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Cumulative risk assessment of five phthalates in European children and adolescents

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ABSTRACT

The European Human Biomonitoring Initiative (HBM4EU) assessed human biomonitoring data on phthalates in children and adolescents, that were sampled between 2014 and 2021, in a harmonised way. These so-called "HBM4EU Aligned Studies" revealed that almost all children and adolescents were exposed to multiple phthalates concurrently. Some phthalates have been shown to act in a dose-additive manner, thus, a mixture risk assessment is warranted. In our study, we determine the risk from combined exposure to five anti-androgenic phthalates, namely DEHP, DiBP, DnBP, BBzP and DiNP by making use of the hazard index (HI) approach. Toxicologically-based human biomonitoring guidance values (HBM-GVs) derived within the framework of HBM4EU served as basis. Our results show that exposures of 17% of children and adolescents from twelve European countries resulted in hazard indices (HI) > 1 with an HI of 1.77 at the 95th percentile (geometric mean,

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GM = 0.44). Main drivers for the mixture risk are DnBP and DiBP. Generalized Linear Model (GLM) analysis including four major exposure determinants (age, sex, European region, sampling year) simultaneously reveal differences for the European regions and between sampling years. Children and adolescents living in the Eastern region of Europe have on average, higher HIs (GM=0.58) than in the Southern region (GM = 0.36) and Western region (GM = 0.42). Moreover, participants from which urine samples were taken in the earlier years (2014–2016) seem to have higher average HI levels than participants from studies with later sampling periods. Strikingly, the majority (63%) of participants with HIs > 1 would have gone unnoticed in single substance risk assessments as individual phthalates levels were below corresponding HBM-GVs. Thus, our results underline the importance of mixture risk assessment approaches to adequately address risks from concurrent chemical exposure.

Abbreviations

3xG	Health - Municipalities - Birth Study (Belgium, BE)
BEA	Biomonitoring in Adolescents Study (Spain, ES)
CELSPAC:TE	Central European Longitudinal Study of Parents and Children: Teenagers (Czech Republic, CZ)
CROME	Cross-Mediterranean Environment and Health Network Study (Greece, EL)
ESTEBAN	Health study on environment, biomonitoring, physical activity and nutrition (France, FR)
FLEHS IV	4th cycle of the Flemish Environment and Health Survey (Belgium, BE)
GerES V-sub (unweighted)	5th cycle of the German Environmental Survey (subsample, unweighted data, Germany, DE)
GM	Geometric mean
HBM	Human Biomonitoring
HBM4EU	The European Human Biomonitoring Initiative
HBM-GV	Human Biomonitoring Guidance Value
HI	Hazard index
ICI/EQUAS	European interlaboratory comparison investigations (ICI) and external quality assurance schemes (EQUAS)
InAirQ	Transnational Adaption Actions for Integrated Indoor Air Quality Management Study (Hungary, HU)
MCR	Maximum Cumulative Ratio
MRA	Mixture Risk Assessment
NEB II	Norwegian Environmental Biobank II (Norway, NO)
OCC	Odense Child Cohort (Denmark, DK)
PCB cohort follow-up	Endocrine disruptors and health in children and teenagers in Slovakia study (follow-up study, Slovakia, SK)
RQ	Risk Quotient
SLO CRP	Exposure of children and adolescents to selected chemicals through their habitat environment study (Slovenia, SI)
SPECIMEn-NL	Survey on Pesticide Mixtures in Europe (The Netherlands, NL)
SVHC	Substances of Very High Concern

1. Introduction

HBM4EU is a joint project funded under the Horizon 2020 programme designed to advance and harmonise Human Biomonitoring (HBM) in Europe (HBM4EU, 2017–2022). A main achievement is the so-called "HBM4EU Aligned Studies". A sampling frame has been developed and later been implemented by aligning existing national and regional HBM studies to meet a common goal, that is the assessment of human biomonitoring data on environmental chemicals in a harmonised way (Gilles et al., 2021, 2022).

One of the first prioritised chemical substance group under HBM4EU were phthalates (Ougier et al., 2021). Phthalates are used as plasticisers to soften poly vinyl chloride (PVC) and are or used to be applied in a variety of consumer products, such as cosmetics, food packages, medicinal products, textiles, toys, and footwear (European Chemicals Agency (ECHA) and Danish Environmental Protection Agency, 2016; EFSA, 2019). Several phthalates have endocrine disrupting properties and are classified in the European Union as reproductive toxicants, category 1B ("May damage fertility and/or the unborn child") under regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures. As a result, they are identified as substances of very high concern (SVHC) (ECHA, 2022) and are subject to various regulations in the European Union. Animal studies have revealed that exposure to certain phthalates affects fertility and reproduction of both sexes (US CPSC, 2014; European Chemicals Agency (ECHA) and Danish Environmental Protection Agency, 2016; NRC, 2008; Yost et al., 2019). Most susceptible for phthalate toxicity is the male offspring if exposed prenatally: *in utero* exposure to e.g. diethylhexyl phthalate (DEHP), diisobutyl phthalate (DiBP), di-n-butyl phthalate (DnBP) and butyl benzylphthalate (BBzP) during the critical window of sexual development induces various irreversible malformations and alterations of the reproductive tract of the male rat offspring, which are summarized under the term "phthalate syndrome" (Conley et al., 2021; European Chemicals Agency (ECHA) and Danish Environmental Protection Agency, 2016; ECHA, 2017a; EFSA, 2019; German HBM Commission, 2011; NRC, 2008; US CPSC, 2014). Observed effects include hypospadias, cryptorchidism, testicular and epididymal malformations, but also reduced sperm count and reduced anogenital distance (Gray et al., 2000; Gray and Butterworth, 1980; Hannas et al., 2011; Howdeshell et al., 2008a, 2015). The occurrence of the same effects after exposure to different phthalates observed in animal experiments led to the assumption that this group of substances may act via the same mechanism and thus might have cumulative effects. Phthalates suppress testosterone and insulin-like 3 hormone production, androgens crucial for male sexual development (Gray et al., 2000; Howdeshell et al., 2008a). More than 10 years ago, the National Academies of Science National Research Council Committee (NRC) recommended to assess the risk from exposure to reproductive phthalates together by using a dose-addition approach (NRC, 2008). Phthalate mixture studies in rodents confirmed the cumulative effects of phthalates already at low doses for the individual chemical (Conley et al., 2021; Furr et al., 2014; Hannas et al., 2011; Howdeshell et al., 2007, 2008b, 2015, 2017). It can be assumed that the assessment of cumulative mixture effects is relevant for humans exposed to several phthalates at the same time. Besides the concurrent exposure to reprotoxic phthalates, scientist have expressed their concerns about the vast number of endocrine disrupting chemicals the general population is exposed to (Howdeshell et al., 2017; Kortenkamp, 2007, 2008; Orton et al., 2014). It has been shown in animal studies that beyond the group of phthalates also other anti-androgenic substances that disrupt male reproductive tract development act in a dose-additive manner and thus contribute to the cumulative risk. This is even true if the individual substances act via different mechanisms of action to disrupt the androgen-mediated pathway or even via

completely different pathways (Conley et al., 2018, 2021; Howdeshell et al., 2017; Rider et al., 2010). To evaluate the risk of possible health effects from exposure to reprotoxic phthalates in European children and adolescents, within HBM4EU, health-related human biomonitoring guidance values (HBM-GVs) for the general population (HBM-GV_{GenPop}) were derived for five phthalates, namely DEHP, DnBP, DiBP, BBzP and di-(2-propylheptyl)phthalate (DPHP) (Lange et al., 2021). These values refer to the urinary concentration of the specific biomarker(s) of a phthalate at and below which, according to current knowledge, no risk of health impairment is anticipated. HBM-GVs can directly be compared with the urinary biomarker concentrations gathered in HBM studies (Apel et al., 2020b). Since the developing organism is most sensitive to the toxicological effects from phthalates, it is necessary to prevent the foetus from phthalate exposure, but also to protect children and adolescents as these are among the most vulnerable populations. Therefore, in the present study, a mixture risk assessment (MRA) was conducted by using the hazard index (HI) approach as straightforward approach making use of harmonised European human biomonitoring data and the HBM-GVs already derived in HBM4EU. Generically, the HI is the sum of risk quotients of the individual substances (RQ_i), which represent the exposure level divided by a toxic potency measure. For HI < 1, it is assumed that there is no concern for cumulative mixture effects (EFSA Scientific Committee et al., 2019; NRC, 2008; Teuschler and Hertzberg, 1995). The five phthalates, included in the MRA (DEHP, DnBP, DiBP, BBzP and DiNP), were selected based on their common reprotoxic properties and on their co-occurrence in the European subpopulations (Cullen et al., 2017; Hond et al., 2015; Husøy et al., 2019; Santé Publique France, 2019; Schoeters et al., 2017; Schwedler et al., 2020). HBM-GV_{GenPop} were utilised to assess the cumulative risk posed by the five reprotoxic phthalates in HBM data of European children and adolescents from the HBM4EU Aligned Studies. We also apply and discuss the use of additional "precautionary factors" on the HI to account for concurrent exposures to other anti-androgenic phthalates and other substances not included in the phthalate MRA as previously suggested by Apel et al. (2020a) and Kortenkamp and Koch (2020).

