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for a healthy future

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AD 15.1

WP 15 - Mixtures, HBM and human health risk

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2 Glossary

AL	Acceptable Level
DA	Dose Addition
EL	Effect Level
HBGV	Health Based Guidance Values
HBM	Human Biomonitoring
HI	Hazard Index
HQ	Hazard Quotient
IA	Independent Action
MRA	Mixture Risk Assessment
MoA	Mode of Action
PCA	Principal Component Analysis
PODI	Point of Departure Index
PCA	Principal Component Analysis
RA	Response Addition
RPF	Relative Potency Factor
TEF	Toxic Equivalence Factor
TEQ	Toxic Equivalence Quotient
TUS	Toxic Unit Summation
WP	Work Package

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3 Abstract/Summary

This deliverable describes the activities in task 15.3 leading up to the development of cases studies for mixture health effects and outlines the proposed case studies. The proposed case studies are:

- Developmental neurotoxicity beyond polybrominated diphenylethers
- Heavy metals and nephrotoxicity
- Anti-androgenic chemicals and male reproductive health
- Chromium (VI), nickel and polycyclic aromatic hydrocarbons and lung cancer
- Addressing exposure misclassification in mixture studies

The Addendum provides further details about multi-year perspective and timing, as well as detailed budgetary aspects per case study.

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4 Introduction and background

4.1 Aims and objectives

The aim of AD 15.1 is to describe the development of case studies of mixture effects of pollutants within the HBM4EU project. Requirements were that the case studies should focus on exposures and on health endpoints of concern, and should incorporate, as much as possible, pollutants defined as priority substances for the HBM4EU project. Moreover, a mix of different, preferable hybrid, approaches from both toxicology and epidemiology was sought.

Another aspiration was to link the case studies to biomarkers of effect elaborated in WP 14.

4.2 Procedures and the Lisbon workshop

At the WP 15 workshop held in November 2017 at RIVM, partners realised that the topic of mixture risk assessment, with its applications in regulatory toxicology, epidemiology and exposure science, is rather diverse. It was deemed necessary to provide a common ground to all partners from which case studies for exploring mixture effects could be developed, and it was agreed that this necessitates an introduction to the state of the art of mixture toxicology. Accordingly, it was decided to hold a workshop in May 2018, which would introduce partners to mixture toxicology and would enable everybody to advance the development of case studies.

The workshop was held on 9-10 May 2018 in Lisbon, on the premises of INSA. During this workshop, a detailed discussion of possible case studies took place, culminating in proposals for a number of such case studies. In the weeks following the workshop, these case study proposals were further developed. This Deliverable details the selection process and documents the proposals for these case studies, together with their aims and objectives.

The Addendum provides further details about multi-year perspective and timing, as well as detailed budgetary aspects per case study.

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5 Why case studies on mixture effects? – The link to potential biomonitoring strategies

Human populations are exposed to rather large numbers of chemicals simultaneously and it is increasingly realised that chemical risk assessment approaches should take account of this reality. As much as possible, human biomonitoring should also integrate knowledge about mixture effects and begin to develop strategies that can cope with the reality of multi-chemical, multi-pathway exposures of humans.

5.1 Problem formulation

The case studies will deal with the question: What are the health risks of mixtures from combined chemical exposures and do predicted risks of such mixtures exceed the levels regarded as acceptable for humans? The following specific aspects will be addressed:

- Are there specific human health endpoints for which acceptable risks are exceeded?
- Are there pollutants that contribute most to defined health endpoints of interest and are therefore drivers of mixture risks?
- Is it possible to devise human biomonitoring strategies that capture those pollutants?

5.2 What should the case studies deliver?

The case studies are intended to structure and organise the work in WP15 in a logical and consistent manner. They should also crystallise questions and issues that are not sufficiently resolved, for debate within the HBM4EU project. Especially, the case studies should:

- Identify methods for the prediction of mixture effects that can be used consistently for human risk assessments and can inform biomonitoring strategies in terms of chemicals to be monitored together,
- Define properties of data required as input for mixture effect predictions, and define the nature of data requirements.

5.3 The structure of this deliverable

An overview of general mixture risk assessment principles will be followed by setting out selection criteria for the case studies. This then provides the foundations for a description of the development of the proposed case studies.

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6 Mixture risk assessment (MRA): General considerations, approaches and methods from a toxicological perspective

6.1 General considerations on combined toxicity and its assessment

The combined effects of several chemicals are often referenced in terms of two fundamentally different modes: similar action and dissimilar action (sometimes also called independent joint action). These distinctions were first introduced by Bliss (1939) and Hewlett and Plackett (1952) on the basis of statistical principles. They have gained wide acceptance for the interpretation of mixture effects and are allied to the assessment concepts of dose addition (DA, linked with similar action) and independent action or response addition (IA, RA, linked with dissimilar action). Many regulatory bodies and competent authorities have used these terms in broadly identical ways, although there are differences in the level of detail specified for each of the two modes (summarised in Kortenkamp *et al.* 2012). There is a consensus that similar action “occurs when chemicals in a mixture act in the same way, by the same mechanism/mode of action, and differ only in their potencies” (EFSA 2008). Conversely, “dissimilar action” occurs with combinations of chemicals that produce a common effect by action through different modes of action, or at different sites.

While these definitions are clear-cut in principle, in practice it is often not straight-forward to distinguish between dissimilar action and similar action. In many cases, the mechanistic information needed to differentiate between the two types of combination effect is not available. Clear distinctions are further complicated by ambiguities concerning the precise meaning of the terms “mode of action” and “site of action” and its implications for assessments of combination effects. For example, two chemicals might affect different sites of an effector chain leading to an adverse outcome, in agreement with a key feature of simple dissimilar action. However, if the same key metabolite or intermediate is affected, the toxicological consequences could be better described in terms of simple similar action.

Dose addition is based on the idea that all components in a mixture behave as if they were dilutions of one another (Loewe and Muischnek 1926). Examples would be combinations of chemicals that all exert their toxicity by binding to the same receptor, e.g. the Ah receptor (polychlorinated dioxins) or the active centre of acetylcholinesterase (organophosphates, carbamates). In these cases, similar action applies because one chemical can be replaced by an equal fraction of an equi-effective concentration (e.g. an EC_{50}) of another, without diminishing the overall combined effect. In mathematical terms, this concept can be formulated as:

$$ECx_{Mix} = \left(\sum_{i=1}^n \frac{p_i}{ECx_i} \right)^{-1} = \left(\sum_{i=1}^n \frac{p_i}{F_i^{-1}(x)} \right)^{-1}$$

with n denoting the number of mixture components, p_i the relative fraction of chemical i in the mixture (percentage of the total dose or concentration), x a common effect level (e.g. 10%), ECx_{Mix} the total dose or concentration causing the effect x , ECx_i the equi-effective doses or concentrations of the individual chemicals, F_i the individual dose or concentration response functions, and F_i^{-1} the corresponding inverse functions.

Dose addition implies that every toxicant in the mixture contributes to the combination effect in proportion to its dose and individual potency. Whether the individual doses are also effective on their own does not matter. Thus, under dose addition, combination effects can be expected when toxicants are present at levels below effect thresholds, but only if the number of components sums up to a total mixture dose sufficiently large to produce effects. For example, two chemicals

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combined at 1/10 of their threshold concentration are not expected to produce a combination effect according to dose addition.

Independent action (response addition) conceptualises mixture effects in a different way. It assumes that a combination effect can be calculated from the responses of the individual mixture components by following the statistical concept of independent random events (Bliss 1939). This can be mathematically expressed by the equation

$$E(c_{Mix}) = 1 - \prod_{i=1}^n [1 - E(c_i)]$$

which can be transformed into

$$x \% = 1 - \prod_{i=1}^n (1 - F_i(p_i \cdot (ECx_{Mix})))$$

with $E(c_i)$ denoting the fractional effects (0-100%) that the individual mixture components would cause if applied singly at that dose or concentration at which they are present in the mixture, and $E(c_{Mix})$ the effect provoked by the total mixture (other parameters as defined for DA above).

On a population level, the idea of independent random events may also apply to one and the same chemical administered in a sequential fashion if non-reversible events such as mortality are investigated within time frames where recovery of the population does not occur. In this situation, the mode of action is identical, but the randomness of events is introduced by exposures that occur in sequence. The overall effect is then accessible by multiplication of the likelihood of independent events (administrations of the chemical). In the case of simultaneous exposure of an individual to several chemicals, the principle of independence of effects is only applicable when all the chemicals in the mixture act through dissimilar modes by affecting different target sites (dissimilar action). Examples would be combinations of chemicals that affect algal reproduction by disrupting photosynthesis, DNA synthesis and multiple other sub-systems. The principles of independence of effects also imply that components present at doses below thresholds and thus associated with zero effects will not contribute to the joint effect of the mixture. If this condition is fulfilled for all mixture components, combination effects are not expected under independent action.

Mixed Models (MM) combine both concepts, DA and IA, in cases where the components of a mixture can be clustered into groups of strictly similar acting substances while the groups cause a common effect by strictly dissimilar modes of action. The MM approach includes completely similar action (DA) and completely dissimilar action of all components (IA) as the two possible extreme situations. The approach presumes that all mixture components can be exclusively assigned to one out of a number different MoA groups. Where mixture components are multi-site inhibitors contributing to different MoAs or where adverse outcome pathways (AOP) do not lead to a common endpoint in completely independent ways, but are interlinked at some point along the route, the MM approach may not be applicable. In such a situation, an exact prediction of the mixture toxicity is not possible on the basis of the concepts of DA and IA, but it can be expected to lie somewhere in the prediction window between the extremes defined by DA and IA. In practice, this prediction window may be rather small, as further detailed in section 2.4 below.

6.2 The additivity expectation

By making the assumption that each mixture component exerts its effects in the same way as it would do when present on its own, i.e. without diminishing or enhancing the toxicity of other components, it becomes possible to formulate a quantitative expectation of the joint effects of multiple chemicals. These so-called additivity expectations are calculated on the basis of the

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effects of each individual chemical in the mixture. Additivity expectations can be derived from independent action or from dose addition or from a mixed model and serve as points of reference for the identification of synergisms or antagonisms. This idea is only workable when **both the mixture and all its components are able to produce the same toxic effect** (when present at sufficiently high concentrations or doses). In cases when some chemicals in the mixture do not affect the endpoint under consideration, it is not possible to predict a joint effect from the effects of mixture components.

Similar difficulties also arise if some or all components interact with each other, e.g. by induction of toxifying or detoxifying metabolic steps. In such situations, the observed mixture effects will differ from the expected additive effects which were calculated on the basis of the effects of its individual components. With reference to the expected additive effects, deviations in the direction of stronger responses can then be classed as synergism or as antagonisms if the observed effects fall short of those predicted. However, the magnitude of deviations from expected additivity cannot be calculated from information about the individual effects of mixture components.

6.3 Dose addition as a default assumption

In the light of the practical difficulties encountered when using considerations of similar or dissimilar action as the starting point for MRA (lack of mechanistic data, ambiguities associated with key terms such as mode of action, mechanism of action), the dichotomous approach to MRA is increasingly considered as problematic.

To deal with this problem, there is now a growing consensus that MRAs should start from the default assumption of dose addition (DA) for all mixture components, regardless of MoAs, as a reasonable worst case estimate. If this indicates a significant risk, refined MoA-based assessments may be conducted where the necessary data are available. Alternatively, precautionary measures may be taken. This way of thinking has guided ecotoxicological MRAs for quite some time already, but is now also gaining increased acceptance in the human arena (Meek et al. 2011, EFSA 2013). This development opened the way for using consistent and coherent approaches across the disciplinary borders.

The use of DA as a pragmatic and precautionary default assumption can be justified by a combination of four arguments:

- Data requirements for a proper application of DA are much easier to fulfil than for IA or MM.
- Usually, the assumption of DA provides a more conservative estimate of mixture toxicity than the alternative assumption of IA. Theoretically, the reverse situation is possible but the practical relevance of such a situation has as yet not been demonstrated.
- Synergistic effects that significantly exceed the DA expectation are exceptions and not the rule, at least for multi-component mixtures.
- The DA assumption is conservative, but not vastly over-conservative. Typically, the prediction window between DA and IA is not very wide. For realistic assessment situations it will rarely exceed an order of magnitude on the concentration axis. Typically, it is much smaller. Even with mixtures composed of up to 100 chemicals, predicted effect concentrations of the mixture derived from CA and IA differing by a factor of less than 5, as explained theoretically and demonstrated practically in Chapter 13.4 of Kortenkamp et al. (2012).

