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Mixture risk assessment of nine reproductive toxic chemicals affecting male sperm quality in a representative sample of children and adolescents living in Germany – results from the German Environmental Survey (GerES V)

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ABSTRACT

Reproductive health is declining globally, with growing evidence linking exposure to endocrine-disrupting chemicals during critical pregnancy stages to adverse male sexual development. This study assessed the cumulative risk of nine such chemicals—acrylamide, PCB 118, DEHP, DnBP, DiBP, BBzP, DiNP, DCHP, and DnPeP—and possible influencing factors such as age, socioeconomic status (SES), and region (former East vs. West Germany). We analyzed cross-sectional data from the German Environmental Survey for Children and Adolescents 2014–2017 (GerES V), including 1090 participants with complete urine and plasma samples.

Using the Hazard Index (HI) method, which combines exposure levels with human biomonitoring guidance values, we found that 31% of participants had an HI above 1, indicating elevated risk from combined chemical exposure. Notably, 26% of these cases would have gone unnoticed in single-substance assessments. DnBP, DiBP, and acrylamide were the main contributors to overall risk.

Stratified analyses revealed that younger children had higher HI levels than older ones. Children from lower SES backgrounds also showed higher risk compared to those from medium or high SES groups. Additionally, residing in former East Germany was associated with increased HI levels compared to former West Germany.

These findings emphasize the importance of considering chemical mixtures in risk assessments and recognizing subgroup-specific vulnerabilities. Future assessments should expand the range of included chemicals and focus on high-risk groups—especially children, individuals with low SES, and residents of former East Germany—to better capture the scope of potential health impacts.

1. Introduction

The decline in reproductive health has become a global concern over recent decades (Lindahl-Jacobsen et al., 2026), evidenced by a more than 50% reduction in sperm counts between 1973 and 2011 (Levine et al., 2017). Further indicators of impairment of male reproductive health, such as decreases in sperm concentration and motility, have been reported and are accelerating (Auger et al., 1995; Levine et al., 2017; Sengupta et al., 2018). This trend results in significant economic and societal burdens, with estimated annual costs of 15 billion EUR in the EU alone (Hauser et al., 2015; Winters and Walsh, 2014). In Germany, which has one of the lowest birthrates in Europe (Population Reference Bureau, 2007), impaired semen quality is a prevalent issue (Paasch et al., 2008) and may contribute to low fertility rates (Jensen et al., 2002). Understanding these factors is crucial for developing effective public health strategies.

So far, the determinants of the decline in male fertility are not fully understood and appear to be complex, with factors such as smoking, alcohol consumption, diet, and genetic predispositions all seeming to play a role (Okonofua et al., 2022; Skakkebaek et al., 2016). Adding to this complexity, exposure to certain chemicals has been hypothesized to effect male fertility (Lahimer et al., 2023). Specifically, exposure to endocrine-disrupting chemicals during critical stages of pregnancy may have effects on male sexual development, for instance by altering sperm function by targeting testicular development or affecting androgen receptors (Jeseta et al., 2021; Lahimer et al., 2023). For several chemicals or chemical groups, such as phthalates (CHAP, 2014; Dobrzynska, 2016; Radke et al., 2018), bisphenol A (BPA) (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids, 2023), or dioxin-like PCBs (Ermler and Kortenkamp, 2022), the effects on sperm quality are well

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documented. While single-chemical assessments provide valuable insights, individuals are exposed to a multitude of chemicals often simultaneously (Bil et al., 2023; Rodriguez Martin et al., 2023). Therefore, using mixture risk assessment (MRA) to evaluate the combined effects of chemicals is more reflective of real-life exposure scenarios. The issue of chemical mixtures has long been a policy concern in Europe, with the European Commission emphasizing the need to account for mixture toxicity and 'cocktail effects' (European Commission, 2012), and more recently embedding this in its zero-pollution ambition (European Commission, 2011). We have previously conducted a MRA on five anti-androgenic phthalates using human biomonitoring (HBM) data from European children and adolescents in 12 countries (Lange et al., 2022). Our results showed that 17% of the participants were at increased risk, and a striking 63% would have gone unnoticed in single-substance evaluations. Including a broader range of anti-androgenic chemicals the general population is exposed to could result in even higher risk estimates (Howdeshell et al., 2017; Orton et al., 2014). Kortenkamp et al. (2022) recently demonstrated that combined exposures to nine jointly monitored chemicals in Danish male volunteers, including phthalates, bisphenols, and paracetamol, exceeded a hazard index (HI) of 1 by more than 17-fold (Kortenkamp et al., 2022). Overall, this underscores the importance of assessing combinations of anti-androgenic chemicals which the general population is exposed to from a wide range of sources.

The aim of this study is to perform a MRA on nine selected chemicals related to male reproductive health, including acrylamide, polychlorinated biphenyl (PCB) 118, and phthalates di(2-ethylhexyl) phthalate (DEHP), di-n-butyl phthalate (DnBP), diisobutyl phthalate (DiBP), butyl benzyl phthalate (BBzP), diisononyl phthalate (DiNP), dicyclohexyl phthalate (DCHP), di-n-pentyl phthalate (DnPeP), using data from a representative sample of children and adolescents residing in Germany. Single-substance assessments have previously been conducted for all nine chemicals (Bandow et al., 2020; Schwedler et al., 2020, 2021), and we previously performed a group-specific MRA of four phthalates (Lemke et al., 2021). However, a comprehensive MRA across different substance groups has not yet been conducted in a representative sample of the young German population. In addition, we investigated determinants of elevated risk by examining potential differences according to sex, age, socio-economic status, and region of residence, and identified the individual chemicals contributing most to mixture risk among children and adolescents in Germany.

2. Materials and methods

2.1. Study population and data collection

We used cross-sectional data from the German Environmental Survey for Children and Adolescents 2014–2017 (GerES V), representative of the German population regarding region of residence (former East and West Germany), community size, age, and sex (Schulz et al., 2021). In a total of 2294 participants, pollutants and other environmental stressors were assessed in human samples and in their homes between 2014 and 2017 in cooperation with the second follow-up of the German Health Interview and Examination Survey for Children and Adolescents, performed by the Robert Koch Institute (RKI) (Mauz et al., 2017). More specifically, examinations were performed in samples of whole blood, blood plasma, morning urine, drinking water, measurements of ultrafine particles and noise levels, standardized interviews and self-administrated questionnaires. Further details on the study design can be found elsewhere (Schulz et al., 2021). For the present study, we selected a subsample of 1090 participants with available measurements of the selected substances in urine and blood plasma described below. These 1090 participants comprise a random subsample of the full GerES V sample (n = 2294). The subsample does not differ from the full sample regarding sociodemographic characteristics. The reason for selecting the subsample only is that blood plasma was only available for half of the study population. All available measurements were used, regardless of e.g. the urinary creatinine content. Although the health-based guidance values applied in the MRA were derived from male reproductive toxicity endpoints, exposure assessment and mixture characterization were conducted in both sexes, as exposure to the investigated chemicals occurs independently of sex and male reproductive effects represent sensitive and protective toxicological benchmarks regarding overall reproductive health.