2. Methods and materials

2.1. Study designs and fieldwork

A sampling strategy for a Europe-wide human biomonitoring survey was established to collect harmonised and quality-controlled HBM data. As few countries already run HBM programs on a regular basis, this strategy intended to build on existing national and regional HBM capacities and infrastructures as much as possible. Therefore, the strategy set up inclusion and exclusion criteria for HBM studies to be harmonised (Gilles et al., 2021). Studies were eligible that i) were already completed, but would provide biobanked samples; ii) that were already initiated before HBM4EU; and iii) studies that hadn't yet started. In addition, the eligible studies were aligned with respect to age group, biomarkers of interest, sampling period (between 2014 and 2021), and were provided with guidelines on post-harmonisation of questionnaires data, matrix, sample population, sample size (maximum n = 300), and sampling process (Gilles et al., 2021). The HBM4EU Aligned Studies include recent phthalate exposure data from two age groups: (i) children aged 6–11 years, and (ii) adolescents aged 12–18 years. Three studies, included children at the age of 12 years in the sample, i.e. GerES V-sub (unweighted), ESTEBAN and PCB cohort (children) as some participants turned 12 at the time of urine collection and the interview in which also age was obtained was conducted prior to sample collection (Gilles et al., 2022). In total, 12 and 11 studies delivered exposure data on up to 15 exposure biomarkers (metabolites) for 10 phthalate diesters in children and adolescents, respectively (Gilles et al., 2021, 2022). Per design recommendation, each study should have sampled as many girls as boys and individuals with different socio-economic status living in cities, towns/suburbs and rural areas should be represented. Different urine sampling types were collected in the participating studies, i.e. random spot urine samples (SU) as well as first morning urine (MU) samples. In the harmonisation processes within the HBM4EU Aligned Studies, MU samples, were defined as samples collected between 6 and 12 o'clock am. Urine samples collected outside this window, were considered as SU. Therefore, for some studies (SLO CRP, GerES V-sub, 3xG, BEA, CELSPAC:TE), both sampling types are applicable as few participants fall outside the time window set for MU. For more details on the study characteristics, see Gilles et al., (2022). To ensure a wide European coverage the sampling strategy stratified Europe into four geographical regions (North, South, West, East) and proposed that the number of studies assigned to a geographical region is proportional to the number of inhabitants for the respective region. It was suggested setting the minimum of studies per European region to be included as follows: at least 2 studies for the Northern region; at least 3 studies for the Southern region; 3–4 studies for the Western region; and at least 1 study for the Eastern region (Gilles et al., 2021). Informed consent was given by all study participants and all studies were approved by ethical committees. For more details, please see Gilles et al. (2022) and Supplementary Material, Table S15.

2.2. Chemical analysis

Prior to chemical analyses, best suitable biomarkers, analytical methods and human matrices for the phthalate substance group was determined by an experts group from the HBM4EU consortium (Vorkamp et al., 2021). Exposure to phthalates were determined by measuring their metabolites (specific exposure biomarker(s)) excreted in urine (Table 1). Within HBM4EU a European network of HBM laboratories was built and a quality assurance/quality control (QA/QC) program was set up, in which HBM laboratories from partner countries were qualified, among others, for phthalate measurements (Esteban López et al., 2021). To ensure the comparability and reliability of the measurements data, chemical analysis should be performed by laboratories that successfully participated in the ICI/EQUAS (European interlaboratory comparison investigations/external quality assurance schemes) of HBM4EU. As the analytical laboratory could choose for which metabolite it participated in the ICI/EQUAS, each metabolite measurement is given a data quality label (A-D). Data that were analysed by an analytical laboratory that passed the ICI/EQUAS were labelled "Biomarker data quality assured by HBM4EU QA/QC" (data quality label A). If participation was not successful, the data quality label "Biomarker data not quality assured by HBM4EU QA/QC" (data quality label C) was given. For some studies, sample analyses were performed prior to the HBM4EU project. To use as much data as possible, the quality assurance unit (QAU) within HBM4EU were asked how to evaluate the analytical results gathered outside HBM4EU. If the analytical laboratory that analysed the samples outside HBM4EU did later successfully participated in the ICI/EQUAS within HBM4EU, the data quality label "Biomarker data generated before HBM4EU QA/QC program but deemed comparable by HBM4EU QAU" (data quality label B) was assigned, otherwise it was labelled "Biomarker data generated before HBM4EU QA/QC program but comparability not guaranteed by HBM4EU QAU" (data quality label D) (Gilles et al., 2021). All laboratories determined phthalate metabolites in urine by liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Vorkamp et al., 2021).

2.3. Study and biomarker selection for the MRA

Only exposure data on metabolites for the five selected phthalates (DEHP, DiBP, DnBP, BBzP and DiNP) included in the MRA were considered (Table 1). As the exposure (i.e. metabolite concentration) is directly compared to HBM-GV_{GenPop} derived for the respective metabolite or metabolite combination (Table 1), only studies that delivered data on a minimum set of metabolites were included: mono-benzyl

Table 1
Phthalate compounds and respective exposure biomarkers included in the mixture risk assessment measured in the HBM4EU Aligned Studies and their corresponding HBM-GV_{GenPop}.

Parent compound	Abbreviation	CAS-Number	Specific exposure biomarker (s)	Abbreviation	HBM-GV_{GenPop} children in mg/L	HBM-GV_{GenPop} adults incl. adolescents in mg/L
Di(2-ethylhexyl) phthalate	DEHP	117-81-7	Mono(2-ethyl-5-hydroxyhexyl) phthalate	5-OH-MEHP	For the sum of 5-oxo- & 5-OH-MEHP:	For the sum of 5-oxo- & 5-OH-MEHP:
			Mono(2-ethyl-5-oxohexyl) phthalate	5-oxo-MEHP	0.34	0.5
			Mono(2-ethyl-5-carboxypentyl) phthalate	5-cx-MEPP	For the sum of 5-cx-MEPP & 5-OH-MEHP: 0.38	For the sum of 5-cx-MEPP & 5-OH-MEHP: 0.57
Butylbenzyl phthalate	BBzP	85-68-7	Mono-benzyl phthalate	MBzP	2.0	3.0
Di-n-butyl phthalate	DnBP	84-74-2	Mono-n-butyl phthalate	MnBP	0.12	0.19
Diisobutyl phthalate	DiBP	84-69-5	Mono-iso-butyl phthalate	MiBP	0.16	0.23
Diisononyl phthalate	DiNP	28553-12-0, 68515-48-0	Mono(4-methyl-7-hydroxyoctyl) phthalate	OH-MiNP	pHBM-$GV_{GenPop\text{-}MRA}$* for the sum of cx- & OH-MiNP:	pHBM-$GV_{GenPop\text{-}MRA}$* for the sum of cx- & OH-MiNP:
			Mono(2,7-methyl-7carboxy-heptyl) phthalate	cx-MiNP	0.34	0.51

CAS = Chemical Abstract Service, HBM-GV_{GenPop} = Human biomonitoring guidance value for the general population.
*These are not HBM-GVs derived within HBM4EU and therefore did not undergo a consolidation process with experts from the member countries. For this reason, there are labelled provisional (p) and with MRA (mixture risk assessment). These values were only derived for the purpose of a mixture risk assessment and cannot be used in single substance risk assessment.

phthalate (MBzP), mono-iso-butyl phthalate (MiBP), mono-n-butyl phthalate (MnBP), mono(2,7-methyl-7carboxy-heptyl) phthalate (cx-MiNP), mono(4-methyl-7-hydroxyoctyl) phthalate (OH-MiNP), mono(2-ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP), and either mono(2-ethyl-5-carboxypentyl) phthalate (5-cx-MEPP) or mono(2-ethyl-5-oxohexyl) phthalate (5-oxo-MEHP). For DiNP and DEHP, the HBM-GV_{GenPop} is given for a sum of two oxidised metabolites (in μg/L), i.e. ∑OH-MiNP, cx-MiNP and either ∑5-OH-MEHP, 5-oxo-MEHP or ∑5-OH-MEHP, 5-cx-MEPP with a preference given to the former metabolite concentration. Therefore, the exposure to DiNP and DEHP expressed as the sum (in μg/L) of the two oxidised metabolite concentrations and calculated as such. For three participants, information on 5-oxo-MEHP

Table 2a
Children data sets and main study characteristics included in the phthalate cumulative risk assessment.

