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The use of human biomonitoring data to address human exposure to real-life mixtures: exploratory study

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EXECUTIVE SUMMARY

Throughout their life-time, humans are exposed to a mixture of environmental stressors and chemicals that independently or in interaction may have an impact on health. These mixtures can consist of an almost infinite number of different combinations of chemicals, which makes the exposure and risk assessment challenging. A detailed assessment of risks for all possible unintentional mixtures under REACH and other regulatory sectors would require a significant increase of information requirements, which would lead to huge additional costs for testing. A simplistic solution to address these complicated issues has been proposed in the form of a mixture assessment factor (MAF) under the REACH Regulation Annex I.

By preference, the magnitude of the MAF should be supported by scientific data. In relation to the MAF discussion for human health, the use of human biomonitoring (HBM) data can be considered since this type of data forms a good reflection of real-life human exposure since HBM data reflect exposures from all routes and sources, and in nearly all HBM studies, exposure to multiple chemicals has been monitored.

In this study, an overview of existing HBM datasets was made, and the relevance and potential use for human health mixtures risk assessment was assessed.

- *Description of existing HBM datasets*

The Horizon 2020 project 'Human Biomonitoring for Europe – science and policy for a healthy future' (HBM4EU; www.hbm4eu.eu) generated an extensive overview of biomonitoring datasets in the EU. There is a wealth of existing HBM datasets (>100) described in the EU, with potential relevance for mixtures in relation to human health risk assessment. For the majority of the studies (fulfilling criteria such as minimal sample size of 50) aggregated data are accessible via the HBM4EU dashboard (https://www.hbm4eu.eu/eu-hbm-dashboard/). These include summary statistics, such as the percentiles of the biomarker levels per data collection for 18 HBM4EU priority substances or substance groups (acrylamide, anilines, aprotic solvents, arsenic, bisphenols, cadmium, chromium, flame retardants, lead, mercury and its organic compounds, mycotoxins, per/polyfluorinated compounds (PFAS), pesticides, phthalates and DINCH, polycyclic aromatic hydrocarbons (PAHs) and UV filters (benzophenones)).
On the one hand, it is recognized that the majority of HBM studies (18 chemical classes, covering 152 biomarkers of exposure) cover only a small subset of the 'real world mixtures', in view of the thousands of chemicals produced by industry. On the other hand, these 18 substance groups should not be minimized too much, since the selection of these groups was a result of a thorough HBM4EU prioritization strategy, considering relevance for human health. Prioritization of chemicals under HBMEU is driven by members of the EU Policy Board and the National Hubs. More information on this prioritization process can be found on the HBM4EU website.[1]

[1] https://www.hbm4eu.eu/wp-content/uploads/2017/03/HBM4EU_D4.3_Prioritisation_strategy_criteria-1.pdf

Some of the chemicals included in the HBM campaigns pertain to legacy chemicals (e.g. hexabromocyclododecane 'HBCDD', Hexachloro-1,3-butadiene 'HCBD', PFOA and PFOS) or chemicals with restrictions (e.g. DEHP, bisphenol A) and are therefore rather a reflection of past exposure, in combination with current exposure to substances present in materials with long lasting uses (e.g. furniture, construction products). The consideration of legacy chemicals in the MAF discussion should therefore be considered.

Recent small pilot studies using suspect and non-target screening on human biomonitoring data also demonstrated that the 'classical' targeted screening (such as biomarkers belonging to the 18 HBM4U priority groups) does not capture a substantial number of industrial chemicals present in human body fluid, and thus the mixtures that are identified using target screening are very likely an underestimate of the real mixtures in human biofluids. The field of suspect and non-target screening is an upcoming field, and once it will be enrolled in several HBM studies (as anticipated under the PARC initiative[2]), it can be very informative to inform on composition of mixtures in HBM samples (Pourchet et al., 2020).

The currently available HBM datasets in EU can be seen as a 'patchwork' of studies, from different regions, with each study having a particular scope and research question, and therefore different population groups (age/gender), periods and different sets of chemicals monitored. The HBM4EU project puts strong efforts in setting up aligned studies, which will enable in the future a better comparison of results across studies. The observation of diversity in HBM studies in several aspects should not hamper the use of individual datasets for mixture risk assessment; though comparison of risk estimates as outcomes of different studies should interpreted with caution, and accounting for the difference characteristics of the studies.

Investigating mixture patterns on human biomonitoring datasets and performing mixtures risk assessment requires access to individual datapoints. Aggregated datasets are useful to perform single substance risk assessment. Aggregated datasets entail information for various single chemicals, however the aggregated level does allow insight in co-occurrence patterns or dependency of distributions patterns of single chemicals. Attempts have been made to use aggregated datasets to create mixtures scenarios, and related mixtures risk assessments (see further). However, the representativeness of such 'constructed mixtures exposure scenarios' in view of realistic mixtures is questionable. The ability to get access to (pseudonymized) individual HBM datasets is much more difficult compared to individual datasets from environmental monitoring campaigns, given the confidentially and privacy concerns of individual's health data. Thus, information at the individual level is not publicly available for any human biomonitoring dataset. Access to this type of data might be requested upon a motivated request submitted to the data owners/controllers of the datasets (the duration of the procedure and conditions to get access currently differ from study to study). Improvements of data sharing of individual records to perform mixtures risks assessment is essential to move forward with human biomonitoring mixture risk assessment (Bopp S, 2016).

[2] PARC = Partnership for the Assessment of Risk from Chemicals (launched under the Horizon Europe Programme)

Despite the large number of humane biomonitoring (HBM) studies, the analysis of mixtures co-occurrence patterns in HBM data, observational studies linking mixtures to health outcomes, and mixtures risks assessments, seem to be less covered in the scientific literature compared to single substance risk assessments and health associations.

- *Evidence on co-occurrence of chemicals in human biofluids*
    - *Co-occurrence in view of simultaneous **presence/detection** of chemicals in human biofluids*

Exploring the HBM4EU dashboard, we noticed that for a first group of chemicals (including several of the 18 HBM4EU priority chemicals or chemical groups), at least one exposure biomarker has been detected in urine or blood in nearly all available samples of general populations (i.e. measurements above LOQ/LOD in more than 95% of the investigated samples). This is the case for acrylamide, anilines, arsenic, bisphenol A, cadmium, chromium, lead, mercury, PFOS and PFOA, some pesticides (3-PBA; glyphosate), phthalates and PAHs. It should be remarked that not all these chemicals are systematically monitored in all datasets, but *if* monitored, it appears that they have a high detection frequency.

There is a second group of chemicals which are present in a part of the population (e.g. DINCH, some organophosphate pesticides, several PFAS: present in > 50 % of samples if LOQ is sufficiently low), they were not detected in another part of the population. For a third, smaller set of chemicals, levels are only detected in a small proportion of the datasets (i.e. P90 or above > LOQ/LOD); e.g. this is the case for benzophenones (UV filters), some pyrethroids pesticides and some BDE's. For the group of BDEs: there is a rather diverse pattern: BDE-153 is detected in most samples (P5 > LOQ) in some databases, while having a smaller detection rate in other databases. Other chemicals belonging to the BDEs are only detected in the minority of the samples of some datasets (e.g. BDE 183).

The detection frequency cannot be fully compared across different studies, since LOQ and LODs might differ across studies (up to 20-fold or more), explaining partly why in some studies a higher proportion of the samples is < LOD/LOQ. Besides the difference in LOQ and LODs across datasets, also other factors play a role in detection frequencies across datasets; e.g. age of the study participants, sampling years, the region of the study and the specific population. For example, DINCH metabolites were only detected in 25 % of urine samples the ESB_2009 study in Germany in 2009; while keeping the same LOQ, DINCH metabolites became ubiquitous in the samples in the next decade (up to presence in > 95%), likely due to the increased use of DINCH as a substitute for DEHP.

In summary, in the majority of the human biofluids in HBM campaigns, a multitude of chemicals have been detected, thus providing evidence for simultaneous exposure to several chemicals.

    - *Co-occurrence in view of **magnitude of exposure** (concentration levels) of chemicals in human biofluids*

For several HBM studies, co-occurrence patterns of chemicals in human biomonitoring datasets in terms of **magnitude of exposure** (concentration levels) have been reported. The co-occurrence patterns in these studies are based on a variety of statistical techniques including linear regression, heat maps, circular plots, principal component analysis (PCA) and network analysis.

Given the diversity of the studies (population group, sampling years and monitored chemicals) conclusions differed from study to study. Overarching over different studies, the following trends and observations were made: ***Mainly levels of chemicals belonging to the same chemical group tend to correlate***. For example, samples with high levels for one PCB, have a likelihood to have also high level of other PCBs, and vice versa (this was demonstrated in several HBM Studies, Rosofsky et al. (2017); Govarts et al. (2020); Ottenbros; Agay-Shay et al. (2015); Tamayo-Uria et al. (2019).

Co-occurrence of magnitude levels between several PCBs can be explained by the presence of several PCBs in technical mixtures, thus PCBs having a common source. Likewise, levels ***within*** the groups of PBDEs, PFAS, phthalates, metals, phenols and pesticides tend to be correlated. Co-occurrence of elevated levels across chemical classes is in general weak or not present unless for chemicals occurring in or originating from the same sources. For example: co-occurrence of elevated levels PCBs and Hg can be explained by fish as common source and co-occurrence of mono-ethyl phthalates and parabens was explained by personal care products as common source (Rosofsky et al., 2017).

A lack of a co-occurrence patterns (mainly across chemical groups) based on ***levels*** should not be interpreted as that the ***presence*** of those chemicals is mutually exclusive. Rather, it indicates that chemicals with lack of co-occurrence patterns (levels) are "randomly" co-occurring. Let's say if chemical A and B are both detected in the entire population, but they lack a co-occurrence patterns based on *levels,* it means that they both occur in every sample in the population, but the concentration levels of A and B are independent of each other. A lack of co-occurrence regarding levels in humans can also be regarded as substances having independent distributions.

When performing human health risk assessment on datasets at individual level (cfr. recommended approach), there is no need to explicitly account for co-occurrence patterns, since this type of information is inherently present in databases. When one wants to perform mixtures risk assessment on aggregated data (not the recommended approach), the dependency of distributions should be considered, for chemicals with co-occurrence patterns based on levels.

- *Mixtures in relation to health effects (epidemiology)*

A limited literature search was performed regarding mixtures in human biomonitoring data in relation to health outcomes observed in epidemiological studies. The majority of these studies focus on perinatal exposure (exposure assessment in cord blood of newborns, breastmilk, urine, blood/serum of pregnant mothers) in relation to perinatal health outcomes (birth weight, placental weight, birth length, head circumference), and sometimes health outcomes in the follow-up study (e.g. BMI at 7 years; early menarche of female offspring). These studies demonstrate the importance of considering mixtures exposures when assessing health outcomes in epidemiological studies: statistically significant negative associations between of levels of chemicals mixtures in humans (assessed by biomonitoring) and the health of (vulnerable groups in) the general population have been reported, such as impact on birth indices (e.g. birth weight, length, head circumference). Some associations were not observed with single chemical models, and associations were stronger when mixtures were considered compared to single chemical exposure. The observation that **epidemiological studies reveals associations between mixture exposure and health outcomes,** should further trigger human health regulatory risk assessment to also consider mixtures.

At the same time, it's important to realize that the **current epidemiological knowledge** on mixtures in relation to health effects is mainly focused on a **specific, vulnerable subpopulation** (newborns), considering **generic health outcomes** and a **limited list of chemicals** (including legacy chemicals, and chemicals under restrictions). Epidemiological studies considering effects of mixtures in other vulnerable populations and life stages (e.g. puberty, elderly) and other, more specific categories of health effects (e.g. effects of mixtures on neurodevelopmental effects, cardiovascular effects, respiratory system, immunological effects, etc.) remain to be investigated. Additionally, the associations between health outcomes and mixtures pertain often to legacy chemicals, which have been used in the past, but are currently no longer used or limited in current applications (e.g. POPS under Stockholm Convention, chemicals under Restriction or Authorization). In view of the discussion of MAF for REACH chemicals, it would be very useful to have this type of analysis performed also on mixtures of non-legacy chemicals.

- *Mixture Risk Assessments using HBM datasets*

Whereas epidemiological studies demonstrate that human exposure to mixtures reveals a better model to explain health effects in the general population compared to single chemical exposure, the statistical models described in these studies cannot be used in regulatory risk assessment concepts such as RCRs under REACH. Instead, a practical instrument to perform mixtures risk assessment is the **Hazard Index (HI)** approach, in combination with **maximum cumulative ratio (MCR)**. The HI of a sample (biofluid sample) is the sum of hazard quotients (HQ) of several chemicals present in the biofluid. In its turn, the HQ is the ratio of the concentration (exposure level) to the health-based guidance value per substance. The MCR is the ratio of the HI of the mixture to the maximum of the HQs of the individual components, and thus represents whether the risk is mainly driven by one substance (MCR =1), a few substances (MCR: 2-3), or caused by a lot of substances all contributing a little bit to the risk (MCR → n ; with n = number of substances present in the mixture). The HI and MCR approaches are based on the hypothesis of dose addition, which is considered a conservative assumption for evaluating mixture effects, especially when applied in a low tier approach without considering communalities in endpoints or mode of action, and thus including all substances in the HI summation. The MCR and HI approach can also be used in a higher tier approach, where the summation of HI considers only chemicals affecting the same health effect or according the same mode of action.

In several publications, the HI and MCR concept have been applied in human biomonitoring data. Nearly all studies calculated HI and MCR on individual datapoints in datasets and relied on reverse dosimetry of HBM data (urinary levels) to external exposure estimates, which are used in HI calculations, in combination with health-based guidance values for external exposure for single chemicals. Nearly all publications deal with mixture risk assessment covering risk of substances *within a chemical group*; hardly any of the publications calculated risks across chemical groups.

The majority of the HBM HI/MCR studies within chemical groups pertain to mixture risk assessment for **phthalates**, with a focus on risk for reproductive and developmental toxicity by anti-androgenic modes of action, since this is the critical and common health effect of several phthalates.

To varying degrees, these studies revealed exceedances both of acceptable single and combined phthalate exposures, and in general the HI was dominated by one or two phthalates (dominance of DBP and DEHP in the HI). However, the outcomes of these various phthalate mixture risk assessments are difficult to compare because of three reasons. Firstly, different phthalates and numbers of phthalates were investigated in the different studies. Secondly, the studies used often different health-based guidance values (EFSA 2005 TDIs for phthalates versus RfD for phthalates derived by Kortenkamp & Faust (2010) and Kortenkamp & Koch (2020)). This factor might lead to a 5-fold difference in HI values, if all other factors are kept constant. Thirdly, there is also evidence that phthalate exposures have undergone changes over the years. Apel et al. (2020) performed HI and MCR calculations for each participant from the German Environmental Specimen Bank, which is a 27-year survey of urinary phthalate metabolite levels in 24-hour urine samples. While Apel et al. (2020) reported strong (about 10-fold) declining HI's over this period, ascribed to decreasing exposure levels, they reported at the same time that the more recent HIs were driven by a greater number of phthalates.

Additionally, studies on application of HI and MCR concept on human biomonitoring data were found for parabens (Moos et al., 2017), dioxins, furans and PCBs (Han and Price, 2013), pesticides (F. Fernández et al., 2020; Katsikantami et al., 2019) and PFAS (Borg et al., 2013). Also, for these chemical groups, one or a few compounds dominated in general the HI (i.e. n-PrP was the dominant substance in the HI for parabens; for PCBs: the MCR ranged from 2 to 5; for pesticides: dominance of dimethoate in the organophosphate group; dominance of PFOS and PFOA for respectively the HI hepatoxicity and reproductive effects). However, since PFOA and PFOS are phasing out, the use of other PFAS substances is increasing, which is visible in more recent HBM studies. Therefore, the pattern of dominance of PFOS and PFOA in the mixture toxicity is likely to decrease, and other PFAS might play a dominant role in the HI in the future.

A few studies have looked at the HI **across different chemical groups**. For example, the human health mixture risk assessment of A. Kortenkamp & Faust (2010) included exposures to a combination of 15 chemicals from several groups: phthalates (DBP, BiBP, BBP, DiNP, DEHP), pesticides (vinclozolin, prochloraz, procymidone, linuron, fenitrothion, pp'DDE), a flame retardant (BDE-99), bisphenol A, and parabens (butyl paraben and propyl paraben), all being substances capable of producing reproductive and developmental toxicity by anti-androgenic modes of action. Based on their data, we estimated MCR values of 2.0 and 3.2 for the median and high intake scenario, respectively. On the one hand, this study had an important shortcoming in the sense that the exposure assessment is based on aggregated data from a variety of sources, and not on individual data. On the other hand, the concept of applying HI and MCR calculations on substances across chemical groups considering common health effects, is interesting. It would be useful to apply their concept of across chemicals groups mixtures risk assessment on individual exposure data records instead of aggregated data.

Several authors point to consider mixtures risk across chemical groups (Borg et al., 2013; Kortenkamp, 2020) in order not to miss part of the mixture toxicity. However, **a 'blind' summing of HQs is not recommended for mixture risk assessment on HBM data**. To proceed, further developments should be made in view of gathering detailed toxicological information for grouping, point of departure values for relevant endpoints, and adverse outcome pathways, and derivation of endpoint specific guidance values.

A discussion of the selection of the health-based guidance values as denominator in the HQ could also be welcome, since this can affect strongly the HI values. This was noted when using Reference Doses (RfD's) from EFSA for phthalates versus guidance values derived by Kortenkamp et al. 2010 and 2020. For many chemicals, the **choice of the health-based reference value**s is likely to have a strong impact on the mixture risk. This discussion should also consider the role of human biomonitoring guidance values (HBM-GV) as denominator in the HI. Using HBM-GV would allow a straightforward use of HBM exposure levels (in urine, blood), instead of back-calculated external exposure using reverse dosimetry or empirical factors. The latter introduce uncertainty in the exposure assessment.

In the majority of the investigated studies, a few compounds drive the toxicity (MCRs typically 2-3). However, two important considerations should be made: this conclusion is often based on results from within group chemicals mixtures risk assessment, with a limited number of substances considered (e.g.; phthalates: often only 4-6 compounds considered). It should be further investigated whether this dominance of a few compounds is still valid when considering across chemical groups risk assessment (thus including substantially more chemicals) and considering time trends where substitution towards more compounds (e.g. PFAS and phthalates) is likely to occur.

As final reflection: mixture risk assessments rely on the assumption of the dose additivity concept and does not capture for other types of interactions (e.g.; synergistic effects). For a practical point of view, it is not feasible to consider other types of interactions in regulatory mixture RA approaches; however, one should realize that dose additivity might underestimate the risk.

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LIST OF ACRONYMS

BMI	Body Mass Index
CAG	Cumulative Assessment Groups
CEC	Chemicals of Emerging Concern
CHMS	Canadian Health Measurement Survey
CNA	Comparative Network Analysis
DNEL	Derived No-Effect Level
EFSA	European Food Safety Authority
EL	Exposure Load
EOA	Environmental Organic Acids
FLEHS	The Flemish Environment and Health Study
Fue	Urinary Excreted fraction
GC	Gas Chromatography
GC/TOF-MS	Gas Chromatography coupled to Time-of-Flight Mass Spectrometry
GerES	The German Environmental Survey
GV	Guidance Value
HBM	Human Biomonitoring
HBM-GV	Human Biomonitoring Guidance Values
HBM4EU	H2020-project, Human Biomonitoring for Europe
HI	Hazard Index
HQ	Hazard Quotients
IPCHEM	Information Platform for Chemical Monitoring
LC-QTOF/MS	Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantitation
MAF	Mixture Assessment Factor
Max HQi	Maximum of the Hazard Quotients of the Individual Components
MCR	Maximum Cumulative Ratio
MoA	Mode of Action
NDs	Non-Detects
NTS	Non-Targeted Screening
OP	Organophosphate Pesticide
PAH	Polycyclic Aromatic Hydrocarbons
PCA	Principal Component Analysis
PFAS	Per/Polyfluorinated compounds
POD	Points of Departure
POP	Persistent Organic Pollutants
RR	Risk Ratio; Relative Risk
RCR	Risk Characterization Ratio
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
SMACH	Similar Mixture Approach
SS	Suspect Screening

CHAPTER 1 INTRODUCTION – APPROACH

Throughout the life-time, humans are exposed to a mixture of environmental stressors and chemicals that independently or in interaction may have an impact on health. These mixtures of exposure can form an almost infinite number of different combinations of chemicals, which makes the exposure and risk assessment challenging. Until recently, chemical risk assessment was mainly focused towards single substances. However, a substance by substance approach cannot capture the complexity of simultaneous exposure to multiple chemicals in real life. Also, at the policy and regulatory level, there are strong incentives to cover and manage risks related to mixtures. As an important aspect, the EU chemical strategy for sustainability towards a toxic-free environment foresees the introduction of mixture assessment factor (MAF), to be adopted under the REACH Regulation Annex I (EC, 2020). A detailed assessment of risks for all possible unintentional mixtures under REACH and other regulatory sectors would require a significant increase of information requirements, which would lead to huge additional costs for testing. A simplistic solution to address these complicated issues has been proposed in the form of a mixture assessment factor (MAF) under the REACH Regulation Annex I, and is likely to be applicable to other regulatory areas as well (EC, 2020). During MAF workshops in 2016 and 2020, the magnitude of the MAF and the feasibility of a generic MAF for human health and the environment (versus a specific 'human' MAF and 'environmental' MAF) was intensively debated and it was recognized that MAF is not underpinned with scientific evidence. It was left open whether and for which substance groups the MAF should be applied.

Scientific underpinning of an appropriate MAF value for human health can be seen as the target on the horizon. Mapping of the existing evidence on the (risks) related to human exposure to mixtures can be seen a first step in this process. In this study, we aim to give an overview of existing datasets on human biomonitoring data in EU, and its potential and shortcomings to use human biomonitoring data to better understand real life exposure to mixtures for humans. Subsequently, the use of human biomonitoring data to scientifically address mixtures risks will be discussed.

The report is structured reflecting this approach:

CHAPTER 2: Overview of existing human biomonitoring datasets in EU

CHAPTER 3: Co-occurrence patterns in human biomonitoring datasets

CHAPTER 4: Exposure to mixtures in relation to health effects

CHAPTER 5: The use of HBM data in human health Mixture risk assessment

CHAPTER 6: Conclusions and recommendations

CHAPTER 2 OVERVIEW OF HUMAN BIOMONITORING DATASETS

Human biomonitoring (HBM) data, when conducted appropriately, are considered as the best reflection of real-life human exposure since HBM data resonate exposures from all routes and sources. HBM data provide an integrated picture of the totality of chemicals a person has been exposed to simultaneously. Other proxies (e.g. indoor dust samples; dietary exposure, …) can be considered as additional but are more indirect and partial reflections of exposure, and use of such exposure-source oriented datasets require assumption of the likelihood of co-exposure to distinct sources.

The ongoing Horizon 2020 project 'Human Biomonitoring for Europe – science and policy for a healthy future (HBM4EU; www.hbm4eu.eu) generated an extensive overview of biomonitoring datasets throughout its 30 participating countries, including mainly EU countries.

In this chapter, a summary of the nature of the existing datasets, the level of access and its characteristics (in term of number of chemicals, participants, age, matrices, …) is briefly described. Given the multitude of datasets, it is out of scope to provide details for each dataset individually.

For more details, it is recommended to consult 1) the IPCHEM platform (https://ipchem.jrc.ec.europa.eu/), and 2) the EU HBM dashboard (https://www.hbm4eu.eu/eu-hbm-dashboard/). The human biomonitoring module of the IPCHEM platform hosts meta-information regarding following aspects of the datasets:

- General information: study year, region, coverage, monitoring season, number of individuals in the human biomonitoring study;
- Study population: age, gender, socio-economic status, lifestyle parameters, environmental exposure factors;
- Data access & responsibilities;
- Sampling and analytical information (matrices, compounds, detection limits, ...).
- Also aggregated HBM data are being integrated in IPCHEM. The aggregated HBM collected within HBM4EU are visualized by the EU HBM dashboard.

2.1. OVERVIEW OF EXISTING DATASETS

The human biomonitoring module of IPCHEM entails 128 human biomonitoring studies with data collections starting from 1981 (earliest study) to 2018 (most recent study), covering mainly general populations studies, a few occupational studies and a few clinical populations. Whereas in the US, the national NHANES biomonitoring programme is an US wide programme, with protocols regarding participants selection, chemicals monitored, age groups, analytical techniques ensuring the comparability of data over time and regions, the existing datasets in EU are rather a 'patchwork' of studies, from different regions, with each study having a particular scope and research question, and therefore different population groups (age/gender), periods and different sets of chemicals monitored and possibly different analytical methods and limits of quantification.

The HBM4EU project puts strong efforts in setting up aligned studies, which will enable in the future a better comparison of results across studies.

There is a large variety in completed and ongoing human biomonitoring studies in the EU with respect to:

Study sizes:

- From small studies (e.g. Danish firefighter study investigating PAHs exposures, 'BIOBRAND'; (Andersen et al., 2018) ; n = 22)
- Medium size studies (majority of studies) (n = 200-2000). e.g. Flemish studies (FLEHS) (Ottenbros et al., 2021; Schoeters et al., 2017), INMA studies (Robinson et al., 2015)
- Large cohorts (minority of the studies) (e.g. NIPH MoBa – HBM within the Norwegian Mother and Child cohort; n = 114.000; (Magnus et al., 2016)

Number and types of chemicals measured in human biomonitoring studies:

- A few chemicals, or specific group of compounds targeted to a specific research question, e.g.;
  - Focus on PAH exposure in the Danish firefighter study BIOBRAND
  - Focus on polybrominated diphenyl ethers (PBDE) in the EXBROM study in Denmark; studying the exposure of pregnant women and their un- and newborn children to polybrominated diphenyl ethers (Frederiksen et al., 2009)
  - Focus on perfluoroalkyl compounds in the PFCHUM study in Spain (Pérez et al., 2013)
  - etc.
- Studies covering a wide range of chemical classes, e.g.;
  - The German Environmental Survey (GerES) and the German Environmental Specimen Bank studies; representative population studies carried out in order to determine the exposure to pollutants of the general population in Germany. Covering the following groups: phthalates, DINCH, PFAS, bisphenols, PAHs, heavy metals, acrylamide, pesticides, aprotic solvents, UV filters and flame retardants
  - The French ELFE study (perinatal study) (Béranger et al., 2020); covering the following groups: phthalates, DINCH, PFAS, bisphenols, flame retardants, heavy metals, pesticides, PCBS, dioxins, furans,
  - The Flemish Environment and Health Studies (FLEHS): covering metals, dioxins, furans, pesticides, PCBs, PAHS, phthalates, bisphenols, PFAS, flame retardants (Govarts et al., 2020)

These are a few examples illustrating the variety in number of chemicals assessed in human biomonitoring studies in the EU. For chemicals monitored in other cohorts, we refer to the IPCHEM platform.

2.2. ACCESS TO HBM DATASETS

Granularity of human biomonitoring datasets can be distinguished at three levels:

- **Meta-information level**, including study year, region, coverage, monitoring season, number of individuals in the human biomonitoring study, study population: age, gender, socio-economic status, lifestyle parameters, environmental exposure factors, data access & responsibilities, sampling and analytical information (matrices, compounds, detection limits, ...).
- **Aggregated data level**: summary statistics, such as the percentiles of the biomarker levels per data collection (P05, P10, P25, P50, P75, P90, P95), limit of detection (LOD) / limit of quantitation (LOQ) information. Summary statistics might also include summary statistics stratified by population subgroups (by gender, age, educational level, etc.).
- **Individual record level**: information on exposure and possibly (if taken along) health-related parameters like birth weight for each individual participant in the dataset. This type of information is not publicly available for most of the human biomonitoring datasets. Access to this type of datasets as well as research use is strictly controlled and has to be compliant with the GDPR in case of pseudonymized individual data. Access to this type of data might be requested upon a motivated research request submitted to the data owner (contact details for each dataset can be found in the IPCHEM platform). Request to get access follows in general a strict procedure (the duration of the procedure and the condition for use are to be investigated for each dataset separately).

Investigating mixture patterns on human biomonitoring datasets and performing mixtures risk assessment requires access to individual datapoints. Aggregated datasets are useful to perform single substance risk assessment, however, for mixtures risk assessment individual datapoints are needed. Use of e.g. P95 values for all individual constituents (chemical substances) will provide an overestimation of the mixture exposure in the cohort. Contrary, use of only P05 or P50 values will lead to an underestimation. One individual may be highly exposed to one chemical but average or low exposed to the other, vice versa.

Improvements of data sharing of individual records to perform mixtures risks assessment is essential to move forward with human biomonitoring mixture risk assessment (Bopp S, 2016).

Since it was not possible within the timeframe of this project to request access and use to individual datasets to perform mixtures analysis on HBM datasets, the analysis of co-occurrence patterns and mixture risk assessment based on human biomonitoring studies is based on previous investigations available in literature (see chapters 3, 4 and 5).

2.3. CHEMICALS AND LEVELS IN HBM DATASETS

2.3.1. CHEMICALS

→ **Target screening**

The EU HBM dashboard (www.hbm4eu.eu/eu-hbm-dashboard/) allows to visualize summary statistics from existing HBM data collections obtained through the HBM4EU project. The data included in the dashboard were obtained in a standardized and comparable way. The dashboard visualizes the percentiles of the biomarker levels per data collection (P05, P10, P25, P50, P75, P90, P95) and also displays limit of detection (LOD) / limit of quantitation (LOQ) information. Moreover, there is a filter function to view biomarker data for specific subgroups such as by sex, age, educational level, etc. The current data is limited to the 18 HBM4EU priority substance groups, i.e. acrylamide, aniline, aprotic solvents, arsenic, bisphenols, cadmium, chromium, DINCH, flame retardants, lead, mercury ant its organic compounds, mycotoxins, per/polyfluorinated compounds (PFAS), pesticides, phthalates, polycyclic aromatic hydrocarbons (PAHs) and UV filters (benzophenones). These 18 substance groups also cover the vast majority of substances monitored in HBM campaigns in EU. Other compounds (outside 18 substance groups) are also measured in some studies (e.g. PCBS, other metals, ...). however, a comprehensive overview is not available in IPCHEM or the HBM4EU dashboard.

A first point of attention is the relation between parent compounds (chemicals as produced, and/or as present in the environment) and the biomarker present in the human samples. Most chemicals (parent compounds), once entered in the human body, are distributed by blood route to several tissues and undergo metabolisation in the liver. Consequently, most chemicals end up as metabolites in urine or faeces. Metabolites in urine are so called 'biomarker' of exposure. As an example: pyrene metabolism in humans starts with hydroxylation of pyrene to 1-hydroxypyrene (1-OH pyrene) by cytochrome P450 iso-enzymes. In the second step, 1-OH pyrene is conjugated and is excreted in urine as a glucuronide-conjugate. Hence, for exposure to pyrene, 2 biomarkers in urine (1-OH pyr and gluc) are relevant. This is an example of simple, unbranched metabolisation pathway. For several chemicals, metabolisation might result to several metabolites (branched or unbranched metabolisation pathways), thus several biomarkers of exposure are relevant. Additionally, metabolites might in some case originate from more than one parent compound. For example, cis-DDCA is a metabolite of both permethrin and cypermethrin. As a result, such biomarkers cannot be linked univocal to one parent compound. This difficulty of use of biomarkers for use of mixtures risk assessment of parent compound is mentioned by several authors (e.g. (F. Fernández et al., 2020)

Appendix 1 provides a mapping of parent compounds and metabolites for the chemicals belonging to the 18 HBMEU priority chemicals. Whenever possible, the main parent compound is mentioned in the table. In cases where biomarkers originate from several parent compounds, the group names for the parent compounds are displayed.

