maternal blood (Li et al., 2013).

In conclusion, the general picture is that levels of PCBs, PFAS, and phenols are lower in the fetus compared to the mother, whereas PBDE levels are higher. Overall, the use of exposure levels in the mother will in most cases lead to an overestimation of mixture risk.

However, as newborns and infants are also susceptible to anti-androgenic chemicals, a comparison of exposure levels in infants and their mothers is relevant as well. Recently, studies of exposure in infants and their parents showed that for phenols, infant levels followed the pattern of the parents, and for some compounds, including benzophenone-3, triclosan and bisphenol A, levels were higher in infants than in their parents (Frederiksen et al., 2022a). The same picture was observed for a number of phthalates (Frederiksen et al., 2022b). Also, PFOS is known to exhibit serum exposure levels among breast-fed newborns which are considerable higher than that of their mothers (Schrenk et al., 2020).

Thus, the newborn child will be exposed to several chemicals to a similar or even higher extent compared to the mother, meaning that for this population segment, the used exposure levels may not be overestimated.

4.3. Over- or underestimation of risk?

Our hypothesis was that an RQ for each chemical can be estimated based on an *in vitro* response (in μM) and a blood HBM level (in μM) for each compound. This hypothesis incorporated several assumptions as mentioned above. In general, there are large uncertainties in all risk assessments of chemical mixtures, and the question is whether the uncertainties will lead to an over- or an underestimation of risk. A table including the acknowledged uncertainties and their potential impact on risk assessment is shown as **Suppl. Table 6**. For many factors, it is not known in which direction they will affect the assessment. However, one factor known to lead to an overestimation of the PODI for most of the population is the use of maximum HBM levels, as these do not reflect the exposure of the general population under usual circumstances. The use of mean HBM data from studies covering several populations, geographical areas and all seasons will also not reflect individual exposures and may lead to an over- or underestimation.

Moreover, the risk will be underestimated due to lack of either hazard or human exposure data for many compounds. Initially, we identified > 200 hAR antagonistic compounds, but most were excluded for this reason. While the availability of systematic and harmonized HBM data is increasing at a fast rate, in this case study we were able to retrieve human exposure data for only 77 (33 %) out of the 231 anti-androgenic chemicals identified. This means that the risk is probably underestimated based on a lower availability of human exposure

information compared to *in vitro* data.

As outlined above, several uncertainties relate to the lack of translating nominal effect concentrations *in vitro* to blood levels, which would be expected to be specific for each chemical. An even greater challenge would be to translate the *in vitro* potencies to intratesticular levels in the male fetus. For risk assessment, however, we need human exposure levels in the nominator of the RQ, which will be blood levels since data on intratesticular levels in the male fetus is not available for the general human population. Thus, a risk assessment for the general population based on intratesticular levels is not feasible and the risk assessment should be based on blood levels as the best proxy. It is a challenge based on available data to really understand the ultimate balance of over- versus underestimation of risk. Thus, results need to be interpreted with caution regarding health risk.

4.4. Challenges in mixture risk assessment

According to our methodological approach no single substance appeared with a RQ exceeding 1 and numerous compounds contributed each with a small RQ to the total PODI. However, it was evident that PCB118, butylbenzylphthalate, PFOS, DBP and benzophenone-3 were the major drivers of the mixture risk for antiandrogenic effects and that the high exposure level to these compounds seemed to drive the mixture effect (Fig. 4).

PCBs as well as some reproductive toxic phthalates (including butylbenzylphthalate), and PFOS have undergone regulatory restrictions, which should result in a reduction of human exposure to these compounds in the future. However, it is a surprise that the UV filter benzophenone-3 contributes relatively much to the total risk, although this is fully in line with the conclusions from a recent systematic, integrative review on this compound stating that human exposure levels achieved after a single whole-body sunscreen application are close to concentrations that have been reported to have endocrine disrupting effects (Mustieles et al., 2023).

The question is how to gain information on human mixture toxicity to understand (1) the magnitude of the problem/risk, (2) what the contributing drivers are, and (3) how to cope with the issue from a preventive and regulatory perspective. Two approaches are commonly used, namely the whole-mixture and component-based approaches. Although the whole-mixture approach provides information on contributions from unidentified compounds in the mixture and account for possible interactions between its constituents, this approach is challenged by the endless mixture combinations and variations in mixture composition over time (Boobis et al., 2011). The component-based approaches, such as concentration addition, rely on toxicological information for single compounds, however this information is lacking for many chemicals, especially when it comes to *in vivo* data. Furthermore, concentration addition modelling at present relies on the assumption that no synergistic interactions between mixture component occur (Boobis et al., 2011). Consequently, both approaches have inherent challenges, thereby pushing alternative approaches to examine human mixture risk.