European Region	Country	Study Name	N	Sampling Year	Urinary sample type
North	Norway	NEB II	300	2016–2017	First morning urine
	Denmark	OCC	300	2018–2019	Random spot urine
South	Slovenia	SLO CRP	149	2018	First morning urine, Random spot urine
	Greece	CROME	161	2020–2021	First morning urine
East	Hungary	InAirQ	262	2017–2018	Random spot urine
West	France	ESTEBAN	286	2014–2016	First morning urine
	Germany	GerES V-sub (unweighted)	300	2015–2017	First morning urine, Random spot urine
	Belgium	3xG	133	2019–2020	First morning urine, Random spot urine
	The Netherlands	SPECIMEn-NL	89	2020	Random spot urine

N = number of study participants. 3xG = Health – Municipalities – Births study (BE), CROME = Cross-Mediterranean Environment and Health Network study (EL), ESTEBAN = Health study on environment, biomonitoring, physical activity and nutrition study (FR), GerES V-sub (unweighted) = German Environmental Survey 2014–2017 subsample (DE), InAirQ = Transnational Adaption Actions for Integrated Indoor Air Quality Management study (HU), NEB II = Norwegian Environmental Biobank II (NO), OCC = Odense Child Cohort (DK), SLO CRP = Exposure of children and adolescents to selected chemicals through their habitat environment study (SI), SPECIMEn-NL = Survey on PEstiCide Mixtures in Europe (NL).

Table 2b
Adolescents data sets main study characteristics included in the phthalate cumulative risk assessment.

European Region	Country	Study Name	N	Sampling year	Urinary sample type
North	Norway	NEB II	181	2016–2017	Random spot urine
South	Greece	CROME	150	2020–2021	First morning urine
	Slovenia	SLO CRP	96	2018	First morning urine, Random spot urine
	Spain	BEA	300	2017–2018	First morning urine, Random spot urine
East	Czech Republic	CELSPAC: TE	300	2019–2020	First morning urine, Random spot urine
	Slovakia	PCB cohort follow-up	287	2019–2020	Random spot urine
West	Belgium	FLEHS IV	300	2017–2018	Random spot urine
	France	ESTEBAN	304	2014–2016	First morning urine
	Germany	GerES V -sub (unweighted)	300	2015–2017	First morning urine, Random spot urine

N = number of study participants. BEA = Biomonitoring in Adolescents study (ES), CELSPAC:TE = Central European Longitudinal Studies of Parents and Children: Teenagers (CZ), CROME = Cross-Mediterranean Environment and Health Network study (EL), ESTEBAN = Health study on environment, biomonitoring, physical activity and nutrition study (FR), FLEHS IV = Flemish Environment and Health Study IV (BE), GerES V-sub (unweighted) = German Environmental Survey 2014–2017 subsample (DE), NEB II = Norwegian Environmental Biobank II (NO), PCB cohort follow-up = Endocrine disruptors and health in children and teenagers in Slovakia (SK), SLO CRP = Exposure of children and adolescents to selected chemicals through their habitat environment study (SI).

was not available, thus, the sum of 5-OH-MEHP and 5-cx-MEPP was calculated instead.

A prerequisite for the HI approach is the availability of concurrent exposure data for all substances included in the MRA. Therefore, three datasets for children (Poland, Slovakia, Italy) and two for adolescents (Sweden, Poland) had to be excluded as they did not measure all required metabolites. As a result, the European coverage as suggested by the HBM4EU sampling strategy is not fully met neither for children, nor for adolescents. For the children subsample, only two studies from the Southern region (instead of three) and for the adolescent subsample, only one study from the Northern region (instead of two) met the requirements. An overview of studies in children and adolescents included in the current MRA and their main study characteristics can be found in Table 2a,b, respectively. For our study to ensure a European coverage, it was therefore decided that at least one study per European region must be included. In very few exceptional cases single metabolite measurements which did not meet HBM4EU QA/QC criteria and/or data were obtained before organising the HBM4EU QA/QC program, and methods were not included (data quality label C&D) were used. Otherwise, the complete data set of that study would have to be excluded although all other metabolite measurement data were quality assured by HBM4EU's QAU. It was decided to include these measurements for an exemplary assessment of the phthalate mixture risk in European children and adolescents to make use of as much quality-assured data as possible. The majority of biomarker data was quality assured by HBM4EU QA/QC (data quality label A&B) with 97.2% of the metabolite measurements in children and 94.1% in adolescents. Analytical results from GerES V-sub (unweighted) were obtained prior to HBM4EU and were deemed comparable by the HBM4EU QAU. Data included in the current analyses with data quality labels C and D were i) for children: MBzP measurements from the OCC cohort (DK) and cx-MiNP measurements from the NEB-II cohort (NO); and for ii) adolescents: OH-MiNP measurements in the PCB cohort follow-up (SK) and CELSPAC:TE cohort (CZ), cx-MiNP measurements in NEB-II (NO) and MiBP measurements from CELSPAC:TE (CZ) and PCB cohort follow-up (SK).

2.4. Human biomonitoring guidance values (HBM-GVs)

Consolidated HBM-GVs for the general population (HBM-GV_{GenPop}) are available for four of the five substances addressed in the current MRA, namely DEHP, DnBP, DiBP and BBzP. Detailed information regarding their derivation can be found in Lange et al. (2021). Briefly, HBM-GV_{GenPop} were based on the most sensitive endpoint of each substance. Effects were within the anti-androgenic effect spectrum seen in rat offspring after prenatal exposure. HBM-GV_{GenPop} were derived for two different age groups: children (age 6–13 years) and adults including adolescents (≥14 years). In this study, HBM-GV_{GenPop} for children were applied to individuals of the age 13 and younger, and the HBM-GV_{GenPop} for adults were used for participants aged 14 years and older. For four adolescents (12-18-year old) the exact age in years was not available, thus the HBM-GV_{GenPop} for adults was applied.