→ **Suspect and non-target screening**

The target screening approach for generation HBM data on some specific chemicals has delivered only data on a relatively limited number of substances. Two alternative approaches under development are suspect screening and non-targeted screening. Where target screening uses reference standards, suspect screening uses only attributes such as mass and isotope patterns but no reference standards and non-targeted screening uses no pre-existing knowledge. Until recently, the majority of human biomonitoring studies investigated levels of priority chemicals using targeted analysis (cfr. previous section). The use of non-targeted analysis (suspect screening and non-target screening) has only been used in the field of human biomonitoring analysis during the last couple of years. ”Suspects” are known compounds (“known unknowns”) in terms of chemical name and structure which are expected (“suspected”) to be present in a sample. The typical approach applied in this case is largescale suspect screening aiming to generate semi-quantitative data and contribute to better prioritization for further targeted developments (Cortéjade et al., 2016). Non-targeted screening aims to detect “unknown unknowns” compounds without any a priori criteria, to identify potential new markers of exposure and toxicological concern. Generally, sample preparation and data acquisition are similar for suspect and non-targeted screening whereas data analysis/mining are different. Although highly challenging, this approach represents the most promising strategy to advance our knowledge of the human chemical exposome (Pourchet et al., 2020).

The techniques of suspect screening and non-target screening are of particular interest for the so-called ‘chemicals of emerging concern’ (CEC). CECs encompass both new compounds recently detected in the environment-food-human continuum (for instance, newly developed substitutes of banned and/or regulated chemicals) and compounds with known presence, yet for which concerns have recently increased (e.g. due to progress of analytical performances, newly identified sources, uses and/ or routes of exposure, particularly exposed sub-population, toxicological evidence, evolution of regulatory dispositions…) (Sauvé and Desrosiers, 2014).

Pourchet et al. (2020) and Wang et al. (2021) noticed that monitoring of CECs through the use of suspect and non-target screening is well established in the field of monitoring in environmental matrices, and becomes also more prominent in the chemical food safety area. According to Pourchet et al. (2020), CECs remain less investigated, via suspect or non-targeted screening approaches, in the field of human biomonitoring, except for particular applications focused on specific classes of compound, such as pesticides. One component of HBM4EU project aims at the development and implementation of large-scale suspect and non-targeted screening methods dedicated to the detection of markers of internal chemical exposure for HBM, environmental health studies and support to risk assessment purposes. While the number of publications on suspect and non-target screening in HBM samples is growing, the majority is currently mainly reporting on the development, validation and pilot demonstration of few samples (e.g. Baduel et al., 2015)). The use of suspect and non-target screening on large number of samples (e.g. full analysis on cohort samples) has not yet been reported. HBM4EU produced a nice leaflet to provide ins and outs as well as future opportunities for SS and NTS (HBM4EU Factsheet NTS and SS) as well as several publicly available deliverables (D16.1, D16.2, AD16.1, AD16.2, AD16.3, AD16.4) available at www.hbm4eu.eu.

While the development and implementation of large-scale suspect and non-targeted screening methods for human biomonitoring in Europe is progressing and is expected to advance a lot to our understanding of human exposure to mixtures in the coming years, the currently available results are too few and scattered and are not yet included in the existing HBM exposure databases. Few publications regarding results of suspect and non-targeted screening application on HBM samples have been published yet. Although the number of applications is increasing in this field, some authors suggest that more efforts are needed to harmonize integral SNTS methodologies used in biomonitoring studies to assess the organism exposure to chemicals (Gonzálezgonz´gonzález-Gaya et al., 2021). However, a few (mainly US) publications on the applications of suspect and non-targeted screening give some first insights:

- Tran et al. (2020) performed non-targeted analysis (using GC x GC/TOF-MS) on human breast milk samples from three mothers (2011). A total of 172 presumably anthropogenic halogenated compounds and non-halogenated cyclic and aromatic compounds were tentatively identified in the breast milk samples through mass spectral database searches. Forty of the compounds were prioritized for confirmation based on halogenation or 100% frequency of detection. 34 (85%) of the prioritized contaminants are not typically monitored using target screening in breast milk surveys, and 31 (77%) are regulated in at least one market worldwide.

- Wang et al. (2021) applied liquid chromatography−quadrupole time-of-flight tandem mass spectrometry (LC-QTOF/MS) to perform suspect screening for ∼3500 industrial chemicals on pilot data from 30 paired matern and cord serum samples (n = 60). They identified 557 unique compounds, and identified tentatively 55 compounds not previously reported in the literature. Some of these new identified substances in cord blood or serum are PFAS, plasticizers, substances in cosmetics and consumer products or high production volume chemicals. However, for the majority (i.e. 42) of these compounds, information regarding the sources and uses was not available (therefore, these compounds could not be classified under the categories of pharmaceuticals, pesticides, flame retardants, PFAS, plasticizers, cosmetics, consumer products, hugh production volume chemicals classified in EPA's Chemical Dashboard.

- Gerona et al. (2018) used LC-QTOF/MS for discovery of previously unmeasured environmental chemicals in human serum. They focused on organic compounds with at least one dissociable proton which are utilized in commerce (Environmental organic acids 'EOA'). EOAs include environmental phenols, phthalate metabolites, perfluorinated compounds (PFCs), phenolic metabolites of polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), and acidic pesticides and/or predicted acidic pesticide metabolites. These authors first identified 282 'suspect' EOAs, and 65 of these suspect EOAs were detected in at least 75 % of the 20 serum samples (pregnant women). Only 19 of these compounds are currently biomonitored in NHANES. Again, This indicates that target screening might miss a substantional number of chemicals in a substantion proportion of samples.

- Miaz et al. (2020) analyses PFAS in pooled serum samples from 1996-2017 from first time mothers living in Uppsala (Sweden). The authors detected a decrease in levels of target PFAS over time and reported an increase to novel PFAS which had not yet been identified before. Using suspect and non-target screening, (Miaz et al., 2020) revealed respectively the presence of perfluoro-ethylcyclohexanesulfonate (PFECHS) and 3 PFAS compound with unidentified features (neutral masses 422.2307, 396.2066, and 436.3554).

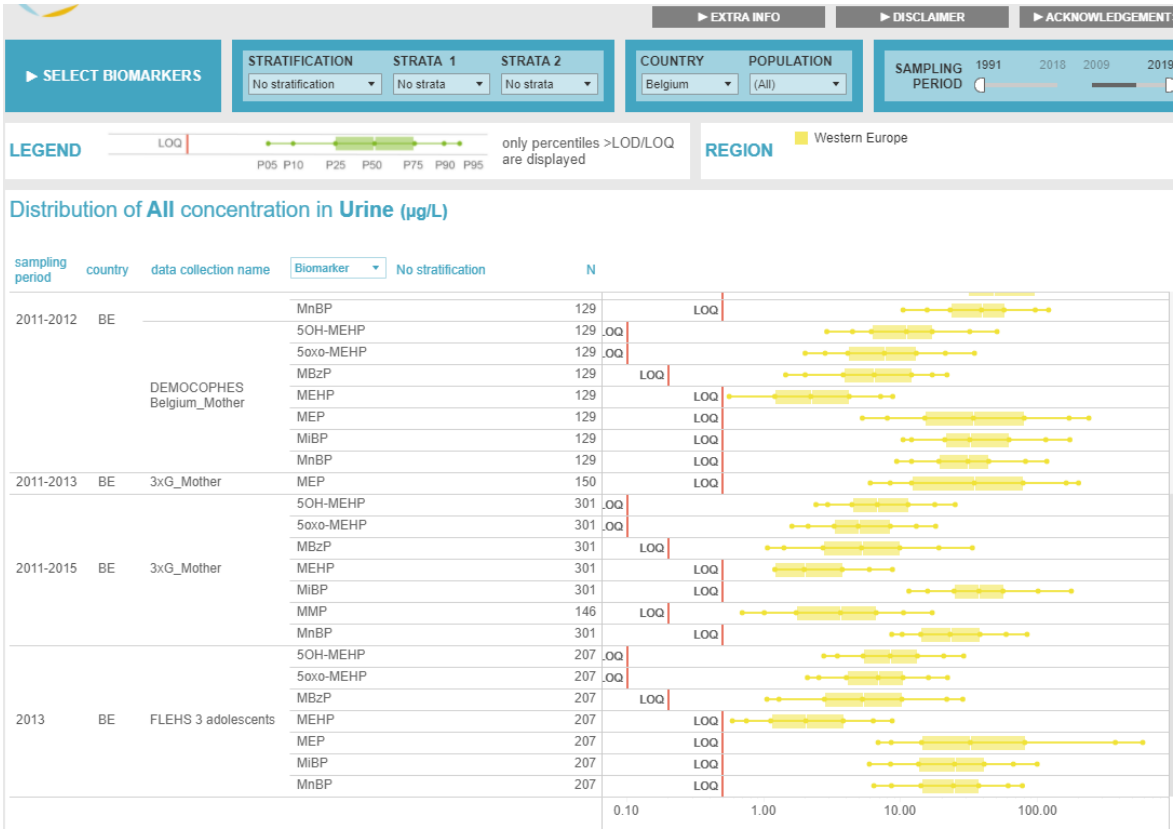
The above mentioned studies should also be regarded as complementary to each other in terms of matrix (breast milk vs. serum and cord blood) and analytical techniques. Wang et al. (2021) used LC-QTOF/MS, primarly focussing on polar and involatile chemicals, Tran et al. (2020) used GC /TOF-MS, capturing nonpolar and (semi)volatile chemicals, Gerona et al. (2018) focused on Environmental organic acids and Miaz et al. (2020) performed suspect and non-target screening of fluorinated compounds.

These studies illustrate that 'classical' target screening of chemicals in HBM is likely to result in only a fragmented picture of the entire mixture that can be present in human matrices. Once the evidence on the application of suspect and non-targeted screening is growing, it can be a very useful source of data to investigate co-occurrence patterns and to perform mixtures risk assessment, as this approach will expand the number of chemicals that may be found in mixtures substantially.

2.3.2. LEVELS OF CHEMICALS

This section is limited to results from target screening monitoring. Firstly, because levels of suspect screening and non-target screening in general do not report levels in a quantitative way. Secondly, because the use of screening and non-target screening is currently at the stage of method development, and demonstration in a pilot case, but it is not yet enrolled as new techniques in completed and ongoing large scale HBM campaigns.

The levels of chemicals measured using target analysis can be easily displayed and extracted from the European HBM dashboard at an aggregated level. An example, using a screenshot of the HBMdasboard, is given here below for the Belgian phthalates dataset (levels of metabolites in urine):



The interactive dashboard available at https://www.hbm4eu.eu/eu-hbm-dashboard/ provides popping-up of infoboxes to show more details (e. LOQ, LOD, value of percentiles, number of datasets,…) when moving the cursor over the boxplots.

The boxplots on levels in urine of the FLEHS adolescents indicate that the majority of the phthalates biomarkers (e.g. MEHP, MEP, MiPB, MnPB, 5OH-MEHP, 5 oxo-MEHP, MBzP) are present in nearly the entire cohort. This is derived from the observation that the lower end of the displayed distribution (e.g. 5th percentile: second left dot outside the bars of 'boxplots') is above LOQ or LOD.

For a detailed description of levels of chemicals in human biomonitoring datasets, we refer to the https://www.hbm4eu.eu/eu-hbm-dashboard/. This dashboard provides a complete picture. We prefer to refer to this dashboard, rather than duplicating the tables and figures in this report.

A screening of the aggregated datasets reported in https://www.hbm4eu.eu/eu-hbm-dashboard/ indicated the following trends in detection frequency in relation to the LOQ/LOD (see Appendix 2) :

- For the majority of the chemicals reported in the HBM4EU aggregated dashboard, at least one biomarker of exposure can be detected in urine or blood in nearly the entire distribution of the available datasets (i.e. present above LOQ/LOD in more than 95% of the investigated samples): this is the case for acrylamide, anilines, arsenic, bisphenol A, cadmium, chromium, lead, mercury, PFOS and PFOA, some pesticides (3-PBA; glyphosate, phthalates and PAHs;
- Some chemicals are present in a substantial subset of the data sets, but not in the vast majority of the population (e.g. DINCH, some organophosphate pesticides, several PFAS);
- For a smaller set of chemicals, levels are only detected in small proportion of the datasets (i.e. only P90 or above > LOQ/LOD); e.g. this is the case for benzophenones (UV filters) some pyrethroids pesticides, and some BDEs;
- For the group of BDEs: there is a rather diverse pattern: BDE-153 is detected in the majority of samples (P5 > LOQ) in some databases, while having a smaller detection rate in other databases. Other chemicals belonging to the BDEs are only detected in the minority of the of samples of some datasets (e.g. BDE 183);
- The detection frequency cannot be fully compared across different studies, since LOQ and LODs might differ across studies (up to 20-fold or more); explaining partly why in some studies a higher proportion of the samples is < LOD/LOQ;
- Besides the difference in LOQ and LODs across datasets, also other factors play a role in detection frequencies across datasets; e.g. sampling years, the region of the study and the specific population. For example, regarding the age of the study: DINCH metabolites was only detected in 25 % of urine samples the ESB_2009 study in Germany in 2009; while keeping the same LOQ, DINCH metabolites became ubiquitous in the samples in the next decade (up to presence in > 95%), likely due to the increased use of DINCH as a substitute for DEHP.

2.3.3. TIME WINDOWS

Majority of the human biomonitoring studies report data of spot samples (urine, blood) or 24h samples (urine). The levels of chemical present in these matrices might represent short term or long-term exposure, depending on the type of matrices and half-lives of the chemicals in the human body. For example, cadmium in blood is a reflection of recent exposure, while Cd levels in urine reflect a

longer history of exposure (Adams and Newcomb, 2014). However, this example for Cd is not a rule of thumb, it is recommended to retrieve information on half-life information case by case.

2.4. IMPRESSION REGARDING THE USE OF EXISTING HBM DATASETS TO ASSESS HUMAN EXPOSURE TO MIXTURES

→ Number and type of chemicals

Industry synthesizes an estimated 130.000 different chemicals (22.000 registered substances are covered by REACH alone). These are all potentially present in our environment, where they can combine in a nearly endless number of potential unintentional mixtures. Chemicals measured in HBM4EU human biomonitoring studies (based on targeted analysis) cover 18 substance groups, and 152 biomarkers of exposure. These numbers show that it's clear that only a small subset of the 'real world mixtures' are covered in HBM. The number of chemicals that will become included in upcoming HBM studies will only increase in the future (e.g. the use of non-target monitoring techniques is a promising field; (Pourchet et al., 2020). Chemicals included in current HBM datasets should indeed be considered as a rather small fraction of all chemicals. On the other hand, these 18 substance groups should not be minimized too much, since the selection of these groups was a result of a thorough HBM4EU prioritization strategy, including consideration of relevance for health and likelihood of sufficient detection frequency. Prioritization of chemicals under HBMEU is driven by members of the EU Policy Board and the National Hubs. More information on this prioritization process can be found on the HBM4EU website.[3]

The substances included in the HBM4EU human biomonitoring datasets thus include both legacy and non-legacy chemicals. PFOA, PFOS and several BDEs are present in the Annex of the Stockholm Convention. The use of DEHP, bisphenol A, several pesticides and metals (Cd,Cr) is subject to authorization or restriction under REACH. Other human biomonitoring substances (several PFAS; phthalates) are currently still produced and used in large quantities in the EU.

→ Comparability of datasets

The nature of the existing EU HBM datasets is rather diverse in terms of age of the study, region, number of subjects, chemicals analyzed, LOD/LOQ, selection and representativeness of individuals (general population, or subgroup). Therefore, it is recommended to perform mixture co-occurrence patterns and mixtures risk assessment study-by-study. Pooling datasets to come to a comparable dataset is an option, however quite demanding. The design of aligned studies (common protocols for new studies set up under HBM4EU) will smooth the path to combine existing datasets.

→ Access to data / implications for the assessment of mixtures

Investigating mixture patterns on human biomonitoring datasets and performing mixtures risk assessment, requires access to individual datapoints. Getting access to individual datasets for human

[3] https://www.hbm4eu.eu/wp-content/uploads/2017/03/HBM4EU_D4.3_Prioritisation_strategy_criteria-1.pdf

biomonitoring data is however not straightforward. Since it was not possible within the timeframe of this project to request access to individual datasets to perform mixtures analysis on HBM dataset, the analysis of co-occurrence patterns and mixture risk assessment in human biomonitoring studies is based on previous investigations available in literature (see chapters 3 and 4).

Notwithstanding that using aggregate data has its limitation for mixtures risk assessment, the distributions for individual levels give us the following insights. First, since for a large part of the HBM4EU priority chemicals, at least one biomarker of exposure is detected in > 95 % of the samples of human biomonitoring studies, it is obvious that there is co-occurrence of several chemicals in human species ('real world mixtures'). At least the following set of chemicals is expected to occur as mixtures in a large part of the general population: acrylamide, anilines, arsenic, bisphenol A, cadmium, chromium, lead, mercury, PFOS and PFOA, some pesticides (3-PBA; glyphosate); phthalates, PAHs. Thus, co-occurrence of chemicals across chemical groups is likely to occur. Second, the correlation between the levels of the detected substances in human biomonitoring cannot be derived from the aggregated data. Hereto, access to individual datasets is required. It is recommended to use individual level datasets, similar to the calculation of hazard indices, in the investigation of chemicals dominating the mixtures.

CHAPTER 3 CO-OCCURRENCE PATTERNS

3.1. APPROACH

Investigating co-occurrence patterns in human biomonitoring datasets requires access to individual datapoints. Since it was not possible within the timeframe of this project to request access to individual datasets to perform analysis of co-occurrence patterns in HBM datasets, this chapter on co-occurrence patterns in HBM studies is based on literature screening of studies in which co-occurrence patterns have been described. The primary focus was on the co-occurrence patterns as such (section 3.2), and extended with studies providing evidence that health effects observed in cohorts can often be explained more accurately by exposure to mixtures rather than exposure to single chemicals (see CHAPTER 4).

A full description of the literature search strategy and findings per individual study can be found in Appendix 3. In this chapter, key findings are described.

3.2. SUMMARY OF KNOWLEDGE CONCERNING HBM CO-OCCURRENCE PATTERNS

3.2.1. GENERAL DESCRIPTION OF STUDIES

Despite the large number of humane biomonitoring (HBM) studies (see CHAPTER 2), the analysis of mixtures co-occurrence patterns in HBM studies seems to be a rather unexplored field. About 10 studies were found in literature reporting co-occurrence patterns of chemicals in human biomonitoring datasets:

- HELIX study – 6 regions in EU (6 birth cohorts: BIB, UK; Eden, France; INMA, Spain; KANC - Lit.; Moba- Norway; Rhea; GR); (Tamayo-Uria et al., 2019)
- Snart Foraeldre/Milieu cohort study in Denmark (Rosofsky et al., 2017)
- FLEHS studies in Belgium (FLEHS I, II, II & 3xG birth cohorts); (Govarts et al., 2020; Ottenbros et al., 2021)
- INMA study; Spain (Robinson et al., 2015)
- EDEN study; France (Philippat et al., 2019)
- ELFE study; France (Béranger et al., 2020)
- Three birth cohorts Greenland, Poland, Ukraine; (Lenters et al., 2016)
- ASLPAC study; UK (Marks et al., 2021)
- EXBROM study; Denmark (Frederiksen et al., 2009)

A summary of description of individual studies (number and characteristics of participants, period of study; matrix, biomarkers of exposure) can be found in Table 1; and more details per study are given in Appendix 3.

The summary in this chapter is limited to conclusions for EU human biomonitoring studies. In Appendix 3, the publications describing mixtures co-occurrence patterns in humane biomonitoring have also been extended towards non-European studies. Mainly studies from US and Canada have been found. The findings from these non-EU studies are consistent with the EU-studies.

Overall, majority of the studies covered chemicals within and across groups of chemicals and pertain in many cases to analysis in urine or blood of newborn-mother pairs, or pregnant women. The studies and the year of human biomonitoring sampling is rather recent (majority is less than 10 years old), thus reflecting probably adequately nowadays exposure.

Cohorts are often country or region representative cohorts of the general population for the age group of interest (e.g. FLEHS, ELFE cohorts). In other cases, there was no explicit description of the representativeness of the recruited participants in view of general population (e.g. region, SES status, ethnicity, etc.). None of the cohorts, except for part of the FLEHS I cohort in Govarts et al. 2020, in Table 1 pertain to populations living in the neighborhood of environmentally polluted sites, nor there were other concerns or indication for elevated exposure to chemicals.

3.2.2. STATISTICAL APPROACHES AND UNDERSTANDING OF THE CONCEPT 'CO-OCCURRENCE' IN HUMAN BIOMONITORING STUDIES

The co-occurrence patterns in these studies are based on a variety of statistical techniques including linear regression, heat maps, circular plots, principal component analysis (PCA) and network analysis (see Appendix 3).

A technical description of these techniques and application in the mentioned studies is elaborated in Appendix 3.

In brief, linear regression/correlation analysis, heat maps and circular plots express the strength of correlation between levels of two parameters. They differ mainly from each other in the visualization mode (respectively Pearson's r value, color map, circular plot). These techniques can reveal correlations between two parameters but are less appropriate to reveal clusters of several parameters/biomarkers, or to find relation between clusters and common sources or explaining variables.

Principal component analysis identifies the maximum amount of mutual correlation between groups of variables that explain latent variables, or components, that cannot be directly observed. Biomarkers are categorized under a given component based on their "loading," which represents correlations between the biomarker and the underlying, latent factor, or component. Because the components are orthogonal, or statistically independent, biomarkers loaded within one component are said to have a low correlation with biomarkers loaded on all other components. The extent to which chemicals load to the final components may indicate common exposure sources within that component, such as chemicals that are found together in diet, consumer products or traffic pollution. PCA results may identify the extent to which chemical exposures share common sources and pathways or jointly contribute to disease" (Rosofsky et al., 2017).

Comparative network analysis (CNA) provide a graphical method to represent groups or communities in the data. Networks facilitates the detection of exposure patterns and allows for the systematic comparison of observed exposure patterns between datasets and strata within datasets. The length of the edges in a CNA is proportional to the inverse of the correlation between exposures.

Irrespective of the technique used, all reported analysis of co-occurrence in the investigated studies (see Table 1) are based on *concentration levels*, and do not pertain to co-occurrence in terms of presence or absence of chemicals. In other words, if a pearson correlation, heat map, circos plot or PCA analysis indicates a strong co-occurrence of 2 chemicals (let's say biomarkers A and B), it can be interpreted as follows: if concentration of biomarker A is high for an individual in the population (high compared to the range of levels A within that population), it is likely that this individual also has a high concentration of biomarker B (high compared to the range of levels A within that population). If there is no co-occurrence between 2 biomarkers (let's say biomarkers C and D) this means that distributions of levels of biomarkers C and D are independent. If biomarkers C and D have both a detection frequency of 100 % in the population, this implies that in individuals have detectable levels of biomarker C and D, but that the magnitude of levels of biomarker C and D are independent of each other.

3.2.3. ACCOUNTING FOR RESULTS BELOW LOD OR LOQ

The treatment of non-detects in HBM datasets is of importance when investigating co-occurrence patterns, especially in datasets where a substantial number of samples is below LOD or LOQ for one or several biomarkers. In some datasets, nearly all analytes had high detection frequencies (e.g. in the EDEN birth cohort: all analytes were between 98-100 % > LOD), thus no treatment of non-detects was needed. In some studies, the analysis of co-occurrence patterns was restricted to that biomarkers were detected in majority of the study population (e.g. 75 % cut off in Snart Foraeldre/ MilieuDenmark (Rosofsky et al., 2017)). The majority of studies did not explicitly excluded data < LOD/LOQ, but applied a method to treat non-detects. Ottenbros et al. (2021), Tamayo-Uria et al. (2019), Govarts et al. (2020), Robinson et al. (2015) and Agay-Shay et al. (2015) imputed biomarker values below LOD based on a imputation techniques, involving e.g. maximum likelihood estimation via single conditional imputation dependent on observed values for the other biomarkers. This approach resulted in a distribution in values below LOD, and not in the same value to replace values < LOD what is usually achieved when treating data with concentrations below the limit of detect (LOD) by using the LOD divided by the square root of two. This latter technique was previously often applied, and also in one of the mentioned HBM studies, i.e. Rosofsky et al. (2017). Replacing <LOD by a fixed value (such as LOD/$\sqrt{2}$) is more prune to 'false' co-occurrence patterns compared to imputation techniques because all values below LOD get the same value using this technique.

In summary, several techniques have been used in the various studies to treat non-detects. Most of the studies used the imputation technique to address missing values (<LOD), which is considered as a better technique than replacing <LOD by a fixed value (such as LOD/$\sqrt{2}$).

In none of the studies, a sensitivity analysis or comparison of different techniques to treat non-detects on the outcome of co-occurrence patterns was made. However, since the majority of the biomarkers are detected in large portions of the population, and the state-of-the-art techniques of imputation is used, it can be anticipated that the non-detects do not drastically influence the conclusions of co-occurrence patterns.

3.2.4. GENERAL TRENDS IN CO-OCCURRENCE PATTERNS

For a detailed description of co-occurrence patterns per study, and graphical illustrations we refer to description in Appendix 3.

Overarching over different studies, the following trends and observations were made:

1. Mainly levels of chemicals belonging to the same chemical group tend to co-occur (based on regression analysis, PCA or network analysis), hereby some examples:

- PCBs co-occur with each other in human samples and this was demonstrated in;
    - in the Snart Foraeldre/Milieu cohort study in Denmark using PCA analysis (Rosofsky et al., 2017);
    - the FLEHS cohorts using heatmaps, circular correlation globes and network detection techniques in Govarts et al. (2020; Ottenbros et al. (2021); (PCB 138; PCB 153, PCB 180)
    - in the INMA cohort in Spain using PCA analysis (Agay-Shay et al., 2015) (PCB 138, PCB 153, PCB 180);
    - in the HELIX cohorts using network analysis (Tamayo-Uria et al., 2019).

    Co-occurrence between several PCBs can be explained by the presence of several PCBs in technical mixtures, thus PCBs having a common source

- PBDEs co-occur with each other in human samples and this was demonstrated in;
    - in the INMA cohort in Spain based on PCA analysis (Agay-Shay et al., 2015) (PBDEs together in PC 1) and based on correlation heatmaps (Robinson et al., 2015);
    - In the EXBROM cohort in Denmark, based on analysis in placental tissue. The loading plot showed groupings of the measured PBDE variables in three groups, representative of Penta-, Octa- and Deca-BDE technical mixtures. Congeners representing the individual technical mixtures were close to orthogonal or inversely correlated, indicating variation in the congener patterns of internal exposure corresponding to the patterns of technical mixtures used in products.

- PFAS co-occur with each other in human samples and this was demonstrated in;
    - in the HELIX cohorts using network analysis (Tamayo-Uria et al., 2019);
    - in the Snart Foraeldre/Milieu cohort study in Denmark using PCA analysis (Rosofsky et al., 2017): PFHxS and PFOS loading PC 2, while PFDeA, PFNA and PFOA loading PC 3;
    - in the FLEHS cohorts using heatmaps, circular correlation globes and network detection techniques (Ottenbros et al., 2021); (PFOA, PFOS, PFNA, PFHXS);

- Phthalates co-occur with each other in human samples and this was demonstrated in;
    - the HELIX cohorts using network analysis (Tamayo-Uria et al., 2019) (the following phthalates metabolites are clustered in the network: MEHP, MNPB, MBZP, ECPP, MEOP, OHMiNP, MIBP)
    - in the Snart Foraeldre/Milieu cohort study in Denmark using PCA analysis (Rosofsky et al., 2017): several phthalates loading PC 3, while mono ethyl butyl phthalate loading PC 3
    - in the INMA cohort in Spain based on PCA analysis (Agay-Shay et al., 2015) (phthlatates constituting PC 1) and based on correlation heatmaps (Robinson et al., 2015).

- Metals co-occur with each other in human samples and this was demonstrated in;
    - in the HELIX cohorts using network analysis (Tamayo-Uria et al., 2019): for mothers: on the one Cd, Hg and As close to each other in the network; on the other side: Cd, Pb, Co, Mn and Mo close to each other in the network;
    - in the Snart Foraeldre/Milieu cohort study in Denmark using PCA analysis (Rosofsky et al., 2017): Cd, Pb, Co in PC 2, and Mn and total Hg in PC 1; while total As and arsenobetaine in PC 3.

- Phenols occur with each other in human samples and this was demonstrated in;
    - in the HELIX cohorts using network analysis (Tamayo-Uria et al., 2019): for mothers: BUPA, ETPA, TRCS, PRA, ETPA, MEPA, OXBE, and BPA (the latter with a longer distance to the other phenols for the mothers analysis)

- Pesticides occur with each other in human samples and this was demonstrated in;
    - in the HELIX cohorts using network analysis (Tamayo-Uria et al., 2019): organophosphate pesticides DEPT, DETP, DMP, DMTP, DMDTP are close to each other in the networks;
    - in the Snart Foraeldre/Milieu cohort study in Denmark using PCA analysis (Rosofsky et al., 2017): persistent pesticides (hexachlorobenzene, pp—DDE- in PC 1; while other pesticides (paranitrophenol, 3,5,6 trichloro-2pyridinol,l diethylphosphate in PC 3).

2. Co-occurrence across chemical families is in general weak or not present unless for chemicals occurring in or originating from the same sources:

- PCBs and Hg co occur in human samples:
    - in the Snart Foraeldre/Milieu cohort study in Denmark using PCA analysis (Rosofsky et al., 2017): Hg and PCBs load PC 1.

- Mono-ethyl phthalates & parabens:
    - in the Snart Foraeldre/Milieu cohort study in Denmark using PCA analysis (Rosofsky et al., 2017): mono-ethyl phthalates & parabens load PC 2; the authors of this study explain this by personal care products as common source.

- Network analysis show that some chemical groups are closer to each other than others (examples of network analysis: see Figures 1 and 5 in Appendix 33) : e.g. in the FLEHS cohorts, PFAS and PCBs are in separate clusters, though the distance between those groups is shorter than e.g. the distance between PCBs and metals (Mn, Cu, Cd) network detection techniques (Ottenbros et al., 2021); (PFOA, PFOS, PFNA, PFHXS). As in the HELIX cohorts, the groups of PCBs and PFAS are close neighboring groups in the overall network (Tamayo-Uria et al., 2019)

3.2.5. INTERPRETATION OF TRENDS IN CO-OCCURRENCE PATTERNS FOR MIXTURES RISK ASSESSMENT

Chemicals within the same chemical groups tend to co-occur, and also may likely cause similar effects, due to similar chemical structures and/or functionalities, and thus act according to dose-addition. Therefore, it is recommend considering chemical groups as a basis for human health risk assessment of mixtures, and to take into account the co-occurrence patterns. When performing human health risk assessment on datasets at individual level, there is no need to explicitly account for co-occurrence patterns, since this type of information is inherently present in databases. However, when attempting to use HBM datasets at aggregated level, one should be cautious and avoid considering distributions of chemicals as independent.

Finally, it is important to be careful in the interpretation of lack of co-occurrence patterns. A lack of a co-occurrence pattern cannot at all be interpreted as the ***presence*** of those chemicals is mutually exclusive. Rather, it indicates that chemicals with lack of co-occurrence patterns (levels) are "randomly" co-occurring. Let's say if chemical A and B are both detected in the entire population, but they lack a co-occurrence patterns based on *levels,* it means that they both occur in every sample in the population, but the concentration levels of A and B are independent of each other. A lack of co-occurrence regarding levels of human can also be regarded as substances having independent distributions.

Table 1: Overview of publications investigating mixtures patterns in European HBM studies.