2.2. Chemical selection for MRA

For the selection of chemicals, we followed a similar approach as to that of Kortenkamp et al. (2022). These included chemicals that met the following criteria: evidence on semen quality reductions in male offspring from animal or epidemiological studies and evidence of a hormonal mode of action (MoA). We included the following overlapping chemicals measured in our subsample: acrylamide, PCB 118, and the phthalates DEHP, DnBP, DiBP, BBzP, and DiNP. In addition, we used the same criteria to select the remaining chemicals measured in our subsample. We screened the literature from peer-reviewed journals and documents from governmental organizations that evaluated the strength of evidence on male reproductive toxicity, including reports from the European Food Safety Authority (EFSA) and European Chemicals Agency (ECHA). This targeted screening was conducted up to April 2024 and was intended to support chemical selection rather than a systematic review. From this screening, we selected DCHP and DnPeP as candidates to be included in the MRA.

2.3. Chemical analysis of selected chemicals

The metabolites of the phthalates DnBP, DEHP, DiBP, DiNP, BBzP, DCHP, and DnPeP, as proxies for exposure to the respective parent substances (see Table 1) were analyzed in urine by the Institute for Prevention and Occupational Medicine at Ruhr-University Bochum, Germany, using on-line high-performance liquid chromatography-tandem mass spectrometry with isotope-labelled standards according to previously published methods (Koch et al., 2003, 2007, 2012, 2017; Preuss et al., 2005). Internal quality control for the phthalate metabolites included analysis of control urines with known concentrations, consistently within the ±3σ range. Blinded repeated measurements showed concentrations within the respective confidence intervals. External quality assurance was verified through biannual participation in the German External Quality Assessment Scheme (G-EQUAS) for metabolites of DEHP, and MnBP, MiBP and MBzP (Schwedler et al., 2020).

Mercapturic acids *N*-acetyl-*S*-(3-amino-3-oxopropyl)-cysteine (AAMA) and *N*-acetyl-*S*-(3-amino-2-hydroxy-3-oxopropyl)-cysteine (GAMA) are specific biomarkers for acrylamide exposure, however, with different toxicokinetics (Fuhr et al., 2006). As AAMA is considered a rather direct indicator of exposure (Poteser et al., 2022) by representing an estimated 50% of administered dose of acrylamide in contrast to only 5% for GAMA (Boettcher et al., 2006; Fuhr et al., 2006), we included AAMA as metabolite of exposure in our MRA. Analysis of AAMA in urine was conducted by the Analytisch-Biologisches Forschungslabor Munich, using validated multiplex liquid chromatography-tandem mass spectrometry as described by Pluym et al. (2015). Internal quality control (QC) measures included analyzing control urines with known biomarker concentrations at three different levels corresponding to the expected concentration range of the analytes. Blinded control samples were randomly included in all analytical cycles. Internal and blinded QCs were evaluated based on Food and Drug Administration (FDA) guidelines, as previously published elsewhere (Schwedler et al., 2021). External quality assurance was confirmed through participation in the G-EQUAS.

PCB 118 concentrations were measured in blood plasma samples. Following blood collection, aliquots of samples underwent protein precipitation and were subsequently analyzed using gas chromatography

Table 1
Selected substances with respective exposure biomarkers and their corresponding HBM-GVs.

Parent compound	Abbreviation	Specific exposure biomarker(s)	Abbreviation	Sample matrix	% ≥ LOQ[a]	Critical endpoint for MRA	Reference key study	HBM-GV_{GenPop} or pHBM-$GV_{GenPop\text{-}MRA}$ for children in µg/L
Polychlorinated biphenyl	PCB	Polychlorinated biphenyl 118	PCB 118	plasma	20.0 %	semen quality	He et al. (2020)	0.24[c]
Diethylhexylphthalate	DEHP	Mono(2-ethyl-5-hydroxyhexyl)phthalate	5-OH-MEHP	urine	100 %	dysgenesis genitalia	Wolfe and Layton (2003)	380[b]
		Mono(2-ethyl-5-carboxypentyl) phthalate	5-cx-MEPP	urine	100 %			
Di-n-butylphthalate	DnBP	Mono-n-butylphthalate	MnBP	urine	100 %	spermatocyte development	Lee et al. (2004)	120[b]
Diisobutylphthalate	DiBP	Mono-iso-butylphthalate	MiBP	urine	100 %	spermatocyte development (via read-across from DnBP)	Lee et al. (2004)	160[b]
Benzylbutylphthalate	BBzP	Mono-benzylphthalate	MBzP	urine	99.6 %	suppression testosterone synthesis; semen quality	Ahmad et al. (2014) Furr et al. (2014)	2000[b]
Diisononylphthalate	DiNP	Mono(4-methyl-7-hydroxyoctyl)phthalate	OH-MiNP	urine	100 %	suppression testosterone synthesis	Clewell et al. (2013)	340[c]
		Mono(4-methyl-7-carboxyheptyl) phthalate	cx-MiNP	urine	100 %			
Dicyclohexylphthalate	DCHP	Mono-cyclohexylphthalate	MCHP	urine	5.1 %	spermatid head count	Hoshino et al. (2005)	1523[d]
Di-n-pentylphthalate	DnPeP	Mono-n-pentylphthalate	MnPeP	urine	5.3 %	suppression testosterone synthesis	Hannas et al. (2011)	1301[d]
Acrylamide		*N*-acetyl-*S*-(3-amino-3-oxopropyl)-cysteine	AAMA	urine	100 %	decrease in sperm number	Ivanski et al. (2020)	447[d]

[a] The percentage of measurements above LOQ refers to the subsample used in this publication and may differ from publications of the full GerES V sample.
[b] HBM-GV_{GenPop} derived by Lange et al. (2021).
[c] HBM-GV_{GenPop} derived by Lange et al. (2022).
[d] pHBM-$GV_{GenPop\text{-}MRA}$ derived for the purpose of this study.

tandem mass spectrometry (GC-MS/MS). Detailed methods for the analysis of PCBs in blood plasma are available in a previous study (Bandow et al., 2020). PCB 118 concentration was determined using an internal standard approach, based on the measurement of 12 calibration solutions with concentrations ranging between 0.01 and 5.0 µg/L. The calibration curves exhibited correlation coefficients above 0.998, and a weighting factor of 1/x was applied.