To include DiNP in the current MRA, provisional HBM-GV_{GenPop} (each for children and adolescents), solely for the purpose of the present MRA were derived and termed pHBM-$GV_{GenPop\text{-}MRA}$ to make the distinction clear. DiNP has been shown to affect male sexual development in the rat, i.e. suppression of foetal testis testosterone after prenatal exposure (Clewell et al., 2013; Furr et al., 2014; Hannas et al., 2011). The potency of DiNP to decrease foetal testis testosterone, however, was lower compared to DEHP (Hannas et al., 2011). DiNP has been suggested to be included in a MRA for male reproductive health as it is regarded to add to the cumulative risk by decreasing foetal testosterone (Apel et al., 2020a; EFSA, 2019; Kortenkamp, 2020; Kortenkamp and Koch, 2020; US CPSC, 2014). EFSA (2019) and Kortenkamp and Koch (2020) identified the study by Clewell et al. (2013) as critical for reproductive effects on DiNP (EFSA, 2019; Kortenkamp and Koch, 2020). Clewell et al. (2013) exposed pregnant rats by gavage to 0, 50, 250 or 500 mg DiNP/kg bw/d from gestational day 12–19. At a dose of 250 mg/kg bw/d decreased foetal testis testosterone production and multinucleated gonocytes were observed and the dose was identified by the authors as lowest observed effect level (LOEL) and 50 mg/kg bw/day as no observed effect level (NOEL). Kortenkamp and Koch (2020) proposed a reference dose for male reproductive toxicity suitable for a phthalate MRA (RfD_{AA}) of 59 μg/kg bw/d based on a benchmark dose lower bound (5% benchmark response: testosterone suppression; $BMDL_{05}$) of 5.9 mg/kg bw/d for foetal testis testosterone synthesis suppression observed in the study by Clewell et al. (2013) (Kortenkamp and Koch, 2020). According to the derivation strategy of HBM-GV (Apel et al., 2020b), pHBM-$GV_{GenPop\text{-}MRA}$ for DiNP were derived for children and adults including adolescents using i) the $BMDL_{05}$ of 5.9 mg/kg bw/d from Kortenkamp and Koch as TRV-like value; ii) toxicokinetic data (i.e. fractional urinary excretion factors for OH-MiNP and cx-MiNP) from Anderson et al., 2011); and iii) a factor of 10 each for inter-and intra-individual variability. It is important to note, that for the derivation of consolidated HBM-GV_{GenPop}, the most sensitive endpoints for the respective compounds were chosen, while for the pHBM-$GV_{GenPop\text{-}MRA}$ derived for DiNP this is not the case. Instead, a common anti-androgenic reprotoxic endpoint was chosen (i.e. suppression of foetal testicular testosterone synthesis).

2.5. Mixture risk assessment and the use of precautionary factors

For the risk assessment, the concentration of a metabolite or of the sum of metabolites (in μg/L) of each individual phthalate (i.e. ∑DEHP metabolites, MiBP, MnBP, MBzP, ∑DiNP metabolites) was divided by their respective HBM-GV_{GenPop}/pHBM-$GV_{GenPop\text{-}MRA}$ to obtain a risk quotient (RQ) for each substance. RQs for each phthalate included in the MRA were then summed to gain the hazard index (HI) per study participant i according to the following formula:

$$HI_i = RQ_{DnBP,i} + RQ_{BBzP,i} + RQ_{DiBP,i} + RQ_{DEHP,i} + RQ_{DiNP,i}$$

Several different classes of chemicals that act by dose addition in mixture models have recently been compiled and reviewed by Howdeshell et al. (2017). Orton et al. (2014) have compiled 24 current use and environmentally relevant pesticides and 17 non-pesticidal pollutants (e.g. parabens, benzophenones, perfluorooctane sulfonate (PFOS), galaxolide, tonalide, BDE100, 4-MBC, and PCB13) that have anti-androgenic properties and produce combination effects. Kortenkamp (2020) concluded, that a minimum set of chemicals to be assessed together with phthalates includes certain pesticides (vinclozolin, prochloraz, procymidone, linuron), pain killers (paracetamol, aspirin and ibuprofen), some pharmaceuticals (finasteride, ketoconazole, and the lipid-lowering drug simvastin), poly-chlorinated dibenzo-dioxins and other dioxin-like pollutants and phenolics (bisphenol A, butylparaben). Thus, "precautionary factors" of 5 and 10 to account for co-occurring anti-androgenic substances that contribute to the risk of adverse effects on reproduction have previously been applied to the HI (Apel et al., 2020a; Kortenkamp and Koch, 2020), yielding adapted HIs of 0.2 and 0.1. As the evaluation of a mixture risk from all these substances goes beyond the scope of our study, we not only evaluated our data towards the HI of 1 but also to adapted HIs of 0.2 and 0.1. The percentage of participants exceeding these adapted HIs were calculated.

The maximum cumulative ratio (MCR) for each participant was calculated by dividing each participant's HI by their individual maximum RQ (RQ_{max}) as introduced by Price et al. (2012) and applied for phthalate mixture risk assessments by Apel et al. (2020a). Scatterplots depicting MCR vs. HI were created. MCRs are calculated to understand whether the risk from combined exposure is driven by a single chemical or multiple chemicals. For participants with MCR < 2, one phthalate contributes to the majority of the combined risk, whereas the opposite is true for participants with MCR > 2.

Finally, to estimate the effect of major exposure determinants (age, sex, European region, sampling year) on individual HI levels a

multivariate linear regression analysis was done using the Generalized Linear Model (GLM) including all four predictors simultaneously. The model was specified with a log link function to account for the non-normal distribution of HIs (not shown) and survey procedures to account for higher similarity of individuals within each study.

Statistical analyses were performed on individual data in RStudio (RStudio Team, 2021). Values below limits of detection (LOD) and limits of quantification (LOQ) and values between LOQ and LOD were imputed per biomarker and study when there were at least 30% of detected values (applicable to all biomarkers reported here) (Lubin et al., 2004). Details will be found in Govarts et al., (*submitted*).

3. Results and discussion

In our study, exposure data from eight phthalate metabolites analysed in urine samples of 4,198 children and adolescents aged 6–18 years from 12 countries is used (see Table 2a,b). Male and female participants are equally represented, but there are slightly more adolescents (n = 2,218) than children (n = 1,980) (see Table 3). In children, quantification frequencies (QFs) are very high (87% or higher ≥ LOQ) for all, except for DiNP metabolites in Belgian (Flemish) children (see supplementary material, Table S1). In European adolescents QFs are even higher, with each phthalate metabolite quantified in at least 91% of the study subsamples (Table S1). The high QFs confirm the concurrent exposure and thereby verify the selection of DEHP, DnBP, DiBP, DiNP and BBzP for our MRA.

3.1. Mixture risk of phthalates in European children and adolescents

A summary of descriptive statistics on the hazard indices per subsample, including GM, 95th percentiles, corresponding 95th confidence intervals and range can be found in Table 3. On average, exposures of European children and adolescents result in HI at GM of 0.44, suggesting no risks from combined exposure to the five phthalates. At the 95th percentile, however, HI are above the limit of acceptable risk (HI = 1.77). Children show slightly higher average HI (GM = 0.47) than adolescents (GM = 0.41) and *vice versa* at the 95th percentile (HI = 1.71 vs. 1.86) (Table 3). No significant difference in the average level of HI between the age groups (Fig. 3, panel A), nor the sexes (Fig. 3, panel B) is observed in GLM models when controlling for the other three variables.

Fig. 1 shows the analysis of combined exposure of all study participants (children in purple, adolescents in green) with individual HI versus MCR depicted in a scatter plot. One child, depicted to the utmost right, is reaching an HI as high as 119. Analysing the percentage of the population with HI > 1, gives an indication of the population share that exceeds exposures deemed tolerable for the above five phthalates. Our analysis show, that while at population level, no indication of a mixture risk is observed, however, at individual level 17% (n = 708) of all children and adolescents investigated exceeded HI of 1, thus have high