Name of Cohort	*# participants*	*Characteristics of cohort*	*# of chemicals/ biomarkers*	*Chemical (groups)*	*Matrix*	*# samples > LOD and treatment in analysis of co-occurrence*	*Reference*
Sub cohort of HELIX (6 EU birth cohorts: BiB, EDEN, INMA, KANC, MoBA and Rhea)	1301	Mother-child pairs	87 (pregnancy) 122 (childhood)	Organochlorine compounds PBDEs PFAS Metals and elements Phthalate metabolites Phenols Organophosphate pesticide metabolites Cotinine	Serum, plasma, blood or urine	/	(Tamayo-Uria et al., 2019)
Snart Foraeldre/ Milieu Denmark	73	Danish women (18-40 years) from the general population who stopped using contraception because they wished to become pregnant	135	Cotinine Metals PAH BFRs Herbicides Insecticides PCBs Pesticides Perchlorates Phenols Parabens Phthalates Phytoestrogens PFAS	Blood, Serum or Urine	Analysis restricted to biomarkers that were detected in 75% of the study population	(Rosofsky et al., 2017)
4 Flemish birth cohort: FLEHS I, II & III and 3XG (Belgium)	1579	Flemish mother-newborn pairs	7	PCBs Hexachlorobenzene p,p'-DDE Cadmium Lead	Cord blood	1.1–27% depending on the compound	(Govarts et al., 2020)
3 Flemish birth cohort: FLEHS I, II & III (Belgium)	281	Flemish mother-newborn pairs	19	Organochlorine compounds PFAS Metals	Cord blood	At least 60% of the measurements above LOD	(Ottenbros et al., 2021)

Name of Cohort	*# participants*	*Characteristics of cohort*	*# of chemicals/ biomarkers*	*Chemical (groups)*	*Matrix*	*# samples > LOD and treatment in analysis of co-occurrence*	*Reference*
INMA, Sabadell Spain	728	Pregnant women	81	Organochlorines PFAS Mercury PBDEs Metals Phthalates BPA Cotinine	Serum Cord blood Breast milk Urine	If analyte was nondetectable in > 85% of samples, biomarkers was excluded	(Robinson et al., 2015)
Flemish birth cohort FLEHS II **(Belgium)**	248	Flemish mother-newborn pairs	15	Metals PCBs P,p'-DDE PFOS POFA MECPP	Cord blood Maternal whole blood Plasma cord	0-21% of n < LOD/LOQ	(Govarts et al., 2016)
INMA, Sabadell Spain	657	Mother-child (7 years of age) pairs	27	BPA Phthalates Metals (arsenic, lead and cadmium, mercury) Organochlorine pesticides PC PBDEs	Uri Maternal blood Cord blood Maternal colostrum	n samples < LOD varies between 0 and 100	(Agay-Shay et al., 2015)
EDEN birth cohort, France	473	Mother-son pairs	20	9 phenols: 4 parabens, 2 dichlorophenols, Triclosan, benzophenone-3 and BPA 11 phthalate metabolites	Urine	All analytes were between 98-100% > LOD	(Philippat et al., 2019)

Name of Cohort	*# participants*	*Characteristics of cohort*	*# of chemicals/ biomarkers*	*Chemical (groups)*	*Matrix*	*# samples > LOD and treatment in analysis of co-occurrence*	*Reference*
ELFE nationwide birth cohort, France	311	Women who gave birth to liveborn singleton ≥ 33 weeks of gestation	64	Organochlorines Organophosphorus Pyrethroids Carbamates Dinitroanilines Thiocarbamates Phenylpyrazoles Acid herbicides Azoles Oxadiazines Triazines/triazones Amide pesticides Strobilurins Carboxamides Urea Neonicotinoids Anilino-pyrimidines	Maternal hair	28 of 64 pesticides and metabolites were detected in > 70% of samples, 10 were detected in 50-70% of the samples, 10 were detected > 50% of the samples	(Béranger et al., 2020)
Three birth cohorts: **Greenland, Poland and Ukraine**	1250	Mother-infant pairs	16	Secondary metabolites of DEHP and DiNP, PFASs organochlorines	Maternal serum	All 16 biomarkers were quantifiable in at least 72% of serum samples	(Lenters et al., 2016)
Avon Longitudinal Study of Parents and Children (ASLPAC), UK	448	Mother-female child pairs	52	8 PFAS 35 PCBs 9 organochlorine pesticides	Maternal serum	EDCs detected in greater than 75% of mothers were included in the main analyses.	(Marks et al., 2021)
EXBROM	50	First time mothers	12	PBDEs	Placental samples	Frequency of detection from 6% (BDE-17); to 16 % (BDE-66; DBE-85, BDE-183) over 22 % (BDE-28) to 72 % (BDE-100), and >= 88 % (BDE-47: BDE-99; BDE-153; BDE-154 and BDE-209)	(Frederiksen et al., 2009)

CHAPTER 4 RELATION BETWEEN MIXTURES EXPOSURE AND HEALTH OUTCOMES

Despite the large number of humane biomonitoring (HBM) studies, analysis of mixtures in HBM seems to be a rather unexplored field.

In this study, we performed a limited literature study on studies describing *chemical co-occurrence patterns in relation with health outcomes*. We focused thereby first on European HBM studies. However, studies from outside EU have also been considered because they may report other techniques and groups of substances. Both the snowball method and the use of key words in Pubmed and Web of science were applied as a search strategy (Chapter 2 in Appendix 3).

The results from this limited literature study should be regarded in view of the selected keywords (Chapter 2 in Appendix 3). A wider scope of keywords and search strategy might have revealed additional studies. However, an extensive literature search was out of scope of this project.

These search results were first screened for relevant titles after which the abstract was read to determine if the particular publication was suitable for inclusion in this report. The majority of these studies focus on perinatal exposure (exposure assessment in cord blood of newborns, breastmilk, urine, blood/serum of the pregnant mothers) in relation to perinatal health outcomes (birth weight, placental weight, birth length, head circumference), or in relation to longitudinal studies (e.g. BMI at 7 years; early menarche of female offspring).

This chapter summarizes findings from key studies in the EU.

Twelve out of a total of twenty-four identified studies (Appendix 3) reported stronger associations for mixture exposure were observed compared to single pollutants. This observation may simply be explained of the fact that the remaining studies did not investigate single pollutant associations and solely focused on mixture effects, except for the study of Reyes et al. (2018). These authors namely concluded that the largest risks tended to occur in individuals whose exposure were dominated by a single phthalate.

In the FLEHS II cohort was arsenic significantly associated with reduced birth weight in single pollutant models (91 g; 95% CI: 17; 164 g). The effect estimate increased when including cadmium and mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP) co-exposure (effect estimate = -121 g; 95% CI: -201; -42 g). Combining exposures by principal component analysis generated an exposure factor loaded by cadmium and arsenic that was associated with reduced birth weight. MECPP induced gender specific effects. In girls, the effect estimate was doubled with co-exposure of thallium, PFOS, lead, cadmium, manganese and mercury (estimate = -235 g; 95CI: -369; -102 g), while in boys, the mixture of MECPP with cadmium showed the strongest association with birth weight (estimate = -129 g; 95% CI: -220; -37 g). Govarts et al. (2016) and Govarts et al. (2020) concluded that some chemicals (i.e.. Cd, Pb, PFOA and MECPP) not showing significant associations at single pollutant level contributed to stronger effects when analyzed as mixtures at the exposure levels occurring in this cohort.

In the IMNA study, exposure to the highest tertile compared with the lowest tertile of the organochlorine factor (factor 3: loaded with organochlorines perinatal exposures – blood and urine of mothers) was associated with a significant increase in the z-BMI of the 7-years old with a RR of 0.37 (95% CI: 0.03 - 0.72) and with an increase in the RRs of overweight of 2.59 (95% CI: 1.19, 5.63) (Agay-Shay et al., 2015).

In the French EDEN mother-sons cohort (Philippat et al., 2019) evidence of possible associations between triclosan, benzophenone-3, MCNP and MCOP and effects on placental weight and placental-to-birth-weight ratio PFR was given. Triclosan (β = -4.11 g; 95% CI: -8.26; 0.05 g) and MNCP (β = -10.9 g; 95% CI: -21.8; 0.09 g) were negatively associated with placental weight while benzophenone-3 (β = 4.76 g; 95% CI: -1.77; 11.3 g) and the sum of parabens (β = 7.12 g; 95% CI: 0.41; 13.9 g) were positively associated with placental weight. MNCP (estimate = -0.20; 95% CI: -0.54; 0.13) and MCOP (βestimate = -0.23; 95% CI: -0.58; 0.11) were negatively associated with PFR. The fact that the direction of the associations with placental weight differed across biomarkers might indicate different mechanisms of action.

The ELFE French nationwide birth cohort (Béranger et al., 2020) demonstrated statistically significant associations between maternal hair concentrations of mixtures of pesticide metabolites and birth measurements (weight: fipronil sulfone; length: TCPy, bitertanol, DEP, and isoproturon; head circumference: tebuconazole and prochloraz). The authors observed statistically significantly higher BW for a medium ($\beta_{adjusted}$ = +150 g; 44, 255), exposure to fipronilsulfone ($\beta_{adjusted}$ = +28 g; −84, 141). A statistically significantly lower body length associated with the intermediate ($\beta_{adjusted}$=−0.64 cm; −1.15, −0.14) exposure to DEP as well as with the hair concentration of TCPy (per 2-SD increase in log-transformed concentration: $\beta_{adjusted}$=−0.42 cm; −0.85, 0.00). Statistically significantly higher body length was also observed among children of women with detectable bitertanol ($\beta_{adjusted}$ = +0.60 cm; 0.09, 1.10), and isoproturon ($\beta_{adjusted}$ = +0.55 cm; 0.11, 1.00) in hair. Finally, a statistically significantly larger head circumference was associated with prochloraz (detected vs. not: $\beta_{adjusted}$ = +0.57 cm; 0.17, 0.97) and tebuconazole (detected vs. not: $\beta_{adjusted}$ = +0.31 cm; 0.01, 0.61). In data from three birth cohorts (Greenland, Poland, Ukraine), mixtures of phthalate metabolites (MEHHP, MOiNP), perfluorooctanoic acid (PFOA), and *p,p´*-DDE were most consistently predictive of term birth weight based on elastic net penalty regression (Lenters et al., 2016). 2-SD increases in natural log–transformed MEHHP, PFOA, and *p,p´*-DDE were associated with lower birth weight: –87 g (95% CI: –137, –340 per 1.70 ng/mL), –43 g (95% CI: –108, 23 per 1.18 ng/mL), and –135 g (95% CI: –192, –78 per 1.82 ng/g lipid), respectively; and MOiNP was associated with higher birth weight (46 g; 95% CI: –5, 97 per 2.22 ng/mL).

Marks et al. (2021) investigated the associations between prenatal exposure to PFAS, PCBs and organochlorine pesticides as mixtures with early menarche among female offspring. No significant relations or interactions were found.

For further details regarding individual studies, and results from similar non-European studies we refer to Appendix 3.

In summary, statistically significant associations between mixtures exposure (from nowadays levels of chemicals present in humans assessed by human biomonitoring) and health effects in the (vulnerable groups of) general population have been reported in several studies.

Not all effects were observed using single chemical models, and associations were stronger when mixtures were considered compared to single chemical exposure associations. Current exposure levels, for example PCBs, in human bodies were often caused by historical contamination, but are still found in human samples due to the persistency and long half lifes.

While some studies reported associations between mixtures exposure and adverse health outcomes, there were also some studies showing presence of mixtures in biofluids, where no adverse effects were observed. An overarching conclusion over different studies is difficult to draw given the differences in characteristics in chemical mixtures, different methodologies and different outcomes and different cohorts investigated across studies.

At the same time, it's important to realize that the current epidemiological knowledge on mixtures in relation health effects is mainly focused on a specific, vulnerable subpopulation (newborns), considering generic health outcomes and a limited list of chemicals Epidemiological studies considering effects of mixtures in other vulnerable populations and life stages (e.g. puberty, elderly) and other types and more specific categories of health effects (e.g. effects of mixtures on neurodevelopmental effects, cardiovascular effects, respiratory system, immunological effects, etc.) remain to be investigated. Additionally, the associations between health outcomes and mixtures pertain often to legacy chemicals, which have been used in the past, but are currently no longer used or only used in exceptions (e.g. POPS under Stockholm Convention, chemicals under Restriction or Authorization). In view of the discussion of MAF for REACH chemicals, it would be very useful to have this type of analysis performed also on mixtures of non-legacy chemicals.

Overall, mixture exposure might lead to adverse effect on human health and this observation should trigger human health regulatory risk assessment to also consider mixtures.

CHAPTER 5 USE OF HBM DATA IN HUMAN HEATLH MIXTURE RISK ASSESSMENT

Whereas some epidemiological studies demonstrate that human exposure to mixtures reveals a better model to explain health effects in the general population compared to single chemical exposure (see CHAPTER 4), the statistical models (or RR) described in these studies cannot be used in regulatory risk assessment concepts such as RCRs under REACH.

In this chapter, we elaborate the mixtures risk assessment case studies using human biomonitoring data, and discuss outstanding issues that should be further investigated to move forward to use human biomonitoring data in risk assessment practices and regulatory risk assessment. A practical instrument to preform mixtures risk assessment is the combined maximum cumulative ratio (MCR) and Hazard Index (HI) approach.

The HI of a sample (biofluid sample) is the sum of hazard quotients (HQ) of several chemicals present in the biofluid. In its turn, the HQ is the ratio of the concentration (exposure level) to the health-based guidance value per substance. The HI thus represents the risk of the mixtures, based on sum of risks of components in the mixtures. Usually, first the HI approach is applied and only in case of a HI > 1, one wants to look further to estimate whether the mixture risk is caused only by one or a few chemicals. This might obviously be of help for indicated risk management measures.

The MCR approach is an extension of the hazard index (HI) which is commonly used as a screening tool for evaluating mixture toxicity (Meek et al., 2011). In addition to HI, MCR quantifies the significance of cumulative toxicity compared to single component toxicity and is a tool for investigating the magnitude of the toxicity potentially missed if a cumulative risk assessment is not performed (Han and Price, 2011). As described in Han & Price (2011), the MCR can be calculated using the hazard quotients (HQs) for each substance present in a mixture and the hazard index (HI) of the mixture. The value of MCR for an individual exposed to a mixture of n substances in an environmental media is calculated by:

$$HQ_i = {}^{C_i}/_{GV_i}$$

$$HI = \sum_i HQ_i$$

$$MCR = {}^{HI}/_{\max HQ_i}$$

where Ci is the concentration or the exposure (dose) of the i^{th} substance to which an individual is exposed and RV_i is the health-based guidance value (GV) of substance i (expressed as a concentration). HQi is the hazard index of the individual's exposure to the i^{th} substance. The MCR of the individual's exposure to the mixture is the ratio of the HI of the mixture to the maximum of the hazard quotients of the individual components (max HQi).

Ideally, in the context of human biomonitoring data, the Ci in the nominator refers to exposure biomarkers levels in biofluids (biomonitoring data), and the GV_i in the denominator is the corresponding health based human biomonitoring guidance (HBM-GV) value for i. However, in practice, we see often, by lack of available HBM values for several substances, an alternative approach: using reverse dosimetry from biomarker levels to external dose (nominator), in combination with HB GV for external exposure (see further).

Both the HI and MCR (based on the HI) approaches are based on the hypothesis of dose addition, which is considered a conservative assumption for evaluating mixture effects of non-carcinogenic substances (Meek et al., 2011), especially when applied in a low tier mixtures without considering communalities in endpoints or mode of action. The MCR ratio is bounded by 1 and n (n= the number of analyzed substances in the mixture). An MCR close to 1 means that one substance is responsible for nearly all the toxicity of the mixture. Exposures to a mixture of n substances with equal toxicities would have an MCR of n. The MCR and HI approach can also be used in a higher tier approach, where the summation of HI considers only chemicals affecting the same health effect or according the same mode of action.

In addition to the HI and MCR approach, also the EFSA 'cumulative assessment groups' (CAG) approach offers a practical approach to perform mixtures risk assessment, and is most applied to the domain of pesticides (EFSA, 2013). This is a clear higher tier approach which makes sense as for pesticides risk assessment huge information requirements exist. In the CAG approach, grouping of substance according to mode of action and information on the toxicological profiles is already taken into account by default in pesticides mixtures risk assessment.

In addition to the HI and CAG approaches, also other approaches to perform mixtures risk assessment (e.g. the use of the HI-interaction method, the use of the Relative Potency Factors, etc.) are described, often to be used in higher tier mixtures risk assessments. For more details, we refer to the review paper of Kienzler et al. (2016).

In this report, we mainly focus on the HI/MCR approach since this is the most widely used method applied on human biomonitoring data, and because the MCR value is most useful in trying to estimate the level of the MAF factor (Backhaus, 2015).

5.1. APPLICATION OF HI AND MCR ON HUMAN BIOMONITORING DATA

In several publications, the HI and MCR concept have been applied in human biomonitoring data.

Nearly all publications deal with mixture risk assessment covering risks of substances *within a chemical group*. Combining risks from chemicals belonging to the same group in a HI make sense since chemicals belonging to the same group of chemicals have similar chemical structures and/or functionalities, and therefore, they may cause similar effects and have similar behaviour in terms of their absorption, distribution, metabolism and excretion, resulting in similar toxicokinetics and so clustering in their fate. Consequently, they are more likely to act according to dose-addition, and thus combining HQs in HI is a reasonable approach. Hardly any of the publications calculated risks across chemical groups.

This make sense since chemicals belonging to different group of chemicals different chemical structures and/or functionalities, and therefore, are likely to cause different effects. The issue is that different chemicals may have completely different toxicity, that is to say one chemical might cause liver damage and the other chemical neurotoxicity. The application of dose addition is in that case a worst-case scenario in a low tier (conservative HI). In this example of only two chemicals, the mixture risk is probably absent. Notwithstanding that it's less likely that several chemicals belong to different groups cause similar effects, it cannot be excluded at priori that chemicals belonging to different groups cause similar effects. Several authors point to consider further mixtures risk across chemical groups (Borg et al., 2013; Kortenkamp, 2020). Hereto, further developments should be made in view of gathering detailed toxicological information for grouping, point of departure values for relevant endpoints, and adverse outcome pathways, to enable mixtures risk assessment across chemical risk assessment in a higher tier approach (e.g.; using the Point of Departure Index, instead of the HI approach).

Calculation of HI and MCR *across chemical groups* in a low tier approach (based on additivity for all compounds in the mixture, irrespective of common health effects or not and combining critical guidance values that likely reflect a diversity of health endpoints across substances) is theoretically possible for human biomonitoring datasets, as was previously done for indoor air mixtures and drinking water mixtures as a relevant matrices for external human exposure (De Brouwere et al., 2014; Han and Price, 2011; Szabados et al., 2021). However, we did not find literature describing the use of the HI/MCR concept in such a low tier, screening approach with no distinction between relevant health endpoints with HBM data. This is likely because a low tier, screening assessment for human biomonitoring would go with a significant higher likelihood of finding individual HBM samples with HI > 1. This would prompt to shift from a low tier, screening assessment to higher tier assessment. For higher tier assessments, considering of grouping based on health effects and endpoint specific PODs for common effects is recommended (Meek et al., 2011); and this type of assessments is also what we found in literature (see further). Additionally, the use of low tier methods for hazard assessment (grouping all substances, irrespective of communalities in health effects) in combination with high tier level for exposure assessment (using HBM data) would rather be unbalancing the tiered levels of hazard versus exposure in the tiered approach (Meek et al., 2011). On the other hand, if HI<1 for the majority of the individuals using the conservative low tier approach, then mixture risks can be reasonably excluded and no further work would be indicated.

5.1.1. HBM MIXTURES RISK ASSESSMENT WITHIN GROUPS OF CHEMICALS

Majority of the HBM HI/MCR studies within chemical groups pertain to mixture risk assessment for phthalates, with a focus on risk for reproductive and developmental toxicity by anti-androgenic modes of action, since this is the critical and common health effect of several phthalates.

Apel et al. (2020) included in their paper a review of mixture risk assessment for phthalates, including 7 EU studies (4 studies in Denmark, 1 study in Belgium, 1 study in Poland, 1 study in Austria and 1 in Germany) and studies in Taiwan, USA, China, Iran, South Korea and Brazil. Results described in this section are based on the EU studies.

The studies calculated HI and MCR on individual datapoints in datasets and relied on back-calculation of HBM data (urinary levels) to external exposure estimates, which are used in HI calculations, in combination with TDIs (external health-based guidance values – HB GV) for single phthalates. To varying degrees, these studies revealed exceedances both of acceptable single as well as (expected) combined phthalate exposures. For example, the 95th percentile HI of a children study in Denmark was 2.3 (Bekö et al., 2013)). Due to their high prevalence and high potency in disrupting male sexual development, DBP and DEHP generally contributed most to the HI. According to Apel et al. (2020), the outcomes of these various phthalate mixture risk assessments are difficult to compare because of three reasons. Firstly, different phthalates and numbers of phthalates were investigated in the different studies. Secondly, the studies used often different HB GV, e.g. TDI for phthalates (EFSA 2005) versus RfD for phthalates from A. Kortenkamp & Faust (2010) or new RfD for anti-androgenicity for phthalates from (Kortenkamp and Koch, 2020). This factor might lead to a 5-fold difference in HI values, if all other factors are kept constant. Thirdly, there is also evidence that phthalate exposures have undergone changes over the years (Apel et al., 2020). This time trend was analyzed by Apel et al. (2020). They performed HI and MCR calculations for each participant from the German Environmental Specimen Bank, which is a 27-year survey of urinary phthalate metabolite levels in 24-hour urine samples.

The decreasing phthalate exposures over the last decades led to declining HIs. Whereas the geometric mean HI was 1.8 in 1993, it dropped to 0.2 in 2015. Similar, the 95th percentile HI dropped from 7.5 in 1993 to 0.55 in 2015 (while keeping the exposure assessment method and RfDs constant over the calculations). Apel et al. (2020) attributed this decrease to the decreasing HQs for DBP and DEHP over time as internal exposure levels (HBM data) decreased over time. By contrast, they found the HQs for the other phthalates fluctuated or even increased slightly (DINP) between 1988 and 2015. As a result, the more recent HIs were driven by a greater number of phthalates, reflected in the slight upwards gradient of the regression lines of MCR versus study year (increase of MCR from 1.5 to 1.8 for the geometric mean, and a value from 2.0 to 2.5 for 95th percentile MCR).

Additionally, studies on application of HI and MCR concept on human biomonitoring data were found for parabens (Moos et al., 2017), dioxins, furans and PCBs (Han and Price, 2013), pesticides (F. Fernández et al., 2020; Katsikantami et al., 2019) and PFAS (Borg et al., 2013).

The study regarding parabens (6 parabens: MeP, EtP, iso-PrP, n-PrP, iso-BuP, n-BuP) was based on the same database (German Environmental Specimen Bank) and method for HI calculations as the case study on phthalates (Apel et al., 2020) (i.e. also using reverse dosimetry from biomarker levels to external exposure). For the hazard assessment, the group ADI from EFSA for MeP + EtP was used. For the other parabens, by lack of an official health based guidance value, (Moos et al., 2017) used the benckmark (NOEL) used by Scientific Committee on Consumer Safety (SCCS), in combination with an Uncertainty Factor of 100. Common health effects are endocrine effects. As a result, median HI within the population (n = 660) was 0.1, while the HI at the 95th percentile and a maximum HI were respectively 1.3 and 4.4. n-PrP was the most influential contributor to the HI, followed by other longer chain parabens. At the 95th percentile the contribution of n-BuP to the HI is ~20%, and the contribution of iso-BuP is ~ 5%. The short-chain parabens (MeP and EtP) only play a small role in the cumulative HI of the parabens. Moos et al., 2017 did not report MCR values. It can be derived from the data that MCR is rather low given the dominance (> 50 %) of n-PrP in the median, P95 and maximal HI.

The study on dioxins and PCBs (26 compounds), performed on HBM samples from the NHANES cohort (US) and two occupational studies in the US, focused on MCR rather than on HI (Han and Price, 2013). Although the two occupational groups have higher total toxicity equivalence (TEQ) levels than the NHANES group, average MCR values of the three groups are similar (3.5, 3.6, and 3.2). The MCR values also indicated that only 2-5 of the 26 chemicals make significant contributions to total TEQ values (Han and Price, 2013).

The Spanish pesticides study of F. Fernández et al. (2020) evaluated cumulative risks using 26 metabolite biomarkers of organophosphate pesticides (OP), herbicides and pyrethroids in urine of 568 children.

F. Fernández et al. (2020) evaluated cumulatieve pesticide risk using two different strategies: one based on the pesticides' mode of action (MoA) as grouping method in combination with the HI appraoch and the other based on cumulative assessment groups (CAGs) approach, proposed by EFSA. Three separate HI were calculated: The HI was calculated for the group of organophosphates (OPs), thus not including the herbicides and pyrothroids with the argumentation that they should be assessed separately since they have different mode of actions. HI for OPs was calculated adding the HQs of chlorpyrifos-ethyl (ΣDEPs) and dimethoate (ΣDMPs) using the their estimated daily intake (external dose), back-calculated from dialkyl phosphates (DAP) concentrations in urine, i.e. the representative metabolites of almost the entire OPs family. The HI for the OP group was <1 in the entire cohort. Dimethoate contributed most to the HI. For the other pesticides (parathion, and ƛ-cyhaolthrin), HQ was calculated (P95 HQ < 1), but these were not included in any HI calculations. The data presentation in paper did not allow to estimate MCR values.

A second study using HBM pesticides data used aggregated data for cumulative exposure to malathion, diazinon, parathion, phorate and dimethoate (Katsikantami et al., 2019). The authors used literature data from various HBM studies. Median HI values for children ranged between 0.016 and 0.618, for pregnant women between 0.005-0.151, for the general population between 0.008-0.206 and for farmers between 0.009-0.979. Combined exposure to dimethoate and phorate was the worst-case scenario. However, the assessment was made on aggregate data, and not in individual data, which makes the assessment more difficult to interpret. Not only the use of aggregated data makes this assessment harder to interpret, also the combination of several studies (different cohorts, age of study, profile of participants) makes the interpretation of relevance for real life exposure difficult. No information on MCR values could be derived.

Borg et al. (2013) assessed the cumulative risk of 17 PFAS substances in the Swedish population (2006-2013). In contrast to the prevous described studies, the HQ was based on the ratio of levels in serum, to the point of departures expressed as internal dose (µg/ml serum), and thus avoiding uncertainties in reserve dosimetry. It is noted that the values they used are no official HBM GV, but toxicogical data obtained from literature researches, performed within that study. For PFAS with lacking information of POD expressed as serum levels, the authors used read acros techniques.

One case study covered urinary levels of the general population, indirectly exposed to PFAS via the environment, and an occupational group of professional ski waxers. They included 6 small HBM studies (n = 9-80) from 2006 onwards.

They did not perform RA on all individuals of the cohorts; instead the highest concentrations at the latest time-point in a temporal study or from a sample in a snapshot study were selected to perform the RA. The toxicological endpoints evaluated were hepatoxicity, reproductive toxicity, immunology and mammary gland development. The HQ of PFOS for immunotoxicity for the general population was very high (HQ = 229), as well as the HQ for mammary gland development for PFOA (HQ = 18). For these two effects/substances, single substances risk assessment points out for concern; therefore mixture risk assessment is less relevant. For the other considered health effects (hepatoxicity and reproductive toxicity) the HQ for the single substances was < 1, and the HI of the mixture was 0.27 and 0.18 respecitevely for hepatoxicity and reproductive toxicity. For both effects, PFOS dominated the HQ (64 % of HI for hepatoxicty and 76 % of HI for reproductive toxicity). In a separate high exposed subpopulation eating PFOS-contaminated fish, the HI were higher (HI for hepatoxicty: 1.4; driven by PFOS; 90 % of HI). For occupational exposed group (ski-waxers), HQ for PFOA was 3.8 for hepatotoxicity, and HQ for PFOA was 0.85 for reproductive toxicity and PFOA was the main contributor the mixtures toxicity. Hence, we also see 1-2 compounds dominating the risk of the mixtures in this study. Nevertheless, since PFOA and PFOS are phasing out, the use of other PFAS is increasing, which is visible in more recent HBM studies. Therefore, the pattern of dominance of PFOS and PFOA in the mixture toxicity is likely to decrease, and more PFAS might play a dominant role in the HI in the future.

5.1.2. HBM MIXTURES RISK ASSESSMENT ACROSS GROUPS OF CHEMICALS

There are some exception on the finding that HI calculations are limited to chemicals within a group. For example, the human health mixture risk assessment of A. Kortenkamp & Faust (2010) included exposures to a combination of 15 chemicals: phthalates (DBP, BiBP, BBP, DiNP, DEHP), pesticides (vinclozolin, prochloraz, procymidone, linuron, fenitrothion, pp'DDE), a flame retardant (BDE-99), bisphenol A, and parabens (butyl paraben and propyl paraben), all being substances capable of producing reproductive and developmental toxicity by anti-androgenic modes of action. In their analysis, A. Kortenkamp & Faust (2010) assessed exposure to those chemicals from a diversity of (mainly) EU sources and approaches (not limited to human biomonitoring; they also included external exposure estimates based on food consumption), and used reference doses for anti-androgenicity (RfD AA) in the denominator of the HI value. Reference doses were retrieved from EFSA opinions and EU documents. For substances where the exposure was assessed using HBM data, the, reverse dosimetry was used to calculate exposure (as nominator in the HQ).

A. Kortenkamp & Faust (2010) calculated a HI of 0.38 for a median intake scenario, and a HI of 2.01 for a high intake scenario. The substances contributing most to the HI were DEHP (24 %), vinclozolin (32 %), butylparaben (16%) in case of the median scenario. Other substances had a contribution < 10%. 6/15 six chemicals made up 91% of the HI for the median scenario. In case of the high intake scenario, substances contributing most to the HI were butylparaben (50%) and prochloraz (14 %). The HQ's of 7/15 chemicals explained 94% of the HI for the combination of the 15 anti-androgens in the high intake scenario.

A. Kortenkamp & Faust (2010) did not calculate MCR values. Based on their data, we estimated MCR values of 2.0 and 3.2 for the median and high intake scenario respectively.

However, it is important to mention that A. Kortenkamp & Faust (2010) did calculate HI on aggregate data, and constructed a median scenario based on median exposures from all chemicals, and a high intake scenario based on a combination of high intakes (mostly based on 95th percentiles) of the 15 chemicals. This is obvious deviating from the standard approach of using individual records in mixture risk assessment. It is questionable if a combination of high intakes (95th) of 15 chemicals is realistic within the population. And therefore, it is also questionable whether the value of HI of 2.01 for the high intake scenario would correspond to the 95th percentile of HI if based on individual data. An indication here can be found in the study of Willey et al. (2021). They developed the Exposure Load (EL) concept. The EL calculates "to how many chemicals, persons are simultaneously internally exposed above a predefined threshold" e.g. EL 50, EL 95; They applied the EL on Canadian Health Measurement Survey (CHMS), a Canadian HBM study 2012-2015 involving 1858 participants aged 12-79 years, and 44 analyte biomarkers representing 26 chemicals. At the threshold of the 95th percentile, the majority of the Canadian population had an EL between 0 and 3. 4 % of the population had an exposure load of 12. Few participants had an EL up to 15 or 17; none had an EL > 18. Notwithstanding this is a study from another region and is limited to one study, the study of Willey et al. 20201 indicates that *combining 95th percentiles of all chemicals does not reflect a realistic P95 scenario (about combining 95th percentiles for half of 2/3 of chemicals would be more realistic*).

Nevertheless, the concept of applying HI and MCR calculations on substances across chemical groups considering common health effects, is interesting. It would be useful to apply their concept on individual exposure data records instead of aggregated data.

5.2. FURTHER REFLECTIONS AND DISCUSSION ON THE USE OF HBM DATA IN MIXTURE RISK ASSESSMENT

5.2.1. DOMINANCE OF LIMITED SET OF CHEMICALS IN MIXTURE TOXICITY?

In the majority the cited references, a few compounds drive the toxicity (MCRs typically 2-3). However, two important considerations should be made: this conclusion is often based results from within group chemicals mixtures risk assessment, with a limited number of substances considered (e.g. phthalates: often only 4-6 compounds considered). It should be further investigated whether this dominance of a few compounds is still valid when considering across chemical groups risk assessment, considering time trends where substitution towards more compounds (e.g. PFAS and phthalates) is likely to occur.

5.2.2. INFLUENCE OF NON-DETECTS ON HI AND MCR

As discussed by Price & Han (2011) non-detects (NDs) are a significant issue in the HI and MCR method. For the majority of the HBM4EU priority chemicals, at least one exposure biomarker is detected > LOD in nearly all samples (see previous chapters). We checked how the authors in the abovementioned HI and MCR papers dealt with this issue. Several replaced values < LOD by a value of LOD/(sqrt 2), or by imputation techniques. Han & Price (2013) trimmed their datasets by removing people where NDs had a significant impact on the MCR. Apel et al. (2020) mentioned that none of their findings was sensitive to their treatments of non-detects (since non-detected scarce in their dataset).