2.4. Human biomonitoring guidance values (HBM-GVs)

Table 1 presents an overview of the selected substances and their biomarkers included in this MRA with their critical endpoints, reference key study and their corresponding human biomonitoring guidance values or provisional values derived for this MRA (HBM-GV_{GenPop} and pHBM-$GV_{GenPop\text{-}MRA}$, respectively).

HBM-GVs are the concentration of a substance or its specific metabolite(s) in human biological media (e.g. urine, blood, hair) at and below which, according to current knowledge, there is no risk of health impairment anticipated. Those toxicologically derived values for the general population are termed HBM-GV_{GenPop} in contrast to those derived for the working population. These values are consolidated values derived within the European Human Biomonitoring Initiative (HBM4EU) and are easy to use as internal exposure levels measured in HBM studies as GerES V can directly be compared to those values (Apel et al., 2020).

For several chemicals selected in this MRA there are already HBM-GV_{GenPop}s for the general population based on anti-androgenic effects seen in rat offspring after prenatal exposure (i.e. DEHP, DnBP, DiBP, BBzP and DiNP). Further details on the derivation of the guidance values for DEHP, DnBP, DiBP and BBzP can be found in our previous work (Lange et al., 2021). For DiNP a pHBM-$GV_{GenPop\text{-}MRA}$ based on suppression of fetal testicular testosterone solely for the purpose of a phthalate MRA was also part of our previous work (Lange et al., 2022). We incorporated these values in this MRA, as the toxicological endpoint (male reproductive toxicity) is the same.

For PCB 118, DCHP, DnPeP, and acrylamide, no HBM-GV based on male reproductive health endpoints were available and thus pHBM-$GV_{GenPop\text{-}MRA}$ for the sole purpose of the current MRA were derived. It must be noted that the values are not meant for single substance risk assessments, as the toxicity reference value (TRV) we selected is for this endpoint and may not necessarily represent the most sensitive endpoint of the substance. In the following the derivation of pHBM-$_{GVGenPop\text{-}MRA}$ for this MRA is described for each substance.

2.4.1. PCB 118

The reference dose (RfD) for PCB 118 was derived from a systematic review by Ermler and Kortenkamp (2022), which examined the impact of PCB exposure on semen quality across human and animal studies. Strong evidence came from a mouse study focused on reproductive function, where pregnant mice were orally exposed to 0, 20, and 100 µg/kg_{bw}/d of PCB 118 from gestational days (GD) 7.5 to 12.5 (He et al., 2020). The deformity rate of sperm in offspring increased compared to control at 20 and 100 µg/kg_{bw}/d. Histological changes in testes were observed in 20 and 100 µg/kg_{bw}/d treatment groups compared to control, including the relative number of spermatogonia in the seminiferous tubules. Lastly, increased testicular coefficient of the male offspring was observed in both treatment groups compared to control (He et al., 2020). As such, the lowest observed adverse effect level (LOAEL) is 20 µg/kg_{bw}/d of PCB 118 for declines in sperm quality (He et al., 2020). Converting LOAEL to no observed adverse effect level (NOAEL) with an Assessment Factor (AF) = 3 as standard approach (ECETOC, 2003) and applying inter- and intraspecies AFs, a daily intake reference dose of

0.0029 µg/kg$_{bw}$/d was reported by Ermler and Kortenkamp (2022). In our study, PCB 118 was measured in plasma so we converted the external daily intake reference dose to the internal concentration in blood following an approach provided by the Federal Institute for Occupational Safety and Health (BAuA) (BAuA, 2017), with the assumptions that the daily intake is constant, and intake and elimination is already in a steady state. The approach by BAuA (2017) is as follows: Considering a blood level of 17 µg/L with a NOAEL of 10 µg/kg$_{bw}$ in rats, the permissible additional blood level for humans with a background blood level of 8 µg/L of higher-chlorinated, non-dioxin-like biphenyls is 9 µg/L (17 µg/L - 8 µg/L = 9 µg/L). With a fat/blood distribution coefficient of 300, 9 µg/L of blood corresponds to 2700 µg per kg of fat. With 20% fat content in body mass, the permissible exposure to inhalation of higher-chlorinated, non-dioxin-like biphenyls is thus 540 µg/kg$_{bw}$.

The half-life of PCB 118 is 3395 days (Ermler and Kortenkamp, 2022). Therefore, the exposure is reached with a daily intake of 0.1103 µg/kg$_{bw}$/d (ln 2 × 540 µg/kg$_{bw}$/3395 days = 110.3 ng/kg$_{bw}$/d). Taken the RfD of 0.0029 µg/kg$_{bw}$/d, the conversion to blood yields 0.237 µg/L blood ((0.0029 µg/kg$_{bw}$/d × 9 µg/L)/0.1103 µg/kg$_{bw}$/d) (BAuA, 2017).

2.4.2. DCHP

Among the pool of evidence on reproductive effects of DCHP, the study by Hoshino et al. (2005) was selected as the one with the lowest reported LOAEL on poor male reproductive health. The authors exposed male and female parental rats to DCHP in the diet to 0, 16, 80, 402 mg/kg$_{bw}$/d, starting from the age of 5 weeks. For the males, administration continued until necropsy through 10 weeks or more of pre-mating and mating periods, while for females, administration lasted until necropsy through 10 weeks or more of pre-mating, mating, gestation, and lactation periods until weaning of the offspring on postnatal day (PND) 21 (i.e. 21 days after birth, a common weaning point in rodent studies). Adverse effects in male offspring included reduced spermatid head counts in the testes observed at 80 mg/kg$_{bw}$/d. Testicular atrophy and decreased anogenital distance were observed at 402 mg/kg$_{bw}$/d. As such, a NOAEL = 16 mg/kg$_{bw}$/d was determined by the authors. By applying an AF of 100 to account for inter- and intraspecies differences, a toxicological reference value of 0.16 mg/kg$_{bw}$/d was set. The urinary excretion fraction (F_{ue}) of DCHP was not available in the literature and was thus estimated by linear regression of log K_{ow}s against the known F_{ue} of other phthalates and taking the average of four approaches (see Supplementary Table 1 for details on F_{ue} estimations), using an approach similar to Moos et al. (2017). By incorporating the metabolic weights of DCHP and MCHP, we used the formula described in Apel et al. (2020) to derive an pHBM-$_{GVGenPop-MRA}$ of 1523 µg/L or 1.5 mg/L for DCHP.