Another challenge for mixture risk assessment is predicting the chemical composition of the mixture in part due to the lack of exposure information (Egeghy et al., 2012). Exposure assessment is commonly based on a single source and/or pathway (Boberg et al., 2021), such as food intake, however there is an inherent risk of underestimating the risk associated with an exposure when based solely on one source. For instance, bisphenol A exposure originates from multiple sources including food intake, dermal contact with thermal paper, and wrongful use of recycled paper, and thus basing the risk assessment solely on one source or exposure pathway will underestimate the effect. To this end, human biomonitoring data could be used to address challenges in aggregated exposure estimation, still not routinely used in risk assessment (Louro et al., 2019). Using internal chemical levels has the advantage that it gives aggregated exposure levels irrespective of

sources and pathways of exposure and is less uncertain than estimated external exposures levels for risk assessment (Louro et al., 2019).

Alternative approaches are needed to assess whether the aggregated mixture exposure in humans is problematic. In HBM4EU, approaches have been examined to address these issues, including experimental approaches, where the bioactivity of extracts/fractions of human samples are examined with the goal of gaining information on the bioactivity of the total mixture (Rodríguez-Carrillo et al., 2021; Vinggaard et al., 2021). Moreover, mixture risk assessments based on European HBM data and Health-Based Guidance Values were recently reported (Socianu et al., 2022).

4.5. Conclusion

The aim of the study was to explore a novel methodology for assessing mixture effects of chemicals. As a case, we investigated combined exposure to antiandrogenic chemicals with direct action on the androgen receptor *in vitro* that may affect human health. Chemicals with this mode of action are known to be involved in a range of reproductive disorders observed in boys and men when exposed during foetal life. Our approach has pinpointed compounds that contribute markedly to the PODI, such as PCBs, phthalates, PFOS, and benzophenone-3. Several uncertainties of the methodology were identified. Although it is not ready to be used for decision making, this approach may be applied to the identification of chemical mixture drivers, thereby providing a basis for a better understanding of the extent to which the combined chemical load will potentially exhibit adverse antiandrogenic effects in humans.

CRediT authorship contribution statement

Yanying Ma: Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Camilla Taxvig:** Data curation, Formal analysis, Writing – review & editing. **Andrea Rodrí- guez-Carrillo:** Data curation, Formal analysis, Visualization, Writing – review & editing. **Vicente Mustieles:** Data curation, Formal analysis, Visualization, Writing – review & editing. **Lena Reiber:** Data curation, Formal analysis, Writing – review & editing. **Anja Kiesow:** Data curation, Formal analysis, Writing – review & editing. **Nathalie Michelle Löbl:** Data curation, Formal analysis, Writing – review & editing. **Mariana F. Fernández:** Conceptualization, Funding acquisition, Methodology, Supervision, Project administration, Writing – review & editing. **Tina Vicky Alstrup Hansen:** Data curation, Formal analysis, Writing – review & editing. **Maria João Valente:** Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Marike Kolossa-Gehring:** Project administration, Funding acquisition, Methodology, Supervision, Writing – review & editing. **Madlen David:** Data curation, Formal analysis, Writing – review & editing. **Anne Marie Vinggaard:** Conceptualization, Funding acquisition, Methodology, Project administration, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2023.107815.

References

Amato-Lourenço, L.F., dos Santos Galvão, L., de Weger, L.A., Hiemstra, P.S., Vijver, M.G., Mauad, T., 2020. An emerging class of air pollutants: Potential effects of microplastics to respiratory human health? Sci. Total Environ. 749, 141676 https://doi.org/10.1016/J.SCITOTENV.2020.141676.

Aylward, L.L., Hays, S.M., LaKind, J.S., Ryan, J.J., 2003. Rapid communication: Partitioning of persistent lipophilic compounds, including dioxins, between human milk lipid and blood lipid: An initial assessment. J. Toxicol. Environ. Health A 66, 1–5. https://doi.org/10.1080/15287390306460.

Aylward, L.L., Irwin, K., St-Amand, A., Nong, A., Hays, S.M., 2018. Screening-level Biomonitoring Equivalents for tiered interpretation of urinary 3-phenoxybenzoic acid (3-PBA) in a risk assessment context. Regul. Toxicol. Pharm. 92, 29–38. https://doi.org/10.1016/j.yrtph.2017.11.002.