Other authors did not report explicitly on the impact of ND treatments on the HI or MCR values. From a few studies, we could derive that the impact is probably rather low. For example, in the PFAS study of Borg et al. (2013), exposures were reported < DL for PFDoDA, PFTrDA, PFTeDA and 6.2 FTS, but the contribution of exposures to HI for hepatoxicity and reproductive toxicity was < 1 %, which the exception of 5.9 % for 6.2 FTS for reproductive toxicity. Moos et al. (2017) had for 2/6 parabens levels < LOD in majority of the samples (iso-PrP, iso-BUP). They substituted values < LOD by LOD/2; however, they did not report on the impact on the HI.

When a lot of non-detects are present in the datasets, it is recommended to use imputation techniques for values < LOD, since imputation techniques are in literature currently regarded as the most appropriate method to deal with value < LOD. It is also recommended to assess the impact of covering non-detects in the HI (cfr. Moos et al., 2017), to get a feeling on the potential importance of non-detects in the mixture risk assessment.

5.2.3. CHOICE OF HEALTH BASED GUIDANCE VALUE IN HI CALCULATIONS

The value for the denominator in the HQs and HI might have an important impact on the outcome of the HI. The GV (denominator in HQ) reflects the health-based guidance value for that substance. In view of HBM data, we see two practices: 1) the use of a RV for internal exposure (human biomonitoring guidance value or HBM GV) and, 2) the use of reference value for external exposure

The first approach is the most straightforward one, since a direct use of HBM monitoring data in the HQ calculations is possible. The second approach requires reverse dosimetry from HBM levels to external dose (for the nominator in HQ). The latter introduces uncertainties given that the reverse dosimetry is based on point estimates for empirical molar extraction factors (Fue), for which the values are rather uncertain. None of the authors explicitly calculated the impact on uncertainty of the Fue value on the HI. However, Fue is very sensitive to fluctuations in the exposure, especially for chemicals with relatively short half-life and at the same time. The second reason, because of the same fact, is that the levels of biomarkers measured in spot-urine – which is the most commonly used approach to sampling urine – will vary very much for these chemicals. Therefore, we anticipate that using point value for Fue (and read across) as done in the cited studies, introduced some uncertainty on the HI values.

While the first approach is the preferred one because of above mentioned reasons, the latter is done in all nearly cases we found in literature (except the PFAS study from Borg et al. (2013). The use of HBM internal guidance values is currently hampered by the limited number of substances from which HBM guidance values are available (Apel et al., 2017).

Also, in case a health based guidance value for external exposure is used, there are often several candidate values for use as GV in HQ calculations. E.g. for phthalates, the choice for either the EFSA 2005 TDIs versus the RfD by A. Kortenkamp & Faust (2010; Andreas Kortenkamp & Koch (2020) has a large (up to 5-fold) impact on the HI values (Apel et al., 2020). For other substances (e.g. parabens) benchmark (NOEL) in combination with an Uncertainty Factor (e.g. of 100) is used by lack of an official health-based threshold value. Also, when using endpoint specific values (e.g. done for PFAS) for endpoint specific HI opens the question for the selection of the most appropriate key study and point of departure.

In conclusion, the outcome of mixtures RA in HBM data is rather sensitive to the choice for GV for individual compounds in the mixtures. Harmonization in choice could be welcomed. Also, the use of DNEL as instead of regulatory established values such as RfD, TDI, … is not yet assessed. However, this would be very relevant in the discussion of the MAF value.

5.2.4. ACROSS CHEMICAL GROUPS MIXTURE RISK ASSESSMENT

Nearly all publications deal with mixture risk assessment covering risks of substances *within a chemical group*; hardly any of the publications calculated risks across chemical groups. Several authors point to consider further mixtures risk across chemical groups ((Borg et al., 2013; Kortenkamp, 2020)) in order not to miss part of the mixture toxicity. However, a 'blind' summing (as in screening tool) is not recommended for mixture risk assessment on HBM data. To shift forward for across chemical group mixture risk assessment forward, further developments should be made for higher tier approaches, including gathering detailed toxicological information for grouping, point of departure values for relevant endpoints, adverse outcome pathways, and derivation of endpoint specific guidance values to enable a higher tier mixture risk assessment.

5.2.5. ADDITIVITY CONCEPT

Above mentioned assessments rely on the assumption of the dose additivity concept and does not capture for other types of interactions (e.g. synergistic effects). For a practical point of view, it is not feasible to consider other types of interactions in regulatory mixture RA approaches; however, one should realize that dose additivity might as well underestimate the risk, e.g. in cases of synergism.

Systematic review analyzed over 700 mixture studies, confirming additive models are a good approximation (Martin et al., 2021). However, it should be relegalized that in some cases dose addition might underestimate the risk. A pregnant Swedish mothers pregnancy cohort (the SELMA study) gives indications in this direction (Bornehag et al., 2019). A reference mixture of these substances was prepared and tested for relevant effects in vitro and in vivo. In a next step they used the Similar Mixture ApproaCH (SMACH) to find out how many mothers in the SELMA cohort had a sufficiently similar mixture detected and would be considered of concern regarding the exposure of the unborn baby. Using this approach, the authors found many more Swedish mothers to be at risk (13 %) compared to using only single substance assessments (1.6 %) and also compared to the dose additivity approach (3 %) (Bornehag et al., 2019).

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

The central research question in this report is whether human biomonitoring (HBM) data can inform us about the magnitude of a future mixture assessment factor (MAF) that is needed in order to protect human health sufficiently regarding risks that go with human exposure to mixtures. In order to investigate this, four basic topics were addressed – in a logical order of needed availability and complexity - from "HBM mixtures data availability", "co-occurrence patterns" and "relation between HBM exposure biomarker levels and health effects" to "HBM-based mixtures risk assessment".

In order to be really useful for mixtures risk assessment, individual HBM data are needed. They are however not publicly available due to ethical and data protection requirements. The data owners need to be involved and user agreements arranged. This was outside the scope of the current project due to time constraints. The HBM part of the project therefore focused completely on aggregated data sets available via public platforms and repositories.

Data sets on human biomonitoring data

More than one hundred human biomonitoring (HBM) data sets already exist in Europe. Many of them are visible and the owners traceable via IPCHEM. The metadata are always visible and for part of the data sets also the measurement data can be accessed directly, albeit only in aggregated form (e.g. P05, P50 and P95). Because of privacy and data protection issues, for individual data, the data owner has to be contacted in all cases to get to a mutual agreement on use of the data, obviously in line with the EU General Data Protection Regulation. The studies as described in chapter 3 illustrate that 'classical' targeted screening of chemicals in HBM is likely to result in only a fragmented picture of the entire mixture that can be present in human matrices. With further development and wider acceptance, suspect and non-targeted screening are expected to become a very useful tools to generate mixtures exposure data. The latter techniques have much more potential to generate data on the co-occurrence of a much wider set of chemicals and to perform mixtures risk assessment.

Recommendation: Based on this conclusion, it is recommended to invest significantly into further development, quality assurance, quality control, acceptance and use of suspect and non-targeted screening in order to generate more data on a wider set of chemicals in human matrices compared to the current targeted monitoring campaigns.

Co-occurrence of chemicals in HBM data

From the results as presented in chapter 4 it can be concluded that substances within the same chemical groups tend to co-occur. Furthermore, due to similar chemical structures and/or functionalities, they may very cause similar effects and thus act according to dose-addition. Importantly, when performing human health risk assessment on datasets at individual level, there is no need to explicitly account for co-occurrence patterns, since this type of information is intrinsically taken into account.

Recommendation: All in all, it can be cautiously recommended to consider chemical groups as a basis for human health risk assessment of mixtures, and to take into account the co-occurrence patterns. However, when attempting to use HBM datasets at aggregated level, one should be cautious and avoid considering distributions of chemicals as independent.

Relation HBM levels and health effects

The studies found on assessment of mixtures found in HBM and health effects demonstrate stronger exposure-effect correlations for mixtures compared to single substances in the mixture. Clear statistical associations have been found between the levels of mixtures of chemical substances in HBM samples and some health effects. These associations were stronger for some combinations of substances than for each of the substances on their own.

Notably, some of the substances found in human bodies (e.g. PCBs) are caused by historical contamination. They are still found in human samples due to the persistency and long half-lives. The observation that epidemiological studies indicate health effects due to mixtures exposure triggers human health regulatory risk assessment to also consider mixtures.

Recommendation: It is recommended to include mixtures exposures when correlating exposure to health outcomes in epidemiological studies. This will provide a better estimate of risks linked to chemicals exposure then just doing substance by substance risk assessment. And as legacy chemicals in our bodies may contribute as well to mixtures risk of currently marketed chemicals it is recommended to include them in assessing overall mixtures risks as long as they are present in human matrices.

Mixtures risk assessment

Quite some studies have been found in which it was attempted to perform mixture risk assessment. However, the approach of many of these studies was different, making it hard to make direct comparisons. Also, in many studies the denominator for the HQ calculations to add up to the HI was an external guidance value such as the TDI and the measured HBM values were calculated back to external exposures. This reverse dosimetry is associated with large uncertainties.

Recommendation: It is recommended to develop health-based HBM guidance values for many more chemicals, even if only preliminary.

Overall discussion and conclusions

Regarding the central question at the basis of the work described in the underlying report, i.e. whether HBM-based mixture risk assessment outcomes could inform discussions regarding the magnitude for a MAF for setting DNELs for individual chemicals under REACH, it is concluded that currently available information in the literature is insufficient in amount as well as in quality to address this question unequivocally. However, some important findings can be reported to aid the discussions.

Some studies do provide HI values for a series of substances determined in HBM samples. But the approaches used to calculate the denominator in the HQ's for each individual chemical are typically associated with large uncertainties. Often, no health-based HBM guidance values exist and HBM exposure biomarker concentrations, assuming steady state exposure and steady state kinetics, are calculated back to modelled external exposure (back-extrapolation or reverse dosimetry) using the urinary excreted fraction (Fue).

Unfortunately, this approach leads to large uncertainties. One important reason is that the Fue is very sensitive to fluctuations in the exposure, especially for chemicals with relatively short half-lives. The second reason for the uncertainty, is that the levels of biomarkers measured in spot-urine – which is the most commonly used approach to sampling urine – will vary very much for these chemicals.

A second problem encountered is that more or less all mixture risk focused studies include a relatively low number of chemicals. This implies a higher odd of finding low MCR values which might lead to the conclusion that only one or two chemicals are causing a mixture risk. But if many more chemicals would be included in the mixture risk assessment, the chances of finding higher MCR values would possibly increase. Which means that, although currently found MCR values tend to be low, it cannot yet be concluded definitely that mixture risk in human matrices is mainly determined by a few chemicals in the mixture.

Current studies suggest the following, although insufficient data are available to draw definitive conclusions: if a lower tier mixture risk assessment (so no knowledge or consideration of target organs or modes of action) of *chemical groups of structurally similar chemicals* shows HI values clearly above 1, there is a high probability that the mixture risk is realistic and not worst case because structurally similar chemicals quite often exhibit similar target organs toxicity.

One clear conclusion is though that in order to generate more knowledge to feed into the MAF discussion, HBM data on more substances are needed. In this respect, more focus on and more commissioning of suspect-screening and non-target screening will probably deliver better quality data, i.e. on larger mixtures.

However, this recommendation should not stop us from using the existing HBM datasets (limited to target screening only) for the MAF discussion. Assuming access to individual data sets, one could consider calculating, based on available HBM datasets, what MAF value would be sufficiently to protect a defined level, e.g. what MAF would be needed to turn HI into HI < 1 for 95th percentile of HBM samples in a cohort/data set (see method T. Backhaus used for MAF for environmental mixtures exposure and risk). This is probably feasible in a higher tier, albeit clearly access to individual data is needed. But it would not make sense at the screening level (where HI considers all chemicals in summation, irrespective of chemical similarity or similar mode of action) since it is expected that screening level HI would result in a majority of HBM samples at HI > 1. Trying to reduce the HI to levels < 1 for 95^{th} percentile with a number of MAF values using screening level data would not be recommended given that other options are preferred, i.e. performing higher tier HI calculations considering grouping of effects (or mode of action is needed). Scrutinizing MAF values theoretically in a higher tier HI approach is recommended. Importantly, in such an exercise, one should pay attention to selection of the value in the denominator (DNEL, HBM-GV, external HB-GV).

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APPENDIX 1: OVERVIEW OF BIOMARKERS OF EXPOSURE TO CHEMICALS MEASURED IN THE EUROPEAN HBM4EU MONITORING DATASETS (TARGET SCREENING)

substance group	*parent compound*	*Biomarker (parent compound or metabolite)*	*matrix*
acrylamide	acrylamide	AAMA (N-acetyl-S-(2-carbamoylethyl)-cysteine)	Urine-spot
acrylamide	acrylamide	GAMA (N-Acetyl-S-(2-carbamoyl -2-hydroxyethyl)-L-cysteine)	Urine-spot
analines	paracetamol	NA4AP (N-acetyl-4-aminophenol, paracetamol)	Urine-spot
aprotic solvents	N-methyl-2-pyrrolidone (NMP)	2-hydroxy-N-ethylsuccinimide	Urine-spot
aprotic solvents	N-methyl-2-pyrrolidone (NMP)	2-hydroxy-N-methylsuccinimide	Urine-spot
aprotic solvents	N-methyl-2-pyrrolidone (NMP)	5-hydroxy- N-methyl-2- pyrrolidone (5-HNMP),	Urine-spot
aprotic solvents	N-methyl-2-pyrrolidone (NMP)	5-hydroxy- N-ethyl-2- pyrrolidone (5-HNEP),	Urine-spot
Arsenic	arsenic	Total arsenic (Tas)	Urine-spot
Arsenic	arsenic	Toxicologically relevant arsenic	Urine-spot
Arsenic	arsenic	arsenic acid (As(V))	Urine-spot
Arsenic	arsenic	arsenous acid (As(III))	Urine-spot
Arsenic	arsenic	monomethylarsonic (MMA)	Urine-spot
Arsenic	arsenic	dimethylarsinic (DMA)	Urine-spot
Arsenic	arsenic	arsenobetaine (AsB)	Urine-spot
Arsenic	arsenic	sum As(III) + As(V) + DMA + MMA	Urine-spot
Bisphenols	bisphenol A	BPA total	Urine-spot
Cadmium	cadmium	Cd	Urine-spot
Chromium	chromium	total chromium Cr	Urine-spot
DINCH	dinch	cyclohexane-1,2- dicarboxylate-mono-(7- hydroxy-4-methyl)octyl ester (OH-MINCH, MHNCH)	Urine-spot

substance group	*parent compound*	*Biomarker (parent compound or metabolite)*	*matrix*
DINCH	dinch	cyclohexane-1,2- dicarboxylate-mono-(7-oxo- 4-methyl) octyl ester (oxo- MINCH, MONCH)	Urine-spot
Flame retardants (brominated diphenyl ethers)	BDE-28	BDE-28	Blood Serum
Flame retardants (brominated diphenyl ethers)	BDE-47	BDE-47	Blood Serum
Flame retardants (brominated diphenyl ethers)	BDE-66	BDE-66	Blood Serum
Flame retardants (brominated diphenyl ethers)	BDE-99	BDE-99	Blood Serum
Flame retardants (brominated diphenyl ethers)	BDE-100	BDE-100	Blood Serum
Flame retardants (brominated diphenyl ethers)	BDE-153	BDE-153	Blood Serum
Flame retardants (brominated diphenyl ethers)	BDE-154	BDE-154	Blood Serum
Flame retardants (brominated diphenyl ethers)	BDE-183	BDE-183	Blood Serum
Flame retardants (brominated diphenyl ethers)	BDE-209	BDE-209	Blood Serum
Flame retardants (brominated)	HBCDalpha	HBCDalpha	Blood Serum
Flame retardants (brominated)	HBCDbeta	HBCDbeta	Blood Serum
Flame retardants (brominated)	HBCD gamma	HBCD gamma	Blood Serum
Flame retardants (novel brominated flame retardants)	TBBPA	TBBPA	Blood Serum

Appendix 1: Overview OF BIOMARKERS of exposure to chemicals measured in the European HBM4EU monitoring datasets (target screening)

substance group	*parent compound*	*Biomarker (parent compound or metabolite)*	*matrix*
Flame retardants (novel brominated flame retardants)	HBB	HBB	Blood Serum
Flame retardants (novel brominated flame retardants)	PBEB	PBEB	Blood Serum
Flame retardants (novel brominated flame retardants)	BTBPE	BTBPE	Blood Serum
Flame retardants (novel brominated flame retardants)	PBT	PBT	Blood Serum
Flame retardants (novel brominated flame retardants)	DBDPE	DBDPE	Blood Serum
Flame retardants (novel brominated flame retardants)	OBIND	OBIND	Blood Serum
Flame retardants (organophosphor)	triphenyl phosphate (TPHP)	Diphenyl phosphate (DPHP)	Urine-spot
Flame retardants (organophosphor)	Tris(2-chloroethyl) phosphate (TCEP)	Bis(2-chloroethyl) phosphate (BCEP)	Urine-spot
Flame retardants (organophosphor)	Tri-n-butyl phosphate (TNBP)	Di-n-butyl phosphate (DnBP)	Urine-spot
Flame retardants	Syn-dechlorane plus	Syn-dechlorane plus	Blood Serum
Flame retardants	Anti-dechlorane plus	Anti-dechlorane plus	Blood Serum
Flame retardants	flame retardants	Dec603 (dechlorane 603)	Blood Serum
Flame retardants	flame retardants	Dec602 (dechlorane 602)	Blood Serum
Flame retardants	hexabromocychlododecane	total HBCD	Blood Serum
Lead	lead	Lead	Urine-spot
Mercury and its organic compounds	mercury	Mercury (total)	Urine-spot
Mercury and its organic compounds	mercury	Mercury (total)	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	PFBA	PFBA	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	PFPeA	PFPeA	Blood Serum

substance group	*parent compound*	*Biomarker (parent compound or metabolite)*	*matrix*
Per-/poly-fluorinated compounds (PFASs)	PFHxA	PFHxA	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	PFHpA	PFHpA	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	PFOA	PFOA	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	PFNA	PFNA	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	PFDA	PFDA	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	PFUnDA	PFUnDA	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	PFDoDA	PFDoDA	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	PFTrDA	PFTrDA	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	PFTeDA	PFTeDA	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	PFBS	PFBS	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	PFHxS	PFHxS	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	PFOS	PFOS	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	PFDS	PFDS	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	FOSA	FOSA	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	N-EtFOSA	N-EtFOSA	Blood Serum
Per-/poly-fluorinated compounds (PFASs)	N-MeFOSA	N-MeFOSA	Blood Serum
Pesticides (organophosphorus)	chlorpyrifos	3,5,6-trichloro-2-pyridinol (TCPy); 3,5,6-trichloro-2-pyridinol (TCPy)	Urine-spot
Pesticides (organophosphorus)	chlorpyrifos	Diethyl phosphate (DEP)	Urine-spot
Pesticides (organophosphorus)	Pesticides (organophosphorus)	Diethyl thiophosphate (DETP)	Urine-spot
Pesticides (organophosphorus)	Pesticides (organophosphorus)	Diethyldithiophosphate (DEDTP)	Urine-spot
Pesticides (organophosphorus)	Pesticides (organophosphorus)	Dimethyl phosphate (DMP)	Urine-spot
Pesticides (organophosphorus)	Pesticides (organophosphorus)	Dimethyl thiophosphate (DMTP)	Urine-spot

Appendix 1: Overview OF BIOMARKERS of exposure to chemicals measured in the European HBM4EU monitoring datasets (target screening)

substance group	*parent compound*	*Biomarker (parent compound or metabolite)*	*matrix*
Pesticides (organophosphorus)	Pesticides (organophosphorus)	Dimethyl dithiophosphate (DMDTP)	Urine-spot
Pesticides (organophosphorus)	Pesticides (organophosphorus)	2,5-dichlorophenol (2,5-DCP)	Urine-spot
Pesticides (organophosphorus)	glyphosate	glyphosate	Urine-spot
Pesticides (organophosphorus)	glyphosate	Aminomethylyphosphonic acid (AMPA)	Urine-spot
Pesticides (pyrethroids)	pesticides (pyrethroids)	3-phenoxybenzoic acid (3-PBA)	Urine-spot
Pesticides (pyrethroids)	pesticides (pyrethroids)	4-fluoro-3-phenoxybenzoic acid (F-3-PBA)	Urine-spot
Pesticides (pyrethroids)	pesticides (pyrethroids)	cis-3-(2,2-dichlorovinyl)-2,2-dimethylcyclo propane-1-carboxylic acid (cis-DCCA)	Urine-spot
Pesticides (pyrethroids)	pesticides (pyrethroids)	trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclo propane-1-carboxylic acid (trans-DCCA)	Urine-spot
Pesticides (pyrethroids)	pesticides (pyrethroids)	cis-3-(2,2-dibromovinyl)-2,2-dimethyl cyclopropane-1-carboxylic acid (cis-DBCA)	Urine-spot
Phthalates	DEHP	Mono(2-ethylhexyl) phthalate (MEHP)	Urine-spot
Phthalates	DEHT	Mono(2-ethyl-5-hydroxy- hexyl) phthalate (5OH- MEHP, MEHHP)	Urine-spot
Phthalates	DEHT	Mono(2-ethyl-5-oxo-hexyl) phthalate (5oxo-MEHP, MEOHP)	Urine-spot
Phthalates	BBzP	Mono-benzyl phthalate (MBzP)	Urine-spot
Phthalates	DnPB	Mono-n-butyl phthalate (MnBP)	Urine-spot
Phthalates	DiBP	Mono-isobutyl phthalate (MiBP)	Urine-spot
Phthalates	DEHP	Mono(2-ethyl-5-carboxy- pentyl) phthalate (5cx- MEPP, MECPP)	Urine-spot
Phthalates	DEP	Mono-ethyl phthalate (MEP)	Urine-spot
Phthalates	DINP	Mono-methyl-octyl phthalate (MiNP)	Urine-spot

substance group	*parent compound*	*Biomarker (parent compound or metabolite)*	*matrix*
Phthalates	DINP	7-OH-(Mono-methyl-octyl) phthalate (OH-MiNP, MHNP, MHiNP)	Urine-spot
Phthalates	DINP	7-Oxo-(Mono-methyl-octyl) phthalate (oxo-MiNP, MONP, MOiNP)	Urine-spot
Phthalates	DINP	7-Carboxy-(mono-methyl- heptyl) phthalate (cx-MiNP, MCOP, MCiOP)	Urine-spot
Phthalates	DnOP (di-n-octyl phthalate)	Mono-n-octyl phthalate (MnOP, MOP)	Urine-spot
Phthalates	DIDP	Mono-propyl-heptyl phthalate (MiDP)	Urine-spot
Phthalates	DnPeP	Mono-n-pentyl phthalate (MnPeP)	Urine-spot
Phthalates	DCHP (di-cyclo-hexyl phtalate)	Mono-cyclo-hexyl phthalate (MCHP)	Urine-spot
Phthalates	DnBP	3-OH-Mono-n-butyl phthalate (OH-MnBP)	Urine-spot
Phthalates	DiBP	2-OH-Mono-iso- butylphthalate (OH-MiBP)	Urine-spot
Phthalates	DiDP	6-OH-Mono-propyl-heptyl phthalate (OH-MiDP)	Urine-spot
Phthalates	DiDP	6-Oxo-Mono-propyl-heptyl phthalate (oxo-MiDP)	Urine-spot
Phthalates	DMP	Mono-methyl phthalate (MMP)	Urine-spot
Polycyclic Aromatic Hydrocarbons (PAHs)	pyrene	1-hydroxypyrene (1-PYR)	Urine-spot
Polycyclic Aromatic Hydrocarbons (PAHs)	phenanthrene	1-hydroxyphenanthrene	Urine-spot
Polycyclic Aromatic Hydrocarbons (PAHs)	phenanthrene	2- hydroxyphenanthrene	Urine-spot
Polycyclic Aromatic Hydrocarbons (PAHs)	phenanthrene	3- hydroxyphenanthrene	Urine-spot
Polycyclic Aromatic Hydrocarbons (PAHs)	phenanthrene	4- hydroxyphenanthrene	Urine-spot
UV-filters (benzophenones)	Benzophenone 1 (BP-1)	Benzophenone 1 (BP-1)	Blood Serum
UV-filters (benzophenones)	Benzophenone 2 (BP-2)	Benzophenone 2 (BP-2)	Blood Serum
UV-filters (benzophenones)	Benzophenone 3 (BP-3)	Benzophenone 3 (BP-3)	Blood Serum
UV-filters (benzophenones)	5-chloro-2-hydroxybenzophenone (BP-7)	5-cholro-2-hydroxybenzophenone (BP-7)	Blood Serum

Appendix 1: Overview OF BIOMARKERS of exposure to chemicals measured in the European HBM4EU monitoring datasets (target screening)

substance group	*parent compound*	*Biomarker (parent compound or metabolite)*	*matrix*
UV-filters (benzophenones)	4-hydroxy-benzophenone (4-HBP)	4-hydroxy-benzophenone (4-HBP)	Blood Serum
UV-filters (benzophenones)	4-methyl-benzophenone (4-MBP)	4-methyl-benzophenone (4-MBP)	Blood Serum
UV-filters (benzophenones)	Benzophenone (BP)	Benzophenone (BP)	Urine-spot
UV-filters (benzophenones)	Benzophenone 1 (BP-1)	Benzophenone 1 (BP-1)	Urine-spot
UV-filters (benzophenones)	Benzophenone 2 (BP-2)	Benzophenone 2 (BP-2)	Urine-spot
UV-filters (benzophenones)	Benzophenone 3 (BP-3)	Benzophenone 3 (BP-3)	Urine-spot
UV-filters (benzophenones)	5-chloro-2-hydroxybenzophenone (BP-7)	5-chloro-2-hydroxybenzophenone (BP-7)	Urine-spot
UV-filters (benzophenones)	4-hydroxy-benzophenone (4-HBP)	4-hydroxy-benzophenone (4-HBP)	Urine-spot
UV-filters (benzophenones)	4-methyl-benzophenone (4-MBP)	4-methyl-benzophenone (4-MBP)	Urine-spot

APPENDIX 2: SUMMARY OF DETECTION FREQUENCIES OF CHEMICALS IN HUMAN BIOMONITORING STUDIES (DATA RETRIEVED FROM HTTPS://WWW.HBM4EU.EU/EU-HBM-DASHBOARD/)

Chemical group	*datasets*	*biomarker*	*Matrix*	*Levels of biomarkers in relation to LOQ/LOD **
Acrylamide	GerES V (2015-2017);	AAMA; GAMA	urine	P5> LOQ*
Anilines and MOCA	Democophes DK	NA4AP	urine	P5> LOQ
Aprotic solvents	ESB 2008-2014; GerES V	2-HESI; 2-HMSI; 5-HNEP	urine	2-HMSI: P5> LOQ; 2HESI: P25 > LOQ; 5-HNEP: detected from P75 or above 5-HNMP: P10 > LOQ
Arsenic	GerES IV; FLEHS II, ESB, 3xG; SLO-HBM-I	As total	urine	P5 > LOQ in FLEHS II; P25 > LOQ > in ESB
	Moba; FLEHS 2, SLO-HBM-I; FLEHS 3	As total	blood	P5 > LOD
	SLO-HBM-I	As total	Breast milk	P5 > LOD
Bisphenols	Several	BPA total; PBS total	urine	BPA: In several datasets: P5 > LOQ; in some P10/P25 > LOQ BPS: detected in some studies (DK; P50 > LOQ); while less in e.g. NO IES Mother: P90 > LOQ
Cadmium	several	Cd	urine	In majority of datasets; P5 > LOQ; in a few datasets LOQ ~ P10, P25, P50
Cadmium	several	cadmium	blood	In majority of datasets; P5 > LOQ; in a few datasets LOQ ~ P10, P25, P50 (especially in database with rather high LOD or LOQ)
	SLO-HBM-I; PRENATAL mother	cadmium	breastmilk	In PRENATAL mother: P5 > LOD In SLO-HBM-I: P75 > LOD (LOD is 2.5 x higher than in PRENATAL study)
Chromium	FLEHS II, III and 3xG	Cr total	urine	In FLEHS 2: P5 > LOD; in FLEHS 3 and 3xG: P50 < LOD; mind different LOD between FLEHS 2 & 3

Appendix 2: Summary of detection frequencies of chemicals in human biomonitoring studies (data retrieved from https://www.hbm4eu.eu/eu-hbm-dashboard/)

Chemical group	*datasets*	*biomarker*	*Matrix*	*Levels of biomarkers in relation to LOQ/LOD **
Chromium	FLEHS II, III and 3xG	Cr total	blood	In FLEHS 2 and 3: P5 > LOD; 3xG: P25 > LOD;
DINCH	several	xc-MINCH, OH-MINCH; oxo-MINCH	urine	In some datasets: P5 > LOQ On other datasets: LOQ ~ P25/P75
OP flame retardants	NO IES, AT Orphos; GerES V	BCEP, NdPB, PDHP, BBOEP, BDCIPP	urine	20 fold difference in LOQ between studies (e.g BCEP); From P5 > LOQ (GerES V DPHP) to LOQ ~ P50 or above
BDEs	several	BDE 28, BDE47, BDE 99, BDE 100, BDE 153, BDE 154, BDE 183, BDE209	Blood	In several databases: several BODs not detected (P90 > LOQ); in other datbases some BDEs, e.g BDE 153: P5 > LOQ
Lead	several	Lead	Urine	P5 > LOQ/LOD in all databases
Lead	SLO-HBM-I	Lead	Breast milk	P75 > LOD
Mercury	several	Hg total	blood	In majority of databases: P5, P10 or P25 > LOQ
Mercury	several	Hg total	urine	In majority of databases: P5 > LOQ; in some P25 > LOQ
Mercury	SLO-HBM-I	Hg total	Breast milk	P5 > LOD
PFAS	several	Several PFAS: FOSA, PFBA, PFBS, PFDA, PFDoDA, PFHpA, PFHxA,PFHxS, PFNA, PFOA, PFOS PFUnDA	blood	Diversity among studies and PFAS; e.f. PFOS and PFAS; P5 > LOQ in all reported studies; other PFAS: more diversity (also due to range in LODs across studies); general findings: a lot of PFAS detected at least above P50 if LOQ is sufficiently low
PFAS	PCBCohort_mother (SK); Prenatal mother (SK); CzechHBM-HM 2014 & 2017	Several PFAS: FOSA, PFBA, PFBS, PFDA, PFDoDA, PFHpA, PFHxA,PFHxS, PFNA, PFOA, PFOS PFUnDA	Breast milk	PFOS and PFAS; P5 > LOQ in all reported studies; other PFAS: in general below LOQ,: except PFNA (P5 > LOD in CzechHBM-HM 2017)
Pesticides (organophosphate)	several	AMPA, glyphosate, DEP, DETP, DMP, DMTP, DMDTP, etc.	urine	For majority of pesticides biomarkers in majority of databases: P5/P10 > LOQ, with some exceptions (e.g. DEDTP, DETP detected less frequently in e.G. FLEHS 2: DK); here also differences in LOD/LOQ explain partly differences

Appendix 2: Summary of detection frequencies of chemicals in human biomonitoring studies (data retrieved from https://www.hbm4eu.eu/eu-hbm-dashboard/)

Chemical group	*datasets*	*biomarker*	*Matrix*	*Levels of biomarkers in relation to LOQ/LOD **
Pesticides (pyrethroids)	GerES IV, KD Doense Child Cohorte; FLESH III Adults	3-PBA; cis DDCA, trans-DDCA, F-3-PBA	urine	3-PBA: P5 > LOQ; cis DCCA and trans DDCA: LOQ ~ P25-P50 in dataset with low LOQ; while LOQ > P75 in datasets with higher LOQ ; F-3-PBA: LOQ ~ P90 or above
Phthalates	>30 datasets	From 7 to > 20 phthalates (depending on database)	urine	Despite the large number of datasets and number of metabolites: for nearly all metabolites and databases: P5 > LOQ (except for MnOP, MnPEP and MCHP; measured in some datasets at only at levels P95~ LOQ)
PAHs	Several datasets	Several metolites of pyrene, phenanthrene, naphthalene and flourene	urine	For nearly all datasets and biomarkers: P5 > LOQ; some exceptions (e.g. FLEHS 1: 1 PYR; P50 ~ LOD, probably because high LOD)
Benzophenones (UV filters)	Several datasets	BP-1; BP-3, BMHB	urine	In general: less frequent detected; Diverse pattern: in some datasets; LOQ > P90 while in others a rather high detection frequency e.g. in Copenhagen puberty study P25 > LOQ for BP-1, BP-3

APPENDIX 3: LITERATURE STUDY ON CO-OCCURRENCE PATTERNS IN HBM DATASETS AND RELATION BETWEEN MIXTURES EXPOSURE AND HEALTH OUTCOMES

See next page

Appendix 3: literature study on co-occurence patterns in HBM datasets and relation between mixtures exposure and health outcomes

Appendix of the technical report Annex E Human Health 'Characterization of real-life co-exposure for humans: analyzing HBM datasets on co-occurrence'

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LIST OF ABBREVIATIONS

BAS	Bayesian Adaptive Sampling
BIC	Bayesian Information Criterion
BIC-BMA	Bayesian model averaging approach using BIC
BKMR	Bayesian Kernel Machine Regression
CAN	Comparative network analysis
ENET	Elastic Net
HBM	Humane biomonitoring
HI	Hazard Index
LOD	Limit of detection
LOQ	Limit of quantification
MCP	Minimax concave penalty
MCR	Maximum Cumulative Ratio
MLR	Multiple linear regression
PCA	Principle Component Analysis
WQS	Weighted quantile sum

CHAPTER 1 INTRODUCTION

Throughout the life-time, humans are exposed to a mixture of environmental stressors and chemicals that independently or in interaction may have an impact on health. These mixtures of exposure can form an almost infinite number of different combinations of chemicals, which makes the exposure and risk assessment extremely challenging. Chemical risk assessment typically evaluates single compounds but generally does not appropriately reflect the complexity of concomitant exposure to multiple chemicals in real life. In addition, little is known regarding commonly occurring exposure mixtures and how these mixtures change between important covariates such as gender, time or country [1]. To date, a few exceptions of studies consider simultaneously more than a couple of chemical groups thereby focusing on 1) co-occurrence patterns, and 2) linkage between mixture patterns and health outcomes such as birth weight, as described in the following sections [2, 3]. However, the available information is still scarce.