2.4.3. DnPeP

We identified the study by Hannas et al. (2011) as the key study for the derivation of a pHBM-GV$_{GenPop-MRA}$ for DnPeP. The study was conducted in pregnant rats who received 0, 11, 33, 100, or 300 mg/kg$_{bw}$/d on GD 14-18. Fetal testicular testosterone production levels on GD 18 were significantly reduced at 33 mg/kg$_{bw}$/d or higher in a dose-responsive fashion. With a NOAEL = 11 mg/kg$_{bw}$/d and AF of 100 for inter- and intraspecies differences, a TRV = 0.11 mg/kg$_{bw}$/d was yielded. F_{ue} of MnPeP was estimated using the same approach as for DCHP, and by incorporating the metabolic weights of DnPeP and MnPeP, the derived pHBM-GV$_{GenPop-MRA}$ = 1301 µg/L or 1.3 mg/L for DnPeP.

2.4.4. Acrylamide

For acrylamide we followed the proposed critical study by Kortenkamp et al. (2022) on sperm production in rodents by Ivanski et al. (2020). Despite missing gestational exposure, Kortenkamp et al. (2022) included this study in the MRA because of the clear hormonal MoA. In specific, prepubertal rats between PND23 – PND60 received either 0, 2.5, or 5 mg/kg$_{bw}$/d of acrylamide by oral gavage. Sperm parameters were affected in a dose-response manner. Exposure to 2.5 and 5 mg/kg$_{bw}$/d reduced total and daily sperm production, sperm reserves, and affected sperm functionality. The LOAEL = 2.5 mg/kg$_{bw}$/d, and by application of a LOAEL to NOAEL extrapolation factor of 3 and AF of 100 for inter- and intraspecies differences, a RfD = 0.008 mg/kg$_{bw}$/d was reported by Kortenkamp et al. (2022). Using the formula described in Apel et al. (2020) incorporating the molar masses of acrylamide and AAMA, and a F_{ue} = 0.5 (Boettcher et al., 2006), we derived a pHBM-GV$_{GenPop-MRA}$ = of 447 µg/L for acrylamide.

2.5. Statistical analysis

Case weights provided by the RKI (Hoffmann et al., 2018) were used to adjust the sample for deviations from the 3-17-year-old population in Germany in terms of region of residence, community size, age, sex, parental education, and German nationality. Results presented here are based on weighted calculations and samples sizes are given as the sum of respective case weights.

2.5.1. Mixture risk assessment

For assessing the mixture risk, we used the hazard index (HI) approach. With this component-based approach, the urine or plasma concentration of each metabolite or the total concentration of metabolites of a substance is divided by their respective HBM-GV (HBM-GV$_{GenPop}$ or pHBM-GV$_{GenPop-MRA}$) to determine a personalized risk quotient (RQ_p) of each substance:

$$RQ_p = \frac{Urine\ or\ plasma\ concentration_p}{HBM\text{–}GV}$$

The RQs of each substance are then summed to determine the HI_p of each study participant, using the following formula:

$$HI_p = RQ_{PCB\text{-}118} + RQ_{DEHP} + RQ_{DnBP} + RQ_{DiBP} + RQ_{BBzP} + RQ_{DiNP} + RQ_{DCHP} + RQ_{DnPeP} + RQ_{Acrylamide}$$

This approach requires the assumption that concentrations can be added due to a common mode of action (MoA) or common toxic effect endpoint (Goodrum et al., 2021). The assumption of dose additivity is justified by the selection of substances based on a shared adverse health outcome, namely effects on male reproductive function and sperm quality and can serve as a proxy for reproductive toxicity. Although the individual substances may act via different mechanisms, they contribute to a common toxicological endpoint. In the absence of detailed information on substance-specific modes of action or interaction effects, dose additivity represents the default and health-protective assumption for mixture risk assessment, as recommended by the European Commission (European CommissionDirectorate-General for Health and Consumers, 2012).

The interpretation is as follows: if the HI is below 1, it does not pose a risk, whilst when above 1 the likelihood of a toxicological response to the mixture is increased (European CommissionDirectorate-General for Health and Consumers, 2012).

2.5.2. Determinants of elevated risk

We included the following potential determinants for subgroup analyses: sex, age, region of residence, and socioeconomic status (SES). Age was categorized into the following 4 groups: 3-5 years-old, 6-10 years-old, 11-13 years-old, and 14-17 years-old. Place of residency was modelled as a dichotomized variable (former East Germany including former East Berlin vs. former West Germany including former West Berlin). SES was defined as an index based on income, education, and job of parents (Lampert et al., 2018), and modelled as a three-level categorical variable (low, medium, and high). To evaluate associations between predictors and HI, a linear regression model was applied using the Generalized Linear Model (GLM) with log link function to account

for the non-normal distribution of the HIs. All predictors were adjusted for each other.

2.5.3. Drivers of risk

We explored the complexity of combined exposures by examining the contribution of individual substances to the total sum of RQs, in total and stratified by subgroups. We further explored this with the Maximum Cumulative Ratio (MCR) as introduced by Price et al. (2012). The MCR for each participant is determined by dividing their HI by their highest RQ (RQ_{max}). Calculating MCR helps to discern if the combined exposure risk is dominated by a single chemical or by multiple chemicals. For participants with an MCR less than 2, a single substance accounts for most of the combined risk, while for those with an MCR greater than 2, multiple substances contribute more significantly to the risk. As such, a scatterplot of MCR vs. HI was generated. We followed the risk management categories according to Price et al. (2012) that include the following scenarios: Region I represents scenarios where combined exposures raise concern because one or more individual chemicals surpass the HBM-GV (RQ > 1, HI > 1); Region II shows combined exposures with minimal concern for both individual chemicals and for their combined effects (all RQs <1, HI < 1); Region III indicates combined exposures with low concern for individual chemicals, but a concern for their combined effects (all RQs<1, but HI > 1).