Barr, D.B., Wilder, L.C., Caudill, S.P., Gonzalez, A.J., Needham, L.L., Pirkle, J.L., 2005. Urinary creatinine concentrations in the U.S. population: Implications for urinary biologic monitoring measurements. Environ Health Perspect 113, 192–200. https://doi.org/10.1289/EHP.7337.

Bauer, A.Z., Swan, S.H., Kriebel, D., Liew, Z., Taylor, H.S., Bornehag, C.G., Andrade, A. M., Olsen, J., Jensen, R.H., Mitchell, R.T., Skakkebaek, N.E., Jégou, B., Kristensen, D. M., 2021. Paracetamol use during pregnancy — a call for precautionary action. Nat. Rev. Endocrinol. https://doi.org/10.1038/s41574-021-00553-7.

Birkhøj, M., Nellemann, C., Jarfelt, K., Jacobsen, H., Andersen, H.R., Dalgaard, M., Vinggaard, A.M., 2004. The combined antiandrogenic effects of five commonly used pesticides. Toxicol. Appl. Pharmacol. 201, 10–20. https://doi.org/10.1016/J.TAAP.2004.04.016.

Boberg, J., Bredsdorff, L., Petersen, A., Löbl, N., Jensen, B.H., Vinggaard, A.M., Nielsen, E., 2021. Chemical Mixture Calculator - A novel tool for mixture risk assessment. Food Chem. Toxicol. 152 https://doi.org/10.1016/j.fct.2021.112167.

Bolon, B., Haschek, W.M., 2020. The exposome in toxicologic pathology. Toxicol. Pathol. 48, 718–720. https://doi.org/10.1177/0192623320912403.

Boobis, A., Budinsky, R., Collie, S., Crofton, K., Embry, M., Felter, S., Hertzberg, R., Kopp, D., Mihlan, G., Mumtaz, M., Price, P., Solomon, K., Teuschler, L., Yang, R., Zaleski, R., 2011. Critical analysis of literature on low-dose synergy for use in screening chemical mixtures for risk assessment. Crit. Rev. Toxicol. 41, 369–383. https://doi.org/10.3109/10408444.2010.543655/SUPPL_FILE/ITXC_A_543655_SM0002.XLS.

Cariou, R., Antignac, J.P., Zalko, D., Berrebi, A., Cravedi, J.P., Maume, D., Marchand, P., Monteau, F., Riu, A., Andre, F., bizec, B. Le, 2008. Exposure assessment of French women and their newborns to tetrabromobisphenol-A: Occurrence measurements in maternal adipose tissue, serum, breast milk and cord serum. Chemosphere 73, 1036–1041. 10.1016/J.CHEMOSPHERE.2008.07.084.

CDC, 2009. Fourth National Report on Human Exposure to Environmental Chemicals.

Chapin, R.E., Harris, M.W., Davis, B.J., Ward, S.M., Wilson, R.E., Mauney, M.A., Lockhart, A.C., Smialowicz, R.J., Moser, V.C., Burka, L.T., Collins, B.J., 1997. The Effects of Perinatal/Juvenile Methoxychlor Exposure on Adult Rat Nervous, Immune, and Reproductive System Function. Fundam. Appl. Toxicol. 40, 138–157. https://doi.org/10.1006/FAAT.1997.2381.

Christiansen, S., Scholze, M., Dalgaard, M., Vinggaard, A.M., Axelstad, M., Kortenkamp, A., Hass, U., 2009. Synergistic disruption of external male sex organ development by a mixture of four antiandrogens. Environ. Health Perspect. 117, 1839–1846. https://doi.org/10.1289/EHP.0900689.

DeKoning, E.P., Karmaus, W., 2000. PCB exposure in utero and via breast milk. A review. Journal of Exposure Science & Environmental Epidemiology 2000 10:3 10, 285–293. 10.1038/sj.jea.7500090.

Danish Environmental Protection Agency, 2017. Exposure of children and unborn children to selected chemical substances. Danish Environ. Protect. Agency.

Domingo, J.L., Nadal, M., 2019. Human exposure to per- and polyfluoroalkyl substances (PFAS) through drinking water: A review of the recent scientific literature. Environ. Res. 177, 108648 https://doi.org/10.1016/J.ENVRES.2019.108648.

EFSA, 2018. Risk for animal and human health related to the presence of dioxins and dioxin-like PCBs in feed and food. EFSA J. https://doi.org/10.2903/j.efsa.2018.5333.