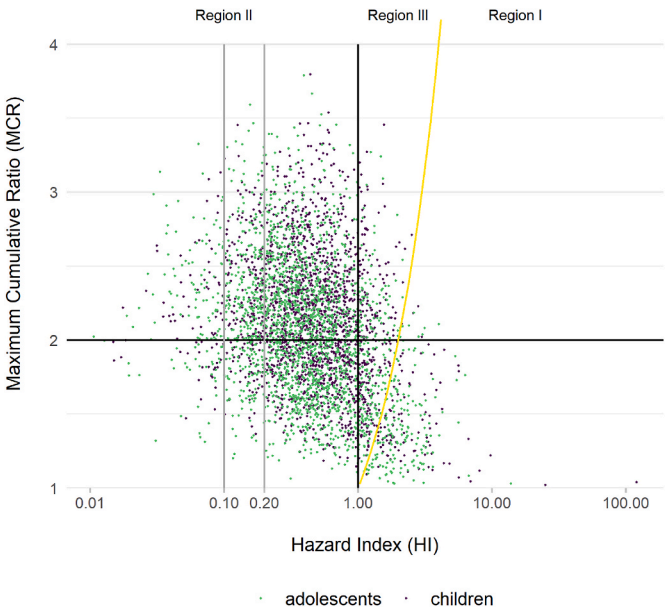

Fig. 1. Analysis of combined exposure to five phthalates in European children and adolescents
Presentation of maximum cumulative ratio (MCR) versus hazard index (HI) as scatter plot according to Apel et al. (2020a) with risk management categories (Region I-III) introduced by Price et al. (2012). Please note, the Region I-III introduced by Price et al. (2012) are not geographical areas as defined in the term "European regions" (referring to East, West, South, North Europe). Dots represent each study participant, with purple dots for children and green dots for adolescents. The horizontal line represents MCR = 2; the black vertical line represents HI = 1; the grey vertical lines represents adapted HI = 0.2 and HI = 0.1; the curved yellow line represents MCR = HI. Definition of regions according to Price: Region I depicts combined exposures of concern as one or more individual chemicals exceed the HBM-GV (area right to yellow line); Region II (area left to black vertical) depicts combined exposures where there is a low concern for both individual chemicals and for their combined effects (HI < 1); Region III (area between black vertical line and yellow curved line) depicts combined exposures with a low concern for individual chemicals, but concern for the combined effects (all RQs < 1, but HI > 1). Dots below the horizontal black line (MCR < 2) represent combined exposures in which one chemical accounts for the majority of combined exposure, whereas dots above the vertical black line (MCR > 2) represent combined exposures driven by multiple chemicals. Individual HIs range from > 0.01 to 119. 17% of the study participants have HIs > 1 of which the majority (~63%) lies in Region III. Thus, they would have gone unnoticed in single substance risk assessment. Approximately 60% of the children and 54% of the adolescents have MCRs above 2, showing that the combined exposure to phthalates is driven by multiple substances instead of only one. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Descriptive statistics for hazard indices (HI) in European children and adolescents.

	N	GM_{HI}	95%th CI	$P95_{HI}$	95%th CI	Min_{HI}	Max_{HI}
Total study sample (6–18 years)	4,198	0.44	0.42–0.45	1.77	1.69–1.86	0.42	1.86
Female	2,098[a]	0.44	0.42–0.46	1.84	1.72–2.07	0.43	2.07
Male	2,097[a]	0.43	0.42–0.45	1.72	1.6–1.84	0.41	1.84
Children (6–11* years)	1,980	0.47	0.45–0.48	1.71	1.58–1.82	0.45	1.82
Adolescents (12–18 years)	2,218	0.41	0.39–0.42	1.86	1.74–1.98	0.38	1.98
Total study by region (6–18 years)							
North	781	0.42	0.4–0.45	1.75	1.48–2.01	0.38	2.01
South	856	0.36	0.34–0.38	1.38	1.19–1.54	0.34	1.54
East	849	0.58	0.55–0.62	2.42	2.16–2.8	0.56	2.8
West	1,712	0.42	0.4–0.44	1.51	1.4–1.67	0.41	1.67

N = number of study participants, GM = geometric mean, CI = confidence interval; Min = minimum value of HI; Max = maximum value of HI. * For ESTEBAN and GerES V-sub (unweighted) 18 and 19 participants, respectively were 12 years old at the time urine samples were collected. [a] For three children, no information on sex is available.

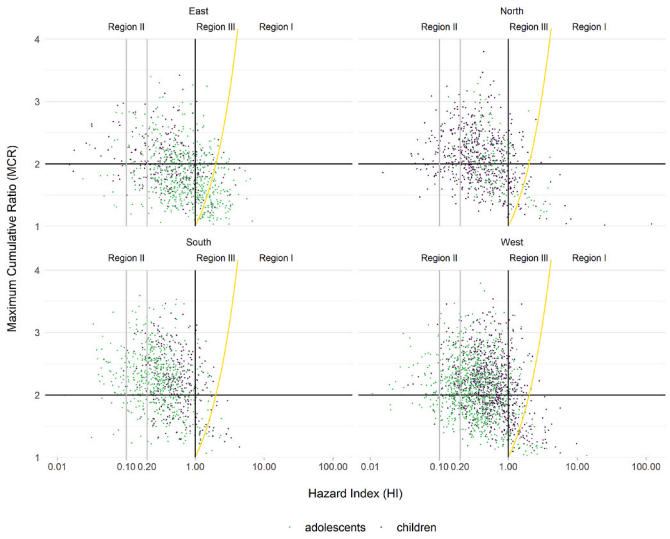

Fig. 2. Analysis of combined exposure to five phthalates per European region

Analysis of combined phthalate exposure for children and adolescents per European region with MCR versus HI scatter plots. The results of the individual study participants are shown as dots (purple = children; green = adolescents). The vertical black line shows HI = 1, the vertical grey lines the adapted HI = 0.2 and HI = 0.1. The horizontal black line depicts an MCR = 2 and the curved yellow line represents MCR = HI. Please note, the Region I-III introduced by Price et al. (2012) are not geographical areas as defined in the term "European regions" (referring to East, West, South, North Europe), but risk management categories (more details can be found in the description of Fig. 2). Highest percentage above HI = 1 is observed for participants from Eastern Europe (31%), whereas the percentages of other European regions with HI > 1 are similar (11–15%). The high percentage in the Eastern region is due to the high percentage of adolescents above HI = 1 (37% versus 17% for children). In Eastern Europe for the majority of children and adolescents, only one phthalate does drive the HI with 64% of the participants having MCR < 2, whereas for Southern Europe the mixture risk is driven by multiple phthalates (with 70% of participants having MCR > 2). For the majority of participants from Northern and Western Europe multiple phthalates contribute to the HI (57% and 60%, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mixture risks from the above five phthalates (Table 4). Depending on age and sex, these exceedances varied between 15 and 19%. The percentage of the two age groups above the hazard indices per study can be found in the supplementary material (Tables S2a and b).

MCRs indicate how many chemicals drive the mixture risk. Slightly more than half of the study population (57%, n = 2,372) have MCR > 2, thus their HI is driven by multiple phthalates rather than by only one compound (MRC < 1) (Fig. 1, MCR = 2 is depicted by the black horizontal line). In comparison, the share of children for which the combined risks are driven by multiple phthalates is higher (60%, n = 1,178) than in adolescents (54%, n = 1,194).

3.2. Drivers of the mixture risk

In the total sample, DnBP and DiBP contribute most to the HIs (Table 5). In about half (52%, n = 2,170) of the study participants the highest risk quotients are observed (RQ_{max}) for DnBP, followed by DiBP in 42% of participants (n = 1,767). The percentage of participants with RQ_{max} for DEHP and DiNP are over ten times lower with 3%. BBzP does not reach RQ_{max} in any of the participants. A similar pattern (DnBP > DiBP) is observed for adolescents (54 and 39%) as for the total sample, while it was slightly different for children. DiBP and DnBP more or less equally contributed to the HI (45% and 49%, respectively, Table 5) of children exposures.

Table 4
Percentage of individuals exceeding respective hazard indices (HIs) per subsample.

	N	% > HI = 1	% > HI = 0.2	% > HI = 0.1
Total study sample (6–18 years)	4,198	17	83	95
Female	2,098[b]	18	82	95
Male	2,097[b]	16	84	96
Children (6–11[a] years)	1,980	17	86	96
Female	974[b]	19	85	96
Male	1,003[b]	16	86	97
Adolescents (12–18 years)	2,218	17	80	94
Female	1,124	18	79	94
Male	1,094	15	81	95
Total study by region (6–18 years)				
North	781	15	83	95
South	856	11	79	94
East	849	31	88	96
West	1,712	14	82	96
Children (6–11[a] years)				
North	600	13	79	93
South	310	18	93	99
East	262	18	78	92
West	808	20	90	99
Adolescents (12–18 years)				
North	181	20	96	100
South	546	7	70	91
East	587	37	92	97
West	904	9	74	93

N = number of study participants; HI = hazard index. All values are rounded.
[a] For ESTEBAN and GerES V-sub (unweighted) 18 and 19 participants, respectively were 12 years old at the time urine samples were collected.
[b] For three children, no information on sex is available.

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

	DnBP	DiBP	DiNP	DEHP
Total study sample (6–18 years)	52	42	3	3
Female	51	43	4	2
Male	52	42	3	3
By region				
North	60	35	4	2
South	43	51	3	4
East	64	30	3	3
West	46	47	4	3
Children *(6–11* years)*	49	45	3	4
Female	47	47	3	2
Male	50	43	2	4
Adolescents *(12–18 years)*	54	39	4	2
Female	55	38	4	2
Male	54	40	3	3

RQ_{max} = maximum risk quotient. The proportion of individuals that reached RQ_{max} are presented for DEHP, DnBP, DiBP and DiNP. BBzP did not reach RQ_{max} in any of the participants. Sums of each row add up to 100%. Deviations are due to rounded values. *For ESTEBAN and GerES V-sub (unweighted) 18 and 19 participants, respectively were 12 years old at the time urine samples were collected.