Statistical analyses were performed in R (version 3.4.3, R Foundation for Statistical Computing, Vienna, Austria) in R studio (version 0.99.903, RStudio Inc., Boston, MA, USA). Biomarker values below limits of quantification (LOQ) were imputed by substituting with LOQ/$2^{1/2}$ following a similar method to Kortenkamp et al. (2022). The percentage of measurements at or above LOQ for each biomarker is indicated in Table 1. Values missing at random of the following covariates were imputed: SES, using mice package in R (Buuren and Groothuis-Oudshoorn, 2011). Sensitivity analyses excluding participants with missing SES yielded comparable results (data not shown).

3. Results

Table 2 presents the baseline characteristics of the study population including the distribution of sex, age group, SES, and region of residence. In total, 1090 individuals had complete urine and plasma samples of all nine substances included in the MRA.

Fig. 1 shows the scatter plot of individual HI vs. MCR with risk management categories. MCRs show the number of chemicals that contribute to the overall mixture risk (Price and Han, 2011). In the plot we can observe that MCR diminishes with increasing HI, along a line of data points descending from left to right. This suggests that among participants with the highest HI, the mixture complexity reduces, as a greater part of the HI is explained by the RQ of a single substance. In total, 13% (142/1090) of participants had MCR<2, indicating single-substance dominance. Among participants with HI > 1, 73% had MCR>2, reflecting more complex mixtures. Region III depicts the combined exposure with a low concern for individual chemicals, but concern for the combined effects (RQs<1 but HI > 1). In total, 31% (334/1090) of the participants had a HI > 1. Among these participants, 83% (276/334) were classified as Region III, corresponding to, 25% (276/1090) of the total population, indicating they would have gone unnoticed in a single substance risk assessment.

Table 2
Weighted baseline characteristics of the study population and percentage of individuals exceeding hazard index (HI) of 1.

		N (%)	% > HI = 1
Total		1090 (100)	31
Sex	Boys	558 (51)	29
	Girls	532 (49)	33
Age group	3-5 years	186 (17)	40
	6-10 years	353 (32)	34
	11-13 years	225 (21)	26
	14-17 years	327 (30)	25
Socioeconomic status	Low	236 (22)	39
	Medium	645 (59)	30
	High	210 (19)	25
Region of residence	Former West Germany	924 (85)	29
	Former East Germany	167 (15)	41

Abbreviations: N: number of study participants; HI: hazard index. All values are rounded.

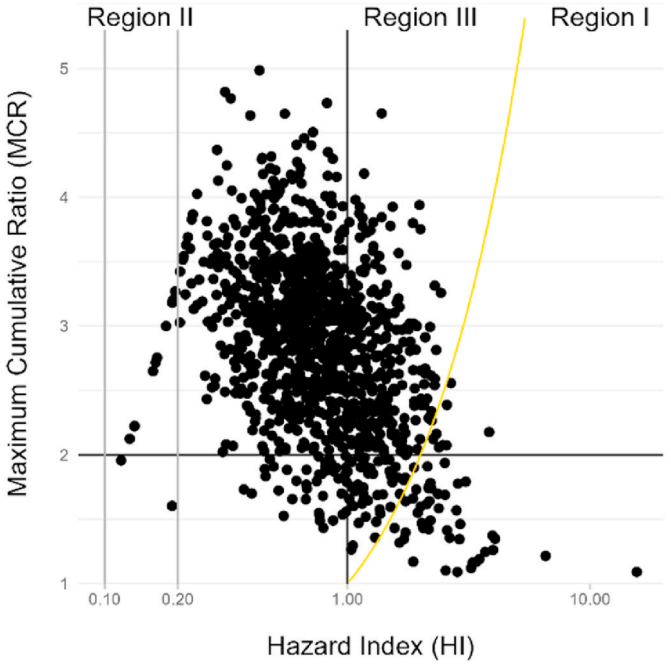

Fig. 1. Scatter plot of Hazard Index (HI) vs. Maximum Cumulative Ratio (MCR) with risk management categories indicated. Each dot represents an individual study participant. Region II (area left of the vertical line for HI = 1, i.e. individuals with HI < 1) depicts individuals with combined exposures that are not of concern. Region III (area enclosed by the vertical line for HI = 1 and the curved yellow line for HI = MCR) depicts individuals with HI > 1, but without exceeding any of the single-substance HBM-GVs, i.e. all RQs <1. Region I (area to the right of the yellow line for HI = MCR) depicts individuals exceeding both, the acceptable combined exposure and HBM-GVs for single substance(s). The horizontal line for MCR = 2 distinguishes individuals for which one substance contributed 50% or more to the MCR (those below the MCR = 2 line) and individuals with multiple substances contributing to the HI (those above the MCR = 2 line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.1. Determinants of mixtures

We first descriptively evaluated geometric means and 95th percentiles of the HIs stratified by sex, age, SES, and place of residency (see Table 3). Boys and girls had similar HIs in terms of geometric means, while boys appeared to have slightly higher HI at the 95th percentile compared to girls [boys vs girls, 95th percentile (95% CI): 2.16 (1.99, 2.57) vs. 2.09 (1.86, 2.21)]. Younger children showed higher HI compared to older children [3-5-year-olds vs. 14-17-year-olds, geometric mean (95% CI): 0.86 (0.75, 0.98) vs. 0.68 (0.62, 0.74); 95th percentile (95% CI): 2.16 (1.99, 2.57) vs. 1.73 (1.50, 2.52)]. Individuals coming from a low SES showed higher HI compared to those with a high SES [low vs high, geometric mean: 0.84 (0.73, 0.96) vs. 0.72 (0.67, 0.78); 95th percentile: 2.21 (2.15, 3.28) vs. 1.86 (1.53, 2.32)]. Those living in former East Germany showed higher HI than those in West Germany [East vs. West, geometric mean: 0.86 (0.78, 0.94) vs. 0.74 (0.70, 0.78); 95th percentile: 2.19 (2.13, 3.39) vs. 2.09 (1.93, 2.21)].

Fig. 2 illustrates the geometric means and variations in HI as

Table 3
Descriptive statistics for hazard indices per subgroup.