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6.4 Data requirements and the distinction between scientific concepts of additivity and pragmatic simplifications for regulatory MRAs

As mentioned before, the data requirements for DA-based MRAs are easier to meet than for IA or MM. Proper application of IA requires knowledge about the slope of dose response functions F of the individual mixture components (see the equation above), MM additionally requires knowledge of MoAs. In contrast, under the assumption of DA, the prediction of effect concentrations (or effect doses) of mixtures only requires that equivalent effect concentrations of single substances are available. Furthermore, the formula for DA can be directly transformed into a risk quotient for mixtures. The algebraic equivalent of the DA formula usually used for this purpose is the so-called toxic unit summation (TUS) as explained in more detail below.

DA and TUS require EC_x or ED_x values that refer to the same endpoint in the same species under comparable conditions. In a regulatory context, even this relatively simple data requirement may be impossible to meet. As a result, a number of pragmatic approaches have been derived from the original DA concept. Partly they have already become established procedures under specific pieces of legislation in the EU or in the US. Some prominent examples are the Hazard Index (HI), Point of Departure Index (PODI), Relative Potency Factors (RPF) and the concept of Toxic Equivalency Factors (TEF). All these MRA methods are simplifications of the DA concept. As a common feature, they make use of the DA formula as a calculation rule, but either they use input data that deviate more or less from the strict requirements of the original concept, or they make additional simplifying assumptions about the individual dose response curves. Below, we will first introduce these MRA methods, and then spell out their simplifying assumptions.

Simplifying regulatory approaches explicitly derived from IA are not developed. In human toxicology, an implicit application of IA as a MRA method is the assumption that mixture effects will not arise when all chemicals in question are present at levels below their ADIs, with the additional tacit assumption that ADIs represent true zero effect levels. It should be emphasised that the implicit application of independent action can only be used for chemicals for which ADIs have been derived and is thus applicable only to the minority of chemicals for which ADIs have been formally established.

6.5 Mixture risk assessment methods in practical use

6.5.1 Toxic unit summation

The method of Toxic Unit Summation (TUS) (Sprague 1970) is a direct application of the DA concept and defined by the formula

$$TUS = \sum_{i=1}^n TU_i = \sum_{i=1}^n \frac{c_i}{ECx_i}$$

where c_i are the actual concentrations (or doses) of the individual substances in a mixture and ECx_i denote equi-effective concentrations (or doses) of these substances if present singly (e.g. EC₅₀_{*i*}). The quotients c_i / ECx_i are termed Toxic Units (TU). Toxic Units rescale absolute concentrations (or doses) of substances to their different individual toxic potencies. They express the concentrations (or doses) of mixture components as fractions of equi-effective individual concentrations (or doses) ECx_i . Typically, $x = 50\%$ (EC₅₀_{*i*}) is chosen as the reference level, but TUS can also be calculated for any other effect level x . If $TUS = 1$, the mixture is expected to elicit the total effect x . If the Sum of Toxic Units ($\sum TU$) is smaller or larger than 1, the mixture is expected to elicit effects smaller or larger than x , respectively.

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6.5.2 The Hazard Index

The Hazard Index (HI) (Teuschler and Hertzberg 1995) is a regulatory approach to component-based mixture risk assessment derived from DA and which can be generally defined by the formula

$$HI = \sum_{i=1}^n \frac{EL_i}{AL_i}$$

where EL is the exposure level, AL is the acceptable level, and n is the number of chemicals in the mixture. The ratio of EL to AL is called the Hazard Quotient (HQ).

Various measures for exposure levels and expectable levels may be applied; the only constraint is that EL and AL must be expressed in the same unit. Input values for AL can be ADIs or reference doses (RfD) for specific endpoints.

If $HI > 1$, the total concentration (or dose) of mixture components exceeds the level considered to be acceptable. The method offers flexibility in applying different assessment factors (AF) when defining AL for the individual substances.

An assumption implicit in the use of the HI approach, and one that derives from the principles of the DA concept, is that the acceptable levels AL for each individual chemical represent exposures associated with the same (small or negligible) effect. In most cases, this is not proven in practice, and will remain unproven in the foreseeable future. For most practical applications, however, the error in making this assumption can be considered small.

6.5.3 The Point-of-departure Index

The Point of Departure Index (PODI) is an approach to component-based mixture risk assessment which is similar to the HI and TUS. In contrast to the HI, however, exposure levels (EL) of chemicals in a mixture are not expressed as fractions of individually acceptable levels (AL) but as fractions of their respective points of departure (PODs) such as NOAELs or benchmark concentrations or doses (BML). In this way, different uncertainty factors that may be included in AL values (see HI) are removed from the calculation (Wilkinson et al. 2000):

$$PODI = \sum_{i=1}^n \frac{EL_i}{POD_i}$$

A PODI lends itself to the estimation of margins of exposure for the mixture of interest. Similar to the HI, there is the implicit assumption that all PODs are associated with the same effect magnitude, a principle derived from the features of DA.

6.5.4 Relative potency factors

The Relative Potency Factor (RPF) approach is another application of the DA concept for mixtures of chemical substances that are assumed to be toxicologically similar (US EPA 2000). The effective concentrations (or doses) of mixture components are scaled relatively to the effective concentration (or dose) of an index compound, and then summed up. The scaling factor is called RPF. The total toxicity of the mixture is assessed in terms of the toxicity of an equivalent concentration of the index compound. In general, the mixture concentration C_m expressed in terms of the index compound for n compounds is

$$C_m = \sum_{i=1}^n (c_i * RPF_i)$$

where c_i is the concentration of the i^{th} mixture component, and $RPF_1 = 1$, as $i = 1$ indicates the index chemical.

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6.5.5 The toxic equivalence factor concept

The Toxic Equivalence Factor (TEF) is a specific type of RPF formed through a scientific consensus procedure (EPA 2000). Based on the assumptions of a similar mechanism of action of structurally related chemicals and parallel concentration (or dose) response curves, they were first developed for dioxins. The total toxicity of the mixture is assessed in terms of the toxicity of an equivalent concentration of an index compound. The total equivalent quantity *TEQ* is estimated by summation of the concentrations (or doses) of mixture components c_i multiplied by the respective TEF_i :

$$TEQ = \sum_{i=1}^n (c_i * TEF_i)$$

6.5.6 Data requirements and applicability of simplified mixture risk assessment methods

All of the above cumulative risk assessment methods require at least rudimentary dose-response information of individual mixture components which is used to derive the input values, be they ADIs, RfDs, POD or information about relative potencies such as RPF or TEF. Information about exposures must also be available.

The HI sums up ratios of exposure levels and ADIs or RfDs of chemicals. These estimates can be arrived at by utilizing different assessment factors (AF) for each mixture component, in order to deal with differences in data quality and sources of uncertainty.

If this is perceived to be inadequate, the PODI method can be used. PODI is based not on reference doses, but on points of departure (NOAELs, benchmark doses). Extrapolation issues (e.g. animal to human) are dealt with either by using one overall AF, or by estimating margins of exposure (MOE).

The TEQ concept is predicated on the choice of a reference chemical and requires parallel dose-response curves for all components. Both these requirements are often not met by chemicals, but the method has been validated for dioxins and dioxin-like substances.

6.6 The principal assumptions and simplifications behind mixture risk assessment methods

In common with the MRA approaches applied in regulatory practice and the WHO/IPCS approach (Meek et al. 2011), the practical application of mixture risk assessment approaches is based on a number of assumptions and simplifications which must be made explicit, as follows:

- **The possibility of synergisms or antagonisms is disregarded.** This assumption is the direct consequence of the fact that the degree of synergism or antagonism cannot be predicted quantitatively on the basis of the toxicity of the mixture components. All mixture effect prediction methods and accordingly, all MRA methods, assume additivity. Considering that the likelihood of synergisms is relatively small when multiple toxicants are present at low regulatory acceptable levels (Boobis et al. 2011; Kortenkamp et al. 2009), the disregard for toxic interactions may be regarded as sufficiently protective.
- **Simultaneous exposure to multiple chemicals is assumed.** In numerous settings encountered by humans and species assemblages there is simultaneous exposure to multiple chemicals. For example, there is consumption of single food items that contain multiple chemicals and, even when food items are consumed sequentially, the subsequent exposure of body tissues to the chemicals contained within the items may be simultaneous.

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Strictly sequential exposures are also a reality, but the risk assessment methods available for MRA are not applicable to sequential exposure to multiple chemicals. In theory and concept, the development of methods for the assessment of sequential exposures is still in its infancy (Altenburger and Greco 2009).

- Potency estimates for mixture components may be derived from different endpoints.** Application of DA requires the use of potency estimates for the same adverse outcome as input values. However, such input values are often not available because chemical safety testing is geared towards identifying critical toxic effects which can be used for the establishment of reference doses (ADI, TDI). In practice, this means that toxicity information for chemicals that occur together in mixtures often derives from disparate endpoints. To enable assessments of cumulative risks, the demand for potency estimates for the same endpoints is therefore relaxed, especially for simplified analyses at lower tiers. This simplification is in line with the principles of the framework analysis suggested by WHO/IPCS (Meek et al. 2011).
- It is assumed that the potency estimates entered into MRA methods (e.g. ADIs, POD) describe doses associated with the same effect magnitude.** The equations for DA are based on single chemical effect doses for identical effect magnitudes. When applied to the PODs that enter the mathematical expressions used in MRA methods such as HI or PODI, this means that all PODs should describe effect doses for the same effect levels. In practice however, this demand cannot always be met, except in the case of benchmark doses which are defined in relation to the same effect levels. In human toxicology, the effects associated with NOAELs that form the basis for ADIs by combination with AFs are normally not known. To make cumulative risk assessment methods workable despite these knowledge gaps, ADIs and PODs are taken as if they described effect doses for the same effect magnitude. Similar considerations apply to ecotoxicology with PNECs.
- Potency estimates can be derived from different tests, performed under different conditions.** In the interest of consistency, the evaluation of experimental mixture effects by using the concept of DA should utilise effect data for all the mixture components that were gathered under the same experimental conditions, with the same test organisms. If this condition is not fulfilled, a bias may be introduced into the analysis, leading to erroneous determinations of mixture effects in terms of additivity, synergy or antagonism. MRA however has to rely on data that were produced in the context of single chemical testing, under widely varying experimental conditions, even when the same organisms were used, so that the demand of consistency of data cannot be realised in practice. To allow continuation of MRA, this demand therefore has to be relaxed.
- Data on exposures and potency must be recorded by using the same dose metric.** To allow utilization of the formula for HI or PODI, for all mixture components both exposure and toxicity data must be expressed in the same unit and must refer to the same route or matrix, either in terms of an external concentration in an environmental medium such water or food, or in terms of an amount taken up per unit of time and biomass via a defined route such as oral, dermal, inhalative etc., or in terms of an aggregated dose via different routes, or in terms of an internal concentration in defined tissue or body fluids such fat or blood, or in terms of a total body burden.

6.7 Principles of a step-wise (tiered) analysis in mixture risk assessment

Tiered approaches to MRA can avoid unnecessary expenditure of resources by offering the possibility of discontinuing the analysis on the basis of crude and simple assumptions about exposures and hazards when cumulative exposures are judged to be tolerable or acceptable. In this way, lengthy, but largely unproductive efforts of refining the analysis can be avoided.

This concept was adopted in the WHO/IPCS framework for assessing combined exposures to multiple chemicals (Meek et al. 2011). The framework is based on a series of four tiers that begins with simple and conservative screening assumptions and moves to higher tiers as necessary. The higher tiers adopt more refined, less conservative and more accurate assessments than the preceding tiers. This requires more resources in terms of additional exposure and toxicity data.

An epidemiological perspective to mixture health effects

6.8 Mixture definition

From an epidemiological point of view, a mixture consists of exposures that occur at the same time and have similar effects on organ level, phenomenological endpoints, mode of action, and/or mechanism of action. Quantifying the risk of disease from environmental chemical mixtures could help identify modifiable exposures that may be amenable to public health interventions (Braun et al. 2016). The risk of these mixtures may be different depending on both their qualitative composition (type of chemicals in the mixtures, see Figure 1), as well as their quantitative composition (at what level are these chemicals in the mixture).