Follow-up GLM analyses investigating the role of sex and age on HI levels reveal no difference between children and adolescents or between sexes (Fig. 3, panel A and B). Median cumulative risk quotients for all five phthalates and age group can be found in Fig. 4a and b.

Our results are in line with previous findings. In the REACH restriction proposal of phthalates, a combined risk assessment of four phthalates (DEHP, DnBP, DiBP, BBzP) was conducted based on 95th percentile urinary biomonitoring exposure levels of children from DEMOCOPHES data (2011–2012). Whereas BBzP did not at all, DnBP and DiBP contributed most to the risk of combined exposures in European children (ECHA, 2017a; 2017b). Furthermore, in a combined risk

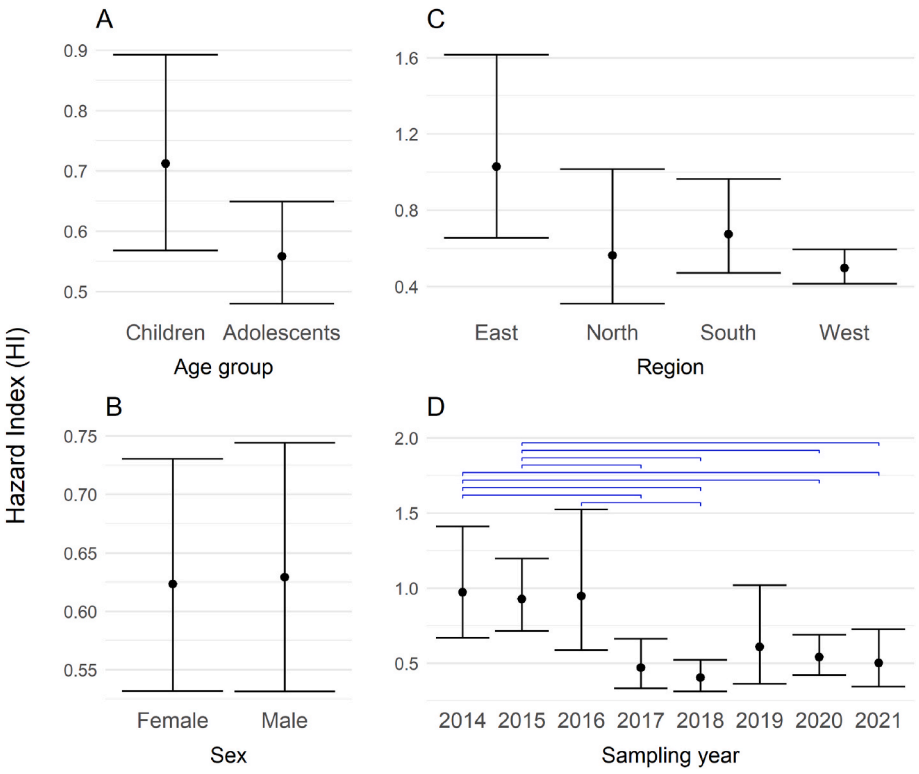

Fig. 3. Results of the multivariate linear regression analysis (GLMs)
Depicted are the results from the multivariate linear regression analysis (GLMs) predicted by age (panel A), sex (panel B), European region (panel C), and sample collection year (panel D) controlling for the other three predictors in each case, respectively. Black dots represent averages of the hazard indices (HI). Black bars represent the corresponding confidence intervals. No statistically significant difference in the level of the HI were obtained for sex or age. Eastern participants have significant higher HI levels than Southern and Western participants. Earlier sample collection years have significant higher HI levels compared to later years (blue brackets). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

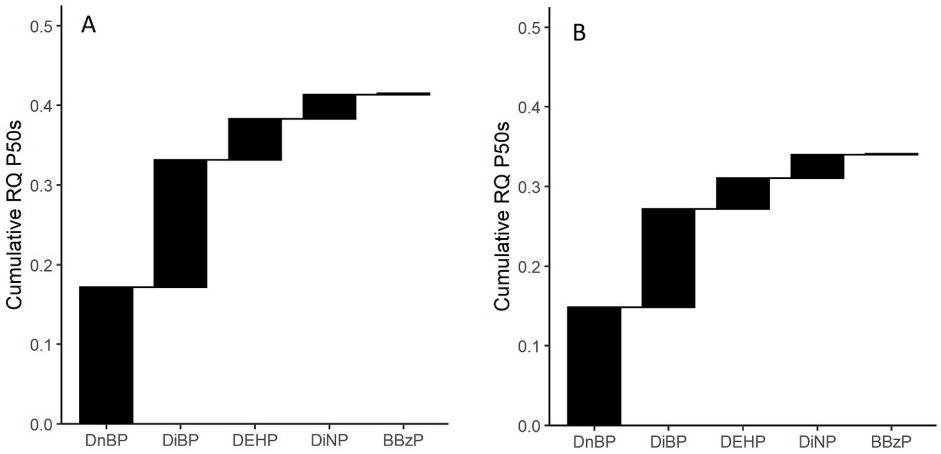

Fig. 4. Median cumulative risk quotients (RQs) for European children and adolescents for five phthalates
The 50th percentiles (P50) of the risk quotients (RQs) per phthalates are displayed (black bars) in a cumulative fashion for the European children (Figure 4Fig. 4a) and adolescent subpopulation (Fig. 4b). DnBP (P50 = 0.17 children; P50 = 0.15 adolescents) and DiBP (P50 = 0.16 in children; P50 = 0.12 in adolescents) are the main drivers for the mixture risk in both subpopulations. Cumulative RQs (corresponds to hazard index) at P50 are for 0.4 children and 0.34 for adolescents.

assessment of the above five phthalates in Finnish adults, also DnBP and DiBP appeared to be the main drivers, whereas in German adults the main risk driver is DEHP, followed by DnBP (Apel et al., 2020a; Porras et al., 2020). As underlying toxicological endpoints and subsequent derived reference doses used as toxic unit values to assess the risk are not the same throughout these risk assessments and these do largely affect which phthalates does drive the mixture risk, comparison need to be done with caution (Kortenkamp and Koch, 2020; Søeborg et al., 2012). Considering only those participants with HI > 1, the two main risk drivers remain DnBP and DiBP. Contributions of phthalates to HIs are very similar: In 51% (n = 364) of the study participants mixture risks are driven by DiBP, followed by DnBP (40%, n = 283), and DEHP and DiNP (4 and 5%). In 6% (n = 260) of the European children and adolescents at least one RQ for any phthalate is > 1, thus having levels exceeding HBM-GVs (Fig. 1, Region I) with more adolescents (7%, n = 161) above respective HBM-GVs, than children (5%, n = 99) (data not shown). Exceedances are mostly observed for DnBP and DiBP with 3% of all participants exceeding the HBM-GV_{GenPop} for DnBP (n = 141) and DiBP (n = 111), and to a lesser extent DiNP and DEHP (≤ 0.5%). Only one child has exposure levels of MBzP above the respective HBM-GV_{GenPop}. Considering only adolescents, the contribution is similar with most exceedances observed for DnBP (5%) > DiBP (2%) > DiNP (0.5%) > DEHP (0.3%). In children, however, most exceedances are observed for DiBP (3%), followed by DnBP (2%) and only few exceeds HBM-GVs for DiNP (0.5%) and DEHP (0.4%). It is noteworthy, that of those individuals who exceeds HBM-GVs, mostly only one phthalate exceeds the respective HBM-GV_{GenPop}. In only few cases an individual exceeds HBM-GV_{GenPop} of two substances at the same time, and only in one case exceeds three substances simultaneously. The pHBM-$GV_{GenPop\text{-}MRA}$ for DiNP is not consolidated within HBM4EU and solely derived for the purpose of the MRA based on a common anti-androgenic endpoint. As for DiNP toxicity the liver is the most sensitive target organ, the HBM-GV is not suitable for single substance risk assessment (EFSA, 2019; Kortenkamp and Koch, 2020). Exceedances of the pHBM-$GV_{GenPop\text{-}MRA}$ were

reported solely for the purpose of reporting the share of DiNP to the mixture risk and do not indicate a risk from adverse effects of DiNP exposure.