Egeghy, P.P., Judson, R., Gangwal, S., Mosher, S., Smith, D., Vail, J., Cohen Hubal, E.A., 2012. The exposure data landscape for manufactured chemicals. Sci. Total Environ. 414, 159–166. https://doi.org/10.1016/J.SCITOTENV.2011.10.046.

Engel, A., Buhrke, T., Imber, F., Jessel, S., Seidel, A., Völkel, W., Lampen, A., 2017. Agonistic and antagonistic effects of phthalates and their urinary metabolites on the steroid hormone receptors ERα, ERβ, and AR. Toxicol. Lett. 277, 54–63. https://doi.org/10.1016/J.TOXLET.2017.05.028.

Ermler, S., Scholze, M., Kortenkamp, A., 2011. The suitability of concentration addition for predicting the effects of multi-component mixtures of up to 17 anti-androgens with varied structural features in an in vitro AR antagonist assay. Toxicol Appl Pharmacol 257, 189–197. https://doi.org/10.1016/j.taap.2011.09.005.

Escher, B.I., Neale, P., Leusch, F., 2021. Dose–response assessment, in: Bioanalytical Tools in Water Quality Assessment. IWA Publishing, pp. 101–118. 10.2166/9781789061987_0101.

Frederiksen, H., Ljubicic, M.L., Upners, E.N., Fischer, M.B., Busch, A.S., Hagen, C.P., Juul, A., Andersson, A.-M., 2022a. Benzophenones, bisphenols and other polychlorinated/phenolic substances in Danish infants and their parents – including longitudinal assessments before and after introduction to mixed diet. Environ Int 169, 107532. https://doi.org/10.1016/j.envint.2022.107532.

Frederiksen, H., Upners, E.N., Ljubicic, M.L., Fischer, M.B., Busch, A.S., Hagen, C.P., Juul, A., Andersson, A.-M., 2022b. Exposure to 15 phthalates and two substitutes (DEHTP and DINCH) assessed in trios of infants and their parents as well as longitudinally in infants exclusively breastfed and after the introduction of a mixed diet. Environ Int 161, 107107. https://doi.org/10.1016/j.envint.2022.107107.

Fromme, H., Schlummer, M., Möller, A., Gruber, L., Wolz, G., Ungewiss, J., Böhmer, S., Dekant, W., Mayer, R., Liebl, B., Twardella, D., 2007. Exposure of an adult population to perfluorinated substances using duplicate diet portions and biomonitoring data. Environ Sci Technol 41, 7928–7933. https://doi.org/10.1021/ES071244N/SUPPL_FILE/ES071244N-FILE002.PDF.

Gennings, C., Hauser, R., Koch, H.M., Kortenkamp, A., Lioy, P.J., Mirkes, P.E., Schwetz, B. a., 2014. Report to the U.S. Consumer Product Safety Commission by the CHRONIC HAZARD ADVISORY PANEL ON PHTHALATES AND PHTHALATE ALTERNATIVES.

Gilbert, D., Mayer, P., Pedersen, M., Vinggaard, A.M., 2015. Endocrine activity of persistent organic pollutants accumulated in human silicone implants - Dosing in vitro assays by partitioning from silicone. Environ Int 84, 107–114. https://doi.org/10.1016/j.envint.2015.07.008.

Hass, U., Scholze, M., Christiansen, S., Dalgaard, M., Vinggaard, A.M., Axelstad, M., Metzdorff, S.B., Kortenkamp, A., 2007. Combined Exposure to Anti-Androgens Exacerbates Disruption of Sexual Differentiation in the Rat. Environ Health Perspect 115, 122–128. https://doi.org/10.1289/ehp.9360.

Hays, S.M., Aylward, L.L., Gagné, M., Krishnan, K., 2009. Derivation of Biomonitoring Equivalents for cyfluthrin. Regulatory Toxicology and Pharmacology 55, 268–275. https://doi.org/10.1016/J.YRTPH.2009.09.002.

Isaacs, K.K., Glen, W.G., Egeghy, P., Goldsmith, M.-R., Smith, L., Vallero, D., Brooks, R., Grulke, C.M., Özkaynak, H., 2014. SHEDS-HT: An Integrated Probabilistic Exposure Model for Prioritizing Exposures to Chemicals with Near-Field and Dietary Sources. Environ Sci Technol 48, 12750–12759. https://doi.org/10.1021/es502513w.