New studies are beginning to jointly assess the effects of chemical mixtures in addition to evaluating the impact of exposure to individual chemicals on human health. Biomarkers of exposure in human biomonitoring studies are typically considered one of the best measures to characterize the total amount of a chemical absorbed via multiple routes. These data can be used to screen for the presence of clusters of correlated exposure. Most investigations are limited to either individual biomarkers or multiple biomarkers within the same chemical class [4]. Many chemical analytes within the same usage or chemical class are densely correlated [5, 6] and the extent to which humans are exposed to multiple chemicals and how these biomarkers of exposure correlate with each other is an active area of research.

Patterns between multiple biomarkers are not commonly presented. Increasingly, graphical representation of (partial) correlation patterns such as heatmaps, correlation globes or circos plots are being used to demonstrate correlations between variables [1]. On the other hand, comparative network analysis (CNA) provide a graphical method to represent groups or communities in the data. Principal Component Analysis (PCA) is an approach to quantify correlations between measured biomarkers of exposure [5, 7, 8].

The field of statistical tools to explore the association of multiple chemical exposures on health outcomes has evolved in recent years. Several methods have been developed, which can be classified into three groups according to Govarts et al. 2020 [2]: dimension reduction (e.g. PCR, partial least square regression), variable selection (e.g. deletion/substitution/addition algorithm, penalized methods like elastic net (ENET) regression modelling, and Bayesian variable selection methods) and

[1]https://www.hbm4eu.eu/work-packages/deliverable-15-3-report-real-life-exposure-profiles-from-re-analysis-of-existing-hbm-mixture-data/

grouping of observations (e.g. cluster analysis, building groups based on an exposure score, Bayesian profile regression, recursive partitioning techniques) [9].

Despite the large number of humane biomonitoring (HBM) studies, analysis of mixtures in HBM seems to be a rather unexplored field. The objective of this report is to provide an overview including concise summaries of publications and ongoing studies investigating the mixtures patterns and potential mixtures effects in HBM studies. We will mainly focus on scientific publications describing *co-occurrence patterns of chemicals in human biomonitoring datasets*. We also extend the search to publications describing *chemical co-occurrence patterns in relation with health outcomes* (several of these publications deal with birth length and weight). We focus thereby first on European HBM studies. However, studies from outside EU have also been considered because they may report other techniques and groups of substances. However, one should be careful to extrapolate the results as such to the EU situation.

CHAPTER 2 SEARCH STRATEGY

2.1. SCOPE OF SEARCH STRATEGY

The objective of this report is to provide an overview including concise summaries of publications and ongoing studies investigating the **mixtures patterns and potential mixtures effects** in HBM studies. We will mainly focus on scientific publications describing co-occurrence patterns of chemicals in human biomonitoring datasets. We also extend the search to publications describing chemical co-occurrence patterns in relation with health outcomes (several of these publications deal with birth length and weight). We focus thereby first on European HBM studies. However, studies from outside EU have also been considered because they may report other techniques and groups of substances.

2.2. SNOWBALL METHOD

First, relevant publications such as Govarts et al. 2020 [2], Ottenbros 2021 [1], Rosovsky et al. 2017 [4] and Tamayo-Uria et al. 2019 [10] were screened for relevant references also investigating mixtures patterns and potential mixtures effects in HBM studies, the so-called snowball method. We mainly focused on scientific publications describing co-occurrence patterns of chemicals in human biomonitoring datasets. Next, we extended the search to publications describing chemical co-occurrence patterns in relation with health outcomes.

2.3. KEY WORDS IN PUBMED AND WEB OF SCIENCE

The following key words were used in the search for applicable studies on Pubmed and Web of Science: “human biomonitoring and mixtures”, “human biomonitoring and mixture effects”, “human biomonitoring and co-occurrence”, “exposure to multiple pollutants”, “exposure to mixture of chemicals”, “principal component analysis mixture of chemicals” and “principal component analysis chemical mixtures”. For each publication, the cohort name, number of participants, number of chemicals, mixtures patterns, the methods used to investigate these mixtures and potential mixtures effects in HBM studies will be summarized.

CHAPTER 3 RESULTS

3.1. RESULT OF SEARCH STRATEGY

The snowball method yielded all scientific publications describing co-occurrence patterns of chemicals in human biomonitoring datasets described in detail in the sections below and most of the publications describing chemical co-occurrence patterns in relation with health outcomes. For the sake of completeness, Pubmed and Web of Science were searched with the key words defined before in chapter 2 and this method yielded the following results: human biomonitoring AND mixtures; 926 results in Pubmed and 225 results in Web of Science, humane biomonitoring AND mixture effects; 540 results in Pubmed and 101 results in Web of Science, humane biomonitoring AND co-occurrence; 29 results in Pubmed and 6 results in Web of Science, exposure to multiple pollutants; 7978 results in Pubmed and 2695 results in Web of Science, exposure to mixture of chemicals; 7197 results in Pubmed and 6540 results in Web of Science, principal component analysis mixture of chemicals; 565 results in Pubmed and 883 results in Web of Science and principal component analysis chemical mixtures; 565 results in Pubmed and 883 results in Web of Science. These search results were first screened for relevant titles after which the abstract was read to determine if the particular publication was suitable for inclusion in this report. Only a few relevant publications describing chemical co-occurrence patterns in relation with health outcomes [11-15] were found using this key word search method.

3.2. OVERVIEW OF STATISTICAL METHODS USED IN STUDIES INVESTIGATING EXPOSURE PATTERNS OR CO-OCCURRENCE

This section provides an overview of statistical methods that are frequently used to investigate exposure patterns or co-occurrence in scientific literature.

3.2.1. LINEAR REGRESSION: PEARSON CORRELATION, HEAT MAPS AND CIRCOS PLOTS

Linear regression a linear approach to modelling the relationship between a response and one or more explanatory variables. The main limitation of this approach is that it does not consider the association with other exposures [16].

→ Multiple linear regression model (MLR)

Ordinary linear regression was applied as most conventional model to estimate exposure-effect associations, by including all exposure biomarkers in a single multiple linear regression model. Statistical significance of the association between the exposure markers and birth weight was assessed by the *p*-value [2].

→ **Model selection and Bayesian model averaging using the Bayesian information Criterion (BIC)**

Model selection among a finite set of models can be performed by comparing BICs, but only when the number of candidate predictors/exposures is not too high, because it requires evaluation of model fit for all (competing/different) regression models. Statistical inference based on estimated regression coefficients and 95% confidence intervals from the selected "best" or "median probability" models is flawed, because it does not consider the selection process. Although BIC is often used to select only a single "best-fitting" model, it can also be used more comprehensively to evaluate model uncertainty by using BIC to approximate Bayesian posterior model probabilities. By summing the posterior probabilities of all models in which a biomarker occurs, it is possible to estimate the so-called marginal posterior probability of inclusion (MPPI) for each exposure biomarker. Either a model that includes only exposures with a MPPI exceeding a certain threshold (for instance 50%; the so-called median probability model) can be included or base our inference on the full range of models as in (Bayesian) model averaging. In Bayesian model averaging (BMA, MPPIs are used as weights to estimate a full posterior distribution for each regression coefficient, which can be summarized using 95% (Bayesian) credible intervals (95% BCI) [2].

→ **Penalized regression**

Penalized regression methods were developed to address the problems of multicollinearity and high dimensionality. In MLR, regression coefficients are estimated by minimizing the residual sum of squares (i.e. maximizing model fit), while in penalized regression models' coefficients are estimated by jointly minimizing the residual sum of squares and a function of the estimated coefficients. For the well-known LASSO penalty that function is the absolute value of the coefficients, and it can be shown that this leads to effective variable selection because some of the coefficients are shrunken to exactly zero thereby effectively removing the variable from the model [2].

→ **Bayesian Adaptive Sampling (BAS)**

BAS is a Bayesian model averaging technique that can use either sampling or enumeration to explore different model structures. Like for BIC-BMA, a threshold of 50% for the MPPIs could be used to select exposures or we could base our inference on the full range of models [2].

3.2.2. PRINCIPAL COMPONENT ANALYSIS

Principal Component Analysis (PCA) is an approach to quantify correlations between measured biomarkers of exposure [5, 7, 8]. PCA reduces a large number of correlated variables to a smaller number of uncorrelated components while retaining as much information as possible of the original variables [16].

According to Rosofsky et al. 2017 [4]: " PCA identifies the maximum amount of mutual correlation between groups of variables that explain latent variables, or components, that cannot be directly observed [17]. Biomarkers are categorized under a given component based on their "loading," which represents correlations between the biomarker and the underlying, latent factor, or component.

Because the components are orthogonal, or statistically independent, biomarkers loaded within one component are said to have a low correlation with biomarkers loaded on all other components. The extent to which chemicals load to the final components may indicate common exposure sources within that component, such as chemicals that are found together in diet, consumer products or traffic pollution. PCA results may identify the extent to which chemical exposures share common sources and pathways or jointly contribute to disease" [4].

By the technique of principal component analysis (PCA), these principal components are created to explain the observed variability in the predictor variables. PCs represent variation in the original data set; the first PC represents the maximum amount of variation possible in one dimension, the second PC represents the maximum amount of the remaining variation in one dimension perpendicular to the first PC, and so on for all remaining PCs [18]. The resulting principal components can also be linked to health outcomes by the same multiple linear regression models as described for single pollutant models.

3.2.3. COMPARATIVE NETWORK ANALYSIS

Comparative network analysis (CNA) provide a graphical method to represent groups or communities in the data. Networks facilitates the detection of exposure patterns and allows for the systematic comparison of observed exposure patterns between datasets and strata within datasets. Networks can be compared on their similarities or their dissimilarities. Ottenbros et al. 2021 focused on exact graph matching, which involves the exact correspondence between two or more graphs with the exact same set of nodes. An edge is "conserved" if it is present in all of the input graphs. The complement of conserved edges is represented in a network graph (network of conserved edges). Comparative network analysis can also assess the presence of edges in network B which are not present in network A. These results can be interpreted as "additional" or different edges and are presented in a network graph as well (network of differential edges).

3.3. OVERVIEW OF STATISTICAL METHODS USED IN STUDIES DESCRIBING CHEMICAL CO-OCCURRENCE PATTERNS IN RELATION WITH HEALTH OUTCOMES

This section provides an overview of statistical methods that are frequently used to investigate chemical co-occurrence patterns in relation with health outcomes in scientific literature.

3.3.1. ELASTIC NET REGRESSION

ENET determines which exposures are independently associated with the outcome and overcomes the limitations of multivariable linear regression models such as; the production of unreliable parameter estimates in case of multicollinearity or the prevention of exploration of datasets with too large number of dimensions. ENET results in a balanced approach that makes it possible to select the strongest predictors, while enabling collinear predictors to be included in the model [19]. ENET is a penalized regression model relying on a weighted mixture of the least absolute shrinkage and selection operator (LASSO) and ridge penalties. The LASSO penalty allows variable selection through shrinkage. The lowest regression coefficients, corresponding to the least informative predictors, are

attributed a zero value and only the most informative predictors are retained by the model. The ridge penalty accommodates correlated exposures and shrinks regression coefficients from corelated predictors proportionally toward zero [20].

3.3.2. BAYESIAN KERNAL MACHINE REGRESSION (BKMR)

BKMR utilizes a non-parametric approach to evaluate dose-response relationships, allowing for possible non-linearity and interactions in exposure outcome associations, which can often occur in the context of endocrine disrupting chemicals [16]. This approach allows for the examination of independent effects of mixture members, interactions among them, and the overall mixture effect [14]. Unlike WQS, BKMR allows the user to visualize individual exposure–response functions, while accounting for the other exposures and allowing potential non-linear relationships and/or differential directions of effect among exposures [21].

3.3.3. WEIGHTED QUANTILE SUM REGRESSION (WQS)

WQS regression allows for the creation of a weighted linear index of correlated predictors that are weighted by their strength of association with the outcome of interest [14]. WQS regression assumes linear exposure-outcome associations across quantiles of each exposure and is a first-order approximation of non-additivity. Additionally, WQS regression focuses inference in a single direction at a time with constrained optimization of the beta parameter. Effects can be estimated for both directions individually by conducting separate analyses constraining the analyses in positive and negative directions [21].

3.4. DESCRIPTION OF RESULTS

In this chapter, we will provide a concise overview and summary of publications and ongoing studies investigating the mixtures patterns and potential mixtures effects in HBM studies. The main European and non-European studies are summarized respectively in Table 1 and Table 2 and are also explained in detail in the following text sections.

Table 1: Overview of publications investigating the mixtures patterns and potential mixtures effects in European HBM studies.

Name of Cohort	# participants	Characteristics of cohort	# of chemicals/ biomarkers	List of chemicals or list of groups	Matrix	# samples > LOD	Method of analysis	Goel of the study: (1) Exposure patterns/co-occurrence or (2) mixture exposure-health effects	Chemicals with high co-occurrence (in same PCA or high correlation coefficient)	Chemicals with low co-occurrence (in orthogonal PCA or Low correlation coefficient)	Explanation	Reference
Sub cohort of HELIX (6 EU birth cohorts: BiB, EDEN, INMA, KANC, MoBA and Rhea)	1301	Mother-child pairs	87 for the pregnancy period 122 for the childhood period	Organochlorine compounds PBDEs PFAS Metals and elements Phthalate metabolites Phenols Organophosphate pesticide metabolites Cotinine	Serum, plasma, blood or urine	/	1) Within correlations with PEARS, polychoric or polyserial correlations 2) PCA	Exposure patterns/co-occurrence	1) Overall correlations tended to be higher than within correlations and the median correlation within exposure groups was>0.3 for many exposure groups 2) Ten principal components explained 45% and 39% of the total variance in the pregnancy and childhood exposome, respectively, while 65 and 90 components were required to explain 95% of the exposome variability		Some of the high correlations may be due to exposures being measured using a common methodology (e.g. GIS variables) or a common biological medium (e.g. variables measured in urine) or because some variables were used to create others.	Tamayo-Uria et al. 2019 [10]
Snart Foraeldre/ Milieu Denmark	73	Danish women (18-40 years) from the general population who stopped using contraception because they wished to become pregnant	135	Cotinine Metals Arsenic metabolites PAH BFRs Herbicides Insecticides PCBs Persistent Pesticides Perchlorate Phenols Parabens Phthalates Phytoestrogens PFAS Dialkylphosphate metabolites DEET metabolites	Blood, Serum or Urine	Analysis restricted to biomarkers that were detected in 75% of the study population	PCA, 3 models created: 1) All markers combined 2) Urinary markers only 3) Serum and whole blood markers only	Exposure patterns/co-occurrence	*Model 1, component 1:* - Persistent pesticides, PCBs, total mercury *Model 1, component 2:* - Blood and urinary metals, PFAS, cotinine, PAHs, perchlorate, phthalate, parabens *Model 1, component 3:* - PFAS, PCB28 PBDE153, PAHs, urinary metals, phthalates, pesticides *Model 2, component 1:* - PAHs, mono-ethyl phthalate, parabens, phytoestrogen, lead *Model 2, component 2:* - Blood and urinary metals, Arsenic and metabolites, phthalates, pesticides, strontium *Model 3, component 1:* - Blood metals, persistent pesticides, PCBs, PBDE153 *Model3, component 2:*	Urinary metals and phytoestrogens	- *Mono-ethyl phthalate and parabens:* used in cosmetic product and food packaging. - *PAHs and perchlorate:* released by combustion processes. - *PFAS:* high intra-class correlations due to exposure to other PFAS biomarkers - *Cotinine and cadmium:* smoking status - *Mercury and PCBs:* living in urban area and consumption of saltwater fish - *Urinary metals and phytoestrogens:* low correlation due to different sources	Rosofsky et al. 2017 [4]

								- PFAS, blood metals, cotinine, PCB28			
4 Flemish birth cohort: FLEHS I, II & III and 3XG (Belgium)	1579	Flemish mother-newborn pairs, population was restricted to live-born singleton births. Outcome of interest was birth weight.	7	PCB-138 PCB-153 PCB-180 Hexachlorobenzene p,p'-DDE Cadmium Lead	Cord blood	1.1–27% depending on the compound	- Pearson correlation for correlations between the different pollutants - Correlation with birth weight using 5 linear regression-based statistical methods: 1) Multiple linear regression (MLR) 2) Bayesian Information Criterion (BIC) 3) BIC Bayesian model averaging (BIC-BMA) 4) Penalized regression using concave penalty (MCP) 5) Bayesian Adaptive Sampling (BAS)	Exposure patterns/co-occurrence	- PCB congeners 138, 153 and 180 were highly correlated (Pearson's r = 0.75-0.83) - PCB 153 and PCB 180 showed an inverse association with birth weight in all multipollutant models while p,p'-DDE levels were associated with an increasing birth weight	Correlations between different pollutants were low to moderate (Pearson's r = 0.12-0.58)	PCB congeners are estrogens and DDE inhibits androgens and both disrupt thyroid function. Estrogens and androgens play important roles in regulating nutrient delivery to the fetus and organ maturation, hence, PCB's and DDE can disrupt fetal development by their endocrine-disrupting characteristics there explaining the association with birth weight.	Govarts et al. 2020 [2]
3 Flemish birth cohort: FLEHS I, II & III (Belgium)	281	Flemish mother-newborn pairs	19	Organochlorine compounds PFAS Metals	Cord blood	60% of the measurements above LOD	Comparative network analysis (CNA): A node in the network represents a biomarker, and an edge reflects conditional dependency given all other variables	Exposure patterns/co-occurrence	/	/	/	Ottenbros et al. 2021
INMA, Sabadell Spain	728	Pregnant women	81	Organochlorines PFAS Mercury PBDEs Metals Phthalates BPA Cotinine	Serum Cord blood Breast milk Urine	If analyte was nondetectable in > 85% of samples, biomarkers was excluded	Pair-wise Pearson's correlations and polychoric correlations were calculated to produce a correlation matrix. Heat map and circos plots were made to display the correlations. Principal components were then derived directly from the correlations.	Exposure patterns/co-occurrence	The mean correlation (r) all exposures was 0.08 (median = 0.02; 5-95th centiles = -0.12-0.54). The four PFOA compounds had the strongest median absolute correlation (r = 0.62) of the individually measured biomarkers. Overall, the median of all between-family absolute correlations was 0.05 (5th–95th centiles, 0.01–0.23).	The other biomarker families, PDBEs, phthalates, metals, and organochlorines, had all median absolute correlations below 0.5, reflecting their more diverse sources.	/	Robinson et al. 2015 [5]
Flemish birth cohort FLEHS II (Belgium)	248	Flemish mother-newborn pairs	15	Lead Manganese Copper Thallium Arsenic Cadmium PCB-138 PCB-153 PCB-180 P,p'-DDE PFOS	Cord blood Maternal whole blood Plasma cord	0-21% of n < LOD/LOQ	1) PCA 2) Algorithm for mixture effects	Mixture exposure-health effects	Subset PCA: a significant negative association with birth weight was found for arsenic and cadmium (p = 0.009). The mixtures with the highest association with birth weight were composed of five chemicals, i.e., PFOA, lead, cadmium, arsenic, and MECPP (p = 0.0019) and cadmium, thallium, arsenic, MECPP, and methylmercury (p = 0.0021).	Whole PCA: none of the principal components were statistically significant (p < 0.05) associated with birth weight.	/	Govarts et al. 2016 [18]

				POFA MECPP Total mercury Methylmercury	 Maternal hair						
INMA, Sabadell Spain	657	Mother-child (7 years of age) pairs	27	BPA Phthalates Metals (arsenic, lead and cadmium) Organochlorine pesticides PCBs Total mercury PBDEs	Urine Maternal blood Cord blood Maternal colostrum	n samples < LOD varies between 0 and 100	PCA	Mixture exposure-health effects	A factor reflecting combined exposure to multiple phthalate metabolites showed weak evidence for an association with reduced BMI.	Exposure to other EDCs in combined multi-pollutant analyses, showed no evidence for an association with child weight status.	/	Agay-Shay et al. 2015 [22]
EDEN birth cohort, France	473	Mother-son pairs	20	9 phenols: 4 parabens, 2 dichlorophenols, Triclosan, benzophenone-3 and BPA 11 phthalate metabolites	Urine	All analytes were between 98-100% > LOD	Elastic Net (ENET)-penalized regression models	Mixture exposure-health effects	- Triclosan and MNCP were negatively associated with placental weight while benzophenone-3 and the sum of parabens were positively associated with placental weight. - Only benzophenone-3 was associated with birth weight - MNCP and MNOP were negatively associated with placental-to-birth weight ratio.		The fact that the direction of the associations with placental weight differed across biomarkers might indicate different mechanisms of action.	Philippat et al. 2018 [20]
ELFE nationwide birth cohort, France	311	Women who gave birth to liveborn singleton ≥ 33 weeks of gestation	64	Organochlorines Organophosphorus Pyrethroids Carbamates Dinitroanilines Thiocarbamates Phenylpyrazoles Acid herbicides Azoles Oxadiazines Trizines/triazones Amide pesticides Strobilurins Carboxamides Urea Neonicotinoids Anilino-pyrimidines	Maternal hair	28 of 64 pesticides and metabolites were detected in > 70% of samples, 10 were detected in 50-70% of the samples, 10 were detected > 50% of the samples	Elastic Net (ENET)-penalized regression models	Mixture exposure-health effects	Significant associations were observed between; - weight and fipronil sulfone - length and TCPy, bitertanol, DEP, and isoproturon - head circumference and tebuconazole and prochloraz	/	/	Béranger et al. 2019 [19]
Three birth cohorts: Greenland, Poland and Ukraine	1250	Mother-infant pairs	16	Secondary metabolites of DEHP and DiNP, PFASs organochlorines	Maternal serum	All 16 biomarkers were quantifiable in at least 72% of serum samples	ENET	Mixture exposure-health effects	Two phthalate metabolites (MEHHP, MOiNP), perfluorooctanoic acid (PFOA), and p,p´-DDE were most consistently predictive of term birth weight based on elastic net penalty regression.	/	- Oxidative stress - Modulation of sex and thyroid homeostasis - Interference with lipid metabolism by PFASs - BPA and phthalates were associated with perturbations of angiogenesis	Lenters et al. 2016 [3]
Avon Longitudinal Study of Parents and Children (ASLPAC), UK	448	Mother-female child pairs	52	8 PFAS 35 PCBs 9 organochlorine pesticides	Maternal serum	EDCs detected in greater than 75% of mothers were included in the main analyses.	1) BKMR 2) WQS	Mixture exposure-health effects	/	1) Weighted quantile sum regression models showed null associations between the indices for mixtures (PFAS, PCBs, OCPs, and all three classes combined) and early menarche.	/	Marks et al. 2021 [14]

				2) In the BKMR model for all three classes combined, no interaction among mixture members was observed.

Table 2: Overview of publications investigating the mixtures patterns and potential mixtures effects in non-European HBM studies.

Name of Cohort	# participants	Characteristics of cohort	# of chemicals/ biomarkers	List of chemicals or list of groups	Matrix	# samples > LOD	Method of analysis	Goel of the study: (1) Exposure patterns/co-occurrence or (2) mixture exposure-health effects	Chemicals with high co-occurrence (in same PCA or high correlation coefficient)	Chemicals with low co-occurrence (in orthogonal PCA or Low correlation coefficient)	Explanation	Reference
NHANES (2009-2010), USA	10537	All ages, but only participants ≥ 6 were required to provide a urine sample and only participants ≥ 12 were required to provide a blood sample	/	Metals Arsenics Perchlorate Nitrate Thiocyanate Phytoestrogens Phenols Pesticides Phtalates PAHs Pyrethroids Herbicides Organophosphate metabolites Caffeine and metabolites DEET and metabolites Polyfluoroalkyl chemicals	Urine Serum	/	Frequent itemset mining	Exposure patterns/co-occurrence	90 chemical combinations consisting of relatively few chemicals that occur in at least 30% of the U.S. population were identified, as well as three super combinations consisting of relatively many chemicals that occur in a small but nonnegligible proportion of the population.	/	/	Kapraun et al. 2019 [23]
MIREC, CANADA	1744	Pregnant women	28	Arsenic Lead Mercury Cadmium Manganese PCBs Organochlorine pesticides PFASs BPA Organophosphate pesticides Phthalate metabolites	Blood Plasma Urine	All chemicals	PCA	Exposure patterns/co-occurrence	- PCA retained eleven components which explained approximately 70% of the variation. - Persistent organic pollutants (PCB118, PCB138, PCB153, PCB180, OXYCHLOR and TRANSNONA) and phthalates (MEOHP, MEHHP and MEHP) dominated the first and second components, respectively, and this explained 25.8% of the source variation. - Prenatal exposure to persistent organic pollutants) were positively associated with women who have lower education or higher income, were born in Canada, have BMI ≥25, or were expecting their first child in our study population.	MEOHP, MEHHP and MEHP, dominating the second component, however, no particular group of pregnant women was identified to be highly exposed to phthalates.	Persistent organic pollutants are highly present in meat and dairy products thereby explaining the clustering and dominance of component 1. MEOHP, MEHHP and MEHP are metabolites of DEHP. DEHP is widely used in food packaging, cosmetics and personal care products hence it is expected that they cluster together.	Lee et al. 2017 [24]
NHANES (2013-2014), USA	2663	General population (≥ 6 years)	6	6 phthalates: DBP and metabolite MBP DIBP and metabolite MIBP BBP and metabolite MBZP DEHP and metabolites MECPP, MEOHP, MEHHP, MEHP	Urine	Majority of the metabolites were detectable in > 97% of the surveyed participants.	MCR	Exposure patterns/co-occurrence	MCR ranged from 1.1 – 3.6, thus none of the exposed participants received the same level of risk from the six phthalates. There were 21 participants (0.8% of the NHANES sample) with HI>1. The mean MCR value was 2.1. HI and MCR values were negatively correlated (p<0.001) indicating that most participants, especially those with elevated HI values, had their cumulative risks		The 6 investigated chemicals are all phthalates and phthalates are used in many consumer products	Reyes et al. 2018 [25]

				DINP and metabolites MINP and MCOP DIDP and metabolite MCNP					driven by relatively large doses of a single phthalate rather than doses of multiple phthalates.			
Baltimore Tracking Health Related to Environmental Exposures (THREE) Study, USA	300	Singleton liveborn babies	24	Organochlorine pesticides Carbamate pesticides Organophosphate pesticides Pyrethroid pesticides	Cord serum	/	PCA	Exposure patterns/co-occurrence	There were four independent pesticide components: DDT (p,p'- DDT+p,p'-DDE), chlordane (trans-nonachlor+oxychlordane), permethrin (trans- and cis-permethrins+PBUT), and carbamate (bendiocarb + propoxur). DDT and chlordane were 6.1 (95%CI: 2.4, 15.5) and 2.1 (95%CI: 1.0, 4.2) times higher for infants of women >35, and 1.8 (95%CI: 1.2, 2.9) and 1.5 (95%CI: 1.1, 2.1) times higher in smoking mothers. DDT and carbamate were 15 (95%CI: 7, 30) and 2 (95%CI: 1, 4) times higher for infants of Asian compared with Caucasian mothers. No significant differences were observed for permethrin	/	/	Neta et al. 2010 [12]
LIFE study USA	501 couples	Married couples, females aged 18-40 years and males aged 18+	128	13 chemical classes: PCBs Organochlorine pesticide PBDEs PFASs Phytoestrogens Phtalates metabolites Phenols Antimicrobial chemical Paracetamol and derivatives Blood metals Cotinine Urinary metals Urinary metalloids	Blood and urine	/		Exposure patterns/co-occurrence	Shared household explained 43% and 41% of the total variance for PFASs and blood metals, respectively, but less than 20% for the remaining 11 EDC classes.		The authors suggest that there are two additional factors affecting the familial coexposure patterns in our investigation. The first one is concerned with how long the couples have been living together. the second factor is potential physiological dampening of exposure variability related to the half-life of the target chemicals.	Chung et al. 2018 [11]
CHMS, Canada	1858	General population aged 12 to 79	44	26 classes: Cadmium Lead Mercury Nicotine BPA Triclosan Inorganic arsenic Benzo(a)pyrene Chrysene Fluoranthene Fluorene Naphthalene Phenanthrene Pyrene Benzene Ethylbenzene Styrene Tetrachloroethylene Toluene Trichloroethylene Bromodichloromethane Tribromomethane Trichloromethane Xylenes	Blood or urine	/	Exposure loading	Exposure patterns/co-occurrence	1) Adolescents aged 12–19 years had significantly lower Exposure Loads than adults aged 40–79 years at all thresholds and adults aged 20–39 years at the 50th and 75th percentiles. 2) Smokers had significantly higher Exposure Loads than nonsmokers at all thresholds except the LOD.	No differences in Exposure Loads were observed between males and females at any threshold.	1) Age is known to play a role in exposure to chemicals and the amounts of chemicals measured in people as persistent pollutants can bioaccumulate, potentially remaining in tissues for decades (cadmium, lead and mercury). 2) Tobacco smoke is a known source of certain chemicals included in our analysis	Willey et al. 2021 [26]