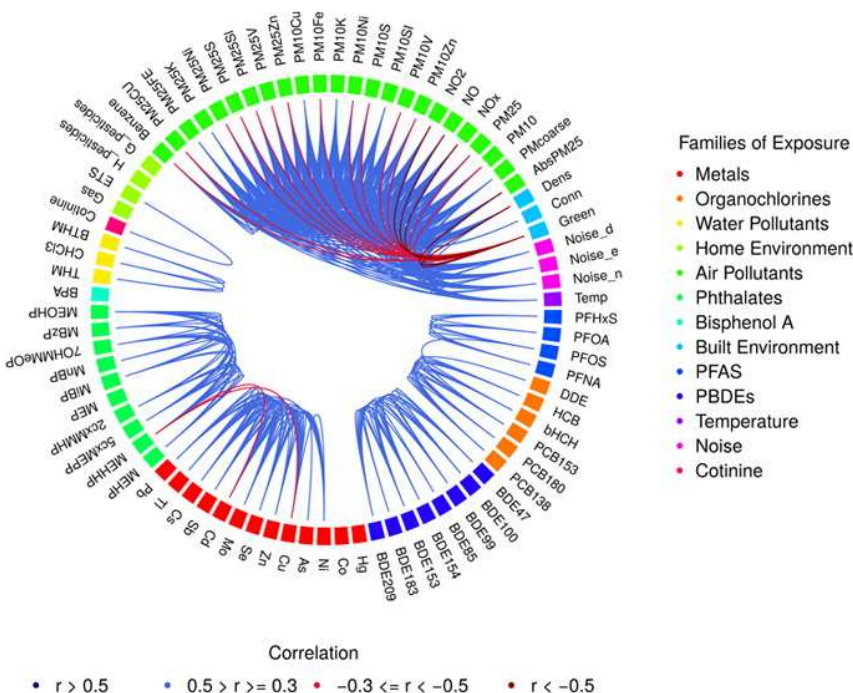


Figure 1: Qualitative composition of a mixture exposure (adopted from Robinson et al. 2015)

Sequential exposures to inhaled environmental pollutants may result in responses not predicted by evaluating exposure to an individual toxicant. For example, individuals with pre-existing lung cancer may be at risk for injury, which may indicate that the lung is damaged or primed by earlier events so that exposure to a nontoxic dose of an environmental pollutant may be sufficient to trigger adverse effects (Johnston, Holm, Finkelstein 2005).

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The concept of acquired 'clinical vulnerability' is related to previous insults/pathophysiological changes that predispose to disease, see Figure 2. An example is the finding of a greater effect of environmental tobacco smoke exposure among ex-smokers compared to non-smokers who have never smoked. Due to existing mutations or epigenetic changes it is plausible that ex-smokers have a greater vulnerability, and further exposure to environmental tobacco smoke leads to selection and clonal expansion of mutated cells (Vineis et al, 2009).

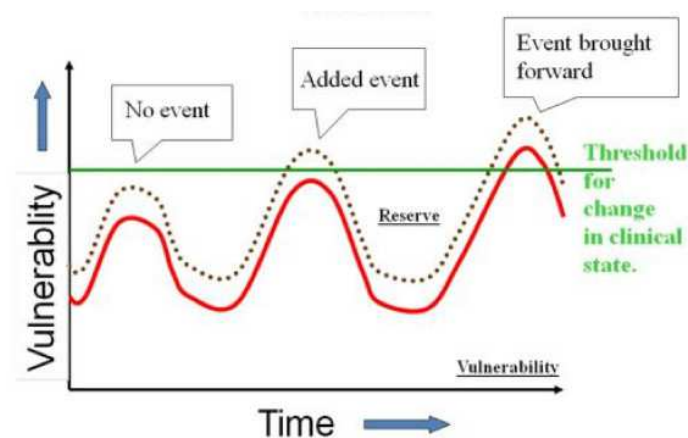


Figure 2: The concept of clinical vulnerability

6.9 Operationalizing mixture analyses

Since humans are exposed to a large number of environmental agents, there is a need to identify exposures that are most strongly associated with adverse health outcomes. Or in other words, by an estimation of the joint effect of relevant components of a mixture. This can be done by assessing each chemical exposure and health outcome separately, plus taking into account correlations between co-pollutants by hierarchical methods, as well as variable selection techniques (either supervised or unsupervised). Due to the correlated nature of many environmental pollutants, it is important to adjust for co-pollutant confounding using appropriate methods when trying to identify single exposure within a mixture that are most important to human health. Failure to do so could result in attributing one exposure to an adverse health outcome, when it might be due to another correlated co-pollutant (Braun et al., 2016).

6.9.1 Supervised variable selection

By assuming the sum of congeners, one assumes the same mechanism and some potency for each congener, mostly by pre-defined groupings.

For example Engel et al. calculated concentrations of total congeners of PCBs by summing the concentrations of all measured congeners (including those below the LOD). Results of grouped analyses based on moles were similar to those based on the weight of the congeners (Engel et al., 2007). A variation on this approach is given by Wolff et al., assuming the same potency within a group. Congeners are grouped by similar mechanism, suggesting the following groups for PCBs in epidemiological studies: Wolff Group 1B (potentially estrogenic, persistent), Wolff Group 2A (potentially anti-estrogenic, persistent), Wolff Group 2B (potentially anti-estrogenic, limited dioxin activity, persistent) and Wolff Group 3 (CYP1A1 and CYP2B inducers, biologically persistent) (Wolf et al. 1997). Another further refinement involves the toxic equivalency quotient (TEQ), which is a toxicity weighted summary parameter. For PCBs TEQs indicating biological activity through the arylhydrocarbon receptor (AhR) pathway, were calculated as the sum of the lipid-adjusted levels of dioxin, furan, coplanar PCB, and mono-ortho-chlorinated PCB congeners, each weighted by its congener-specific toxic equivalency factor (TEF).

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6.9.2 Unsupervised variable selection

Dimensionality reduction techniques

Dimensionality reduction techniques use the correlation among the predictors to define a summary of the original variables into few(er) synthetic variables (the principal components, PCs) that capture the latent structure of the data. This is achieved by searching for linear combinations of variables that optimize some measure of diversity among observations. The i^{th} principle component PC_i is then defined as:

$$PC_i = \alpha_{i1}X_1 + \alpha_{i2}X_2 + \dots + \alpha_{ip}X_p$$

Where X_1, \dots, X_p denote the p original variables in the predictor matrix and $\alpha_{i1}, \dots, \alpha_{ip}$, the vector of linear coefficients or weights defining the contribution of each of the original variables to the i^{th} component.

The principle of dimensionality reduction techniques is to identify the fewest components that minimally distort the original dataset. The use of dimensionality reduction techniques can be reduced to the search of the p weight vectors, which relies on so-called eigen analysis. While considering p components a simple rotation of the original dataset is performed (i.e. no loss of information).

Across the dimensionality reduction techniques, different measures of the information are considered: the variance for Principal Component Analysis (PCA), or the χ^2 distance for Correspondence Analysis (CA) (Dagnino & Macherone, 2018). From the eigen decomposition it is possible to rank the components with respect to the proportion of information each explains. For instance, for PCA a scree plot represents the information restitution, as measured by the proportion of the variance of the original dataset explained by each component (Figure 3).

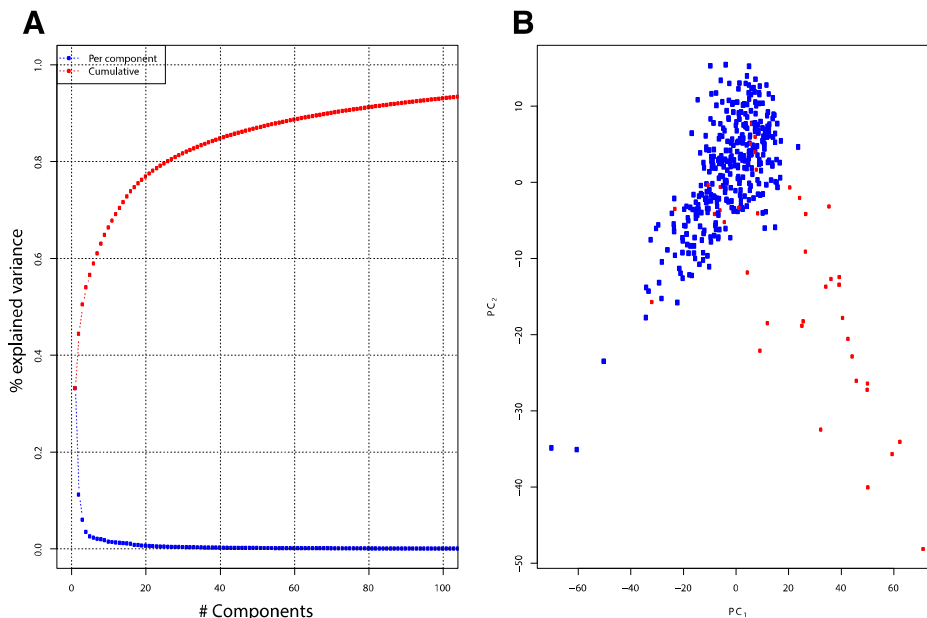


Figure 3: Scree plot of a PCA for 745 transcripts found associated with risk of Chronic lymphocytic leukemia (A). In the score plot (B) individuals are coloured according to their prospective disease status: cases in red and controls in blue. (From Chadeau-Hyam et al. 2014)

From Figure 3-A it is possible to identify the number of PCs that are require to explain more than a certain proportion of the original variance: 72 components are necessary to explain 90% of the variance (% of cumulative explained variance, red line). Projection of the data on the first two

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components (score plot, Figure 3-B), suggests that the 45% explained variance by these two sole components yield good discrimination between cases and controls.

The latent variables can subsequently be used in a (possibly multivariable) regression model to assess how the main drivers of the variation in the original set of variables are related to an outcome of interest. This may result in numerically tractable inference, while characterisation of potential associations remains conditional on the interpretability of the components. Loadings plots (Figure 4) precisely represent the contribution of each of the original variables to the PCs and can help understanding the latent structure captured by each component (Dagnino & Macherone, 2018). In the example presented in Figure 4 none of the two first components were driven by a specific set of transcripts, which hampers the biological understanding of the underlying associations. One way to improve interpretability of the components is to ensure sparsity in the loadings coefficients, which can be done through penalisation.

PCA has proved useful in genetics and has become standard in genome-wide association studies (GWAS), where it is used to correct for population stratification. PCA can accommodate both continuous and discrete data, and is not affected by potential correlation between predictors or by a larger number of variables than observations. However, while PCA has proven efficient in summarising large datasets in (far) fewer dimension, representing the latent structures in the data driving most of the variability in the original dataset, nothing guarantees that this variation is relevant to an outcome of interest. As an alternative to PCA, Partial Least Square (PLS) approaches have been proposed (Wold et al., 1984). PLS components are defined such that they maximise the covariance between the predictors and response variables. As such not only PLS components are capturing as much variance of the original variable as possible, but also focuses on the variance that is relevant to the outcome of interest. PLS-based methods are extremely popular in chemometrics and have been successfully applied in metabolomics. Application to other OMICs data were successful in epigenomics, transcriptomics, and proteomics.

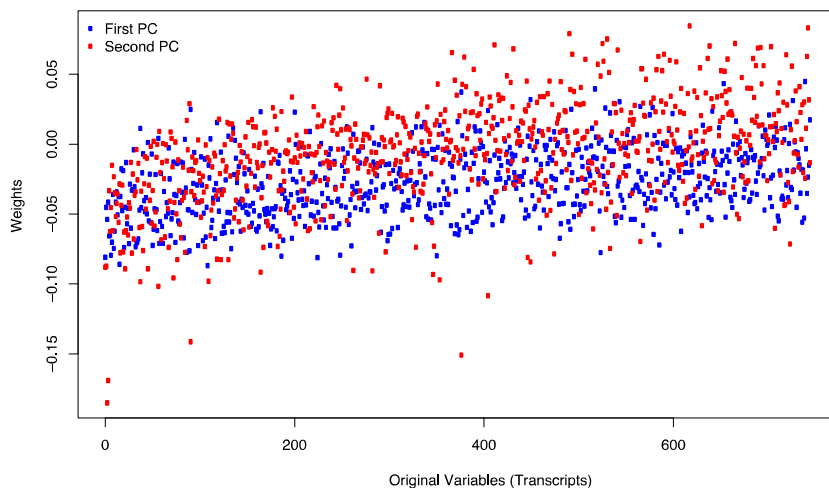


Figure 4: Loadings plots for the first two components of the PCA of the 745 CLL-associated transcripts. (From Chadeau-Hyam et al. 2014)

Irrespective of the type of analysis, the use of latent variables a regression context requires the specification of the number of latent variables to be considered, which usually relies on cross-validation procedures aiming at the identification of the number of components that optimizes both interpretability and prediction error.