Junqué, E., Garcia, S., Martínez, M.Á., Rovira, J., Schuhmacher, M., Grimalt, J.O., 2020. Changes of organochlorine compound concentrations in maternal serum during pregnancy and comparison to serum cord blood composition. Environ Res 182, 108994. https://doi.org/10.1016/j.envres.2019.108994.

Kampa, M., Castanas, E., 2008. Human health effects of air pollution. Environ. Pollut. 151, 362–367. https://doi.org/10.1016/J.ENVPOL.2007.06.012.

Kjærstad, M.B., Taxvig, C., Andersen, H.R., Nellemann, C., 2010. Mixture effects of endocrine disrupting compounds in vitro. Int J Androl 33, 425–433. https://doi.org/10.1111/J.1365-2605.2009.01034.X.

Koch, H.M., Becker, K., Wittassek, M., Seiwert, M., Angerer, J., Kolossa-Gehring, M., 2007. Di-n-butylphthalate and butylbenzylphthalate - Urinary metabolite levels and estimated daily intakes: Pilot study for the German Environmental Survey on children. J Expo Sci Environ Epidemiol 17, 378–387. https://doi.org/10.1038/sj.jes.7500526.

Kojima, H., Katsura, E., Takeuchi, S., Niiyama, K., Kobayashi, K., 2004. Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells. Environ Health Perspect 112, 524–531. https://doi.org/10.1289/EHP.6649.

Kojima, H., Takeuchi, S., Uramaru, N., Sugihara, K., Yoshida, T., Kitamura, S., 2009. Nuclear hormone receptor activity of polybrominated diphenyl ethers and their hydroxylated and methoxylated metabolites in transactivation assays using Chinese hamster ovary cells. Environ Health Perspect 117, 1210–1218. https://doi.org/10.1289/EHP.0900753.

Kojima, H., Takeuchi, S., Sanoh, S., Okuda, K., Kitamura, S., Uramaru, N., Sugihara, K., Yoshinari, K., 2019. Profiling of bisphenol A and eight of its analogues on transcriptional activity via human nuclear receptors. Toxicology 413, 48–55. https://doi.org/10.1016/J.TOX.2018.12.001.

Koo, J.W., Parham, F., Kohn, M.C., Masten, S.A., Brock, J.W., Needham, L.L., Portier, C. J., 2002. The association between biomarker-based exposure estimates for phthalates and demographic factors in a human reference population. Environ Health Perspect 110, 405–410. https://doi.org/10.1289/EHP.02110405.

Kortenkamp, A., Faust, M., 2010. Combined exposures to anti-androgenic chemicals: steps towards cumulative risk assessment 33.

Krause, M., Frederiksen, H., Sundberg, K., Jørgensen, F.S., Jensen, L.N., Nørgaard, P., Jørgensen, C., Ertberg, P., Juul, A., Drzewiecki, K.T., Skakkebaek, N.E., Andersson, A.M., 2018. Presence of benzophenones commonly used as UV filters and absorbers in paired maternal and fetal samples. Environ Int 110, 51–60. https://doi.org/10.1016/j.envint.2017.10.005.

LaKind, J.S., Berlin, C.M., Sjödin, A., Turner, W., Wang, R.Y., Needham, L.L., Paul, I.M., Stokes, J.L., Naiman, D.Q., Patterson, D.G., 2009. Do human milk concentrations of persistent environmental chemicals really decline during lactation? Chemical concentrations during lactation and milk/serum partitioning. Environ Health Perspect 117, 1625–1631. https://doi.org/10.1289/EHP.0900876.

Li, L.-X., Chen, L., Meng, X.-Z., Chen, B.-H., Chen, S.-Q., Zhao, L.-F., Liang, Y., Zhang, Y.-H., 2013. Exposure Levels of Environmental Endocrine Disruptors in Mother-Newborn Pairs in China and Their Placental Transfer Characteristics. PLoS One 8, e62526.

Lioy, P.J., Hauser, R., Gennings, C., Koch, H.M., Mirkes, P.E., Schwetz, B.A., Kortenkamp, A., 2015. Assessment of phthalates/phthalate alternatives in children's toys and

childcare articles: Review of the report including conclusions and recommendation of the Chronic Hazard Advisory Panel of the Consumer Product Safety Commission. Journal of Exposure Science & Environmental Epidemiology 2015 25:4 25, 343–353. 10.1038/jes.2015.33.