Characteristics		Hazard Index			
		Geometric mean (95% CI)	P95 (95% CI)	Min_{HI}	Max_{HI}
Sex	Boys	0.77 (0.72, 0.83)	2.16 (1.99, 2.57)	0.13	15.6
	Girls	0.74 (0.69, 0.80)	2.09 (1.86, 2.21)	0.11	6.57
Age group	3-5 years	0.86 (0.75, 0.98)	2.16 (1.99, 2.57)	0.22	2.87
	6-10 years	0.81 (0.74, 0.88)	2.20 (2.12, 3.07)	0.17	4.09
	11-13 years	0.70 (0.64, 0.77)	1.68 (1.57, 2.36)	0.11	6.57
	14-17 years	0.68 (0.62, 0.74)	1.73 (1.50, 2.52)	0.13	15.6
Socioeconomic status	Low	0.84 (0.73, 0.96)	2.21 (2.15, 3.28)	0.16	4.03
	Medium	0.74 (0.69, 0.78)	1.99 (1.80, 2.19)	0.13	4.09
	High	0.72 (0.67, 0.78)	1.86 (1.53, 2.32)	0.12	15.6
Region of residence	Former West Germany	0.74 (0.70, 0.78)	2.09 (1.93, 2.21)	0.12	4.03
	Former East Germany	0.86 (0.78, 0.94)	2.19 (2.13, 3.39)	0.13	15.6

Abbreviations: CI: confidence interval; Max: maximum value of HI; Min: minimum value of HI; P: Percentile.

determined through GLM analyses across (A) sex, (B) age, (C) SES, and (D) place of residency. Children aged 3-5-years- and 6-10-years exhibited higher HI levels compared to those aged 14-17-years. Individuals with lower SES showed higher HI levels compared to those from medium or high SES backgrounds. Residing in former East Germany was associated with higher HI levels compared to former West Germany. No notable differences were observed by sex (see Supplementary Table 2 for effect estimates and p-values).

These findings were further supported by the percentage of individuals exceeding HI thresholds within each subgroup, as shown in Table 2. Specifically, 40% of children aged 3-5-years showed HI values above 1, compared to 25% among those aged 14-17-years. A similar pattern was observed across SES, with 39% of individuals from low SES backgrounds exceeding HI > 1, compared to 24% among those with high SES. Geographical differences were also evident, with 41% of individuals residing in former East Germany having HI > 1, compared to 29% of those in former West Germany.

3.2. Drivers of mixture risk

Table 4 presents the geometric means of individual chemical concentrations alongside their corresponding RQs including the number of individuals with RQmax >1 for the respective substance. Notably, DnBP, DiBP and acrylamide emerge as the chemicals with the highest average risk quotient, with geometric means of 0.17, 0.16, and 0.17, respectively, and consequently the highest number of individuals with RQmax >1 with 14, 23, and 12, respectively. In contrast, DCHP, DnPeP and BBzP exhibit the lowest risk quotients, with geometric means close to 0 and no individual with RQmax >1. In total there were 52 individuals with RQmax >1. There was only one individual identified with RQmax >1 for more than one substance, i.e. exceeding an RQmax of 1 for DnBP and DiBP.

Table 5 presents the variability in maximum RQs (RQ_{max}) for individual substances across subgroups. In younger children, higher RQ_{max} values were observed for substances PCB 118, DEHP, DnBP, DiBP, while acrylamide showed a higher prevalence of RQ_{max} in older children. Among individuals with lower SES, there was a higher prevalence of RQ_{max} for acrylamide, whereas those with higher SES showed a higher prevalence of RQ_{max} for DnBP. Geographic differences were also evident, with individuals residing in former West Germany showing higher RQ_{max} for acrylamide, and those in former East Germany having higher prevalence of RQ_{max} for DnBP and DiBP.

4. Discussion

In this study we evaluated the combined (cumulative) risk of nine chemicals related to male sexual development as consistent endpoint and as proxy for reproductive toxicity in general in children and adolescents living in Germany across various subgroups, including sex, age, SES, and geographic location. 31% of our participants had a HI above 1 and thus potential elevated risk due to exposure to multiple chemicals. Overall, DnBP, DiBP and acrylamide contributed most to the elevated risk. Exposure patterns varied by age, SES, and region of residence. Specifically, younger children, individuals with lower SES, and individuals living in former East Germany had significantly higher cumulative exposure. Our results reveal notable differences across subgroups in both cumulative exposure and the types of chemicals driving mixture risks. Children seem to be more vulnerable for exposure to environmental toxins than adolescents and adults (Hauptman and Woolf, 2017), and indeed in our study we found that 40% of 3-5 year olds had HI > 1 compared to 25% in 14-17 year olds. Younger children had higher RQs for PCB 118 and phthalates. This may reflect differences in metabolism and exposure behaviors in young children, i.e., increased contact with environmental contaminants (Etzel, 2020). Higher phthalate exposure may result, for instance, from the use of plastic baby bottles, teething rings, toys (Braun et al., 2013). PCBs persist in household dust and on surfaces (Harrad et al., 2006), and because younger children often play on the floor and engage in hand-to-mouth behaviour, exposure levels of PCBs may be higher. In contrast, older children showed higher RQ for acrylamide, potentially linked to tobacco smoke or dietary factors such as the consumption of fried starch-based foods (Etzel, 2020). In the previous cumulative risk assessment on anti-androgenic phthalates in the HBM4EU Aligned Studies (Lange et al., 2022), no significant age-related differences in risk were found. However, the analysis used a binary age variable, grouping children aged 6-11 years and adolescents aged 12-18 years. The current study includes younger children (ages 3-5 years), where we observed the highest prevalence of elevated phthalate exposure and the largest age-related differences in HI, highlighting the importance of including younger age groups in mixture risk assessments.

In addition to age, socioeconomic disparities were observed, with 39% of individuals from lower SES backgrounds exhibiting HI > 1, compared to 25% in the high SES group. Acrylamide exposure appeared to be particularly higher in the lower SES group. This is likely attributable to the higher tobacco smoke exposure in the low SES group as both active smoking and second-hand tobacco smoke exposure is highest in low SES individuals (Hahn et al., 2023), and AAMA exposure in smokers was more than double compared to non-smokers (Schwedler et al., 2021). SES is also known to influence dietary choices, and studies have reported higher consumption of (ultra-)processed foods among lower SES groups (Baraldi et al., 2018; Hosseinpour-Niazi et al., 2024; Marchese et al., 2022). Limited access to affordable, healthy foods may lead to greater reliance on (ultra-)processed foods, which are typically high in acrylamide (Martinez Steele et al., 2023). Additionally, other environmental factors, e.g. poor housing quality or proximity to certain