				Acrylamide							
EARTH study, USA	300	Mother-infant pairs Mothers age between 18-45 years	9	DEHP MEHP MEHHP MEOHP MECPP MiBP MBP MBzP MEP	Urine	/	1) Linear regression 2) PCA 3) Bayesian Kernal Machine Regression	Mixture exposure-health effects	All metabolites were separately negatively but not significantly associated with birth weight. PCA identified two principal components accounting for respectively for 53% and 18% of the variance. Both components were inversely associated with birth weight [−23 (−68, 22), −27 (−71, 17) grams respectively]. BKMR further identified that MEP and MEHP and phthalate concentrations were linearly related to lower birth weight [−51(−164, 63) and −122 (−311, 67), respectively].	None of the individual phthalates or phthalate mixtures were significantly associates with birth weight using the three selected approaches	The lack of significance may due to small samples, high within-person variability of urinary biomarkers, survival bias and unmeasured or/and residual confounding	Chiu et al. 2018 [16]
HOME study, USA	272	Pregnant women	54	Phthalates BPA, PFAS PCBs PBDEs Organochlorine pesticides Metals	Blood or urine	/	Bayesian Hierarchical Linear Models	Mixture exposure-health effects	There was some evidence that PFAS, DMP and Pb were associated with small reductions in birth weight.	For a 10-fold increase in chemical concentration, the mean differences in birth weights (95% credible intervals (CI)) were 1 g (-20, 23) for phthalates, -11 g (-52, 34) for PFAS, 0.2 g (-9, 10) for PCBs, -4 g (-30, 22) for PBDEs, and 7 g (-25, 40) for OCPs. Hence, exposure to these chemicals had null or small associations with birth weight.	This may be explained by the fact that PFAS interact with estrogen receptors, disrupt hormonal balances and alter lipid levels thereby potentially affecting fetal growth and development. PFAS may also affect adipose tissue development and the regulatory systems in body weight homeostasis, which may impact fetal growth outcomes.	Woods et al. 2017 [27]
HOME study, USA	389	Mother-infant pairs	Not specified	Phenols Phthalates Metals Pesticides PCBs PBDEs PFAS Cotinine	Blood or urine	< 10% except for cadmium (36% < LOD) and cotinine (21% < LOD)	1) K-means clustering 2) PCA 3) Linear regression	Mixture exposure-health effects	Select organochlorine compounds, phenols and cadmium demonstrated inverse associations with birth length		Mixtures of environmental chemicals could disrupt mesenchymal stem cell differentiation, and these are predecessors to bone, cartilage and fat cells. Consequently, a decrease in osteogenic proliferation may explain a smaller infant length at birth.	Kalloo et al. 2020 [28]
LIFECODES birth cohort USA	390	99 cases of preterm birth 291 controls	17	Arsenic Barium Beryllium Cadmium Copper Chromium Mercury Manganese Molybdenum Nickel Lead Selenium Tin Thallium Uranium Tungsten Zinc	Urine	14 metals with > 80% > LOD	1) ENET 2) PCA	Mixture exposure-health effects	1) ENET selected Cu as the most important trace metal associated with PTB. 2) PCA identified 3 principal components (PCs). PC2 for essential metals was associated with an increased risk of overall (OR 1.36, 95% CI 1.05, 1.76) and spontaneous (OR 1.58, 95% CI 1.14, 2.20) preterm birth.	/	/	Kim et al. 2018 [15]
Project Viva, Boston, USA	726 465	Mothers Neonates	6 PFAS	PFOA PFOS PFNA PFHxS EtFOSAA MeFOSAA T_4 T_4 index	Plasma	All six PFAS were detected in 99–100% of plasma samples	1) Weighted quantile sum (WQS) 2) Bayesian Kernel Machine Regression (BKMR)	Mixture exposure-health effects	1) Higher concentrations of PFAS mixture were associated with significantly lower maternal FT4I, with MeFOSAA, EtFOSAA, PFOA, and PFHxS contributing most to the overall mixture effect. 2) In infants, higher concentrations of the PFAS mixture were associated with lower T4 levels, primarily in males, with PFHxS and MeFOSAA	/	The mechanisms by which PFAS alter thyroid function are unclear. Proposed mechanisms include reduced responsiveness to the hypothalamic-pituitary-thyroid axis, increased hepatic clearance of T4, increased conversion of T4 to T3 by type 1 deiodinase, and	Preston et al. 2020 [21]

				TSH					contributing most in WQS, and PFHxS contributing the most.		competitive binding to thyroid hormone binding proteins.	
Michigan Mother-Infant Pairs (MMIP) birth cohort USA	56	Pregnant women between 18 and 42 years with a naturally conceived singleton pregnancy	41	12 phthalate metabolites 12 phenol metabolites 17 heavy metals	Urine	/	1) Spearman correlations 2) PCA	Mixture exposure-health effects	Ten of eleven PC groupings demonstrated statistically significant associations with inflammatory cytokines. Using the same 11 PC variables, there were no significant associations between EDC mixtures with infant birth weight or gestational age at delivery.	/	/	Kelley et al. 2019 [29]
EARTH study, USA	384 females 211 males 203 couples	Men (18-55 years) and women (18-46 years) were eligible to participate either independently or as a couple.	15	BPA 3 Parabens 11 Phtalates	Urine	All biomarkers had detection frequencies ≥70%	1) PCA 2) BKMR	Mixture exposure-health effects	1) PCA identified the same four factors for maternal and paternal preconception mixtures. Each unit increase in PCA scores of maternal (adjusted Risk Ratio (aRR): 1.36, 95%CI: 1.00, 1.84) and paternal (aRR: 1.47, 95%CI: 0.90, 2.42) preconception DEHP-BPA factor was positively associated with preterm birth. 2) BKMR models further showed that maternal preconception BPA and mono(2-ethyl-5-hydroxyhexyl) phthalate, and paternal preconception mono(2-ethylhexyl) phthalate were positively associated with preterm birth when the remaining mixture components were held at their median concentrations.		exposure to phenols and phthalates may affect the male and female germline, probably as a result of epigenetic regulation during gametogenesis. Moreover, both BPA and DEHP have been shown to alter the epigenetic regulation of imprinted genes in gametes	Zhang et al. 2021[30]

3.5. EUROPEAN HBM STUDIES FOCUSING ON EXPOSURE PATTERNS OR CO-OCCURRENCE IN HBM DATASETS

This section focuses solely on scientific publications describing co-occurrence patterns of chemicals in human biomonitoring datasets. First, we review European HBM studies.

3.5.1. TAMAYO-URIA ET AL 2019: THE EARLY-LIFE EXPOSOME: DESCRIPTION AND PATTERNS IN SIX EUROPEAN STUDIES

→ Objective

The authors aimed to describe the early-life exosome and focused thereby on correlations between multiple environmental exposures, their patterns and their variability across European regions and across time pregnancy and childhood exposures). This study is part of the HELIX project which aims to characterize the exposome during early-life [10].

→ Methods

HELIX is based on six European birth cohort studies: BiB (Born in Bradford), UK; EDEN (Etude des Déterminants pré et postnatales du development et de la santé de l'Enfant), France; INMA (INfancia y Medio Ambiente), Spain; KANC (Kaunus cohort), Lithuania; MoBa (Norwegian Mother and child Cohort Study), Norway; and Rhea (Greece). Data were used from the HELIX sub cohort consisting of 1301 mother-child pairs with information of environmental exposures during pregnancy and between 6 and 11 years of age. 87 environmental exposure variables for the pregnancy period, of which 49 variables were related to HBM, and 122 for the childhood period, of which 50 variables were related to HBM, were included. Chemical exposures were measured in serum, plasma, blood or urine samples using maternal samples collected during pregnancy or at birth and included; organochlorine compounds, PBDEs, PFAS, metals and elements, phthalate metabolites, phenols, organophosphate pesticide metabolites and cotinine.

Within-cohort correlations were investigated using Pearson, polychoric or polyserial correlations as appropriate. PCA was first conducted within each of 19 pre-defined exposure groups and retained only the first principal component for all of them. In this way, a composite index variable (principal component scores) for each exposure group were created. Next, all the exposures from the different exposure families were included in the same analysis (separately for pregnancy and postnatal period), but the authors focused only on the number of components needed to explain 70% or 95% of the variation.

→ Results

1) Overall correlations tended to be higher than within correlations and the median correlation within exposure groups was > 0.3 for many exposure groups. Correlations between maternal (pregnancy) and childhood exposures were low and even close to zero for some chemical exposures.

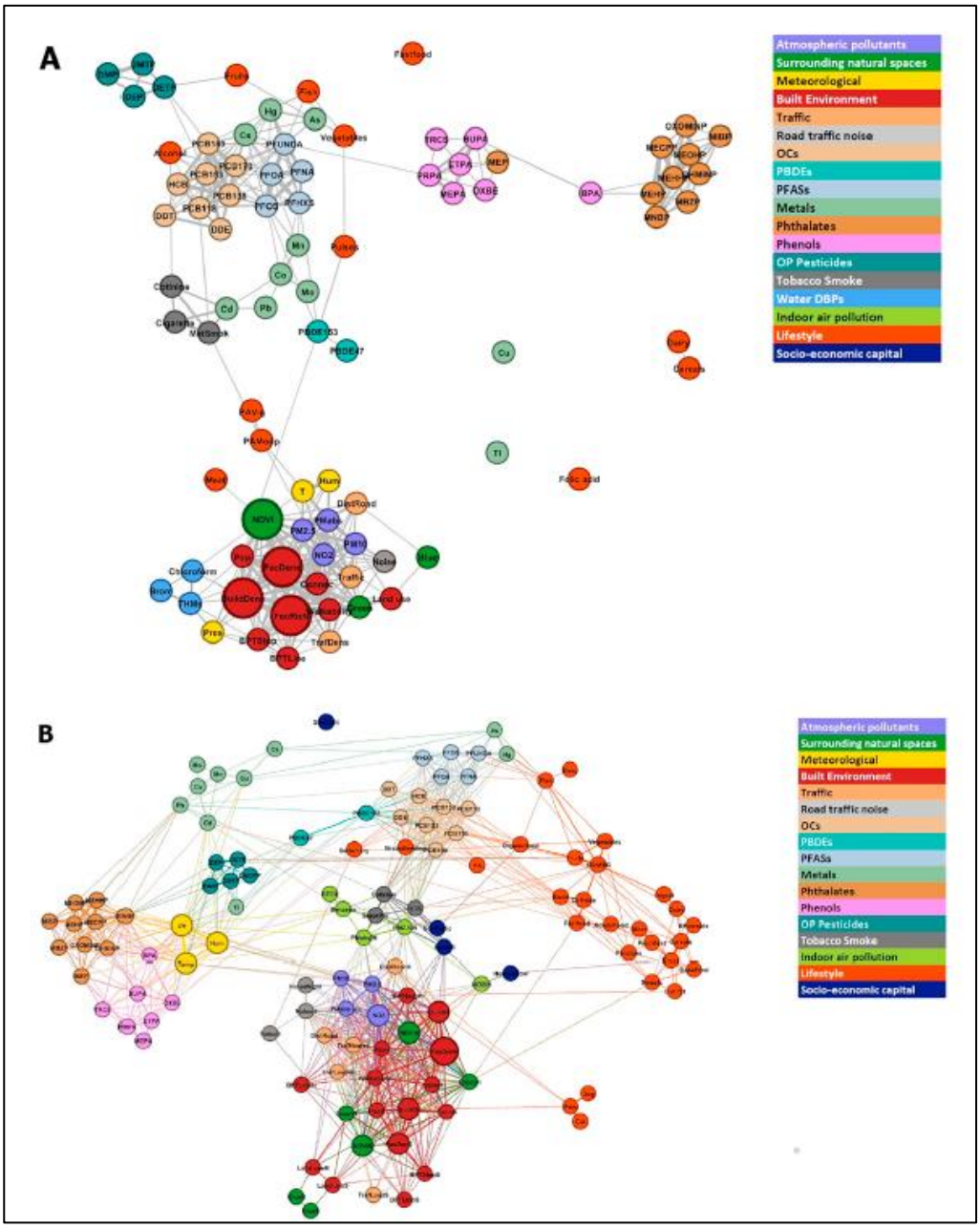

Figure 1: network visualization of the exposome.
The size of the nodes is proportional to the number of correlations were > 0.05 outside the exposure group and the length of the edges is proportional to the inverse of the correlation between exposures. The color of the nodes represents the pre-defined exposures groups. Figure adapted from [10].

2) The exposome correlation structure, using individual exposures and within-cohort correlations, is visualized as a network in for the pregnancy network (Figure 1A) and in for the childhood network above (Figure 2B). Exposures that are close together in the network are more correlated than more distant ones. Moreover, the childhood network appears more compact than the pregnancy network with more links between exposure families or chemical groups as defined above (organochlorine compounds, PBDEs, PFAS, metals and elements, phthalate metabolites, phenols, organophosphate pesticide metabolites and cotinine).

3) Ten principal components explained 45% and 39% of the total variance in the pregnancy and childhood exposome, respectively, while 65 and 90 components were required to explain 95% of the exposome variability.

→ **Discussion**

It was demonstrated that correlations within the same exposure group can be high, but that correlations between exposures from different chemical groups were low. High correlations between exposures from the same exposure (chemical) group are to be expected as these groups are predefined and have also been described previously (Lenters et al., 2015 [3]; Robinson et al., 2015 [5]). The finding of generally low correlations between exposures from different groups, especially after removing cohort effects, is important as it would support the notion that, if this finding is generalizable to all populations, epidemiological studies focusing on a single family of exposures may not be confounded by exposures from other groups. However, although many between-group correlations were low, they cannot be neglected.

Some of the high correlations may be due to exposures being measured using a common methodology (e.g. GIS variables) or a common biological medium (e.g. variables measured in urine) or because some variables were used to create others [10]. It is recommended that investigators conducting exposome research conduct a thorough exploration of the structure of the exposome before evaluating exposome-health associations [10].

3.5.2. ROSOFSKY ET AL. 2017: EXPOSURE TO MULTIPLE CHEMICALS IN A COHORT OF REPRODUCTIVE-AGED DANISH WOMEN

→ **Objectives**

The extent to which women of childbearing age are exposed to multiple chemicals and how these biomarkers of exposure correlate with each other is unclear. A better understanding of the extend of exposure in reproductive-aged women is necessary due to the potential for in utero exposure and fetal susceptibility. The objectives of the study of Rosofsky et al. 2017 were to characterize concentrations of chemical biomarkers during preconception and examine correlations between and within chemical classes [4].

→ **Methods**

Concentrations of 135 markers from 16 chemical classes were measured in blood, serum and urine samples from 73 women (18-40 years) enrolled in the prospective Snart Foraeldre/Milieu cohort study in Denmark (2011-2014). Biomarkers included metals, organochlorines, phthalates, phytoestrogens (a plant-derived dietary estrogen not generated by the own endocrine system but derived from consuming phytoestrogenic plants) and pesticides.

Biomarker correlations between and within chemical classes were examined using principal component analysis (PCA). Three models for the PCA were created: all biomarkers combined (Model

1), urinary biomarkers only (Model 2) and serum and whole blood biomarkers only (Model 3). Analysis were restricted to biomarkers that were detected in 75% of the study population to enhance reliability in the PCA estimates.

The optimal number of principal components were determined for each model. The authors restricted Model 1 to three principal components and Models 2 and 3 to two components (Figure 2). A factor loading greater than 0.25 indicated a high loading.

→ **Results**

	Component 1	Component 2	Component 3
Model 1: Blood and Urine	**Blood Metals:** manganese, Total Hg	**Blood Metals**: cadmium, lead **Urinary Metals:** cobalt, lead **PFAS**: PFHxS, PFOS **Cotinine**	**PFAS**: PFDeA, PFNA, PFOA **PCB28** **PBDE153** **PAHs**: 1-hydroxyphenanthrene
	Persistent Pesticides: hexachlorobenzene, pp'-DDE	**PAHs**	**Urinary Metals:** total arsenic, arsenobetaine
	PCBs	**Perchlorate** **Phthalate**: Monoethyl phthalate **Parabens**: methyl-paraben, propyl-paraben	**Phthalates** **Pesticides**: para-Nitrophenol, 3,5,6-Trichloro-2-pyridinol, diethylphosphate
Proportion Variance Explained	23%	16%	8.1%
Model 2: Urine Only	**PAHs** **Phthalate**: Monoethyl phthalate **Parabens**: methyl-paraben, propyl-paraben **Phytoestrogen**: Equol	**Arsenic and Arsenic Metabolites** **Phthalates** **Pesticides**: para-Nitrophenol, 3,5,6-Trichloro-2-pyridinol, diethylphosphate	N/A
	Urinary Metals: lead	**Urinary Metals:** strontium	
Proportion Variance Explained	24%	14.9%	
Model 3: Serum and Whole Blood Only	**Blood Metals**: manganese, Total Hg **Persistent Pesticides:** hexachlorobenzene , pp'-DDE	**All PFAS biomarkers** **Blood Metals**: cadmium, lead	N/A
	PCBs	**Cotinine**	
	PBDE153	**PCB28**	
Proportion Variance Explained	24%	15.3%	

[a] Biomarkers with loadings < 0.25 are not listed.

Figure 2: Summary of correlations among 135 analytes measured in urine and blood among Snart Foraeldre/Milieu women (n=56). Only biomarkers detected in more than 75% of the study population were included. Figure adapted from [4].

Model 1: biomarkers measured in blood, serum and urine from 56 women

Persistent pesticides, PCBs and total mercury loaded were loaded on Component 1.

- Within component 1, PCBs demonstrated high intra-class correlation, indicating that PCBs correlate well reciprocally (0.33 to 0.98).
- Both hexachlorobenzene and pp'-DDE were moderately correlated with PCBs (0,39-0.88).

PAHs, parabens, urinary metals and cotinine were loaded on Component 2.

- PAHs demonstrated high intra-class correlations (0.62-0.96) and were only highly correlated with perchlorate.
- Within component 2, PFAS exhibited high intra- but not inter-class correlation.

Phthalate metabolites were the primary chemical class loaded on Component 3.

- Phthalate metabolites demonstrated high intra-class correlation but only moderate correlations with PFAS and organophosphate metabolites.

Model 2: biomarkers measured in urine

PAHs, mono-ethyl phthalate and parabens were loaded on Component 1.

- PAHs were moderately correlated with mono-ethyl phthalate.

Arsenic and arsenic metabolites, all other phthalates and non-persistent pesticide metabolites were loaded on Component 2.

- Total arsenic and arsenic metabolites had strong intraclass correlation and were moderately correlated with urinary strontium.
- Diethyl phosphate, a nonspecific metabolite of organochlorine pesticides, was also moderately correlated with 3,5,6-trichloro-2-pyridinol, a specific metabolite.

Model 3: biomarkers measured in blood and serum

Blood manganese, total mercury, PCBs and persistent pesticides were loaded on component 1.

- The strongest correlations in this Model were PCBs 99, 238-158, 146, 153, 183 and 187 with p,p'-DDE.

PFAS biomarkers, blood metals, cotinine and PCB28 were loaded on component 2.

- Blood metals had low intra- and inter-class correlation, while PFAS had high intra-class correlation.
- Cotinine and blood cadmium were moderately correlated (0.58).

→ **Discussion**

Many of the component loadings reflect shared lifestyle patterns in this study population being consumer products, or as combustion byproducts according to the authors [4];

- Mono-ethyl phthalate, a breakdown product of diethyl phthalate and parabens loaded high on the same components. Both are commonly used in cosmetic products, such as hair and nail products, fragrances, make-up, and soaps, and in food packaging.
- PAHs and perchlorate were also found to be strongly correlated, which may be explained by the fact that they are both released by combustion processes.
- The correlations found between phthalates and PAH biomarkers with other chemicals classes may be attributed to their cooccurrence in indoor settings [31].
- PFAS chemicals are primarily used as stain and grease repellants in cookware and furniture, and have been commonly detected in drinking water, with PFOS and PFOA being the most ubiquitous. The observed high intra-class and low inter-class PFAS correlations explain Model 3, Component 2, where high exposure to one PFAS may be related to exposure to other PFAS biomarkers.

- Cotinine is frequently used as a surrogate of cigarette consumption in epidemiological studies and cadmium and cotinine loading was observed on the same component, indicating that smoking status may contribute to a proportion of the variance explained by Component 2 in Models 1 and 3.
- In model 1 and 3 it was found that total mercury loaded with PCB congeners that are commonly found in food (138, 153, 180). It was previously demonstrated that living in an urban area and consumption of saltwater fish were the most predictive factor of mercury in hair and PCB concentrations [32].
- Low correlations between urinary metals and phytoestrogens suggest that metals and phytoestrogens are not derived from the same set of sources as biomarkers that explained the highest proportion of variation in the data. For instance, phytoestrogens are almost exclusively found the diet, specifically fruits, vegetables, soy products and cereal products. Metals are released into the environment from a diverse set of sources, including the natural geography and industry.

3.5.3. GOVARTS ET AL. 2020: EARLY-LIFE EXPOSURE TO MULTIPLE PERSISTENT ORGANIC POLLUTANTS AND METALS AND BIRTH WEIGHT: POOLED ANALYSIS IN FOUR FLEMISH BIRTH COHORTS

→ Objectives

Prenatal chemical exposure has frequently been associated with reduced fetal growth although results have been inconsistent. The objective was to investigate the association between prenatal exposure to a mixture of persistent environmental chemicals and birth weight in the pooled dataset of four Flemish birth cohorts by comparing results using multipollutant models [2].

→ Methods

Concentrations of biomarkers were measured in cord blood samples of 1579 women from four Flemish birth cohorts (FLEHS I, II & III and 3XG) collected of a 10 years' time period. Biomarkers included PCB congeners (138, 153, 180), hexachlorobenzene, pp'-DDE, cadmium and lead.

Four linear regression-based statistical methods, e.g. Multiple linear regression (MLR), Bayesian Information Criterion (BIC), BIC Bayesian model averaging (BMA), penalized regression using minimax concave penalty (MCP) and Bayesian Adaptive Sampling (BAS) were applied to assess the influence of multiple pollutants in a single analysis on birth weight, adjusted for *a priori* selected covariates.

→ **Results**

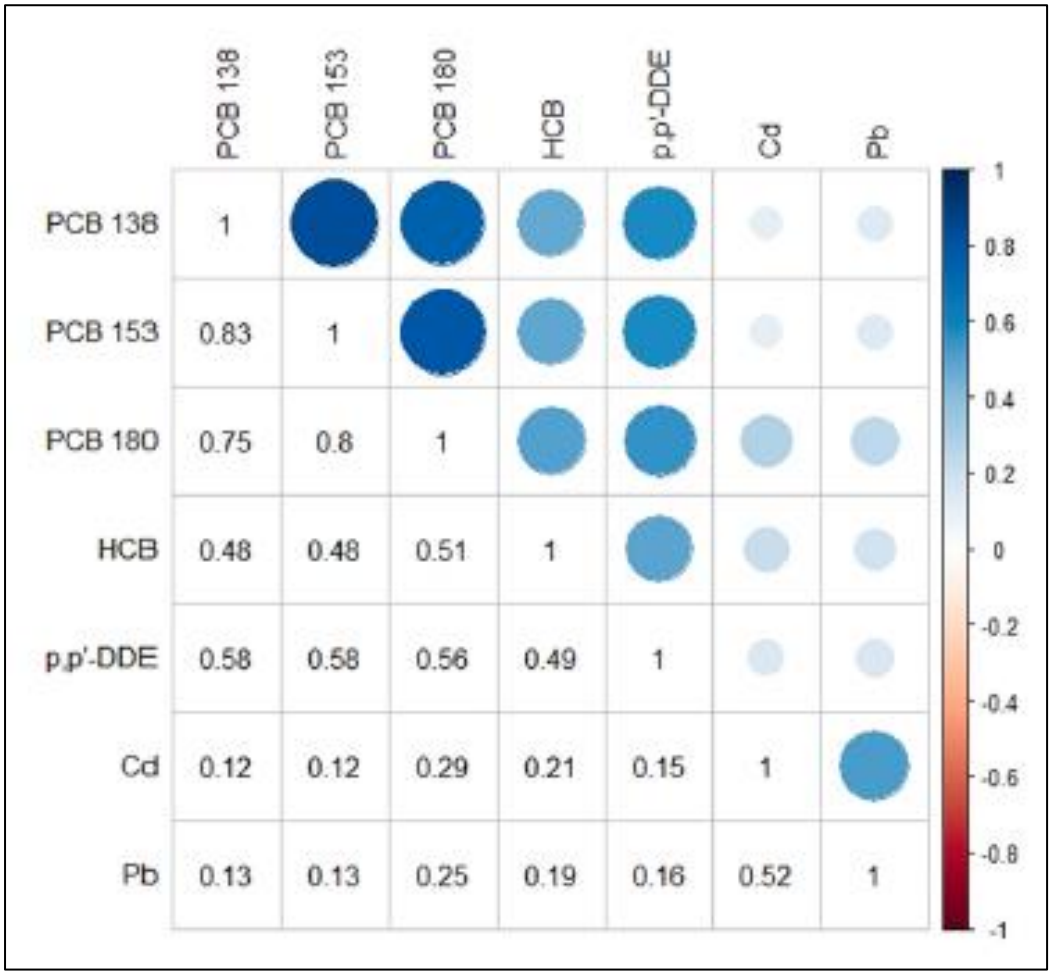

Figure 3: Pearson correlation matrix between the ln-transformed exposure biomarkers, figure adapted from [2].

Correlations between the different pollutants were low to moderate (Pearson's r = 0.12-0.58), except for the three PCB congeners that were highly correlated (Pearson's r = 0.75-0.83) (Figure 3). In single pollutant models, only levels of PCB congeners were significantly associated with reduced birth weight.

Only PCB 180 and p,p'-DDE were significantly associated with birth weight in the MLR model. Using BIC as the selection criterion, the model with PCB 180 and p,p'-DDE was selected as the best model and this was also confirmed in the BMA approach using BIC for which PCB 180 and p,p'-DDE were both associated with birth weight.

PCB 153, PCB 180 and p,p'-DDE were selected in CP models after stability selection with p,p'-DDE having the highest selection probability, followed by PCB 180 and PCB 153.

PCB 153 and PCB 180 showed an inverse association with birth weight in all multipollutant models while p,p'-DDE levels were associated with an increasing birth weight as shown in Figure 4.

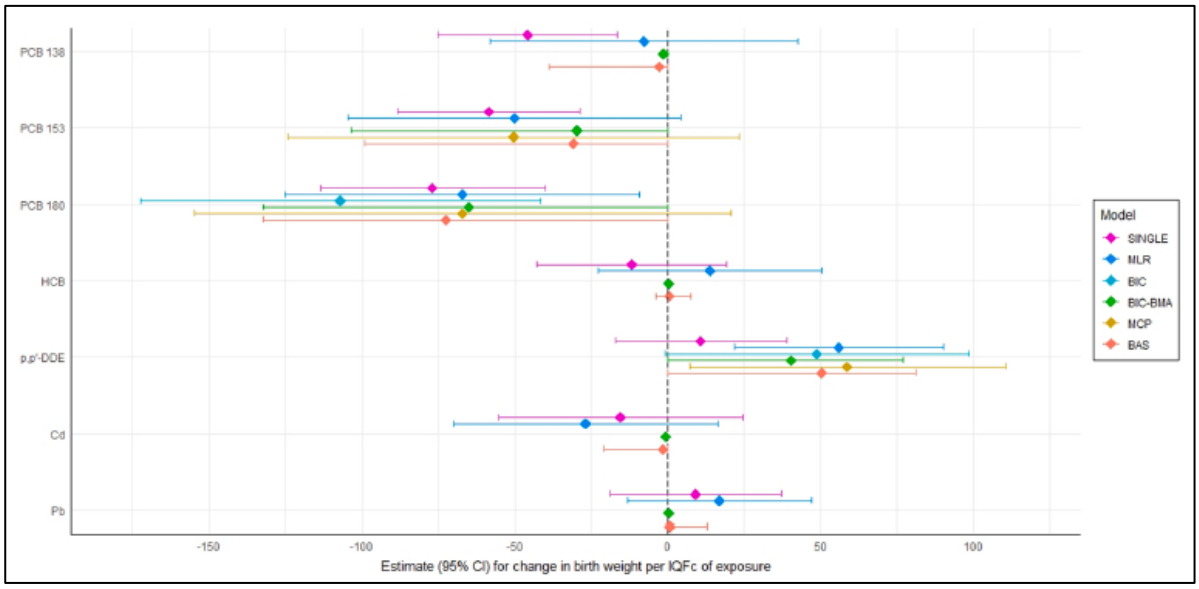

Figure 4: estimated association between exposure biomarkers and birth weight from the single pollutant models (SINGLE) and five different multiple-exposure models (MLR, BIC, BIC-BMA, MCP and BAS) for birth weight. Figure adapted from [2].

→ **Discussion**

p,p'-DDE and PCB 180 were most consistently associated with birth weight according to five different multipollutant modeling approaches. In addition, PCB 153 was selected when applying MCP and BAS. An inverse association with birth weight was found for the PCB congeners, while an increased birth weight was observed for elevated levels of p,p'-DDE. P,p'-DDE had the highest selection probability, followed by PCB 180 and PCB 153. The lower selection probability for the PCB congeners is likely at least partially due to the strong correlation between them.

PCB congeners act mainly as estrogens, but also anti-estrogenic, androgenic and anti-androgenic activities are described depending on the metabolite and concentration. PCB's bind to thyroid transport proteins thereby disrupting thyroid function [33]. DDE inhibits androgens due to binding to their receptors [34] and also disrupts thyroid function [35]. As estrogens and androgens play important roles in regulating nutrient delivery to the fetus and organ maturation [36], PCB's and DDE may disrupt fetal development by their endocrine-disrupting characteristics. Additionally, there is strong evidence indicating that estrogenic EDCs can program gene activity via epigenetic changes during critical periods in development, with long-term consequences that impact the health status of the individual throughout the remainder of life [37].

3.5.4. OTTENBROS ET AL. 2021: NETWORK ANALYSIS TO IDENTIFY COMMUNITIES AMONG MULTIPLE EXPOSURE BIOMARKERS MEASURED AT BIRTH IN THREE FLEMISH GENERAL POPULATION SAMPLES

→ Objectives

Graphical representation of correlation patterns between biomonitoring data such as heatmaps or circulation correlation globes is increasing. However these methods have some challenges and the distinction between groups of correlated compounds is not always straightforward. Networks provide a graphical method to represent groups or communities in the data. Networks facilitates the detection of exposure patterns and allows for the systematic comparison of observed exposure patterns between datasets and strata within datasets [1].

→ Methods

The use of network techniques in HBM data from cord blood samples collected in three campaigns of the Flemish Environment and Health Studies (FLEHSI, II & III). Measured biomarkers were multiple organochlorine compounds, PFAS and metals. Comparative network analysis (CNA) was conducted to systematically compare networks between sampling campaigns, smoking status during pregnancy, and maternal pre-pregnancy BMI.

→ Results and conclusion

According to Ottenbros et al. 2021, network techniques offer an intuitive approach to visualize complex correlation structures within human biomonitoring data. The identification of groups of highly connected biomarkers, "communities," within these networks highlighted which biomarkers should be considered collectively in the analysis and interpretation of epidemiological studies or in the design of toxicological mixture studies [1].

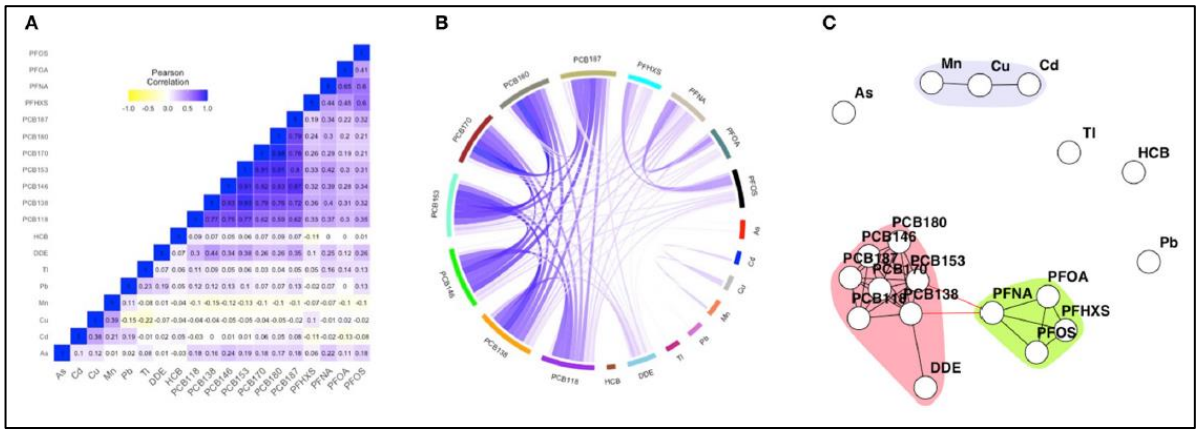

Figure 5: example of a heatmap (A), circular correlation globe (B) and network including community detection (C).
The heatmap is based on Pearson correlation between the biomarkers. Within the circular globe each biomarker is presented as a color-block on the circular axis. Within the network, each dot or node

represents a biomarker, each edge represents a connection between the biomarkers and each different color represents a community within a subnetwork. Figure adapted from [1].