Louro, H., Heinälä, M., Bessems, J., Buekers, J., Vermeire, T., Woutersen, M., van Engelen, J., Borges, T., Rousselle, C., Ougier, E., Alvito, P., Martins, C., Assunção, R., Silva, M.J., Pronk, A., Schaddelee-Scholten, B., del Carmen Gonzalez, M., de Alba, M., Castaño, A., Viegas, S., Humar-Juric, T., Kononenko, L., Lampen, A., Vinggaard, A.M., Schoeters, G., Kolossa-Gehring, M., Santonen, T., 2019. Human biomonitoring in health risk assessment in Europe: Current practices and recommendations for the future. Int J Hyg Environ Health. https://doi.org/10.1016/j.ijheh.2019.05.009.

Mamsen, L.S., Jönsson, B.A.G., Lindh, C.H., Olesen, R.H., Larsen, A., Ernst, E., Kelsey, T. W., Andersen, C.Y., 2017. Concentration of perfluorinated compounds and cotinine in human foetal organs, placenta, and maternal plasma. Sci. Total Environ. 596–597, 97–105. https://doi.org/10.1016/j.scitotenv.2017.04.058.

Mannetje, A. t., Coakley, J., Mueller, J.F., Harden, F., Toms, L.M., Douwes, J., 2012. Partitioning of persistent organic pollutants (POPs) between human serum and breast milk: A literature review. Chemosphere 89, 911–918. 10.1016/J. CHEMOSPHERE.2012.06.049.

Manzano-Salgado, C.B., Casas, M., Lopez-Espinosa, M.-J., Ballester, F., Basterrechea, M., Grimalt, J.O., Jiménez, A.-M., Kraus, T., Schettgen, T., Sunyer, J., Vrijheid, M., 2015. Transfer of perfluoroalkyl substances from mother to fetus in a Spanish birth cohort. Environ Res 142, 471–478. https://doi.org/10.1016/j.envres.2015.07.020.

Marchitti, S.A., Fenton, S.E., Mendola, P., Kenneke, J.F., Hines, E.P., 2017. Polybrominated diphenyl ethers in human milk and serum from the U.S. EPA MAMA study: Modeled predictions of infant exposure and considerations for risk assessment. Environ Health Perspect 125, 706–713. https://doi.org/10.1289/EHP332.

Mes, J., Doyle, J.A.A., Adams, B.R.R., Davies, D.J.J., Turton, D., 1984. Polychlorinated biphenyls and organochlorine pesticides in milk and blood of Canadian women during lactation 13, 217–223. https://doi.org/10.1007/BF01055879.

Metzdorff, S.B., Dalgaard, M., Christiansen, S., Axelstad, M., Hass, U., Kiersgaard, M.K., Scholze, M., Kortenkamp, A., Vinggaard, A.M., 2007. Dysgenesis and Histological Changes of Genitals and Perturbations of Gene Expression in Male Rats after In Utero Exposure to Antiandrogen Mixtures. Toxicol. Sci. 98, 87–98. https://doi.org/10.1093/TOXSCI/KFM079.

Mundy, W.R., 2004. Accumulation of PBDE-47 in Primary Cultures of Rat Neocortical Cells. Toxicol. Sci. 82, 164–169. https://doi.org/10.1093/toxsci/kfh239.

Mustieles, V., Balogh, R.K., Axelstad, M., Montazeri, P., Márquez, S., Vrijheid, M., Draskau, M.K., Taxvig, C., Peinado, F.M., Berman, T., Frederiksen, H., Fernández, M. F., Marie Vinggaard, A., Andersson, A.-M., 2023. Benzophenone-3: Comprehensive review of the toxicological and human evidence with meta-analysis of human biomonitoring studies. Environ Int 107739. https://doi.org/10.1016/j.envint.2023.107739.

Orton, F., Rosivatz, E., Scholze, M., Kortenkamp, A., 2011. Widely used pesticides with previously unknown endocrine activity revealed as in Vitro antiandrogens. Environ Health Perspect 119, 794–800. https://doi.org/10.1289/EHP.1002895.

Orton, F., Ermler, S., Kugathas, S., Rosivatz, E., Scholze, M., Kortenkamp, A., 2014. Mixture effects at very low doses with combinations of anti-androgenic pesticides, antioxidants, industrial pollutant and chemicals used in personal care products. Toxicol Appl Pharmacol 278, 201–208. https://doi.org/10.1016/J.TAAP.2013.09.008.