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6.10 Interactions of individual components

Interaction refers to the situation where the effect of one exposure on a certain outcome is different across strata of another exposure. This means that if interaction between two exposures is present, these exposures are not independent in causing a certain outcome. A classical example is the interaction between smoking and asbestos on the risk of lung cancer (Rothman 2002). The presence and direction of interaction depends on the scale, e.g. additive or multiplicative, that is used. Interaction on an additive scale means that the combined effect of two exposures is larger (or smaller) than the *sum* of the individual effects of the two exposures, whereas interaction on a multiplicative scale means that the combined effect is larger (or smaller) than the *product* of the individual effects. Arguments are given that biological interactions should be assessed on an additive scale rather than a multiplicative scale (Rothman 2002; Andersson et al., 2005; Vanderweele et al., 2007). Interaction on an additive scale can be calculated using relative risks and different measures quantifying this interaction have been described. Provided that the odds ratio approximates the relative risk, these measures can be used to assess interaction on an additive scale even with case-control data. Moreover, methods to quantify interaction on an additive scale in the case of continuous determinants have been presented (Knol et al., 2007). (From Knol et al. 2011)

6.10.1 The impact of timing of exposure on interactions between individual components

Interactions between two chemicals can occur if exposures occur simultaneously, but also when exposure occurs sequentially. Kawaguchi et al. described a scenario with a population exposed to two carcinogens and examined two exposure scenarios: 1) sequential exposure and 2) simultaneous exposure (Kawaguchi et al., 2006). The target population was divided into three states, based on the condition of the target tissue (normal, intermediate or malignant; see Figure 5). The synergy index was used to quantify the interaction between the two carcinogens on the risk of cancer.

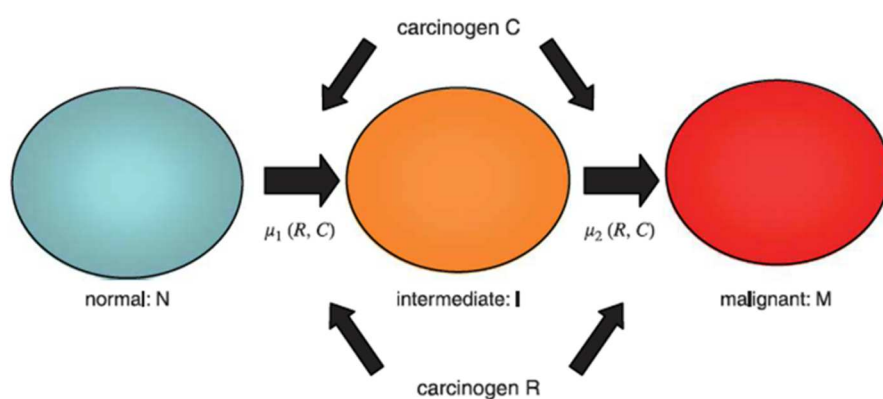


Figure 5: Model scheme: Each individual changes from normal (susceptible or healthy) status to intermediate status, and intermediate to malignant status. Both carcinogens promote each translational step between states.

In their simulation analysis, Kawaguchi et al. observed that:

- 1) If the two carcinogens affect the same steps, the effects of exposure are slightly antagonistic for both carcinogens at any dose of the two carcinogens, whether exposure occurs simultaneously or sequentially.

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- 2) In the case in which carcinogens affect different steps, the combined effects are synergistic when 1) exposure to the carcinogens occurs simultaneously or 2) exposure to the carcinogen that affects step 1 occurs first, subsequently exposure to the carcinogen that enhances step 2 occurs next. However, if the exposure sequence is reversed, the effects of combined exposure become additive.
- 3) In the case in which one carcinogen enhances both mutational steps, whereas the other carcinogen enhances only one step, then a) if the carcinogens are exposed simultaneously, the effect of the combined exposure is synergistic. And b) if exposure to the carcinogens takes place serially the effects depend on the exposure sequence. The effects are antagonistic when a carcinogen that enhances a step is treated in reversed orders of exposure (e.g. a carcinogen that affects both steps during the first period and another carcinogen that affects step 1 exposure during the second period). However, the effects are synergistic when the doses are administered in the sequence of the corresponding affected step.
- 4) The general case in which two carcinogens affect both steps (with low carcinogenic doses) the effects are synergistic, even if the exposure sequence is exchanged.

These results indicate that, in an epidemiological analysis in which interactions between individual components is assessed, the sequence of exposure can have an impact on the degree of interaction that is observed, and should preferably be incorporated in the epidemiological analysis.

6.10.2 The impact of measurement error on the potential to identify interactions between individual components

Measurement error is one of the major sources of bias in epidemiological studies. Measurement error in the components of the mixture will bias the multiplicative interaction effect towards the null value. Under special conditions, in multivariate analysis, correlated independent variables with classical error and Berkson-type error can also lead to bias away from the null (Lebreit, 1990). The impact of misclassification in the study of additive interactions is more difficult to predict and less well understood.

As a result of misclassification the required sample size to detect a departure from the null hypothesis of no multiplicative interaction with a given statistical power will be increased. The increase in sample size also depends on the prevalence of the exposures assessed and on the type and magnitude of the interaction being evaluated. When the interaction effect is moderate to small, even relatively small biases to the interaction parameter can lead to large increases in sample size. Efforts to improve the accuracy of exposure assessment for environmental factors can greatly reduce sample size requirements to study interactions and are critical for accurate assessment interactions in case-control studies (Garcia-Closas et al., 1999; Greenwood et al., 2006).

6.10.3 Different statistical approaches to detect interactions

Many methods have been recommended to examine potentially interacting exposures or, more generally, the effects of mixtures of exposures. Recently, Barrera-Gomez et al. conducted a simulation study in an exposome context comparing the performance of several statistical methods that have been recommended to detect interactions (Barrera-Gomez et al., 2017). In addition, two methods that are not able to detect interactions (LASSO and DSA₁) were also considered for comparison purposes. The results are presented in Table 1.

Table 1: Characteristics and performance measures available for each statistical method (From Barrera-Gomez et al. 2017)

Feature	EWAS ₂	DSA ₁	DSA ₂	Sun3step	LASSO	GLINTERNET	BRT
Model structure							
Provides regression coefficients		✓	✓	✓	✓	✓	
Able to include interaction terms	✓		✓	✓		✓	✓
Able to include confounder covariates	✓	✓	✓	✓	✓		
Able to capture non-linear associations			✓	✓			✓
Measures of performance							
RMS	✓	✓	✓	✓	✓	✓	
RW	✓	✓	✓	✓	✓	✓	✓
R^2_{fit}		✓	✓	✓	✓	✓	✓
Sens	✓	✓	✓	✓	✓	✓	
AltSens	✓	✓	✓	✓	✓	✓	✓
Senvar	✓	✓	✓	✓	✓	✓	✓
Sens ₂	✓		✓	✓		✓	
AltSens ₂	✓		✓	✓		✓	
FDP	✓	✓	✓	✓	✓	✓	
AltFDP	✓	✓	✓	✓	✓	✓	✓
FDPvar	✓	✓	✓	✓	✓	✓	✓
FDP ₂	✓		✓	✓		✓	
AltFDP ₂	✓		✓	✓		✓	

Of the tested methods, GLINTERNET and DSA₂ showed the best overall performance, with GLINTERNET having better properties in terms of sensitivity and predictive ability, and DSA₂ giving lower values of false discovery measures. GLINTERNET and DSA₂ also performed best when capturing interaction terms, with the same trade-off between sensitivity and false discovery proportion. When interactions were not present in the data, using variable selection methods that allow for interactions had almost no cost in sensitivity and only a slight reduction in false discovery rate, compare to methods that only search for main effects.

DSA (Deletion/Substitution/Addition) algorithm is an iterative process that starts with an empty model and uses deletion (removing a variable from the model), substitution (replacing a variable in the model by another not in the model), or addition (adding a variable in the model) moves to find the final model. The final model is selected by minimizing the residual mean squared error (RMSE) using 5-fold cross-validation (Sinisi and van der Laan, 2004). DSA₂ searches for main effects and 2-way interactions between exposures.

Group-Lasso INTERaction-NET (GLINTERNET) is a variable selection algorithm that fits linear pairwise-interaction models that satisfy strong hierarchy: if an interaction coefficient is estimated to be nonzero, then its two associated main effects also have nonzero estimated coefficients (Lim and Hastie, 2015). It considers groups that include main effects and two-way interactions. The particular case in which each group consists of only one variable corresponds to LASSO. Groups of variables are allowed to overlap, in the sense that one variable can be present in more than one group (e.g. the same variable can be present in two or more groups corresponding to different two-way interaction terms). In such cases, the final coefficient for a given variable is the sum of the coefficients of the groups in which the variable is present.

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6.11 Measuring joint effects, the concept of allostatic load

A new and quickly developing approach to studying joint effects of exposure mixtures involves studying biological perturbations, in particular, with so-called OMICS technologies that collectively characterize and quantify pools of biological molecules that translate into the structure, function, and dynamics of the human biological system (Vlaanderen et al., 2010). Prominent OMICS technologies include DNA methylomics, that measures how epigenetic mechanisms regulate gene expression; transcriptomics, that measures gene expression itself; and metabolomics, that measures all small molecules (including lipids) in body fluids.

Perturbations in the OMICS markers can be seen as integrated exposure markers reflecting the total stress on the biological system or the response to the joint exposure, also known as allostatic load. Allostatic load is “the wear and tear on the body” which grows over time when the individual is exposed to repeated or chronic stress. It represents the physiological consequences of chronic exposure to fluctuating or heightened neural or neuroendocrine response that results from repeated or chronic stress (McEwen and Stellar, 1993).

For example preliminary studies have demonstrated that the biological response due to the exposure to indoor air pollution mixture due to the use of smoky coal is remarkably similar to the biological response due to cigarette smoking. Indicating that these two exposures with separate sources, might occur on similar pathways and collectively contribute to the allostatic load (unpublished).

6.12 Where does human data fit in the future of risk assessment of chemical mixtures?

Epidemiological data can contribute to the risk assessment of chemical mixtures at many levels. It can provide insight in the occurrence of chemical mixtures, both across populations and across life stages. Epidemiology can also contribute by studying the health impact of mixtures by conducting epidemiological analyses. However, as described above such analyses can be challenging due to the large amount of data that is required. Finally, the measurement of biological perturbations is an promising and quickly developing avenue to measuring the biological impact of chemical mixtures on human health, also including the concept of allostatic load. Further developments are necessary before this field can contribute fully to risk assessment. (Braun et al., 2016; National Academies of Sciences, 2017)

The streetlight effect, or effect of observational bias, has limited the number of chemicals studied. Epidemiologists have typically measured only a few chemicals, choosing from those known to be of concern or those for which measurement methods currently exist (Braun et al. 2016). When studying large numbers of exposure, the risk of false-positive results is a concern. Epidemiologists must assess the validity, magnitude and precision of observed associations rather than just the statistical significance of associations (Braun et al. 2016).

Epidemiology can also play a role as a triangulation approach to explore to what extent the main drivers of PODI or HI mixture risk occur in human populations and also as a means to test in, for instance as nested-case control study in existing cohorts, hypothesis from toxicological mixture risk assessments about such drivers of mixture risks.

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7 Criteria for the selection of proposed case studies

In discussions among the partners contributing to WP 15, two 'pipelines of reasoning' emerged for the selection of potential case studies: one was a selection based on health endpoints of concern, the other was selection based on chemicals of concern. Although there is no clear black/white division between the two, below they are discussed separately.

Another aspect was the potential to 'triangulate' potentially high mixture risks from a toxicological MRA perspective through analyses of existing data in cohort studies. E.g. when certain chemicals drive the HI/PODI, the same chemicals and related endpoint can be tested in cohorts where such data is available. Also, more methodology oriented topics were considered.

7.1 Health endpoints of concern

In discussions among the partners contributing to WP 15, the following human health endpoints were selected as of concern:

- Developmental neurotoxicity
- Cancers, including e.g. lung cancer
- Nephrotoxicity
- Endocrine disruption, especially disruptions of male reproductive health

A key aspect for consideration included the interest to align the case studies as much as possible to the work ongoing in WP 14, with biomarkers of effect. As this focuses on using *in vitro* assays for the monitoring of total internal exposure to estrogen receptor agonists and androgen receptor antagonists, the choice of endocrine disruption (anti-androgens) as a health endpoint seemed especially appropriate.