3.5.5. ROBINSON ET AL. 2015: THE PREGNANCY EXPOSOME: MULTIPLE ENVIRONMENTAL EXPOSURES IN THE INMA-SABADELL BIRTH COHORT

→ Objectives

Robinson et al. 2015 aimed to describe the correlation structure of the exposome during pregnancy to better understand the relationships between and within families of exposure and to develop analytical tools appropriate to exposome data [5].

→ Methods

Data from the Environment and Childhood Project (INMA) in Sabadell, Spain, were used of 728 pregnant women. 81 chemicals covering the pregnancy period were collated into a single data set. Biomonitoring data included organochlorines and PFAS in serum, mercury in cord blood, PBDEs in breast milk and metals, phthalates, BPA and cotinine in urine.

Pair-wise Pearson's correlations (for continuous variables) and polychoric correlations (for correlations involving binary variables) between each individual exposure were calculated to produce a correlation matrix. Heat map and circos plots were made to display the correlations. Principal components were derived directly from the correlations.

→ Results

- The mean correlation (*r*) all exposures was 0.08 with a standard deviation of 0.21 (median = 0.02; 5-95th centiles = -0.12-0.54).
- The four PFOA compounds had the strongest median absolute correlation (r = 0.62) of the individually measured biomarkers. The other biomarker families, PDBEs, phthalates, metals, and organochlorines, had all median absolute correlations below 0.5, reflecting their more diverse sources.
- The strongest correlation within the home environment exposures was between use of home and garden pesticides (r = 0.16). Overall, the median of all within-family absolute correlations was 0.45 (5th−95th centiles, 0.07−0.85).
- "Blocks" of high correlation within families of exposure were observed along the main diagonal of the heat map (Figure 6), with certain groups such as the organochlorines and phthalate metabolites showing less dense within-family correlations than more closely linked exposures such as the PFAS. With respect to between family correlations, no exposure had an absolute correlation higher than 0.6 with an exposure outside its family.
- Only three pairwise correlations between biomarker measured exposures in separate families were above 0.3 with the strongest correlation observed between perfluorooctanesulfonic acid and PCB-153 (r = 0.32). Overall, the median of all between-family absolute correlations was 0.05 (5th−95th centiles, 0.01−0.23).

- Only three principal components were required to explain 50% of variance across the hole data set, while six components explained 70% of variance and 22 components explained 95% of the variance.

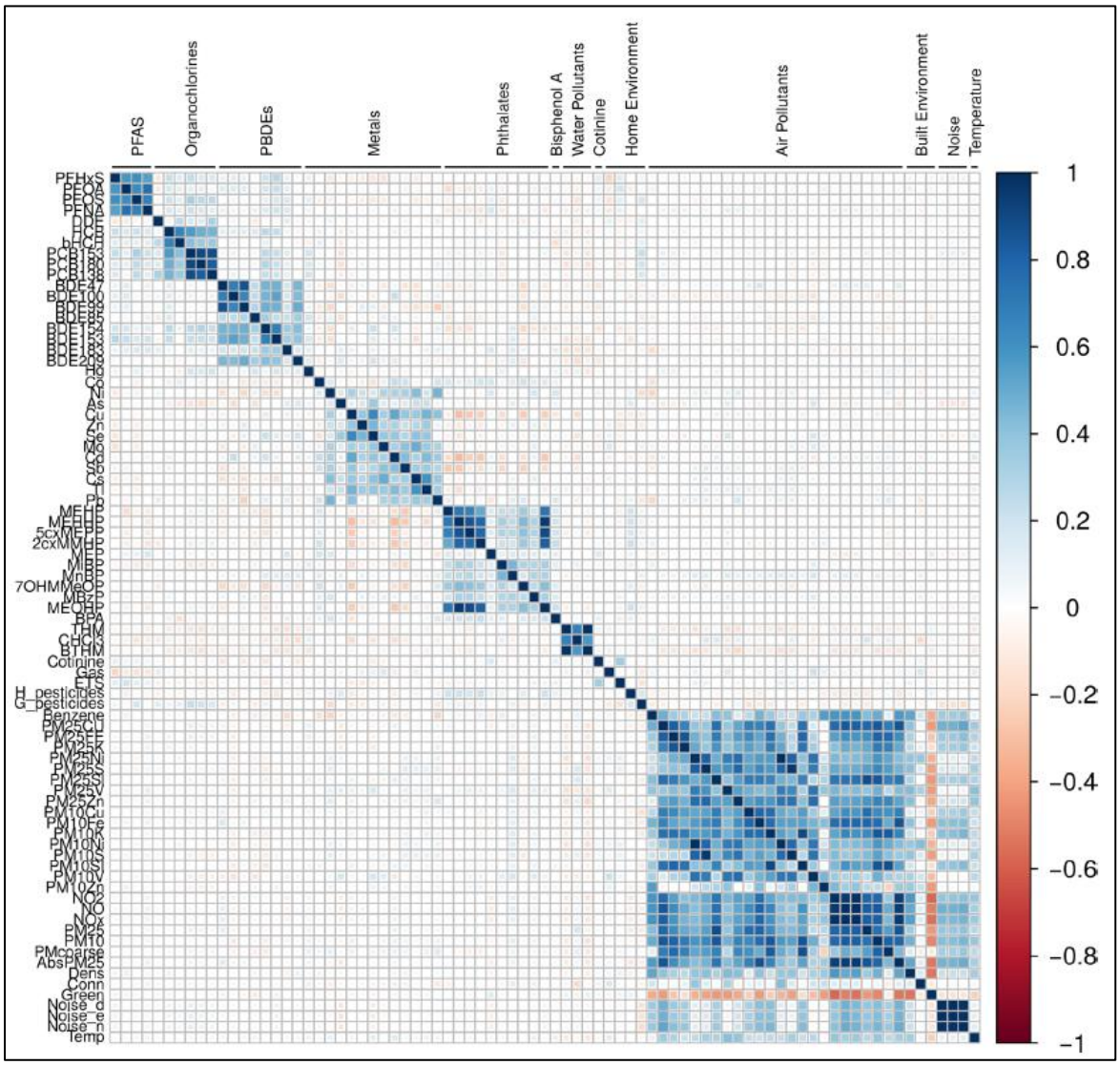

Figure 6: Correlation heatmap, showing pair correlations across all exposures, with blue color indicating positive correlations and red color indicating negative correlations. Figure adapted from [5].

→ **Discussion**

Robinson et al. 2015 concluded that they found strong levels of correlations within families of exposure. Hence, it is important that results reported for single exposures need to be interpreted in light of their correlations to other exposures within their respective families.

3.6. EUROPEAN HBM STUDIES DESCRIBING CHEMICAL CO-OCCURRENCE PATTERNS IN RELATION WITH HEALTH OUTCOMES

3.6.1. GOVARTS ET AL. 2016: COMBINED EFFECT OF PRENATAL EXPOSURES TO ENVIRONMENTAL CHEMICALS ON BIRTH WEIGHT

→ Objectives

Prenatal chemical exposure has frequently been associated with reduced fetal growth. The goal of this study was to investigate the effects of exposure to single pollutants and mixtures on birth weight in mother-child pairs [18].

→ Methods

Data from FLEHS II mother-child cohort were used of 248 newborn-mother couples with an uncomplicated liveborn singleton pregnancy. The birth outcome of interest was birth weight. 15 chemicals were measured in individual samples. Lead, manganese, copper, thallium and arsenic were measured in cord blood and cadmium was measured in maternal whole blood samples. PCB-138, PCB-153, PCB-180, p,p'-DDE, PFOS, PFOA and mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP) were measured in plasma cord samples. Total mercury and methylmercury were measured in maternal hair samples.

Linear regression models were used to quantify the effect on birth weight associated with one pollutant. Two different statistical methods were used to investigate the effects of combined exposure on birth weight; principal component regression and an algorithm to explore the effect of mixtures.

→ Results

Single pollutant multiple linear regression models showed a significant inverse association ($p = 0.016$) between increasing arsenic concentrations in cord blood and lower birth weight.

For the PCs composed from the whole PCA, none of the principal components were statistically significant ($p < 0.05$) associated with birth weight. However, since the models were built for a subset of 152 from 248 individuals, statistical power may be reduced. For the PCs resulting from the subset PCA, a significant negative association with birth weight was found for PC4 ($p = 0.009$) constituted by arsenic and cadmium (Figure 7).

Mixtures with arsenic, cadmium and MECPP showed a strong association with birth weight as can be derived from the estimates (algorithm). The mixtures with the highest association with birth weight were composed of five chemicals, i.e., PFOA, lead, cadmium, arsenic, and MECPP ($p = 0.0019$) and cadmium, thallium, arsenic, MECPP, and methylmercury ($p = 0.0021$). A strong effect of mixture exposures in girls was identified when combining six chemicals, i.e., PFOS, lead, cadmium, manganese, thallium, and methylmercury ($p = 0.0006$); and PFOA, lead, cadmium, thallium, arsenic,

and methylmercury (p = 0.0007). In boys, the mixture of MECPP with cadmium (p = 0.0061) showed a stronger association with birth weight as compared to MECPP alone (p = 0.06).

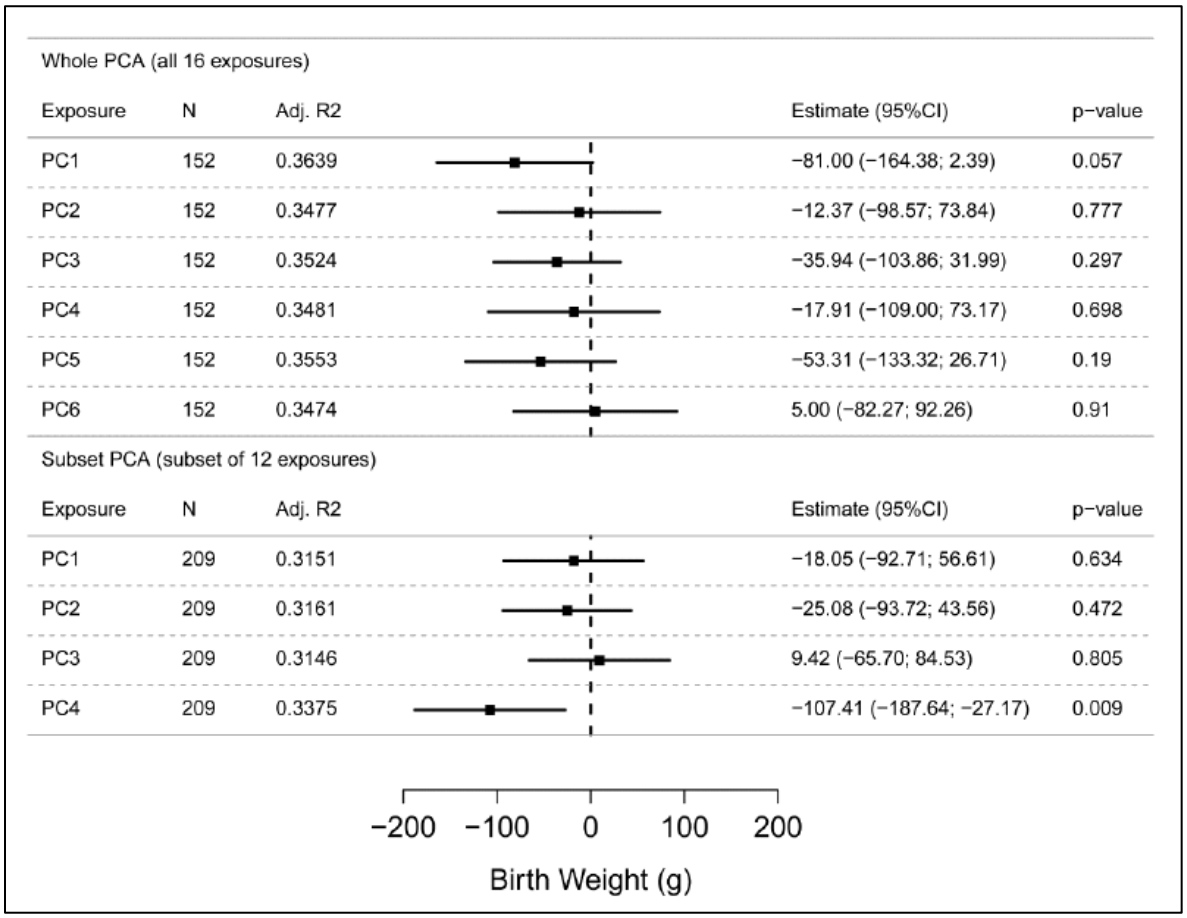

Figure 7: results of the principal component regression for the whole PCA (all 16 exposures) and subset PCA (subset of 12 exposures). Figure adapted from [18].

3.6.2. AGAY-SHAY ET AL. 2015: EXPOSURE TO ENDOCRINE-DISRUPTING CHEMICALS DURING PREGNANCY AND WEIGHT AT 7 YEARS OF AGE: A MULTI-POLLUTANT APPROACH

→ Objectives

Prenatal exposure to endocrine-disrupting chemicals (EDCs) may induce weight gain and obesity in children, but the obesogenic effects of mixtures have not been studied. Therefore, the authors evaluated the associations between pre- and perinatal biomarker concentrations of 27 EDCs and child weight status at 7 years of age [22].

→ Methods

Data from the Environment and Childhood Project (INMA) in Sabadell, Spain, were used of 657 mother-child pairs. The birth outcome of interest was weight and height of the children at approximately 7 years of age. 27 chemicals were measured in individual samples; BPA, ten phthalate

metabolites and metal concentrations (arsenic, lead and cadmium) were measured in maternal urine samples. Organochlorine pesticides and PCBs were measured in maternal blood samples. Cord blood was used to measure total mercury and maternal colostrum samples were used to measure PBDEs.

PCA was applied to evaluate multiple chemical exposures simultaneously and four factors (or components) were chosen of which three were clearly defined by specific exposure groups (PBDEs, phthalates, organochlorines). The fourth factor was a mix of chemicals from different exposure groups, reflecting a pattern of higher MEP, As, Hg, BPA, and PBDE exposure and lower DDE and βHCH exposure. The reasons for this patterning are not clear and require further evaluation.

- Factor 1: PBDEs
- Factor 2: phthalates
- Factor 3: organochlorine
- Factor 4: MEP, As, HG, BPA, PBDE153, PBDE-154

→ **Results**

A factor reflecting combined exposure to multiple phthalate metabolites showed weak evidence for an association with reduced BMI. Exposure to other EDCs, whether in single-pollutant or combined multi-pollutant analyses, showed no evidence for an association with child weight status.

- In the model that simultaneously included all four factors, exposure to the highest tertile compared with the lowest tertile of the organochlorine factor (factor 3) was associated with significant increase in the z-BMI RR of 0.37 (95% CI: 0.03, 0.72) and with an increase in the RRs of overweight of 2.59 (95% CI: 1.19, 5.63). In tertile 2, z-BMI and overweight demonstrated a nonsignificant increase (adj β tertile 2 vs. 1: 0.12; 95% CI: –0.19, 0.43; adj RRs tertile 2 vs. 1: 1.86; 95% CI: 0.92, 3.76).
- Exposure to the phthalate factor (factor 2) showed a decrease in the RRs for overweight of 0.49 (95% CI: 0.25, 0.96) in tertile 2 and nonsignificant negative associations in tertile 3 compared with tertile 1 of 0.63 (95% CI: 0.33, 1.19). Similar, nonsignificant negative associations were observed with z-BMI for exposure to the phthalate factor (factor 2) in tertile 3 and tertile 2 compared with tertile 1.
- Exposure to the PBDE factor (factor 1) showed a nonsignificant decrease in the RRs for overweight (adj RRs tertile 2 vs. 1: 0.61; 95% CI: 0.28, 1.34; adj RRs tertile 3 vs. 1: 0.54; 95% CI: 0.25, 1.17). Similar, nonsignificant negative associations with z-BMI were observed for exposure to the PBDE factor (factor 1) in tertile 3 and tertile 2 compared to tertile 1.

3.6.3. PHILIPPAT ET AL. 2019: PRENATAL EXPOSURE TO SELECT PHTHALATES AND PHENOLS AND ASSOCIATIONS WITH FETAL AND PLACENTAL WEIGHT AMONG MALE BIRTHS IN THE EDEN COHORT (FRANCE)

→ **Objective**

The placenta performs crucial physiological functions to ensure normal fetal development. The goal was to explore the associations between urinary concentrations of phthalate and phenol biomarkers

during pregnancy and placental weight and placental-to-birth weight ratio (PFR) among male newborns. Associations between biomarkers and birthweight were also investigated [20].

→ **Methods**

Data from a subgroup of the EDEN mother-child cohort, consisting of 473 mother-son pairs recruited from april 2003 to March 2006 in France, were used. The birth outcomes of interest were placental weight, birth weight and PFR. 9 phenols (4 parabens, 2 dichlorophenols, triclosan, benzophenone-3, bisphenol A) and 11 phthalate metabolites were measured in spot urine samples collected between week 23 and 29 of gestation.

Adjusted Elastic Net penalized regression models (ENET) was used to select biomarkers associated with the birth outcomes of interest. Unpenalized effect estimates were then obtained by fitting linear regression models simultaneously adjusted for the ENET-selected biomarkers and a priori chosen confounders.

→ **Results**

The ENET model retained four biomarkers for placental weight: triclosan (β = -4.11 g [95% CI: -8.26;0.05]) and MNCP (β = -10.9 g [95% CI: -21.8;0.09]) which were negatively associated with placental weight while benzophenone-3 (β = 4.76 g [95% CI: -1.77;11.3]) and the sum of parabens (β = 7.12 g [95% CI: 0.41;13.9]) were positively associated with this outcome.

Regarding birth weight, benzophenone-3 was the only biomarker selected by the ENET model and the associated unpenalized effect estimate was 21.0 g [95% CI: -3.45;45.5] .

The ENET model retained two phthalate metabolites for PFR, MNCP (β = -0.20 [95% CI: -10.54;0.13]) and MCOP (β = -0.23 [95% CI: -0.58;0.11]) which were negatively associated with this outcome.

→ **Discussion**

These results provide preliminary evidence of possible associations between other compounds such as triclosan, benzophenone-3, MCNP, and MCOP and both placental weight and PFR. The fact that the direction of the associations with placental weight differed across biomarkers might indicate different mechanisms of action [20].

3.6.4. BÉRANGER ET AL. 2019: MULTIPLE PESTICIDES IN MOTHERS' HAIR SAMPLES AND CHILDREN'S MEASUREMENTS AT BIRTH: RESULTS FROM THE FRENCH NATIONAL BIRTH COHORT (ELFE)

→ **Objective**

The general population is continuously and ubiquitously exposed to numerous pesticides. However, studies investigating the possible role of environmental exposure to pesticides on fetal growth have focused on a limited set of substances despite a range of pesticides are present today. Therefore,

the authors explored the relation between maternal hair concentrations of multiple pesticides and metabolites and the measurements at birth of their newborns.

→ **Methods**

Data from the ELFE French nationwide birth cohort, consisting of 311 women who gave birth to liveborn singleton at ≥ 33 weeks of gestation in France in 2011, were used. The birth outcomes of interest were birth weight, length and head circumference. 64 chemical substances were detected in hair samples; organochlorines, organophosphorus, pyrethroids, carbamates, dinitroanilines, thiocarbamates, Phenylpyrazoles, acid herbicides, azoles, oxadiazines, Trizines/triazones, amide pesticides, strobilurins, carboxamides, urea, neonicotinoids, anilino-pyrimidines.

ENET was applied to simultaneously select the strongest predictors of measurements at birth. Selected variables were multiply imputed for missing values and unpenalized estimators were assessed by standard linear regression.

→ **Results**

Statistically significant associations were observed between maternal hair concentrations of seven pesticides or pesticide metabolites and birth measurements (weight: fipronil sulfone; length: TCPy, bitertanol, DEP, and isoproturon; head circumference: tebuconazole and prochloraz).

Analyses restricted to boys identified twelve additional compounds: eight independently associated with birth weight (3Me4NP, DCPMU, DMST, fipronil, mecoprop, propoxur, fenhexamid, and thiabendazole), two with birth length (dieldrin and β-endosulfan), and six with head circumference (β-endosulfan, β-HCH, fenuron, DCPMU, propoxur, and thiabendazole).

3.6.5. LENTERS ET AL. 2016: PRENATAL PHTHALATE, PERFLUOROALKYL ACID AND ORGANOCHLORINE EXPOSURES AND TERM BIRTH WEIGHT IN THREE BIRTH COHORTS: MULTI-POLLUTANT MODELS BASED ON ELASTIC NET REGRESSION

→ **Objective**

Associations between multiple corelated biomarkers of environmental exposure and birth weight were investigated [3].

→ **Methods**

Data from three birth cohorts were used with in total 1250 term singleton infants; 513 mother-infant pairs from Greenland, 180 from Poland and 557 from Ukraine. Outcome of interest was birth weight. Secondary metabolites of DEHP and DiNP, eight perfluoroalkyl acids and organochlorines (PCB-153 and p,p'-DDE) were measured in maternal serum samples. Associations were identified by elastic net penalty to linear regression models.

→ **Results**

Two phthalate metabolites (MEHHP, MOiNP), perfluorooctanoic acid (PFOA), and *p,p´*-DDE were most consistently predictive of term birth weight based on elastic net penalty regression. In an adjusted, unpenalized regression model of the four exposures, two standard deviation increases in natural log–transformed MEHHP, PFOA, and *p,p´*-DDE were associated with lower birth weight: -87 g (95% CI: -137, -340 per 1.70 ng/mL), -43 g (95% CI: -108, 23 per 1.18 ng/mL), and -135 g (95% CI: –192, -78 per 1.82 ng/g lipid), respectively; and MOiNP was associated with higher birth weight (46 g; 95% CI: -5, 97 per 2.22 ng/mL).

→ **Discussion**

It is suggested that several of these environmental chemicals, belonging to three chemical classes, may be independently associated with impaired fetal growth. Lenters et al. 2016 suggested that increased oxidative stress and modulation of sex or thyroid hormones homeostasis may play a role in development and growth velocities. Additionally, PFASs may interfere with lipid metabolism via activation of the peroxisome proliferator-activated receptor alpha (PPARα) [38]. BPA and a few phthalates on the other hand were associated with perturbations of biomarkers of angiogenesis linked to placental development and function, which may have in turn adverse consequences on fetal growth [39].

3.6.6. MARKS ET AL. 2021: PRENATAL EXPOSURE TO MIXTURES OF PERSISTENT ENDOCRINE DISRUPTING CHEMICALS AND EARLY MENARCHE IN A POPULATION-BASED COHORT OF BRITISH GIRLS

→ **Objective**

The associations between prenatal exposure to PFAS, PCBs and organochlorine pesticides as mixtures with early menarche among female offspring were investigated [14].

→ **Methods**

Data from a nested case-control study within the Avon Longitudinal Study of Parents and Children (ASLPAC) recruited in the UK in 1991-1992 were used. Concentrations of 52 chemicals (8 PFAS, 35 PCBs, and 9 OCPs) were quantified in 448 maternal serum samples collected during pregnancy. Daughter's age at menarche was ascertained through questionnaires.

Bayesian kernel machine regression (BKMR) was used to visualize the exposure-response function and verify assumptions. In the case of no identification of non-linearity and or interaction within the mixture through BKMR, weighted quantile sum (WQS) regression was used to estimate associations of maternal EDC mixtures with early menarche.

→ **Results**

1) Weighted quantile sum regression models showed null associations between the indices for mixtures (PFAS, PCBs, OCPs, and all three classes combined) and early menarche. The odds ratio for early menarche for a one-decile increase in chemical concentrations for all three classes combined was 0.89 (95% CI: 0.76, 1.05).
2) In the BKMR model for all three classes combined, no interaction among mixture members was observed. Some chemicals had slightly positive associations (PFHxS, EtFOSAA), some appeared to have negative associations (MeFOSAA, PCB206, β-HCH), but most showed no association with early menarche.

→ **Discussion**

Almost all models suggested that higher prenatal exposure to persistent EDCs was not associated with early menarche, though effect sizes varied.

Associations were further away from the null in WQS regression models than in BKMR. WQS regression assumes that all associations are in the same direction; if this assumption is not met, results can be biased away from the null. This potential for bias could explain the differences in magnitude between WQS regression and BKMR.

3.7. NON-EUROPEAN HBM STUDIES FOCUSING ON EXPOSURE PATTERNS OR CO-OCCURRENCE IN HBM DATASETS

This section focuses solely on scientific publications describing co-occurrence patterns of chemicals in human biomonitoring datasets extended to non-European HBM studies.

3.7.1. KAPRAUN ET AL. 2017: A METHOD FOR IDENTIFYING CHEMICAL COMBINATIONS IN THE U.S. POPULATION

→ **Objectives**

People are exposed daily to a variety of chemicals but the number of mixtures that can be formed from these thousands of environmental chemicals is enormous and testing all of them would be impossible. Therefore, Kapraun et al. 2017 sought to develop and demonstrate a method for identifying those mixtures that are most prevalent in humans [23].

→ **Methods**

Frequent itemset mining [23], a technique traditionally used for market basket analysis, was applied on biomonitoring data from the 2009-2010 cycle of the continuous National Health and Nutrition Examination Survey (NHANES) to identify combinations of chemicals that frequently co-occur in people.

→ **Results**

The authors identified 90 chemical combinations consisting of relatively few chemicals that occur in at least 30% of the U.S. population, as well as three super combinations consisting of relatively many chemicals that occur in a small but nonnegligible proportion of the population.

→ **Conclusion**

It was demonstrated how FIM can be used in conjunction with biomonitoring data to narrow a large number of possible chemical combi- nations down to a smaller set of prevalent chemical combinations.

3.7.2. LEE ET AL. 2017: IDENTIFICATION OF CHEMICAL MIXTURES TO WHICH CANADIAN PREGNANT WOMEN RE EXPOSED: THE MIREC STUDY

→ **Objective**

Although the importance of chemical mixtures has been recognized for some time, rigorous study of their levels and impact has been slow. The goal of this study was to identify the pattern of chemical mixtures to which women are exposed and to characterize women with elevated exposure to various mixtures [24].

→ **Methods**

Data from the Maternal-Infant Research on Environment Chemicals (MIREC) study were used of 1744 participants. Arsenic, lead, mercury, cadmium, manganese, PCBs, organochlorine pesticides, and PFAS were measured in blood samples while BPA, organophosphate pesticides and phthalates were measured in urine samples.

Cluster analysis was implemented to categorize participants based on their socio-demographic characteristics, while principal component analysis (PCA) was used to extract the chemicals with similar patterns and to reduce the dimension of the dataset.

→ **Results**

PCA retained eleven components which explained approximately 70% of the variation. Persistent organic pollutants (PCB118, PCB138, PCB153, PCB180, OXYCHLOR and TRANSNONA) and phthalates (MEOHP, MEHHP and MEHP) dominated the first and second components, respectively, and the first two components explained 25.8% of the source variation.

Prenatal exposure to persistent organic pollutants (first component) was positively associated with women who have lower education or higher income, were born in Canada, have BMI ≥25, or were expecting their first child in our study population.

MEOHP, MEHHP and MEHP, dominating the second component, were detected in at least 98% of 1744 participants in our cohort study; however, no particular group of pregnant women was identified to be highly exposed to phthalates.

→ **Discussion**

Similar as in the studies of Agay-Shay et al. 2015 and Robinson et al. 2015 which were described above, it was demonstrated that POPs dominate one component in PCA. The chemicals (PCB118, PCB138, PCB153, PCB180, OXYCHLOR and TRANSNONA) that dominated PC1 are persistent organic pollutants, which are mainly found in meat and dairy. The highest concentrations are found in animals at the top of the food chain, including humans [24]. Therefore, it is not surprisingly that these chemicals were highly correlated and dominate one component.

Three phthalates (MEOHP, MEHHP ANDMEHP) that dominated the second component are also highly and linearly correlated and are metabolites of di-2-ethylhexyl phthalate (DEHP) [40]. DEHP is widely used in food packaging, cosmetics and personal care products including fragrances, soft PVC products, building and furniture materials, and medical devices [41]. Hence, one would expect them to be clustered together so it is not surprising that human exposure to DEHP is nearly ubiquitous [24].

3.7.3. REYES ET AL. 2018: AN ANALYSIS OF CUMULATIVE RISKS BASED ON BIOMONITORING DATA FOR SIX PHTHALATES USING THE MAXIMUM CUMULATIVE RATIO

→ **Objective**

In this study, MCR was used to evaluate co-exposures to six phthalates [25].

→ **Methods**

Biomonitoring data from 2663 participants of the 2013-2014 cycle of the NHANES study were used. Hazard Index(HI) and MCR were determined. The Hazard Index (HI) is a screening tool for estimating cumulative risks from exposures to multiple chemicals from a common mechanism group. This approach assumes dose addition. MCR values are constructed based on hazard information. The Maximum Cumulative Ratio (MCR) quantifies the degree to which a single chemical drives the cumulative risk of an individual exposed to multiple chemicals.

→ **Results**

The MCR calculated ranged from 1.1 to 3.6. Because six phthalates were considered, MCR can range between 1 and 6. The finding that MCR values were all at or below 3.6 indicated that none of the exposed participants received the same level of risk from the six phthalates.

There were 21 participants (0.8% of the NHANES sample) with HI>1. The mean MCR value in the 21 participants was 2.1. HI and MCR values were negatively correlated ($p<0.001$) indicating that most participants, especially those with elevated HI values, had their cumulative risks driven by relatively large doses of a single phthalate rather than doses of multiple phthalates. The dominate phthalate varied across participants.

3.7.4. NETA ET AL. 2010: DISTRIBUTION AND DETERMINANTS OF PESTICIDE MIXTURES IN CORD SERUM USING PRINCIPAL COMPONENT ANALYSIS

→ Objective

The aims of this study were to identify mixtures of cord serum concentrations of organophosphorus, carbamate, pyrethroid and organochlorine pesticide using PCA and to identify demographic and socioeconomic factors associated with in utero mixtures among a population of babies born in Baltimore, USA [12].

→ Methods

Data of 300 singleton live births from a cross-sectional study of newborn deliveries of the Baltimore Tracking Health Related to Environmental Exposures (THREE) Study were used. Mixtures were identified using PCA.

→ Results

There were four independent pesticide components: DDT (p,p'- DDT+p,p'-DDE), chlordane (trans-nonachlor+oxychlordane), permethrin (trans- and cis-permethrins+PBUT), and carbamate (bendiocarb + propoxur).

- DDT and chlordane were 6.1 (95%CI: 2.4, 15.5) and 2.1 (95%CI: 1.0, 4.2) times higher for infants of women >35, and 1.8 (95%CI: 1.2, 2.9) and 1.5 (95%CI: 1.1, 2.1) times higher in smoking mothers.
- DDT and carbamate were 15 (95%CI: 7, 30) and 2 (95%CI: 1, 4) times higher for infants of Asian compared with Caucasian mothers.
- No significant differences were observed for permethrin.

→ Discussion

Fetal exposures to pesticides are widespread, occur as mixtures, and differ by maternal race, age, and smoking status.

Concentrations of the two chlordane-related chemicals were highly positively correlated with each other and were both detected in the majority of cord serum samples, suggesting that fetal exposures to this chlordane mixture are prevalent in the Baltimore area. p,p'-DDT and p,p'-DDE also were highly

correlated and detected in nearly every sample, suggesting that fetal exposures to DDT in the Baltimore area are prevalent and attributable to a mixture in which the environmental transformation product p,p'-DDE is a predominant compound. DDT mixtures with a predominance of DDE are considered to be from less recent DDT usage. Concentrations of permethrin cis- and trans-isomers were highly correlated with each other and with PBUT. Permethrin house and garden products are formulated with cis-and transisomers as well as with PBUT, a permethrin synergist. This suggests that fetal exposures to permethrin in this area may be related to house and garden products.