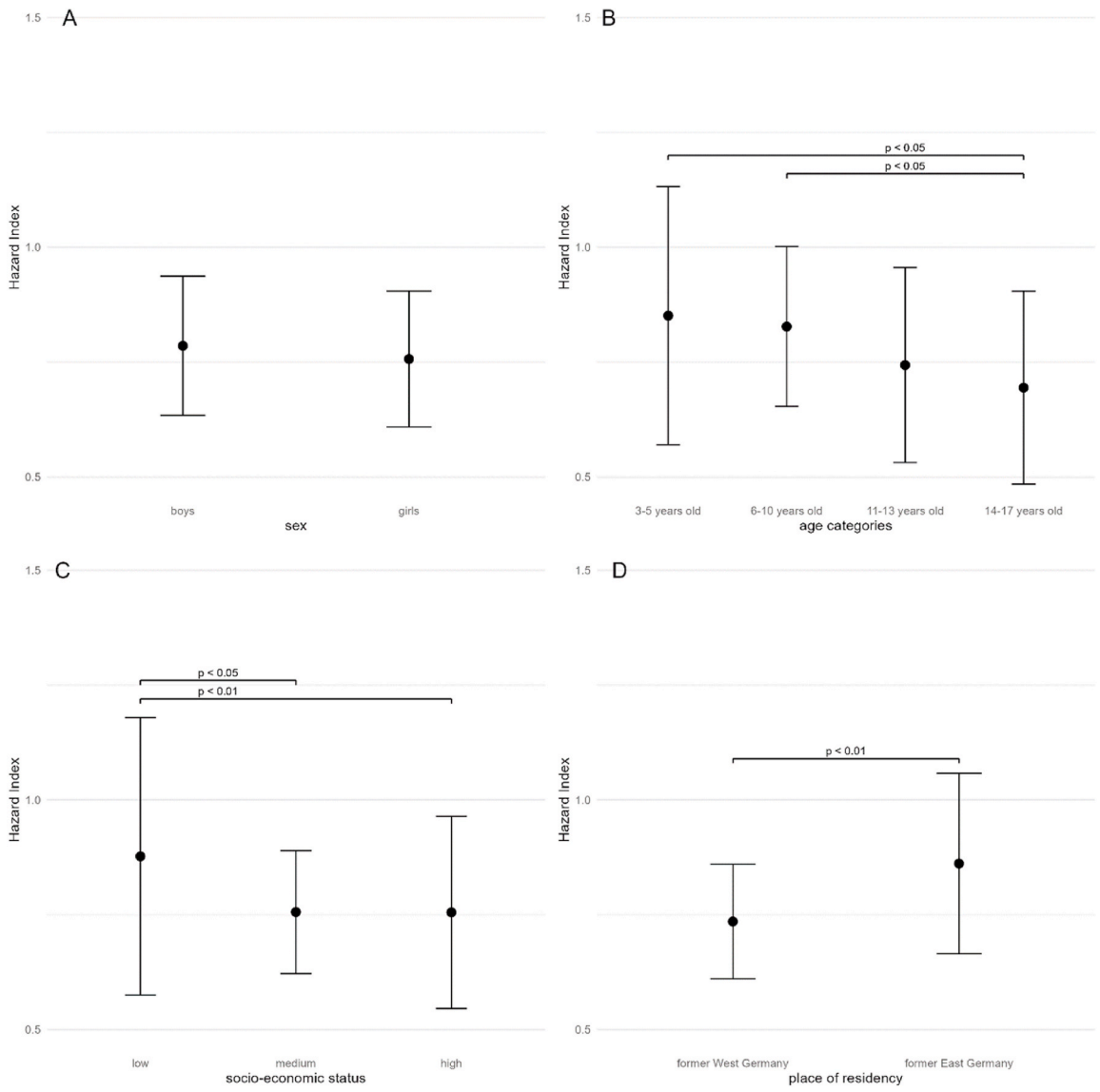

Fig. 2. Results of the multivariate linear regression analysis using GLMs for various socioeconomic subgroups. Dots represent the geometric mean (GM), vertical bars the 95% confidence interval of the GM. Statistically significant differences between subgroups are given by the horizontal bars.

industries, may contribute to increased acrylamide exposure in lower SES groups. Conversely, higher SES groups showed greater exposure to DnBP, possibly due to more frequent use of consumer products such as personal care products containing phthalates (Bloom et al., 2024). These patterns suggest that SES influences not only lifestyle choices but also access and use of products, which can impact exposure to various environmental chemicals. Understanding these nuances is essential for developing targeted interventions that address both economic and exposure inequalities.

Geographic differences in HI point to regional factors influencing exposure. Higher exposure to acrylamide in former West Germany and higher DnBP and DiBP levels in former East Germany may reflect variations in industrial practices, food processing, or consumer habits. In previous analyses within this study population, elevated DnBP in former East Germany were positively associated with the presence of polyvinyl chloride (PVC) flooring (Schwedler et al., 2020). Our findings on geographic differences align with European-wide patterns. In the previous study on cumulative phthalate exposure, the Eastern European region had the highest HI among European regions, and DnBP was clearly the main driver (Lange et al., 2022). These findings suggest that phthalate usage patterns may have persisted beyond reunification of former East and West Germany, and that specific applications, i.e., the presence of materials like PVC flooring common in Eastern European countries, may still play a significant role in current exposure levels. As of 2020, plasticizers like DnBP and DiBP have faced stricter regulation under REACH due to their classification as substances of very high concern, given their reproductive toxicity and potential endocrine-disrupting effects. With the Partnership for the Assessment of Risks from Chemicals (PARC, www.eu-parc.eu) currently underway, recent assessments of phthalate exposure in Europe will help determine whether the regulatory changes are effective towards reducing exposure of the general population to regulated phthalates. Overall, the observed patterns highlight the complex interaction between age, socioeconomic factors, geographic region, and environmental exposure, suggesting that mixture risk assessments must account for these factors.

Our findings shed light on the critical need to assess mixtures of substances rather than evaluating substances on an individual level. Traditional single-substance risk assessments often overlook combined effects of multiple exposures (Bopp et al., 2019). This is evident in our study, where 26% of participants in Region III (those with a HI >1 but individual RQs of <1) would not have been flagged as at-risk under a single-substance approach. Such gaps highlight the limitations of

Table 4
Risk Quotients and Hazard Index for the individual chemicals.