7.2 Pollutants and chemicals

It was decided to align the pollutants and groups of chemicals to be selected for case studies to the priority pollutants chosen for HBM4EU. We realised this by picking out the following pollutant groups and chemicals:

- Polybrominated diphenylethers
- Diverse phenolic substances including BPA, BPS, phthalates, parabens etc.
- Heavy metals such as: lead, mercury, cadmium, arsenic (in various oxidation states)
- Chromium (VI)
- Polycyclic aromatic hydrocarbons
- PFAS

7.3 Cross-cutting issues

As an issue that applies to all health endpoints and all chemicals, it was decided to address exposure misclassification that can result from biomonitoring spot samples. When applied to multiple chemicals (exposures) with a variety of half-lives, this can seriously complicate exposure assessment, leading to exposure measurement error and exposure misclassification. This in turn will negatively impact the assessment of risks in observational studies, through bias in the statistical analyses, with the risk of a potential bias towards the null (false negatives) and a loss of statistical power. Accordingly, this will be the topic of a separate case study.

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8 Proposed case studies

In the light of the above selection criteria, several case studies of mixture effects and mixture risk assessment were proposed. Below the outline and indicative timeline, participants and person month are given for the first phase of case study development. We anticipate that on the basis of first results in 2019, a second phase for further development will be proposed for several of the case studies. The presented timelines, partners and person month are indicative at the time of writing. Further details need to be elaborated and final agreement on the case studies requires a decision in the Management Board. The AWP2019 need to be adapted accordingly and possibly the AWP2018 too.

8.1 Developmental neurotoxicity beyond polybrominated diphenylethers (PBDEs)

8.1.1 Rationale

This will build on a recent congener-specific mixture risk assessment for PBDEs that was based on European intake assessments via food and dust, and utilised health-based guidance values for developmental neurotoxicity developed from recommendations of the EFSA Contam Panel (Martin et al. 2017). The case study will expand to incorporate the 12 developmental neurotoxicants defined by Landrigan and Grandjean (2014) which include certain heavy metals, PCBs, certain pesticides and VOCs, many of which are among the priority substances defined for HBM4EU. Considering that Martin et al. (2017) have established that acceptable exposures to combinations of certain PBDE congeners are already exceeded for many age groups, it can be anticipated that risk estimates will increase further by incorporating additional developmental neurotoxicants. The case study will enable us to rank the pollutants in terms of their contribution to combined exposures and will provide the foundations for HBM strategies of combined exposures to developmental neurotoxicants.

8.1.2 Selected chemicals

The case study will investigate combined exposures to the following developmental neurotoxicants (Landrigan and Grandjean 2014):

PBDEs, chlorpyrifos, PCBs, DDT, toluene, tetrachloroethylene, methyl mercury, lead, manganese, arsenic, fluoride, and ethanol. While not all of these are priority substances in themselves, the mixture perspective of these chemicals requires the inclusions of such known developmental neurotoxicants.

8.1.3 Approach and methods

Following on from Martin et al. (2017), the Hazard Index method will be used, in parallel with the Point of Departure Index approach. This will require assembling acceptable levels for developmental neurotoxicity not only for PBDEs, but also for all the other chemicals. We will retrieve the necessary data from the literature, and/or, if necessary establish acceptable levels by using accepted hazard assessment procedures.

Similarly, the required exposure data (intake levels) will be retrieved from the literature, with an emphasis on data from the European Union. It is foreseen that the exposure assessment arm of the mixture risk assessment case study will proceed in tiers, starting from worst case assumptions and proceeding to more realistic scenarios. The comparison of HI and PODI approaches will give an impression of the overall safety margin between the two indices. It may also reveal which substances drive the safety margin and to what extent.

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8.1.4 Link with other HBM4EU work packages

We foresee links with Pillar 2: WP 9 (analysis of heavy metals in blood and/or urine) and Pillar 3: WP 12 (from HBM to exposure, modelling body burden), and WP 13 (exposure health relationships, AOP), WP15.1 (analysis of existing mixture HBM data).

8.1.5 Indication of time lines and work phases within HBM4EU

In 2018, we expect to assemble hazard data for all selected chemicals, together with some exposure data.

In 2019, the calculation of Hazard Indices within a tiered assessment will begin; completion is expected in the first half of 2019. When strong drivers of HI/PODI are identified, proposals for follow up analysis in existing cohort data will be developed for 2020 and later.

8.1.6 Indicative person months

2018: 4 person months

2019: 2 person months

8.1.7 Indication of team / partners

The case study will be led by Brunel, with some involvement from RIVM and other partners (to be specified).

8.2 Heavy metals and nephrotoxicity

8.2.1 Rationale

Humans are exposed to various heavy metals via different sources and through different exposure routes. The most well-known sources are food, water, air, cigarette smoke, dust and soil. In Europe, exposure to e.g. lead has decreased because of the abolishment of leaded petrol, lead containing paint and leaded pipes. Nevertheless, a major source of exposure throughout the years has been (and still is) food. Dietary intake assessments of heavy metals such as arsenic, cadmium, lead and mercury throughout the years in various European countries have shown that the exposure through food is close to or even exceeding so-called health based guidance values (HBGVs). In some cases exposure to (certain) heavy metals has increased (because of dietary changes in consumed, imported products) and in other cases the HBGVs of (certain) heavy metals have been lowered (because of new, scientific insights). An adverse health outcome common to the selected heavy metals is nephrotoxicity due to inhibition of various enzymes responsible for reabsorption of components of the primary glomerular filtrate. This kind of nephrotoxicity is of considerable concern. Many of the listed heavy metals are among the priority pollutants defined for HBM4EU.

8.2.2 Selected chemicals

Heavy metals: cadmium, mercury and lead. We will possibly also consider arsenic.

8.2.3 Approach and methods

We determine the Hazard Index (HI) for these four metals for the European and the Dutch consumer (the latter with respect to the specific request of the Dutch Ministry of Health, co-financer). In a tiered approach, this will be followed by a determination of the Point of Departure Index (PODI) for these four metals for the European and the Dutch consumer. Finally, we will develop an adverse outcome pathway for these four metals in order to establish a (more specific) adverse outcome and biomarkers of effect. The comparison of HI and PODI approaches will give an impression of the overall safety margin between the two indices. It may also reveal which metals drive the safety margin and to what extent.

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8.2.4 Link with other HBM4EU work packages

We foresee links with Pillar 2: WP 9 (analysis of heavy metals in blood and/or urine) and Pillar 3: WP 12 (from HBM to exposure, modelling body burden), WP 13 (exposure health relationships, AOP) and WP 14 (effect biomarkers), WP15.1 (analysis of existing mixture HBM data).

8.2.5 Indication of time lines and work phases within HBM4EU

2018: determine HI and PODI, start with the development of the AOP; 2019: determine AOP and biomarkers of effect.

8.2.6 Indicative person months

2018: for HI and PODI: 1 person month, for AOP: 1 person month

2019: for AOP: 1 person month

8.2.7 Indication of team / partners

The case study will be led by RIVM, with the involvement of IRAS and Karolinska Institute. The following partners from other WP will also contribute, in an advisory capacity:

- WP 9: INSA, RIKILT, RIVM
- WP 12: ANSES, RIVM
- WP 13: Karolinska Institute, RIVM
- WP 14: BfR, RIVM

8.3 Anti-androgenic chemicals and male reproductive health

8.3.1 Rationale

Male reproductive health and fertility is currently in the spotlight: sperm quality is low and continually decreasing in many countries, there is a higher demand for assisted reproductive techniques, more and more boys are born with malformed sex organs and the incidence of testicular cancer is increasing. The cause is hypothesized to arise during fetal life and to be due to lifestyle and/or environmental factors including exposure to 'antiandrogenic' chemicals (Skakkebaek et al. 2016). This health endpoint is of considerable concern. Therefore, the aim of this case study will be to evaluate the potential for a human health risk due to the present exposure to complex mixtures of 'anti-androgenic' chemicals based on our current knowledge. The group of anti-androgenic chemicals is expected to be very diverse, including phthalates, phenolic substances, certain pesticides and even some pharmaceuticals such as analgesics. This case study will be ideal to link in with the work on biomarkers of effect that is ongoing on WP 14. These biomarkers focus on responses to anti-androgens in cell-based bioassays.

8.3.2 Selected chemicals

Phthalates, bisphenols, PAHs, PCBs, PAPs, FTOHs, pesticides (incl. PANDA pesticides), analgesic drugs (11 drugs). While not all of these are priority substances in themselves, the mixture perspective of these chemicals requires the inclusions of such known developmental neurotoxicants.

8.3.3 Approach and methods

We will first identify the known and widespread 'antiandrogenic' chemicals and drugs which humans are exposed to and gather existing literature on 'antiandrogenic' mixtures.

The next work step will be in collecting hazard data from available sources including in vitro assays (AR reporter gene assay and the H295R steroidogenesis assay), ex vivo assays and, if available, in vivo data.

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This will be followed by compiling relevant exposure data from available sources (human exposure levels, C_{max} values for drugs, µM internal exposure levels). UGR has access to human exposure data and will gather information from human biomonitoring studies.

We will then employ the Hazard Index approach and calculate hazard quotients. The analysis will be refined in the light of data on the likelihood of co-exposures.

8.3.4 Link with other HBM4EU work packages

We will link this work to investigations of antiandrogenic effects in placenta extracts which is ongoing in WP14 (in AR reporter gene assay and H295R steroidogenesis assay). This work will also generate further leads for chemicals to include in this case study.

8.3.5 Indication of time lines and work phases within HBM4EU

Identification of anti-androgenic chemicals and compilation of anti-androgenic mixtures by December 2018.

Collection of hazard data will be completed by March 2019.

The compilation of exposure data will be completed by May 2019, with Hazard Indices and Point of Departure available by June 2019. The remainder of 2019 will be reserved for refinements of the analysis.

8.3.6 Indication of team / partners

The case study will be led by DTU, with the involvement of Brunel and UGR.

8.3.7 Indicative person months

DTU: 2018: 1.5 person months; 2019: 1.5 person months (further person months via co-financing)

Brunel: 2018: 3 person months; 2019: 2 person months

UGR: 2018: 1.5 person months; 2019: 1.5 person months

8.4 Chromium (VI), nickel and polycyclic aromatic hydrocarbons and lung cancer

8.4.1 Rationale

There is data evidencing human exposure to PAHs, chromium (VI) and nickel. Concerning biomarkers of effect, the micronucleus analysis in human peripheral lymphocytes is a validated and relevant biomarkers of effect for carcinogenicity, including lung cancer (Bonassi et al., 2011) and micronuclei are induced by the previously referred mixtures (Peng et al 2015; Muthusamy et al 2018). One common adverse outcome of these exposures is lung cancer. Several studies in North American (Proctor et al. 2016, Beveridge et al. 2010), European (Singh et al. 2018, Bugge et al. 2010, Bjoer et al. 2008, Rønneberg et al. 1999) and Asian (Singh et al. 2018, Lee et al. 2010, Hara et al. 2010, Leem et al. 2010) populations showed associations between exposure to PAHs or to chromium and this form of cancer. However, none of those studies analysed or interpreted the results or health outcomes from a mixture perspective, i.e., none considered the potential combined effects of those substances as determinants of lung cancer.

On the other hand, there are several lines of evidence from in vitro and in vivo experimentation that PAHs/chromium/nickel mixtures lead to adverse effects that are different from the ones observed after exposure to each single substance, namely: more-than-additive effects in vitro (Feng et al., 2003; Peng et al 2015; Muthusamy et al 2018) and in vivo (Sánchez-Martín et al. 2015); synergistic effects in ecotoxicity studies (Gauthier et al, 2015; Fleeger et al. 2007; Chigbo et al. 2013).

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Within the HBM4EU, a study on the occupational exposure to chromate has been planned to start in mid-2018 and involves the characterization of several workplaces where exposure to Cr(VI) occurs. In addition, it is known that there is no single exposure to chromate and that, in those workplaces, exposure to nickel and, in some of them to PAHs, also occurs. In that study, apart from the environmental characterization, also biomarkers of exposure to Cr(VI) and biomarkers of effect (oxidative damage, micronuclei and epigenetic effects) will be analysed in order to link exposure to health effects (see AD 8.2). Although the inclusion of biomarkers of exposure to nickel or to PAHs has not been planned, their measurements in post-shift urine samples (preserved at -20 °C) could be proposed, so that a more complete exposure scenario could be obtained. This would be a very good opportunity to link this new study to WP 15, and to look at the results from the mixtures point of view, both in terms of co-exposure and combined effects.