Strikingly, of those participants who have HIs >1, the majority (63%, n = 446) would have not been identified as being at risk in traditional single compound risk assessment as all single substances are below HBM-GVs (see Fig. 1, Region III). Comparing the subsamples, this contribution is higher in children with 70% (n = 239), whereas considering only adolescents about half (56%, n = 207) would have gone unnoticed in single substance risk assessments. This highlights the urgent need to include mixture risk assessment approaches in current risk assessment practices on a regular basis. The restriction of four reprotoxic phthalates in consumer articles serves as good example. In the joint proposal by ECHA and the Danish Environmental Agency a cumulative risk assessment based on exposure data for DEHP, DnBP, DiBP and BBzP from the DEMOCOPHES program was conducted and based on its outcome were further restriction in plasticised articles (EC, 2018; European Chemicals Agency ECHA and Danish Environmental Protection Agency, 2016; ECHA 2017a, 2017b).

3.3. Comparison of European regions

Comparison of European regions reveal the Eastern region is markedly different with highest HI at GM of 0.58 (P95 = 2.42). Lowest HI (GM = 0.36, P95 = 1.38) is found in the Southern region, and the Western and Northern region have similar HIs at GM (GM = 0.42, P95 = 1.51 and GM = 0.42, P95 = 1.75, respectively). Results from GLMs indicates that Eastern participants have significant higher average HI levels than Southern and Western participants, when controlling for sex, age group and sampling year (see Fig. 3, panel C). No significances emerged from the pairwise comparison of the Eastern region with the Northern region. Despite the alignment and post-harmonisation of the different studies in the HBM4EU Aligned Studies, some differences remain (Gilles et al., 2021) and consequently, caution must be given in regard to the comparability of results of single data sets. No conclusions can be drawn from single data sets of a country to the whole country itself as data sets used are not nationally representative, even though some subsets used here were based on nationally representative HBM programs (i.e. ESTEBAN, GerES V-sub). Furthermore, the single studies differ in sampling periods, age distribution and study design including urine sampling method (Gilles et al., 2022). Therefore, only European regions comprised of data sets from different countries are compared to one another in this analysis. The influence of differences in study design will decrease to a certain extent by pooling the single data sets into European regions, however, uncertainties remain with regard to the validity of the extrapolation of the results per European region that might lead to over- or underestimation of the real risk for that European region. A major limiting factor for comparability of the HBM4EU Aligned Studies is the large time span of the sample collection period (2014–2021) that might confound the metabolite levels. Indeed, certain phthalates, i.e. DEHP, DnBP, DiBP and BBzP have been shown to decrease considerable over time in European adult populations, whereas a clear trend for DiNP across European adult populations cannot be observed. While DiNP exposures decreased over time in Denmark, no such trend can be observed in recent years in Germany and Sweden (Frederiksen et al., 2020; Gyllenhammar et al., 2017; Koch et al., 2017). However, these time trend studies only included exposures until 2014 (Sweden), 2015 (Germany) and 2017 (Denmark). To investigate the possible impact of temporal changes in exposures to the level of HIs, sampling year as predictor was included in GLM analyses. No linear effect of sampling year on the HI was found, controlling for age, sex and European region, but differences in the pairwise comparison of sampling years emerged as significant (Fig. 3, panel D). Participants whose urine samples were taken in the earlier years (2014–2016) seem to have higher average HI levels than participants from studies with later sampling periods (2017–2021). In detail, the pairwise comparisons suggest that participants whose urine samples were collected in 2014 have, on average, higher HI levels than participants that were sampled in 2017, 2018, 2020 and 2021. The same effects are observed for urine samples from 2015, whereas the only pairwise comparison emerged significant for participants sampled in 2016 is that they have higher average HI levels than participants sampled in 2018. Most of the data from the earlier sampling years (2014–2016) are from studies of the Western region, whereas for later sampling years (2017–2020) pooled data come from all geographical regions. Models also indicate an interaction between European region and sampling year suggesting that for some European regions differences between sampling years are stronger than others. Since the data is based on cross-sectional data from studies with different sampling frames we refrain from going into detail here. The reported effects might be confounded with study characteristics. The sampling years 2014 and 2021 only have data from one study each (ESTEBAN and CROME), not allowing comparisons between data collections. For the sampling years 2016–2020 data from at least three studies per year are pooled, thereby increasing the validity of the observed temporal differences in average HI levels.

Considering the individual level, participants from the Eastern region are most at risk from phthalates mixture exposure with one third (31%) having HIs >1 (Table 4). The other European regions are in the range of 11–15% with the lowest percentage with HI > 1 observed for participants living in Southern Europe (11%). A higher share of children with HI > 1 is observed for children from Southern (18%) and Western Europe (20%) than adolescents from that region (7 and 9%, respectively) and *vice versa* for the Eastern (18 vs. 37%) and Northern (13 vs. 20%) regions (Table 4). Interpretation needs to be drawn with caution since only one study each representing European children in Eastern Europe (InAirQ) and European adolescents in Northern Europe (NEB II). In addition, not all studies provided data on both, children and adolescents. Data on both age groups is only available for GerES V-sub (unweighted), ESTEBAN, SLO-CRP and CROME. Again, apparent differences between the age groups at regional level might be confounded with study characteristics.

Further differences in Eastern Europe are observed as for the majority of Eastern children and adolescents, only one phthalate drives the HI, namely DnBP, with 64% of the participants having MCR < 2, whereas for Southern Europe the mixture risk is driven by multiple phthalates (with 70% of participants having MCR > 2). For the majority of participants from the North and West of Europe multiple phthalates contributes to the HI (57% and 60%, respectively) (see Fig. 2).

In all four European regions, DiBP and DnBP contribute mostly to the HI explaining more than 90% of the HI, whereas the contribution of DEHP and DiNP are negligible (Table 5). In the Eastern and Northern region, the proportion of RQ_{max} for DnBP is twice as high as for DiBP, whereas in the Western region RQ_{max} for DnBP and DiBP are equally distributed. When considering only the subsample of children and adolescents with HI > 1, for most regions DiBP is the main driver of the mixture risk, followed by DnBP, except for the Eastern region (data not shown). Here, DnBP is by far the main driver for which 76% of the children and adolescents reach RQ_{max}. Taken together, this might indicate different usage patterns of phthalates in the European regions, that result in higher exposure levels for Eastern children and adolescents, but this is rather speculative and follow-up studies are needed to investigate possible differences in exposure sources in the European regions. Nevertheless, our results on the identification of risk drivers can serve as basis for setting priorities for further research in terms of regional differences and specifically for risk managers where to start for possible refinement of mitigation measures. DEHP, DnBP, DiBP and BBP are already strictly regulated under REACH. The extension of the REACH restriction to plasticised articles came into force only 2020, thus, data from the HBM4EU Aligned Studies used in our analysis collecting samples in 2014–2021 is not able to show the effectiveness of this restriction in the management of risk. Only one study sampled participants in 2021 (CROME). In addition, these phthalates are still allowed to be used in

food packaging and medicinal products, and with our study it is not possible to inform on the contribution of these (or other) exposure sources to total exposure.

Most exceedances of single guidance values are observed by far in the Eastern region, with 14% (n = 122) of Eastern children and adolescents are above single HBM-GVs. From those, the majority are adolescents (89%) and most exceedances are observed for DnBP (79%). For the other European regions, percentages of exceedance are similar, ranging from 3 to 5% (see Fig. 2, Region I). Here, in contrast to the subsample from Eastern Europe, children have most exceedances (on average 60%) and for all three geographical regions most exceedances are observed for DiBP. More extended information and details on single substance risk assessment of phthalates in European children and adolescents will be found in Vogel et al., (submitted). As can be seen in Fig. 2 in Region III, especially in the Southern and Western region, children and adolescents with high risks from cumulative exposure to the five phthalates would not have been detected in single substances risk assessment (74 and 69%, respectively).