Pimentel, D., Cooperstein, S., Randell, H., Filiberto, D., Sorrentino, S., Kaye, B., Nicklin, C., Yagi, J., Brian, J., O'Hern, J., Habas, A., Weinstein, C., 2007. Ecology of Increasing Diseases: Population Growth and Environmental Degradation. Hum Ecol Interdiscip J 35, 653–668. https://doi.org/10.1007/S10745-007-9128-3.

Proença, S., Escher, B.I., Fischer, F.C., Fisher, C., Grégoire, S., Hewitt, N.J., Nicol, B., Paini, A., Kramer, N.I., 2021. Effective exposure of chemicals in in vitro cell systems: A review of chemical distribution models. Toxicol. In Vitro. https://doi.org/10.1016/j.tiv.2021.105133.

Punt, A., Aartse, A., Bovee, T.F.H., Gerssen, A., van Leeuwen, S.P.J., Hoogenboom, R.L.A. P., Peijnenburg, A.A.C.M., 2019. Quantitative in vitro-to-in vivo extrapolation (QIVIVE) of estrogenic and anti-androgenic potencies of BPA and BADGE analogues. Arch Toxicol 93, 1941–1953. https://doi.org/10.1007/S00204-019-02479-6/FIGURES/5.

Rajapakse, N., Silva, E., Kortenkamp, A., 2002. Combining xenoestrogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action. Environ Health Perspect 110, 917–921. https://doi.org/10.1289/EHP.02110917.

Rochester, J.R., Bolden, A.L., 2015. Bisphenol S and F: A systematic review and comparison of the hormonal activity of bisphenol a substitutes. Environ Health Perspect 123, 643–650. https://doi.org/10.1289/EHP.1408989.

Rodríguez-Carrillo, A., Rosenmai, A.K., Mustieles, V., Couderq, S., Fini, J.B., Vela-Soria, F., Molina-Molina, J.M., Ferrando-Marco, P., Wielsøe, M., Long, M., Bonefeld-Jorgensen, E.C., Olea, N., Vinggaard, A.M., Fernández, M.F., 2021. Assessment of chemical mixtures using biomarkers of combined biological activity: A screening study in human placentas. Reprod. Toxicol. 100, 143–154. https://doi.org/10.1016/j.reprotox.2021.01.002.

Rosenmai, A.K., Dybdahl, M., Pedersen, M., van Vugt-Lussenburg, B.M.A., Wedebye, E. B., Taxvig, C., Vinggaard, A.M., 2014. Are Structural Analogues to Bisphenol A Safe Alternatives? Toxicol. Sci. 139, 35–47. https://doi.org/10.1093/TOXSCI/KFU030.

Scholze, M., Taxvig, C., Kortenkamp, A., Boberg, J., Christiansen, S., Svingen, T., Lauschke, K., Frandsen, H., Ermler, S., Hermann, S.S., Pedersen, M., Lykkeberg, A.K., Axelstad, M., Vinggaard, A.M., 2020. Quantitative in Vitro to in Vivo Extrapolation (QIVIVE) for Predicting Reduced Anogenital Distance Produced by Anti-Androgenic Pesticides in a Rodent Model for Male Reproductive Disorders. Environ Health Perspect 128, 117005. https://doi.org/10.1289/EHP6774.

Schrenk, D., Bignami, M., Bodin, L., Chipman, J.K., del Mazo, J., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L. (Ron), Leblanc, J., Nebbia, C.S., Nielsen, E., Ntzani, E., Petersen, A., Sand, S., Vleminckx, C., Wallace, H., Barregård, L., Ceccatelli, S., Cravedi, J., Halldorsson, T.I., Haug, L.S., Johansson, N., Knutsen, H.K., Rose, M., Roudot, A., van Loveren, H., Vollmer, G., Mackay, K., Riolo, F., Schwerdtle, T., 2020. Risk to human health related to the presence of perfluoroalkyl substances in food. EFSA Journal 18. 10.2903/j.efsa.2020.6223.

Schwartz, C.L., Christiansen, S., Vinggaard, A.M., Axelstad, M., Hass, U., Svingen, T., 2019. Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders. Arch Toxicol. https://doi.org/10.1007/s00204-018-2350-5.

Shen, O., Du, G., Sun, H., Wu, W., Jiang, Y., Song, L., Wang, X., 2009. Comparison of in vitro hormone activities of selected phthalates using reporter gene assays. Toxicol Lett 191, 9–14. https://doi.org/10.1016/J.TOXLET.2009.07.019.

Silva, E., Rajapakse, N., Kortenkamp, A., 2002. Something from "nothing" - Eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environ Sci Technol 36, 1751–1756. https://doi.org/10.1021/es0101227.