Thus, considering: i) the above mentioned lack of data on PAHs-chromium-nickel mixture effects in humans; ii) the fact that this co-exposure can happen in several occupational settings, such as welding, cars and aircrafts maintenance and much others implicating exposure of a high number of workers; iii) the published epidemiological data about exposure to each substance and lung cancer without taking into account possible interactive effects; iv) the existence of suitable biomarkers of exposure and effect regarding these substances; v) the planned study on the occupational exposure to chromate providing an opportunity to obtain new data on exposure and effect, we propose this topic to be addressed within the new case studies in WP15.3.

8.4.2 Selected chemicals

PAH, chromium (VI) and nickel

8.4.3 Approach and methods

Phase 1: We will first conduct a literature review of epidemiological studies on existing cohorts, including the available WP10 databases.

This will be followed by a search for occupational cohorts in the settings mentioned above, with the aim of assessing whether there are differences in lung cancer incidences relative to cohorts where exposure to only one of the contaminants, PAH, chromium (VI) or nickel, is reported.

We will compile relevant exposure data to construct hazard quotients derived by using relevant occupational exposure limits for PAHs, chromium (VI) and nickel, followed by a determination of Hazard Indices. We foresee a tiered assessment, where in the lower tiers of the analysis occupational limits set for different health endpoints are combined.

Phase 2: We will refine the exposure assessment arm of the mixture risk assessment by a detailed analysis of the hygiene data present in reports of the companies enrolled in the chromate occupational exposure study. In this way we can verify and characterize the exposure to PAHs and nickel.

We envisage the possibility of expanding the study on occupational exposure to chromate (WP 8), to include complementary measurements of PAHs (and Ni) in the environment and biological fluids, if feasible. Post-shift urine samples from Cr(VI) exposed workers could be preserved for future Ni and PAHs analyses, reshaping the chromate study into a chromate, nickel and PAHs mixtures study.

Effect biomarkers such as micronucleus assay in human lymphocytes are planned to be analysed in the above mentioned chromate study, which have been associated with increased risk of cancer. There are large datasets available relating micronuclei with cancer risk that could be re-analysed to look for evidences of mixtures effects.

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8.4.4 Link with other HBM4EU work packages

- WP5: Review on RA of PAHs using HBM
- WP8: Chromate Occupational Study
- WP9: Laboratory analysis
- WP10: Data management and analysis – data available for PAHs and Cr(VI) but probably not consider as co-exposure in the different studies.
- WP14: Biomarkers of effect

8.4.5 Indication of time lines and work phases within HBM4EU

Phase 1 is expected to be completed in 2018; Phase 2 in 2019.

8.4.6 Indication of team / partners

- INSA (Henriqueta Louro, Bruno Gomes, Célia Ventura)
- ESTeSL (Susana Viegas)
- UU (Roel Vermeulen)
- RIVM (Marcel Mengelers) tbc.
- UGR (Vicente Mustieles, Nicolas) tbc.
- FIOH (Tiina Santonen) tbc

8.4.7 Indicative person months

Phase 1: 1.5 PM

Phase 2: 3.0 PM

8.5 Addressing exposure misclassification in mixture studies

8.5.1 Rationale

The characterization of health effects of modern high production chemicals in humans is hampered by the strong variability of their levels in humans, and the fact that most past epidemiological studies assessed exposures on spot biospecimens, leading to exposure measurement error and exposure misclassification (Slama et al. 2017). Exposure misclassification can strongly impact exposure-response relationships. In the case of classical-type error, i.e. when the estimated exposure randomly varies around the true exposure, naïve models not accounting for measurement error suffer a loss of statistical power and provide estimates that are attenuated towards the null (Perrier et al. 2016). In multivariate analysis, correlated independent variables with classical error and Berkson-type error can also lead to bias away from the null (Lebreton, 1990).

Design related options to correct for exposure misclassification exist. When exposures are assessed through biomarkers, the within-subject biospecimens pooling, consisting in collecting several biospecimens per subject during a toxicologically relevant time window and pooling them, has been shown to be an efficient way to limit attenuation bias and increase power, compared to studies based on a spot biospecimen, in the context when the focus is on a single exposure.

In an exposome (or mixture) context, the effect of measurement error may be compounded. The mis-selection issue that has been observed in an exposome context is expected to be amplified in the case of exposure misclassification: variable selection algorithms may mistakenly attribute the effect of an exposure measured with substantial error to another correlated exposure measured with smaller error, an issue called differential exposure misclassification (Slama et al 2015).

In the case study, we aim to assess the performances of within-subject biospecimen pooling in handling differential exposure misclassification and reducing bias in dose-response functions in an

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exposome context, i.e. a situation in which one wants to simultaneously characterize the impact of a large number of exposure on a health outcome.

8.5.2 Selected chemicals or health endpoints

The case study and its results apply to potentially all types of health effects, although the simulation will focus on health or biological parameters assessed on a continuous scale. The case study will not be specific to a chemical but will apply to the situation of an exposome (or mixture) study in which a large number of chemicals with varying toxicokinetic properties are assessed (typically including persistent compounds such as PCBs) and compounds with highly temporally variable urinary concentrations such as members of the phenols and phthalates families.

8.5.3 Approach and methods

The approach consists of a simulation study, which will allow comparing the results of hypothetical studies with exposure misclassification to what would be observed in studies with a perfect assessment of all exposures. We will expand and combine two former simulation studies from our group dealing 1) with the identification of the effect of exposures on health in an exposome context but without exposure misclassification by Agier et al. (2016) and 2) with the efficiency of the within-subject biospecimens pooling approach to reduce bias due to exposure misclassification developed in a single-exposure context by Perrier et al (2016). More specifically:

1. We will simulate realistic exposome data in a fictitious population; the correlation structure between the individual exposures will be based on realistic exposome data (Agier et al 2016). We will assume that exposures are assessed with biospecimens, with classical type error, the amount of error varying between compounds, some having low and other high within subject variability, using realistic data on the variability of chemical urinary concentrations during pregnancy.
2. We will assume that a small number of exposures impact the health outcome and
3. Compare the efficiency of an approach in which a single biospecimen is collected in each subject to assess exposures, with an approach in which an increasing number of biospecimens are being collected and pooled. The efficiency of each approach will be characterized by the sensitivity and specificity to detect compounds really affecting health, as well as bias in dose-response relations, and compared to what would be observed if the true exposure levels had been available.
4. In a further stage, this simulation will be extended to consider the case of “synergy” (e.g., supra-additive effects) between exposures, again expanding a previous simulation study in an exposome context ignoring exposure misclassification (Barrera-Gomez et al. 2017).

8.5.4 Link with other HBM4EU work packages

Pillar 3: WP 13 (exposure health relationships, AOP)

8.5.5 Indication of time lines and work phases within HBM4EU

2018 Simulation study (steps 1-3 above)

2019: Extension to the case of synergy (step 4 above) and manuscripts writing.

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8.5.6 Indication of team / partners

- Inserm
- ISGlobal
- RIVM

8.5.7 Indicative person months

2018: 6 PM

2019: 6 PM

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Annex 1: Workshop scope and purpose/ workshop programme

The main aim of the workshop is to define, outline and plan the activities of WP15.3, i.e. the development of three cases studies on the health effects of mixtures. According to the AWP2018 task 15.3 is outlined as in the text box below:

Task 15.3A: Identification of mixture health effects (M13-M24)

Leader: UBRUN Partners: RIVM, IRAS, VITO, INSERM, ISGLOBAL, MU, AUTH, DTU, INSA, KI, CULS, CGLs, NHCPs

- Task 15.3 will build on the inventories of WP11, 13 and 14, and other relevant sources , to identify viable indicators of mixture health effects and to make an inventory of current approaches for the identification of mixture effects, combining toxicological and epidemiological studies (UBRUN with IRAS, RIVM, VITO, INSERM, ISGLOBAL, KI, MU and CGLs and NHCPs , MX).
- AUTH, INSA, DTU and CULS will provide comments on intermediate results and drafts.
- Together with relevant partners from Pillar 3, we will convene a 2-day workshop to select and further develop selected case studies on health effects. This workshop is needed to allow a thorough interaction among task 15.3 partners on an optimal decision on health effect case studies.

In this workshop, we will critically discuss AD15.1 (Plan for development of case studies, M18, June 5, 2018) and define work to be done in Y3 and, if relevant, elements to be further developed through an internal call. Case studies will be selected to include a mix of approaches and different types of mixtures, e.g. involve work in existing cohorts (WP11, WP13), and new biomarkers pertinent for mixture effect from WP14. We will liaise with the other Horizon2020 projects to build synergy, to avoid duplication of effort and to benefit from earlier work. The outcome of the workshop will be a specified set of topical case studies (AD15.4, M20).

In the discussion, we will build on the discussion of the Nov 2017 WP15 Workshop (refer to minutes for more detail).

The aim of the first section of the workshop was to introduce participants to thinking about risk assessment of mixtures in (regulatory) toxicology, in epidemiology and to update partners on the developments in WP14 (biomarkers of effect). Following on from this, criteria for selection of case studies were discussed in detail, together with a list of proposed case studies and their possible topics.

On the second day, the case studies were further developed in the plenary feedback, and detailed plans were developed for deliverable AD15.1 (see below). It was agreed to prepare drafts to be forwarded to the WP 15.3 coordinator. The workshop ended with the backbone of AD15.1 defined.

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Wednesday, May 9 2018

Time	Topic	Lead
12.00 – 13.00	Arrival and sandwiches	
13.00 – 13.15	Opening, welcome and introductions	Maria
13:15 – 13.30	Outline, objectives and output for the meeting	Erik
13:30 – 14.30	Introduction to predictive approaches in mixture toxicology (Andreas K); Mixture risk assessment methods: Hazard Index and Point-of-Departure Index	Andreas
14.30 – 15.30	Introduction to epidemiological approaches to mixture health effects	Roel
15.30 – 16.30	Update on the WP14 biomarkers of effect	Nicolas?
16:30 – 17.00	Criteria for selection of case studies	Andreas
17.00 – 17.35	Outlines of possible topics for case studies (5 min each) <ul style="list-style-type: none"> • Differential exposure misclassification (Remy) • Developmental neurotoxicants beyond PBDEs (Andreas, Remy) • Nephrotoxicity Cd, Pb, Hg, ... (Marcel, Agneta) • Liver toxicity, thyroid PFAS etc (Johan) • Cumulative assessment groups pesticides (?) • Lung carcinogenicity PAH, Cr(VI), asbestos (?) 	Andreas
17.45	Adjourn	
19.00	Dinner	

Thursday, May 10 2018

Time	Topic	Lead/comment
9.00 – 9.45	Continuation of discussion of topics for case studies	
9.45 – 10.45	Continuation of discussion of topics for case studies	
10.45 – 11.00	Coffee break	
11.00 – 12.30	Discussion on output of breakout sessions day 1 and selection of 3-5 candidate topics for case studies	Erik
12.30 – 13.30	Lunch	
13.30 – 15.00	Discussion of plan for AD15.1 (Plan for development of case studies), due June 5, 2018 To be further developed in concrete workplans for AWP2019 and beyond (AD15.4, M20)	Defining content, interactions other WPs, planning, budget
15.00 – 16.00	Review of work, distribution of further tasks	Andreas
16.00 – 16.30	Wrap up	
16:30	Close	

Organisational matters:

The workshop was hosted by INSA, Lisbon, Portugal, by Maria João Silva.

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Annex 2: List of workshop participants

For GDPR reasons, no participant list is included, since explicit agreement for listing of name and contact information in this document is not available.

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Addendum to Deliverable Report AD 15.1
**Plan for development of case studies – further
details and clarifications**
WP 15 - Mixtures, HBM and human health risk

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1 Authors and acknowledgements

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We gratefully acknowledge the input of the participants to the Work Package 15.3 (WP15) Workshop on May 9-10, 2018, Lisbon, during which this deliverable was discussed. We also acknowledge the input of the WP15 partners on drafts of the manuscript. We thank INRA for hosting the workshop.

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2 Summary

This document is an addendum to deliverable AD15.1 that describes the development of proposed case studies for mixture health effects under task 15.3. This addendum provides further details about use of information, general approach, links to other WPs, capacity per partner involved, timing of activities and output.