Besides the differences between studies in their sample collection periods, there are other uncertainties that affect the comparability of the studies and consequently the comparability of European regions. In about half of the studies included in our analysis, spot urine samples were collected, and the other half did sample morning urine samples (GerES V-sub, SLO CRP, ESTEBAN, CROME, BEA, 3xG, CELSPAC:TE). Considering the short-half lives of the phthalate metabolites in the human body, both spot and morning urine measurements can only reflect recent exposure and temporary intra-individual variability cannot be assessed using single samples. This may lead to over- or underestimation of exposure depending on the time span between exposure and sampling. Given the likely longer time span between exposure and sample collection with morning urine samples compared to spot urine samples, there might be a difference in dilution of the metabolite concentration between these two sampling methods. However, this is assumed to be negligible as morning urine as well as spot urine sample seem to be as useful as 24h-urine samples for the assessment of phthalate metabolites exposure in population studies (Frederiksen et al., 2013).

3.4. Uncertainties in the health risk assessment

The HBM-GVs are based on the most sensitive effects which are on male reproductive health when the male foetus was exposed gestationally. As the current MRA is conducted for both, the male and (non-pregnant) female subpopulation of children and adolescents and not the foetus, the predicted risk for female participants and for adolescents might be overestimated. However, phthalate exposure at environmental concentrations has been associated with male and female reproductive impairment in humans when exposed as a child or adult (Frederiksen et al., 2012; Jurewicz and Hanke, 2011; Radke et al., 2018, 2019). Despite toxic effects on reproduction and development, epidemiological studies indicate a possible association of phthalate exposure (DEHP, DnBP, DiBP) and obesity, diabetes and insulin resistance (Dales et al., 2018; Jurewicz and Hanke, 2011; Kim et al., 2013; Radke et al., 2018; Zhang et al., 2022). There is also increasing evidence that phthalates have a negative effect on the immune system, in particular an increased risk of developing asthma has been postulated (Bornehag and Nanberg, 2010; Franken et al., 2017; Wu et al., 2020). Likewise, negative effects on cognitive and neurological development are possible (Benjamin et al., 2017; Olesen et al., 2018), but the data on this is not clear (Benjamin et al., 2017; Hyland et al., 2019; Radke et al., 2020). The risk assessment committee (RAC) of the European Chemicals Agency acknowledged that it cannot be excluded that effects on the immune system and/or metabolic system might be equally or even more sensitive than the effects on male reproductive health (ECHA, 2017a; EFSA, 2019). Overall, uncertainties remain, whether the risk from concurrent phthalate exposure is over- or underestimated based on the selected common endpoint on male reproductive development after prenatal exposure.

3.5. Practical considerations in assessing the mixture risk: using precautionary factors

Already without including other anti-androgenic chemicals in this analysis, exceedances are substantial. When comparing the data to the adapted HIs (HI = 0.2 and HI = 0.1) to account for these substances, the percentage exceeding these HIs increase to 83% (HI = 0.2) and 95% (HI = 0.1) (Table 4). Although the amount of these precautionary factors is currently under discussion, they are not implausible (Apel et al., 2020a; Kortenkamp and Koch, 2020; KEMI, 2015; Van Broekhuizen et al., 2016). Structural analysis data suggests that phthalates with a linear ester side length of 4–7 carbon atoms in total, such as DnBP, di-n-pentyl phthalate (DnPeP), di-n-hexyl phthalate (DnHP), di-n-heptyl phthalate (DHP) and phthalates with a branched or non-linear side chain of 4–9 carbon atoms in length, such as DEHP, DiNP, BBzP, DiBP, di-isopentyl phthalate (DiPeP), diisoheptyl phthalate (DiHP), and dicyclohexyl phthalate (DCHP), are toxic to male development (Furr et al., 2014; Kortenkamp and Koch, 2020; Li et al., 2019). Thus, in the group of phthalates alone, six other substances not included here could add to the risk not assessed in the current analysis. DnPeP and DCHP are excluded from our MRA, as metabolites were either not assessed (in 3xG, CELSPAC:TE, PCB cohort follow-up, FLEHS IV), or metabolites were only detected in very few or no samples at all (0–32% > LOQ) and co-exposure is not given for the majority of the study participants (Vogel et al., *submitted*). For those individuals with exposure levels > LOQ these substances could, however, potentially add to the mixture risk. As previously mentioned, beside phthalates, other anti-androgenic substances can contribute to the risk of reproductive malformations, such as pesticides, parabens, pharmaceuticals (Conley et al., 2018, 2021; Kortenkamp, 2020; Rider et al., 2010). Although it can be assumed that a more complex mixture interaction than mere dose addition is present in humans, it has been shown that dose addition best predicts the mixture effects of some of these anti-androgens, although they do not act via the same mechanism of action or even the same pathway (Christen et al., 2012; Howdeshell et al., 2017; Rider et al., 2010). In reality the exact composition of real mixtures within a human body, i.e. individual chemicals that may act together adversely is unknown, nor are sensitive analytical methods in place for the comprehensive analysis of all chemicals. It is therefore not feasible to assess the real risk from anti-androgenic chemical mixtures on reproductive health. To reach the goals of the EU's chemicals strategy for sustainability towards a toxic-free environment (EC, 2020), risks from chemical mixtures need to be assessed and recommendations to risk managers and policy makers need to be formulated to protect the European population. The precautionary factors are a tool to approximate to the real mixture risk to reproductive health from concurrent exposure to multiple chemicals and to get an impression of the level of concern. In the future, more knowledge will become available on real-life mixtures in the human body and their biological interactions that may lead to an adverse outcome. For the time being, lowering the HI can serve as an easy and practical approach to identify priorities for risk managers and policy makers. Our analysis suggest that risks are heavily underestimated given the vast number of anti-androgenic chemicals present in the human body not included in the current MRA. Consequently, our findings highlight the need to adapt current risk assessment practices to truly protect children and adolescents from irreversible effects that might only become apparent later in life.

4. Conclusion

Our results indicate that 17% of the European children and adolescents are at risk from concurrent exposure to five reprotoxic phthalates. We could show that while there was no significant influence of sex (male vs. female) or age (children vs. adolescents), the geographical region

and the sampling year seem important with highest average HI in the Eastern European region and in the earlier sampling year (2014–2016). The two phthalates DnBP and DiBP were the clear drivers of the mixture risk in all cases. Strikingly, for about 63% the risk from combined phthalate exposure would have gone unnoticed in a single substance evaluation. This demonstrates the urgent need to incorporate mixture risk assessment into current regulatory practice at a regular basis. The overall HI of 0.44 at GM of the aligned study population also shows, that the buffer to potential exceedances is rather small. Consequently, if considering likely co-exposures to other anti-androgenic chemicals by adjusting the acceptable HI by a factor of 5 or 10, substantial exceedances of 83% and 95% are observed. While acceptable HIs lowered to 0.1 or 0.2 might be tackled as too conservative, a wealth of data proves dose additivity, especially of anti-androgenic substances (Conley et al., 2021; Howdeshell et al., 2017; Orton et al., 2014). Thus, the more anti-androgens are assessed in human biomonitoring studies, the higher the actual average HI for the population would become. It remains to be seen what HI will be reached in these population samples, if exposures to other chemicals were to be included. Recently derived reference doses for anti-androgenic mixture risk assessments of polybrominated diphenyl ethers (PBDEs) (Ermler and Kortenkamp, 2022) and bisphenol A (Kortenkamp et al., 2022a) in conjunction with known population exposures indicate substantial contributions of these substances to the mixture HI of anti-androgenic substances. Just recently, Kortenkamp et al., 2022a showed that the combined exposures to 29 chemicals including bisphenols, polychlorinated dioxins, paracetamol, and phthalates substantially exceeds HI of 1 to more than 100-fold in an individual and 17-fold at median for 9 chemicals alone. Each of the participants exceeded an HI of 1 for the 9 chemicals jointly measured in urine samples. For the total study population and all 29 chemicals a median HI of 20 was observed (Kortenkamp et al., 2022b). The results observed in the Kortenkamp et al. (2022b) study, supports our practical approach to use a precautionary factor to account for other anti-androgenic chemicals. Further, it strengthens the hypothesis that the actual risk on reproductive health from mixture exposure of anti-androgenic chemicals is higher than indicated in this or previously conducted MRA including only substances from similar chemical classes. Our study underlines the need for follow-up investigations of human internal exposures of the European population to chemical mixtures by continuous HBM studies harmonised at EU level.

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Declaration of competing interest

The authors declare no conflict of interest related to this work.

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Appendix A. Supplementary data

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