Socianu, S., Bopp, S.K., Govarts, E., Gilles, L., Buekers, J., Kolossa-Gehring, M., Backhaus, T., Franco, A., 2022. Chemical Mixtures in the EU Population: Composition and Potential Risks. Int J Environ Res Public Health 19, 6121. https://doi.org/10.3390/ijerph19106121.

Todaka, T., Hirakawa, H., Kajiwara, J., Hori, T., Tobiishi, K., Yasutake, D., Onozuka, D., Sasaki, S., Miyashita, C., Yoshioka, E., Yuasa, M., Kishi, R., Iida, T., Furue, M., 2010. Relationship between the concentrations of polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans, and polychlorinated biphenyls in maternal blood and those in breast milk. Chemosphere 78, 185–192.

Todaka, T., Hirakawa, H., Kajiwara, J., Onozuka, D., Sasaki, S., Miyashita, C., Yoshioka, E., Yuasa, M., Kishi, R., Iida, T., Uchi, H., Furue, M., 2011. Concentrations of polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans, and polychlorinated biphenyls in blood and breast milk collected from pregnant women in Sapporo City, Japan. Chemosphere 85, 1694–1700. https://doi.org/10.1016/J.CHEMOSPHERE.2011.09.014.

Trasande, L., Shaffer, R.M., Sathyanarayana, S., 2018. Food additives and child health. Pediatrics 142. https://doi.org/10.1542/PEDS.2018-1408/37584.

Tsang, H.L., Wu, S., Leung, C.K.M., Tao, S., Wong, M.H., 2011. Body burden of POPs of Hong Kong residents, based on human milk, maternal and cord serum. Environ Int 37, 142–151. https://doi.org/10.1016/J.ENVINT.2010.08.010.

U.S. Environmental Protection Agency, 2020. CompTox Chemicals Dashboard [WWW Document]. URL https://comptox.epa.gov/dashboard/ (accessed 3.20.21).

van der Ven, L.T.M., van Ommeren, P., Zwart, E.P., Gremmer, E.R., Hodemaekers, H.M., Heusinkveld, H.J., van Klaveren, J.D., Rorije, E., 2022. Dose Addition in the Induction of Craniofacial Malformations in Zebrafish Embryos Exposed to a Complex Mixture of Food-Relevant Chemicals with Dissimilar Modes of Action. Environ Health Perspect 130. https://doi.org/10.1289/EHP9888.

Vinggaard, A.M., Niemela, J., Wedebye, E.B., Jensen, G.E., 2008. Screening of 397 chemicals and development of a quantitative structure–activity relationship model for androgen receptor antagonism. Chem Res Toxicol 21, 813–823. https://doi.org/10.1021/tx7002382.

Vinggaard, A.M., Bonefeld-Jørgensen, E.C., Jensen, T.K., Fernandez, M.F., Rosenmai, A. K., Taxvig, C., Rodriguez-Carrillo, A., Wielsøe, M., Long, M., Olea, N., Antignac, J.P., Hamers, T., Lamoree, M., 2021. Receptor-based in vitro activities to assess human exposure to chemical mixtures and related health impacts. Environ Int. https://doi.org/10.1016/j.envint.2020.106191.

Wang, Y., Zhu, H., Kannan, K., 2019. A Review of Biomonitoring of Phthalate Exposures. Toxics 2019, Vol. 7, Page 21 7, 21. 10.3390/TOXICS7020021.

Ward, M.H., Jones, R.R., Brender, J.D., de Kok, T.M., Weyer, P.J., Nolan, B.T., Villanueva, C.M., van Breda, S.G., 2018. Drinking Water Nitrate and Human Health: An Updated Review. International Journal of Environmental Research and Public Health 2018, Vol. 15, Page 1557 15, 1557. 10.3390/IJERPH15071557.

WHO, 1989. Aldrin and Dieldrin. Environmental Health Criteria 91. World Health Organization, Geneva.

Wittsiepe, J., Fürst, P., Schrey, P., Lemm, F., Kraft, M., Eberwein, G., Winneke, G., Wilhelm, M., 2007. PCDD/F and dioxin-like PCB in human blood and milk from German mothers. Chemosphere 67, S286–S294. https://doi.org/10.1016/J.CHEMOSPHERE.2006.05.118.

Wolff, M.S., 1983. Occupationally derived chemicals in breast milk. Am J Ind Med 4, 259–281. https://doi.org/10.1002/ajim.4700040120.