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3 Proposed case studies

Several case studies of mixture effects and mixture risk assessment were proposed. Many of these case studies will involve application of mixture risk assessment methods (such as the Hazard Index, Point of Departure Index, etc) in a tiered framework.

We will strive to develop a consistent, uniform tiered framework that can be applied to four of the five case studies; the fifth case study is an overarching methodologically oriented case study on the effect of measurement error and exposure misclassification in HBM and the bias this creates in exposure-response functions. UBRUN will take the lead on the development of this uniform tiered framework and will provide input from the results of the EU SOLUTIONS project. The framework will include the following:

- A **problem formulation step** that evaluates the need for mixture risk assessment in each of the case studies (i.e. is there potential for co-exposure? Are common adverse outcomes likely to arise?, etc) and clearly defines the scope of the studies
- Clear **rules for tiering** of the exposure and hazard assessment arms of the framework
- Clear **decision rules** that define when further refinement of the analysis is to be conducted, and when the analysis can be stopped
- Consistent **application of assessment factors** in each of the tiers
- A concept that will remove in a step-wise fashion the distortions introduced through the use of different assessment factors in regulatory values for pollutants, with the aim of **approaching the scientific principles of Dose Addition** in higher tiers (points of departures for the same adverse outcome, representative of similar effect magnitudes, e.g. benchmark doses for 5% effects and based on the same test species)
- A consistent approach to **bridging toxicity data gaps** (e.g. by using the TTC concept)
- Clear **grouping criteria**, based on AOP considerations

It is anticipated that this common framework is available by Dec 2018, for use in each of the case studies (no HBM4EU resources consumed, as these are SOLUTIONS results!!). In the intervening time, each case study will commence work with data assembly.

In parallel to the development of the common framework, case study leaders will explore to what extent hazard and exposure information from other EU projects can be used.

Case study leaders will also liaise with the Chemical Group Leaders to help identify hazard and exposure information.

3.1 Developmental neurotoxicity beyond polybrominated diphenylethers (PBDEs)

3.1.1 Rationale

This will build on a recent congener-specific mixture risk assessment for PBDEs that was based on European intake assessments via food and dust, and utilised health-based guidance values for developmental neurotoxicity developed from recommendations of the EFSA Contam Panel (Martin et al. 2017). The case study will expand to incorporate the 12 developmental neurotoxicants defined by Landrigan and Grandjean (2014) which include certain heavy metals, PCBs, certain pesticides and VOCs, many of which are among the priority substances defined for HBM4EU. Considering that Martin et al. (2017) have established that acceptable exposures to combinations of certain PBDE congeners are already exceeded for many age groups, it can be anticipated that risk estimates will increase further by incorporating additional developmental neurotoxicants. The case study will enable us to rank the pollutants in terms of their contribution to combined

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exposures and will provide the foundations for HBM strategies of combined exposures to developmental neurotoxicants.

3.1.2 Selected chemicals

The case study will investigate combined exposures to the following developmental neurotoxicants (Landrigan and Grandjean 2014):

PBDEs, chlorpyrifos, PCBs, DDT, toluene, tetrachloroethylene, methyl mercury, lead, manganese, arsenic, fluoride, and ethanol.

3.1.3 Approach and methods

Following on from Martin et al. (2017), the Hazard Index method will be used initially, followed by the PODI method at higher tiers. This will require to assemble acceptable levels for developmental neurotoxicity not only for PBDEs, but also for all the other chemicals. We will retrieve the necessary data from the literature, and/or, if necessary establish acceptable levels by using accepted hazard assessment procedures. To this end, we will consult with the CGL's and also explore availability of relevant information from other EU projects.

Similarly, the required exposure data (intake levels) will be retrieved from the literature, with an emphasis on data from the European Union. It is foreseen that the exposure assessment arm of the mixture risk assessment case study will proceed in tiers, starting from worst case assumptions and proceeding to more realistic scenarios. Parallel to the assemblage of acceptable levels, output of task 15.1 and data obtained through WP7 Inventory of existing surveys and data from repository and IPCheM will be used to develop realistic exposure scenario's including the correlation patterns in co-exposures.

3.1.4 Link with other HBM4EU work packages

We foresee links with Pillar 2: WP 8 and WP10 (results from newly collected HBM data) and Pillar 3: WP 12 (from HBM to exposure, modelling body burden), and WP 13 (exposure health relationships, AOP). End results will be shared with WP5 for risk assessment.

3.1.5 Indication of time lines and work phases within HBM4EU

The time line for this case study is depicted in the Gantt chart at the end of this document (best seen on A3 format paper). In 2018, we expect to start to assemble hazard data for all selected chemicals, together with some exposure data.

In 2019, the calculation of Hazard Indices within a tiered assessment will begin; progress will be reported in an interim progress report in August 2019.

A report in the drivers of DNT will be finished by November 2019. We expect an update on the exposure information based on the analysis in task 15.1 particularly with respect of the correlation pattern amongst chemicals in the mixture; this will be used for an update in HI/PODI assessment and for the driver of risks. Data from existing cohort studies and exposome studies allow to mimic the results of the HI/PODI in analysis of cohort data. This can serve as a triangulation/reality check in the human population of the mixture risk assessment. This work is foreseen in 2020, with a report in November 2020.

3.1.6 Indication of team / partners

The case study will be led by UBRUN, with involvement from RIVM and ISGLOBAL. ISGLOBAL and RIVM will assist assembly of the exposure information. RIVM will provide information from 15.1 from the analysis of existing HBM mixture data. ISGLOBAL will perform an analysis of their cohort data to assess the effects of the main drivers of DNT risks.

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3.1.7 Products generated

It is anticipated that towards the end of 2018 the assembly of data for the exposure and hazard assessment arm of the mixture risk assessment will be completed. We anticipate a first interim report using the HI approach by the end of the first half of 2019. By the end of 2019 further refinements of the analysis will have been completed, with identification of those chemicals that are the 'risk drivers', i.e. the chemicals that make a disproportionately large contribution to the modelled mixture risks. The identification of drivers will allow us to pinpoint those developmental neurotoxicants that should be targeted by joint biomonitoring.

Some of the heavy metals analysed in the case study on nephrotoxicity also play a role as developmental neurotoxicants (e.g. lead), and RIVM will contribute their work on exposure assessment of heavy metal to this case study.

We strive to disseminate the results of this work in a high impact open access journal; drafting of the manuscript is anticipated towards the end of 2019.

3.1.8 Multi-year perspective

Towards the end of 2019 we should have a clear picture of the developmental toxicants that should be targeted by combined biomonitoring. At that time, also results of 15.1 Analysis of existing HBM data will be available that can provide more realistic exposure scenarios, including the correlation structure among chemicals in the mixture. Moreover, newly generated data from WP8.1 will be available and brought to the repository through task 10.6A. In 2020, these results will be used to update the earlier HI and PODI and re-assess the drivers of risk. The early results from 2019 on HI/PODI and drivers of risk can also be used in 2020 to analyse existing cohort data in other countries, as a way of 'triangulation' in the human population of the mixture risk assessment.

3.2 Heavy metals and nephrotoxicity

3.2.1 Rationale

Humans are exposed to various heavy metals via different sources and through different exposure routes. The most well-known sources are food, water, air, cigarette smoke, dust and soil. In Europe, exposure to e.g. lead has decreased because of the abolishment of leaded petrol, lead containing paint and leaded pipes. Nevertheless, a major source of exposure throughout the years has been (and still is) food. Dietary intake assessments of heavy metals such as arsenic, cadmium, lead and mercury throughout the years in various European countries have shown that the exposure through food is close to or even exceeding so-called health based guidance values (HBGVs). In some cases exposure to (certain) heavy metals has increased (because of dietary changes in consumed, imported products) and in other cases the HBGVs of (certain) heavy metals have been lowered (because of new, scientific insights). An adverse health outcome common to the selected heavy metals is nephrotoxicity due to inhibition of various enzymes responsible for reabsorption of components of the primary glomerular filtrate. This kind of nephrotoxicity is of considerable concern (in particular for the elderly). Many of the listed heavy metals are among the priority pollutants defined for HBM4EU.

3.2.2 Selected chemicals

Heavy metals: cadmium, mercury and lead. We will possibly also consider arsenic.

3.2.3 Approach and methods

We will collect relevant hazard and exposure information in a similar fashion as described in the previous case and outlined in section 3.1.3. Cadmium and mercury have already been studied in the Democophes project (European project on human biomonitoring carried out in the 7th Framework Programme). We will also address occupational exposures. We then determine the

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Hazard Index (HI) for these four metals for the European consumer, as well as for exposure scenarios for occupational exposures. In a tiered approach, this will be followed by a determination of the Point of Departure Index (PoDI) for these four metals for the European consumer. Furthermore, we will develop an adverse outcome pathway for these four metals in order to establish a (more specific) adverse outcome (together with WP13). We will share the outcome with WP14 to help identify possible biomarkers of effect.

3.2.4 Link with other HBM4EU work packages

We foresee links with Pillar 2: WP10 Overview of metadata, and Pillar 3: WP 12 (from HBM to exposure, modelling body burden), WP 13 (exposure health relationships, AOP) and WP 14 (effect biomarkers). End results will be shared with WP5 for risk assessment.

3.2.5 Indication of time lines and work phases within HBM4EU

The time line for this case study is depicted in the Gantt chart at the end of this document (best seen on A3 format paper). In 2018 we will start the assembly of exposure data and of the hazard data. This is expected to continue into the first part of 2019. HI and PoDI determination will start early 2019. The development of the AOP will commence mid 2019 (June). Based on an update of existing HBM data and correlation patterns therein, we will update the HI/PODI on nephrotoxicity in the fall of 2019 and report in December 2019.

3.2.6 Indication of team / partners

The case study will be led by RIVM, with the involvement of INSA and Karolinska Institute. Input for occupational exposures will be obtained through the partners FIOH and IRAS. The following partners from other WP will also contribute:

- WP 9: INSA, RIKILT, RIVM
- WP 12: ANSES, RIVM
- WP 13: Karolinska Institute, RIVM
- WP 14: BfR, RIVM

3.2.7 Products generated

It is anticipated that in the second semester of 2018 the HI and PODI for cadmium and lead will be described in an interim report (August 2019). Depending on the availability of data this will be extended to arsenic and mercury in the first semester of 2019. This means that a final report on HI and PODI of 2-4 heavy metals will be delivered at the end of the first semester of 2019. This will take the form of a manuscript for journal publication (open access).

In the fall of 2018 a literature search will start to trace the elements needed to draw up an AOP for nephrotoxicity in 2019. This will be reported in an internal report the spring of 2019. In the second half of 2019 an AOP will be described in the final report (Dec 2019). On the basis of that report also recommendation to WP14 will be made about possible effect biomarkers for nephrotoxicity.

3.2.8 Multi-year perspective

At the end of the first semester of 2019 it should be clear what the overall safety margin between HI and PODI is with respect to the exposure of 2 to 4 heavy metals in Europe. At the end of the second semester it should be clear what the AOP for heavy metals is with respect to nephrotoxicity. These two deliverables obtained from cooperation between WPs in pillar 3 can then potentially be used in 2020 and 2021 to analyse existing cohort data on heavy metals in specific countries in order to determine to what extent the risk assessed in WP15 is reflected in the existing cohort studies. This is currently not captured in the planning for WP15.

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3.3 Anti-androgenic chemicals and male reproductive health

3.3.1 Rationale

Male reproductive health and fertility is currently in the spotlight: sperm quality is low and continually decreasing in many countries, there is a higher demand for assisted reproductive techniques, more and more boys are born with malformed sex organs and the incidence of testicular cancer is increasing. The cause is hypothesized to arise during fetal life and to be due to lifestyle and/or environmental factors including exposure to 'antiandrogenic' chemicals [1]. This health endpoint is of considerable concern. Therefore, the aim of this case study will be to evaluate the potential for a human health risk due to the present exposure to complex mixtures of 'anti-androgenic' chemicals based on our current knowledge. We build on the expertise of the involved partners, who over the last decades have published several papers on the identity, mixture effects and human exposure to antiandrogenic chemicals [2,3,4,5].

The group of anti-androgenic chemicals is expected to be very diverse, including phthalates, bisphenols and other phenolic substances, certain pesticides, PAHs, fluorinated chemicals and even some pharmaceuticals such as analgesics. Thus, several of the prioritized chemical groups in HBM4EU belong to the 'antiandrogenic' chemicals. This case study will be ideal to link with the work on biomarkers of effect that is ongoing in WP 14. These biomarkers focus among other things on responses to anti-androgens in cell-based bioassays.