3.7.5. CHUNG ET AL. 2018: TOWARDS CAPTURING THE EXPOSOME, EXPOSURE BIOMARKER VARIABILITY AND COEXPOSURE PATTERNS IN THE SHARED ENVIRONMENT

→ Objective

The authors examined the influence of a shared household and partner's sex in relation to the variation in 128 endocrine disrupting chemical (EDC) exposures among couples [11].

→ Methods

501 couples planning to become pregnant were recruited in the LIFE study, USA between 2005-2009. Participants were eligible for inclusion if they were married or in a committed relationship, and females were between 18-40 years old and men were +18 years. 128 persistent and nonpersistent EDC from 13 chemical classes were measured including PCBs, organochlorine pesticide, PBDEs, PFASs, phytoestrogens, phthalates metabolites, phenols, antimicrobial chemicals, paracetamol and derivatives, blood metals, cotinine, urinary metals, urinary metalloids.

Sex specific differences, variance explained by shared household were estimated by Spearman's rank correlation coefficients (R_s) for females, males and couples' exposures. Correlation between exposures were visualized by an exposome globe.

→ Results

1) Sex was correlated with 8 EDCs including per- and polyfluoroalkyl substances (PFASs) ($p < 0.05$).
2) Shared household explained 43% and 41% of the total variance for PFASs and blood metals, respectively, but less than 20% for the remaining 11 EDC classes.
3) For females, two larger positively correlated "clusters" across EDC classes were observed: A) a dense cluster with serum persistent organic compounds such as PCBs and OCPs B) another loosely packed cluster with urinary EDCs such as phytoestrogens, phthalates, phenols, and antimicrobial compounds (Figure 8).
4) For males, there were similar coexposure patterns to females. While we found similar correlations in the population of males and females separately, we found that correlations

in couples living in the same household were, in fact, less densely packed and with values attenuated toward the null.

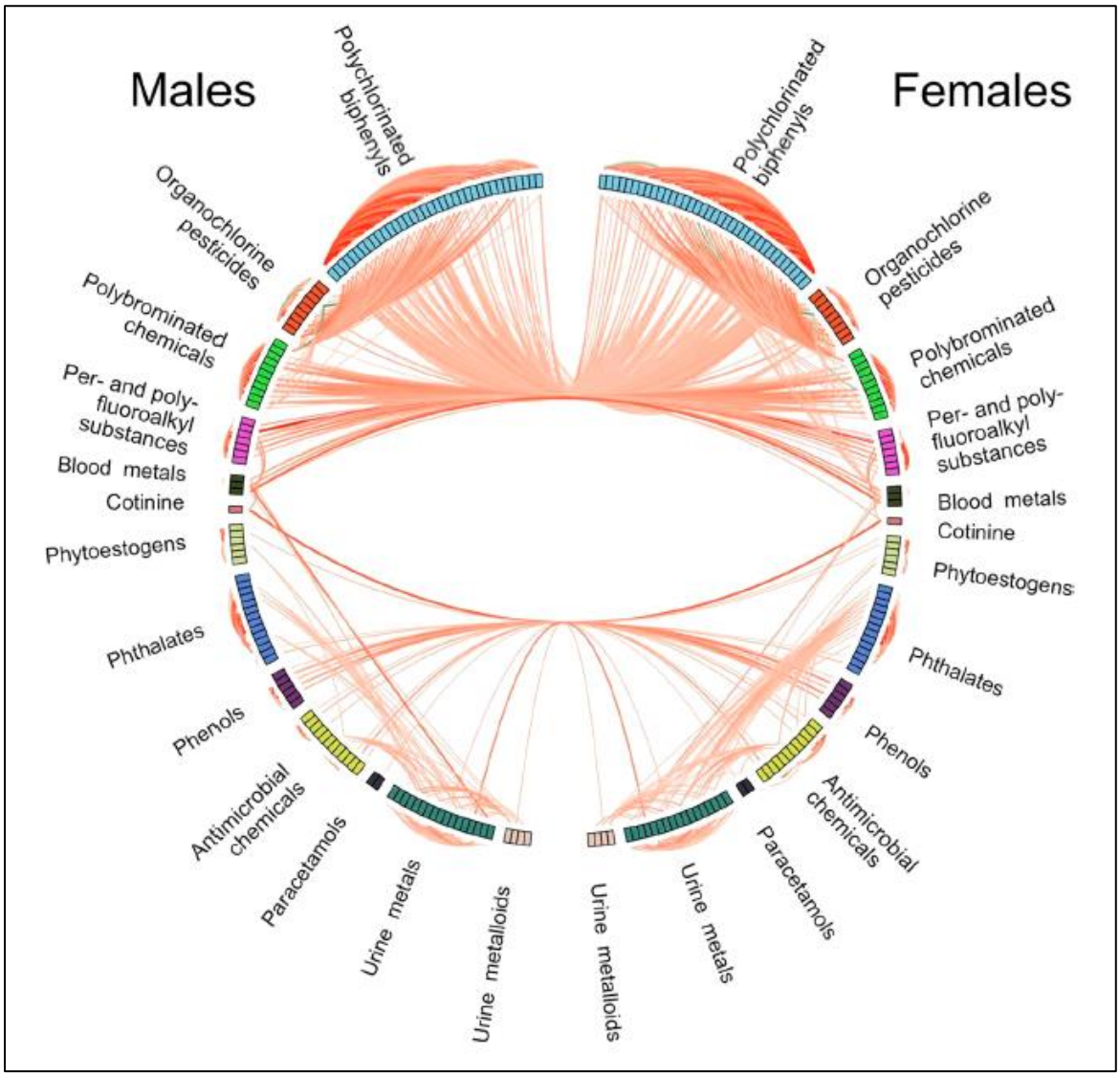

Figure 8: Exposome correlation globe showing the relationships of biomarkers between females, males, and couples.
Right-half represents biomarkers in females; left-half represents biomarkers in males. Only Spearman's rank correlations greater than 0.25 and smaller than -0.25 were shown as connections in the globe. Red line denotes positive correlation, and dark green line denotes a negative one. Color intensity and line width are proportional to the size of the correlation. Within-class and between-class correlations are shown outside and inside of the track respectively. Correlations in couples are indicated by the lines linking females and males (i.e., crossing the vertical-half of the globe).

→ **Discussion**

These findings suggest that individual, rather than shared environment, could be a major factor influencing the covariation of the exposome.

Although couples in this study hypothetically potentially share a large degree of dietary and indoor environmental factors, their exposures were only modestly correlated (low R_S). The authors suggest that there are two additional factors affecting the familial coexposure patterns in our investigation. The first one is concerned with how long the couples have been living together. the second factor is potential physiological dampening of exposure variability related to the half-life of the target chemicals.

3.7.6. WILLEY ET AL. 2021: EXPOSURE LOAD: USING BIOMONITORING DATA TO QUANTIFY MULTI-CHEMICAL EXPOSURE BURDEN IN A POPULATION

→ Objective

Exposure Load may provide an indication of overall exposure burden in a population and may be used to identify potentially vulnerable subpopulations that may be disproportionality exposed to multiple chemicals, as well as to characterize intrinsic and extrinsic factors that may contribute to heterogeneity in chemical exposure profiles across a population [26].

→ Methods

Data were used from cycles 3 (2012-2013) and 4 (2014-2015) from the Canadian Health Measures Survey (CHMS). 44 biomarkers from 26 chemical classes were measured in blood and urine samples of 1858 participants aged 12 to 79. Chemicals classes were: cadmium, lead, mercury, nicotine, BPA, triclosan, inorganic arsenic, benzo(a)pyrene, chrysene, fluoranthene, fluorene, naphthalene, phenanthrene, pyrene, benzene, ethylbenzene, styrene, tetrachloroethylene, toluene, trichloroethylene, bromodichloromethane, tribromomethane trichloromethane, xylenes, acrylamide.

Exposure Load is defined as the number of chemicals measured in an individual above a defined concentration threshold. Exposure Load was calculated based on five concentration thresholds; LOD, and the 50^{th}, 75^{th}, 90^{th} and 95^{th} percentile.

→ Results

1) At higher thresholds, such as the 95th percentile, the majority of Canadians had an Exposure Load between 0 and 3, although some people had an Exposure Load of up to 15, indicating high exposures to multiple chemicals.
2) Adolescents aged 12–19 years had significantly lower Exposure Loads than adults aged 40–79 years at all thresholds and adults aged 20–39 years at the 50th and 75th percentiles.
3) Smokers had significantly higher Exposure Loads than nonsmokers at all thresholds except the LOD, which was expected given that tobacco smoke is a known source of certain chemicals included in our analysis.
4) No differences in Exposure Loads were observed between males and females at any threshold.

→ **Discussion**

These findings broadly suggest that Canadians are concurrently exposed to many chemicals at lower concentrations and to fewer chemicals at high concentrations.

The youngest age group (12–19 years) had a significantly lower Exposure Load than older age groups. Age is known to play a role in exposure to chemicals and the amounts of chemicals measured in people as persistent pollutants can bioaccumulate, potentially remaining in tissues for decades (cadmium, lead and mercury). Moreover, changes in regulations and the phase out of chemicals can translate into younger people having less exposure to certain legacy chemicals, while older people can retain persistent chemicals from past exposures when their use was more widespread. Finally, also reduction in homeostatic reserves and decreased function of organ systems responsible for eliminating xenobiotics due to aging may contribute to higher exposures in older people [26].

3.8. NON-EUROPEAN HBM STUDIES DESCRIBING CHEMICAL CO-OCCURRENCE PATTERNS IN RELATION WITH HEALTH OUTCOMES

3.8.1. CHIU ET AL. 2018: EVALUATING EFFECTS OF PRENATAL EXPOSURE TO PHTHALATE MIXTURES ON BIRTH WEIGHT: A COMPARISON OF THREE STATISTICAL APPROACHES

→ **Objective**

Three statistical approaches were applied to evaluate associations between prenatal urinary concentrations of a mixture of phthalate metabolites and birth weight [16].

→ **Methods**

Data of the Environment and Reproductive Health (EARTH) Study (USA) are used of 300 mother-infant pairs. Women were eligible if they were age 18 to 45 years at enrollment and delivered a singleton live born infant between 2005-2016. Outcomes of interest were birth weight and gestational age. Urinary concentrations of DEHP, MEHP, MEHHP, MEOHP, MECPP, MiBP, MBP, MBzP and MEP were measured.

The authors applied 1) linear regressions; 2) PCA and structural equation models (SEM) and 3) Bayesian Kernel Machine Regression (BKMR). This method utilizes a non-parametric approach to evaluate dose-response relationships, allowing for possible non-linearity and interactions in exposure outcome associations.

→ **Results**

When analyzing one metabolite at a time, all metabolites were negatively but not significantly associated with birth weight.

PCA identified two principal components accounting for respectively for 53% and 18% of the variance. The first component had high loading factors for MEHP, MEHHP, MEOHP and MECPP while the second component had high loading factors for MEP, MBP, MiBP and MBzP. Both components were inversely associated with birth weight [-23 (-68, 22), -27 (-71, 17) grams respectively].

These inverse associations were confirmed when applying SEM. BKMR further identified that MEP and MEHP and phthalate concentrations were linearly related to lower birth weight [-51(-164, 63) and -122 (-311, 67), respectively], and suggested no evidence of interaction between metabolites.

Unfortunately, none of the individual phthalates or phthalate mixtures were significantly associates with birth weight using the three selected approaches.

→ **Discussion**

The lack of significance may due to small samples, high within-person variability of urinary biomarkers, survival bias and unmeasured or/and residual confounding.

As PCA is agnostic approach to reduce the dimension of exposure without considering the correlations with the outcome measure, it is unclear whether the group of phthalate metabolites or specific phthalate metabolites within the group was responsible for the inverse association with birth weight.

3.8.2. WOODS ET AL. 2017: GESTATIONAL EXPOSURE TO ENDOCRINE DISRUPTING CHEMICALS IN RELATION TO INFANT BIRTH WEIGHT: A BAYESIAN ANALYSIS OF THE HOME STUDY

→ **Objectives**

The association of gestational exposure to five chemical classes of potential EDCs with infant birth weight was investigated [27].

→ **Methods**

Data from 272 pregnant women of the Health Outcomes and Measures of Environment (HOME) Study (USA) were used. The outcome variable was birth weight. Nine phthalates, BPA, five PFAS, twenty-three PCBs, nine PBDEs, six organochlorine pesticides and two heave metals were measured in blood and urine samples. Bayesian Hierarchical Linear Models (BHML) was used to examine the association between exposure to these chemicals and birth weight.

→ **Results**

For a 10-fold increase in chemical concentration, the mean differences in birth weights (95% credible intervals (CI)) were 1 g (-20, 23) for phthalates, -11 g (-52, 34) for PFAS, 0.2 g (-9, 10) for PCBs, -4 g (-30, 22) for PBDEs, and 7 g (-25, 40) for OCPs. Hence, exposure to these chemicals had null or small associations with birth weight.

→ **Discussion**

There was some evidence that PFAS, DMP and Pb were associated with small reductions in birth weight. This may be explained by the fact that PFAS interact with estrogen receptors and disrupt hormonal balances [42]. Moreover, serum lipid levels are also altered by PFAS thereby potentially affecting fetal growth and development. PFAS may also affect adipose tissue development and the regulatory systems in body weight homeostasis, which may impact fetal growth outcomes [42].

3.8.3. KALLOO ET AL. 2020: EXPOSURE TO CHEMICAL MIXTURES DURING PREGNANCY AND NEONATAL OUTCOMES: THE HOME STUDY

→ **Objectives**

Three different methods were used to investigate the association between 43 environmental chemical biomarkers and neonatal outcomes [28].

→ **Methods**

Data from the HOME study, a birth cohort in the USA, were used and 389 mothers who delivered a live born singleton infant were included. Outcomes of interest were birth weight, length, head circumference and gestational age. The broad class of chemicals included phenols, phthalates, metals, pesticides, PCBs, PBDEs, PFAS and cotinine measured in urine or blood samples. K-means clustering, principal components (PC), and one-chemical-at-a-time regression were used to investigate the associations.

→ **Results**

1) Using k-means clustering, three chemical mixture profiles were identified. Women in cluster 1 had higher concentrations of most phenols, three phthalate metabolites, several metals, organophosphate/organochlorine pesticides, polychlorinated biphenyls, and several PFAS than women in clusters 2 and 3. On average, infants born to women in clusters 1 (−1.2 cm; 95% CI: −1.9, −0.5) and 2 (−0.5 cm; 95% CI: −1.1, 0.1) had lower birth length than infants in cluster 3.
2) Six PCs explained 50% of the variance in biomarker concentrations and biomarkers with similar chemical structures or from shared commercial/industrial settings loaded onto commons PCs. Birth length was weakly inversely associated with all PCs, except for PC 5. The largest difference in birth weight was observed for PC 1 and 6; each standard deviation increase in PC 1 (organochlorine pesticides, some phenols) and PC 6 (cadmium, bisphenol A) was associated with 0.2 cm (95% CI: −0.4, 0.0) and 0.1 cm (95% CI: −0.4, 0.1) lower birth length, respectively.
3) Organochlorine compounds, parabens, and cadmium were inversely associated with birth length in the one-chemical-at-a-time analysis.

Cluster membership, PC scores, and individual chemicals were not associated with other birth outcomes.

→ **Discussion**

All three methods of characterizing multiple chemical exposures in this cohort identified inverse associations of select organochlorine compounds, phenols, and cadmium with birth length. Mixtures of environmental chemicals could affect infant birth length by disrupting mesenchymal stem cell differentiation and these are predecessors to bone, cartilage and fat cells. Consequently, a decrease in osteogenic proliferation may explain a smaller infant length at birth [28].

3.8.4. KIM ET AL. 2018: URINARY TRACE METALS INDIVIDUALLY AND IN MIXTURES IN ASSOCIATION WITH PRETERM BIRTH

→ **Objective**

The associations between 17 urinary trace metals individually and in mixtures in relation to preterm birth were investigated [15].

→ **Methods**

Data from women of the LIFECODES birth cohort (USA) were used. 99 cases of preterm birth were selected, and 291 unmatched controls were also included. Outcome of interest was gestational age to determine preterm birth. 17 trace metals were analyzed in urine samples: arsenic, barium, beryllium, cadmium, copper, chromium, mercury, manganese, molybdenum, nickel, lead, selenium, tin, thallium, uranium, tungsten and zinc.

because this study has the largest number of trace metal analytes to date to address this research question, two approaches were used to investigate the effect of mixtures. First elastic net (ENET) regularization was used to identify the individual metals and pairwise interactions from the mixture that are most strongly associated with preterm birth. Second, PCA was applied to examine associations with correlated grouping.

→ **Results**

1) ENET selected Cu as the most important trace metal associated with PTB.
2) PCA identified 3 principal components (PCs). PC1 was characterized by high loading from primarily toxic metals, Cd, Mn, and Pb PC2 was characterized by high loading from essential metals, Cu, Se, and Zn. PC3 had high loading from metals that have previously been linked to seafood intake, As, Hg, and Sn. The PC for essential metals was associated with an increased risk of overall (OR 1.36, 95% CI 1.05, 1.76) and spontaneous (OR 1.58, 95% CI 1.14, 2.20) preterm birth.

→ **Discussion**

Maternal urinary copper in the third trimester was associated with increased risk of PTB, and statistical analyses for mixtures indicated that after accounting for correlation this metal was the most important statistical predictor of the outcome.

3.8.5. PRESTON ET AL. 2020: PRENATAL EXPOSURE TO PER- AND POLYFLUOROALKYL SUBSTANCES AND MATERNAL AND NEONATAL THYROID FUNCTION IN THE PROJECT VIVA COHORT: A MIXTURES APPROACH

→ **Objectives**

The associations between exposure to multiple PFAS during early pregnancy with maternal and neonatal thyroid function were investigated [21].

→ **Methods**

Data from 726 mothers and 465 neonates from the Project Viva, a Boston area longitudinal pre-birth cohort were used. Six PFAS (PFOA, PFOS, PFNA, PFHxS, EtFOSAA, MeFOSAA) and thyroxine (T_4), Free T4 Index (FT_4I) and thyroid stimulating hormone (TSH) were measured in maternal plasma samples collected during early pregnancy and neonatal T_4 in postpartum heel sticks.

Individual and joint effects of PFAS exposure with thyroid hormone levels were estimated using weighted quantile sum (WQS) regression and Bayesian kernel machine regression (BKMR) and evaluated potential non-linearity and interactions among PFAS using BKMR.

→ **Results**

1) Higher concentrations of the PFAS mixture were associated with significantly lower maternal FT_4I, with MeFOSAA, EtFOSAA, PFOA, and PFHxS contributing most to the overall mixture effect in BKMR and WQS regression.
2) In infants, higher concentrations of the PFAS mixture were associated with lower T_4 levels, primarily in males, with PFHxS and MeFOSAA contributing most in WQS, and PFHxS contributing most in BKMR.
3) The PFAS mixture was not associated with maternal T_4 or TSH levels.

→ **Discussion**

In this relatively large cohort of pregnant women and their neonates, the PFAS mixture was inversely associated with maternal FT_4I and neonatal T_4 levels in male infants. BKMR and WQS regression identified PFOA, PFHxS, EtFOSAA, and MeFOSAA as contributing to the negative joint association of PFAS exposure with maternal FT_4I. Conversely, WQS identified both PFHxS and MeFOSAA exposure in males as contributing to the negative joint association of PFAS exposure on neonatal T_4 levels,

while BKMR only identified PFHxS as an important contributor to the suggestive negative association with T_4 levels.

The mechanisms by which PFAS alter thyroid function are unclear. Proposed mechanisms include reduced responsiveness to the hypothalamic-pituitary-thyroid axis, increased hepatic clearance of T_4, increased conversion of T_4 to T_3 by type 1 deiodinase, and competitive binding to thyroid hormone binding proteins.

3.8.6. KELLEY ET AL. 2019: EARLY PREGNANCY EXPOSURE TO ENDOCRINE DISRUPTING CHEMICAL MIXTURES ARE ASSOCIATED WITH INFLAMMATORY CHANGES IN MATERNAL AND NEONATAL CIRCULATION

→ Objectives

The association between mixtures of early pregnancy exposures and distinct changes in the maternal and neonatal inflammasome are investigated [29].

→ Methods

Data from 56 women of the Michigan Mother-Infant Pairs (MMIP) birth cohort, USA, were used. Those eligible for MMIP participation are between 18 and 42 years old and have a naturally conceived singleton pregnancy. Birth outcomes of interest were gestational age at delivery, mode of delivery infant sex, and birth weight. 41 exposure chemicals were quantified for each participant: 12 phthalate metabolites, 12 phenol metabolites and 17 heavy metals were measured in urine samples. Additionally, inflammatory biomarkers were also measured.

Spearman correlations and linear regression were used to relate individual exposures with inflammatory cytokines. Principal component analysis was used to assess the effect of weighted EDC mixtures on maternal and neonatal inflammation.

→ Results

1) First trimester measures of several individual EDCs, particularly metals and phthalates, were independently associated with maternal first trimester and term inflammatory markers using Spearman correlation coefficients.
2) Ten of eleven PC groupings demonstrated statistically significant associations with inflammatory cytokines. For interpretation of these PCA results, the color heat map demonstrates the relative contribution of each EDC to a specific PC group (Figure 9). For instance, in PC1, metals and phthalates are positively weighted (red color), suggesting that higher metal and phthalate levels may be positively associated with first trimester IL-8 and IFN-γ. Using the same 11 PC variables, there were no significant associations between EDC mixtures with infant birth weight or gestational age at delivery.

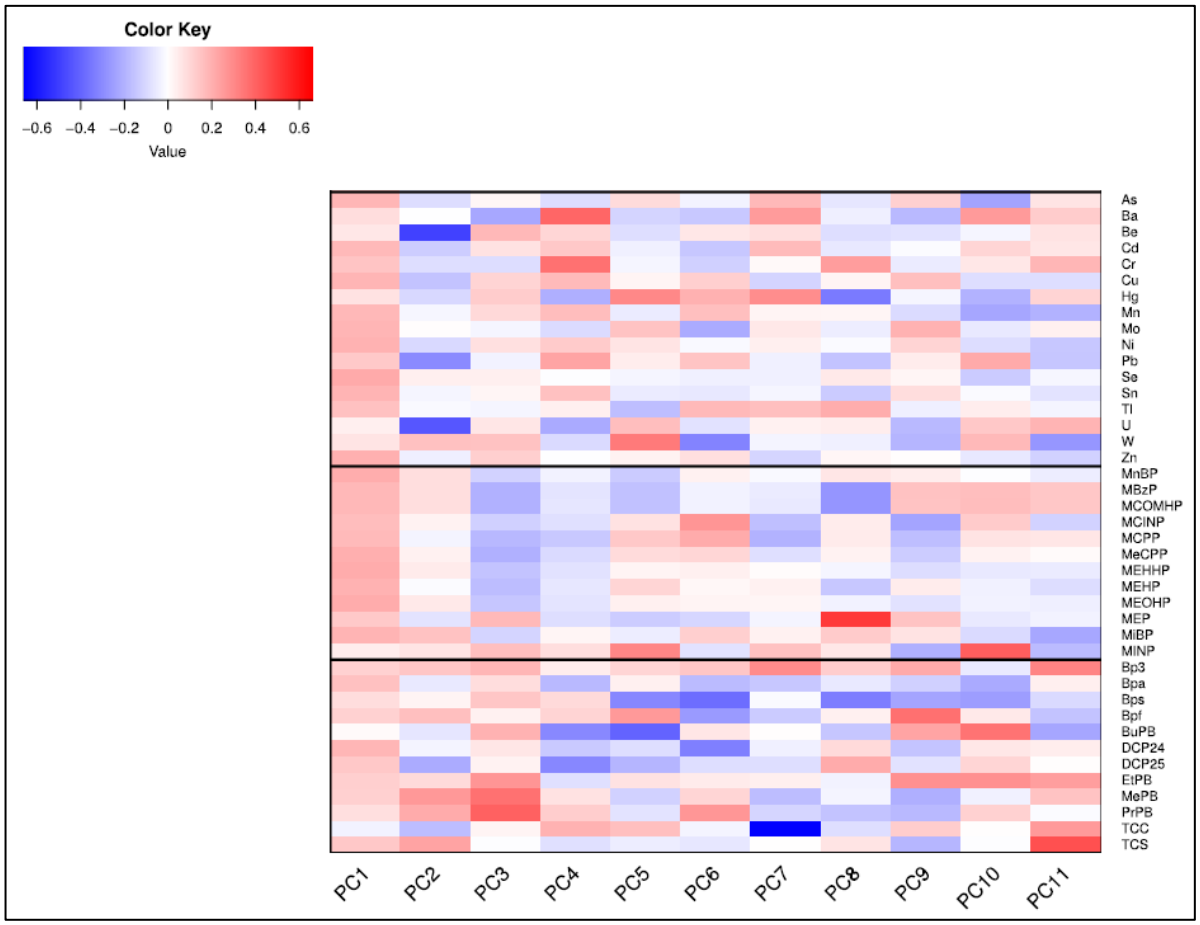

Figure 9: Principal component loading coefficients.
This color heat map shows the loading coefficients of the exposures for each of the principal components (PC), demonstrating the relative contribution of each EDC to a specific PC group. Exposures that have a higher positive weight are darker red, whereas exposures that are negatively weighted are blue.

3.8.7. ZHANG ET AL. 2021: PARENTAL PRECONCEPTION EXPOSURE TO PHENOL AND PHTHALATE MIXTURES AND THE RISK OF PRETERM BIRTH

→ Objective

This study examined whether maternal and paternal preconception urinary concentrations of complex mixtures of phenols and phthalate metabolites interact to influence the risk of singleton preterm birth among couples attending a fertility clinic [30].

→ Methods

384 female and 211 male (203 couples) participants seeking infertility treatment in the Environment and Reproductive Health (Earth) Study who gave birth to 384 singleton infants between 2005 and 2018 were included. Men (18-55 years) and women (18-46 years) were eligible to participate either

independently or as a couple. Urinary concentrations of BPA, 3 parabens, and 11 phthalates were measured. Outcome of interest was gestational age.

PCA was used to classify phenol and phthalate biomarkers into uncorrelated components based on their correlations. This method reduces the number of components while retaining information from the original variables. Factors with eigenvalues greater than one were identified as principal components. Next, BKMR was applied to separately examine maternal and paternal preconception phenol and phthalate mixtures in relation to preterm birth.

→ **Results**

1) PCA identified the same four factors for maternal and paternal preconception mixtures. Each unit increase in PCA scores of maternal (adjusted Risk Ratio (aRR): 1.36, 95%CI: 1.00, 1.84) and paternal (aRR: 1.47, 95%CI: 0.90, 2.42) preconception DEHP-BPA factor was positively associated with preterm birth.
2) BKMR models further showed that maternal preconception BPA and mono(2-ethyl-5-hydroxyhexyl) phthalate, and paternal preconception mono(2-ethylhexyl) phthalate were positively associated with preterm birth when the remaining mixture components were held at their median concentrations. Couple-based BKMR models showed a similar relative contribution of paternal (PIP: 61%) and maternal (PIP: 77%) preconception mixtures on preterm birth.

→ **Discussion**

Maternal BPA and DEHP, and paternal DEHP exposure before conception were positively associated with preterm birth. The authors hypothesized that couples' preconception exposure to phenols and phthalates may affect the male and female germline, probably as a result of epigenetic regulation during gametogenesis [43]. This may continue during embryogenesis, decidualization and/or placentation, thereby predisposing to adverse pregnancy and birth outcomes. Moreover, both BPA and DEHP have been shown to alter the epigenetic regulation of imprinted genes in gametes, potentially explaining the contribution of both parents to birth outcomes such as preterm birth [30]. Genomic imprinting, the monoallelic parent-of-origin-dependent expression of a subset of specific genes, is required for normal development, fetal growth, gestation length and metabolism among other functions [30]. Finally, the fact that DEHP and BPA are in the same factor suggests that both chemicals share exposure sources such as packaged food and beverages [44].

3.9. PRIME PROGRAM (USA)

Given the complexity of mixtures and the effects of individual chemicals within the mixture, interactions among those chemicals, and the combined effect the mixture may have on human health., scientists need improved and innovative statistical methods to understand how exposure to real-world chemical mixtures may affect human health. The Powering Research through Innovative

Methods for mixtures in Epidemiology (PRIME) program created by NIEHS supports projects that develop innovative statistical methods that incorporate information into statistical models[2].

PRIME encourages team science. Experts in epidemiology, biostatistics, toxicology, data science, informatics, and related fields are working together to develop and compare novel approaches. he expected outcomes of PRIME include:

- Improving quantitative methods to better understand the complex relationships between environmental exposures and health outcomes.
- Stimulating new interdisciplinary methods for mixtures research in epidemiology.
- Comparing existing and new approaches to identify the strengths and weaknesses across methods for various exposure and disease contexts.
- Developing informatics tools and related software for broad implementation of methods.
- Providing resources for the research community including publications, webinars, example datasets, and training.

Publications from the PRIME program focus mostly on the development of novel statistical methods or expansion of existing methods such as BKMR or WQS that are deployed to investigate mixture effects. Hence, this research does not investigate mixture effects. Al full list op publications can be consulted on the website[3].

[2] https://www.niehs.nih.gov/research/supported/exposure/mixtures/prime_program/index.cfm

[3] https://www.niehs.nih.gov/research/supported/exposure/mixtures/prime_program/publications/index.cfm

CHAPTER 4 CONCLUSION

As mentioned above, the exposome potentially consists of hundreds of exposures and mixtures may interact with each other leading to additive, synergistic and/or antagonistic effects. To date, most research on environmental determinants of disease has focused on single exposures. However, the traditional one-chemical-at-a-time strategy is not appropriate to represent exposure scenarios as humans are exposed to many chemical families at the same time [30].

Based on the available scientific literature summarized in the sections above, we can conclude that only a few publications investigated interactions and described correlation structures between chemical substances or chemical classes, the so-called *co-occurrence patterns*, including; Tamayo-Uria et al. 2019 [10]. Rosofsky et al. 2017 [4], Govarts et al. 2020 [2], Ottenbros et al. 2021 [1], Robinson et al. 2015 [5], Kapraun et al. 2017 [23], Lee et al. 2017 [24], Reyes et al. 2018 [25], Neta et al. 2010 [12], Chung et al. 2018 [11] and Willey et al. 2021 [26]. Based on these publications, we can summarize a few key findings;

- Most publications focus on commonly monitored substances or chemical groups in HBM datasets: e.g. metals, phthalates, pesticides, PCBs, phenols PFAS and BPDEs;
- Mainly children or newborn and mother pairs are the subject of these mixture investigations;
- Chemicals belonging to the same chemical group/family mainly tend to co-occur;
- Co-occurrence across chemical families is in general weak/not present unless for chemicals occurring in or obtained from the same sources: e.g. PCBs & Hg in fish; phthalates & parabens;
- Similar findings with respect to co-occurrence patterns are demonstrated across, these multiple studies;
- A difference in co-occurrence patterns between age groups such as mothers and children is described [10].
- Chemicals within a family may likely cause similar effects, due to similar chemical structures and/or functionalities, and thus act according to dose-addition. Therefore, we recommend considering chemical groups as a basis for human health risk assessment.
- It is important to be careful in the interpretation of lack of co-occurrence patterns. A lack of a co-occurrence pattern is not mutually exclusive, and it may be possible that chemicals are “randomly” co-occurring. A lack of co-occurrence can also be regarded as substances having independent distributions.

The remainder of publications consider more than a couple of families of exposures simultaneously but focus hereby not in detail on the co-occurrence patterns but rather on the correlation between *mixture effects in relation with health outcomes*; e.g. birth outcomes such as birth weight, birth length or head circumference. Wider studies, including a broader range of measured exposures, multiple lifetime periods of exposure assessment and larger populations covering multiple regions are needed to more fully understand the complexity of human exposure [10].

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