Chemical	Metabolite concentration (µg/L)		Risk Quotient		
	Geometric mean (95% CI)	Max	Geometric mean (95% CI)	Max	n RQmax >1
PCB 118	0.02 (0.02, 0.02)	0.097	0.07 (0.07, 0.07)	0.40	0
DEHP	22.23 (20.86, 23.70)	426	0.06 (0.05, 0.06)	1.12	1
DnBP	20.13 (18.93, 21.41)	361	0.17 (0.16, 0.18)	3.00	14
DiBP	24.95 (23.25, 26.76)	2280	0.16 (0.15, 0.17)	14.25	23
BBZP	2.98 (2.74, 3.25)	156	0.00 (0.00, 0.00)	0.08	0
DiNP	12.44 (11.52, 13.43)	1832	0.04 (0.03, 0.04)	5.39	2
DCHP	0.15 (0.15, 0.15)	88.7	0.00 (0.00, 0.00)	0.06	0
DnPeP	0.15 (0.15, 0.16)	13.3	0.00 (0.00, 0.00)	0.01	0
Acrylamide	75.83 (71.22, 80.75)	1260	0.17 (0.16, 0.18)	2.82	12
Hazard Index (sum)			0.76 (0.72, 0.80)	15.56	

Abbreviations: CI: confidence interval.

current regulatory frameworks in capturing the full extent of chemical exposure across Europe, particularly for vulnerable populations. By implementing mixture-based risk assessment models, we can achieve a more comprehensive evaluation of cumulative exposure risks. Although the vast number of chemicals in use and the limited toxicity information makes mixture risk assessment challenging (Bopp et al., 2019), ongoing initiatives like the PANORAMIX project are advancing this field (Escher et al., 2022).

Despite the high HI observed in our study population, and despite being one of the first studies to include such a broad range of chemicals measured within the same individuals, it is likely that our assessment did not capture the full extent of the issue. This suggests that the actual HI under real-world conditions may be even higher than what we observed. As our approach required exposure and toxicity data, we were not able to include all relevant substances contributing to reduced semen quality risk. For instance, we included PCB 118 as it was measured in our sample, but Ermler and Kortenkamp (2022) and Kortenkamp et al. (2022) have suggested that other PCB congeners also play a role. We selected the largest sub-sample with the greatest number of relevant chemicals measured to maximize our data robustness. However, this choice limited our ability to include other known anti-androgenic substances, such as bisphenol A, which have established evidence of contributing to mixture risk (Kortenkamp et al., 2022). Although these substances were measured in this study, they were assessed in different subsamples and thus excluded from our mixture risk assessment. Future biomonitoring studies could aim to include a wider range of chemicals within the same sample of individuals, enabling a more comprehensive evaluation of exposures to all relevant substances.

Further methodological limitations that affect the interpretation of our findings should be acknowledged. For DCHP and DnPeP, there is considerable uncertainty in the derivation of pHBM-GV$_{GenPop-MRA}$, as no experimentally determined F_{UE} was available. Although these substances are known to be highly potent (Furr et al., 2014), their derived values appear relatively high compared to other phthalates included in this study. This discrepancy may be explained by the limited toxicological data available for these substances and therefore also uncertainty surrounding their toxicokinetic properties. In addition, their detection frequencies were low, which could indicate either limited exposure or concentrations below LOQ. Among the few samples with concentrations above the LOQ, the levels were mainly low. Therefore, these substances did not contribute meaningfully to the overall mixture risk in our study. A similar issue applied to PCBs, for which the reference value from the literature (Ermler and Kortenkamp, 2022) was not defined for internal exposure. We therefore approximated the compatible value, adding further uncertainty to the risk estimate. Another limitation is that each participant in the study was sampled only once, with no repeated measurements. As such, intra-individual variability may lead to exposure misclassification. In addition, since the HBM-GVs we used to estimate the HI are based on male reproductive health effects from gestational exposure, the risk may be conservatively overestimated for non-pregnant individuals, such as children and adolescents, and females that have a different hormonal profile. While our approach may not precisely reflect the specific risks for these groups, it provides a protective benchmark by accounting for potential cumulative effects, which also applies to vulnerable populations such as individuals with pre-existing health conditions. This limitation highlights the need for population-specific guidance values that could improve the precision of risk assessments across different age groups and demographic profiles.

5. Conclusion

In this work we performed a mixture risk assessment for nine anti-androgenic substances measured in a subsample of the population-representative GerES V. 31% of the individuals had a HI greater than 1, i.e. elevated risk for adverse effects regarding reproduction. The majority of those, i.e. 26% of the sample, did not exceed any of the

Table 5
Percentage of study population in which the respective substances reached RQ_{max}.

	PCB 118	DEHP	DnBP	DiBP	BBzP	DINP	DCHP	DnPeP	Acrylamide
Total	5.1	1.8	32.1	22.2	0	2.3	0	0	36.6
Sex									
Boys	4.3	2.1	30.7	23.5	0	1.4	0	0	37.9
Girls	5.7	1.4	33.5	20.9	0	3.2	0	0	35.2
Age group									
3-5 years	10.1	4.0	35.1	25.4	0	2.8	0	0	22.6
6-10 years	2.9	1.9	39.1	25.2	0	2.6	0	0	28.3
11-13 years	1.8	1.3	29.6	19.4	0	1.3	0	0	46.8
14-17 years	6.8	0.8	24.4	19.2	0	2.4	0	0	46.5
Socioeconomic status									
Low	4.9	2.0	27.6	21.9	0	5.1	0	0	38.5
Medium	5.0	1.6	31.8	23.6	0	1.1	0	0	36.8
High	5.4	2.1	37.6	18.3	0	2.6	0	0	33.9
Region of residence									
Former West Germany	5.4	2.0	29.4	21.4	0	2.4	0	0	39.5
Former East Germany	3.2	0.9	46.8	26.9	0	1.8	0	0	20.5

individual HBM-$GV_{GenPop-MRA}$ and would thus have gone unnoticed in a single substance risk assessment. This highlights the need for MRA complementing traditional single substance approaches. To facilitate MRA, a broad assessment of multiple biomarkers in the same sample population is key. Measuring substances only in a subpopulation saves valuable sample material and reduces costs of HBM programs, but hampers MRA. The MRA for anti-androgenic effects performed here already reveals elevated risk for a considerable fraction of the population, but real-world conditions pose even greater risk due to exposure to further anti-androgenic substances. Interestingly, among the main risk drivers of our analysis were DnBP and DiBP, two phthalates that have been banned from consumer products in 2020 (and thus only after the sampling of GerES V which ended in 2017). Recent population surveys such as the German Environmental Survey for Adults 2023–2024 (GerES VI) and the PARC Aligned Studies conducted 2023–2026 on children, adolescents, and young adults, will reveal how exposure to these anti-androgenic phthalates, among many others, since developed within Germany and Europe.

CRediT authorship contribution statement

Liselot Koelman: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Rosa Lange:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Marike Kolossa-Gehring:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Aline Murawski:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Appendix A. Supplementary data

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