3.3.2 Selected chemicals

Phthalates, bisphenols, phenolic compounds, PAHs, PCBs, fluorinated chemicals, pesticides, various drugs

3.3.3 Approach and methods

The approach will follow the general path from the two previous case studies, assembling exposure and hazard data in similar fashion. This includes the steps below:

1. Identify the known and widespread 'antiandrogenic' chemicals and drugs which humans are exposed to and gather existing literature on 'antiandrogenic' mixtures. To this end, we will build on earlier work in WP14 and scoping documents of priority substances. Chemicals will include, but are not limited to: Phthalates, bisphenols, PAHs, PCBs, fluorinated chemicals, FTOHs, pesticides, drugs. Deadline: M12, 2018
2. Collecting hazard data from available sources including in vitro assays (Androgen Receptor reporter gene assay and the H295R steroidogenesis assay), ex vivo assays and, if available, in vivo data. Priority will be given to human-relevant experimental data. Deadline: M6, 2019
3. In parallel collect relevant exposure data from available sources (human exposure levels, Cmax values for drugs, internal exposure levels). UGR will use WP10 Overview of Metadata, explore IPCHEM sources and existing EU projects, to gather relevant exposure and HBM data. Deadline: M6, 2019
4. Calculate Hazard Quotients and Hazard Index over the summer of 2019. Deadline: Oct 2019
5. Refinement and update according to likelihood of co-exposure. Deadline: Dec 2019
6. Sharing and integrating of the outcomes with other WPs. Final cases study report Deadline: March, 2020

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3.3.4 Link with other HBM4EU work packages

We will use available exposure and hazard information from WP10 and WP14 and we will link this work to investigations of antiandrogenic effects in placenta extracts, which is ongoing in WP14 (in AR reporter gene assay and H295R steroidogenesis assay). This work is expected to generate further leads for chemicals to include in this case study. This biomarker activity will be linked to HBM data obtained for the extracts, and in WP16 emerging chemicals are identified in the extracts. The sources of the integrated mixture effects in the placentas will be investigated and may inform the overall mixture evaluation. Results will be shared with WP5.

3.3.5 Indication of time lines and work phases within HBM4EU

Time line is already indicated in section 3.3.3 and depicted in the Gantt chart at the end of this document (best seen on A3 format paper).

3.3.6 Indication of team / partners

The case study will be led by DTU, with the involvement of UBRUN, UGR and UBA.

3.3.7 Products generated

The outcome will be a comprehensive, up-to-date mixture assessment on antiandrogenic chemicals with the purpose of evaluating human safety to these chemicals. For the first time such an assessment will be based on HBM data as the exposure measure.

Milestone August 2019: Internal report and catalogue/database summarizing hazard and exposure data for antiandrogenic chemicals under study

Deliverable, March 2020: A report on the mixture risk assessment of antiandrogenic chemicals and a draft for a publication.

3.3.8 Multi-year perspective

In the beginning of 2020 we would have obtained a human health risk assessment to all currently known antiandrogenic chemicals. This knowledge is expected to feed into the output of WP5 that is focussing on risk assessment and links to the regulatory system for both single chemicals as well as mixtures. Furthermore the collaboration in Pillar 3 in WP14, WP15 and WP16 has the potential to create synergy with respect to identification of new antiandrogenic chemicals, tools how to use HBM data for mixture risk assessment and tools on how to use bioassays for measuring the integrated antiandrogenic effect in human samples.

3.3.9 References

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2. Kortenkamp, A. Ten Years of Mixing Cocktails: A Review of Combination Effects of Endocrine-Disrupting Chemicals. *Environ Health Perspect.* 115 (1): 98–105, 2007
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3.4 Chromium (VI), nickel and polycyclic aromatic hydrocarbons and lung cancer

3.4.1 Rationale

There is data evidencing human exposure to polycyclic aromatic hydrocarbons (PAHs), chromium (VI) and nickel. Concerning biomarkers of effect, the micronucleus analysis in human peripheral lymphocytes is a validated and relevant biomarker of effect for carcinogenicity, including lung cancer (Bonassi et al., 2011) and micronuclei are induced by the previously referred mixtures (Peng et al 2015; Muthusamy et al 2018). One common adverse outcome of these exposures is lung cancer. Several studies developed in North American (Proctor et al. 2016, Beveridge et al. 2010), European (Singh et al. 2018, Bugge et al. 2010, Bjor et al. 2008, Rønneberg et al. 1999) and Asian (Singh et al. 2018, Lee et al. 2010, Hara et al. 2010, Leem et al. 2010) populations showed associations between exposure to PAHs or to chromium and this form of cancer. However, none of those studies analysed or interpreted the results or health outcomes from a mixture perspective, i.e., none considered the potential combined effects of those substances as determinants of lung cancer.

On the other hand, there are several lines of evidence from *in vitro* and *in vivo* experimentation that PAHs/chromium/nickel mixtures lead to adverse effects that are different from the ones observed after exposure to each single substance, namely: more-than-additive effects *in vitro* (Feng et al., 2003; Peng et al 2015; Muthusamy et al 2018) and *in vivo* (Sánchez-Martín et al. 2015); synergistic effects in ecotoxicity studies (Gauthier et al, 2015; Fleeger et al. 2007; Chigbo et al. 2013).

Within the HBM4EU, a study on the occupational exposure to chromate has been planned to start in mid 2018 and involves the characterization of several workplaces where exposure to Cr(VI) occurs. In addition, it is known that there is no single exposure to chromate and that, in those workplaces, exposure to nickel and, in some of them to PAHs, also occurs. In that study, apart from the environmental characterization, also biomarkers of exposure to Cr(VI) and biomarkers of effect (oxidative damage, micronuclei and epigenetic effects) will be analysed in order to link exposure to health effects (see AD 8.2).^{iv}

Thus, considering: i) the above mentioned lack of data on PAHs-chromium-nickel mixture effects in humans; ii) the fact that this co-exposure can happen in several occupational settings, such as welding, cars and aircrafts maintenance and much others implicating exposure of a high number of workers; iii) the published epidemiological data about exposure to each substance and lung cancer without taking into account possible interactive effects; iv) the existence of suitable biomarkers of exposure and effect regarding these substances; v) the planned study on the occupational exposure to chromate providing an opportunity to obtain new data on exposure and effect, a new case study is proposed

3.4.2 Selected chemicals

PAH, chromium (VI) and nickel

^{iv} Although the inclusion of biomarkers of exposure to nickel or to PAHs has not been planned, their measurements in post-shift urine samples (preserved at -20 °C) could be proposed, so that a more complete exposure scenario could be obtained. This would be a very good opportunity to link this new study to WP 15, and to look at the results from the mixtures point of view, both in terms of co-exposure and combined effects. However, this is currently not included in this case study and would need further elaboration.

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3.4.3 Approach and methods

The approach will be similar to the ones described in the earlier described case studies.

Phase 1: We will first screen the literature of for available epidemiological studies on existing cohorts, including the available WP10 databases and build on existing reviews, the WP14 activities and input from CGL's in the second half of 2018.

This will be followed by a search for occupational cohorts in the settings mentioned above, with the aim of assessing whether there are differences in lung cancer incidences relative to cohorts where exposure to only one of the contaminants, PAH, chromium (VI) or nickel, is reported.

We will compile relevant exposure data and hazard (by April 2019) to construct hazard quotients derived by using relevant occupational exposure limits for PAHs, chromium (VI) and nickel, followed by the determination of the Hazard Index (HI). We foresee a tiered assessment, where in the lower tiers of the analysis occupational limits set for different health endpoints are combined.

Phase 2: We will refine the exposure assessment arm of the mixture risk assessment by close analysis of the occupational hygiene data present in reports of the companies enrolled in the chromate occupational exposure study. In this way, we can verify and characterize the exposure to PAHs and nickel.^v Start of this phase depends on the availability of the data from WP8.5, expected by the end of 2019. Integration of these data will then start early 2020, with triangulation against epidemiological information over the summer 2020 with an end report by November 2020.

3.4.4 Link with other HBM4EU work packages

We foresee links with Pillar 1: WP5 (review on RA of PAHs using HBM); Pillar 2: WP8 (the chromate occupational study) and WP10 (databases for PAHs and Cr(VI)); Pillar 3: WP13 (exposure-health relationships) and WP14 (effect biomarkers).

3.4.5 Indication of time lines and work phases within HBM4EU

Time line is already indicated in section 3.4.3 and depicted in the Gantt chart at the end of this document (best seen on A3 format paper).

3.4.6 Indication of team / partners

The case study will be led by INSA, with the involvement of ESTeSL and UU. The following partners will also be expected to contribute and liaise with other WP's: FIOH (WP8), RIVM (WP13) and UGR (WP14).

3.4.7 Products generated

We foresee a first interim report using the HI approach towards the end of the first semester of 2019 August 2019.

By the end of the second semester of 2020 further refinements of the analysis using some real exposure values measured in occupational settings (for chromium and nickel) will have been completed and reported in November 2020.

We strive to disseminate the results of this work in a high impact open access journal; drafting of the manuscript is foreseen for 2020.

^v We envisage the possibility of expanding the study on occupational exposure to chromate (WP 8), to include complementary measurements of PAHs (and Ni) in environmental and biological samples, if feasible. Post-shift urine samples from Cr(VI) exposed workers could be preserved for future Ni and PAHs analyses, reshaping the chromate study into a chromate nickel and PAHs mixtures study. Effect biomarkers such as the micronucleus assay in human lymphocytes are planned to be analysed in the above mentioned chromate study, which have been associated with increased risk of cancer. There are large datasets available relating micronuclei with cancer risk that could be re-analysed to look for evidences of mixtures effects. However, this is currently not included in this case study and would need further elaboration.

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3.4.8 Multi-year perspective

Prospectively, the new data generated from the perspective of a mixture risk assessment will be used to reanalyse the epidemiologic data currently available for occupational exposure to chromium. This case study will profit from a new exposure and effect biomarkers data to refine the HI previously defined and to disclose some pitfalls of using the variables commonly considered in the epidemiologic data, i.e., single exposure-single effect instead of multiple exposure-combined effect. This will allow to refine occupational data inputs and to foresee the delineation of other studies considering the mixture toxicity in specific occupational settings.

3.5 Addressing exposure misclassification in mixture studies

3.5.1 Rationale

The characterization of health effects of modern high production chemicals in humans is hampered by the strong variability of their levels in humans, and the fact that most past epidemiological studies assessed exposures on spot biospecimens, leading to exposure misclassification (Slama et al. 2017). Exposure misclassification can strongly impact exposure-response relationships. While there is a wide literature on this topic in general, few studies have addressed this issue for HBM. To our knowledge, in the literature on mixture health effects the issue has been largely ignored. In the case of classical-type error, i.e. when the estimated exposure randomly varies around the true exposure, naïve models not accounting for measurement error suffer a loss of statistical power and provide estimates that are attenuated towards the null (Perrier et al. 2016). Design related options to correct for exposure misclassification exist. When exposures are assessed through biomarkers, the within-subject biospecimens pooling, consisting in collecting several biospecimens per subject during a toxicologically relevant time window and pooling them, has been shown to be an efficient way to limit attenuation bias and increase power, compared to studies based on a spot biospecimen, in the context when the focus is on a single exposure.

In an exposome (or mixture) context, the effect of measurement error may be compounded. The mis-selection issue that has been observed in an exposome context is expected to be amplified in the case of exposure misclassification: variable selection algorithms may mistakenly attribute the effect of an exposure measured with substantial error to another correlated exposure measured with smaller error, an issue called differential exposure misclassification (Slama et al 2015).

In the case study, we aim to assess the performances of within-subject biospecimen pooling in handling differential exposure misclassification and reducing bias in dose-response functions in an exposome context, i.e. a situation in which one wants to simultaneously characterize the impact of a large number of exposure on a health outcome. We will also assess the effect of measurement error and misclassification on the correlation structure and patterns in mixtures of co-pollutants in the same individual, linking our work to the activities in task 15.1.

3.5.2 Selected chemicals or health endpoints

The case study and its results apply to potentially all types of health effects, although the simulation will focus on health or biological parameters assessed on a continuous scale. The case study will not be specific to a chemical but will apply to the situation of an exposome (or mixture) study in which a large number of chemicals with varying toxicokinetic properties are assessed (typically including persistent compounds such as PCBs) and compounds with highly temporally variable urinary concentrations such as members of the phenols and phthalates families.

3.5.3 Approach and methods

The approach consists of a simulation study, which will allow comparing the results of hypothetical studies with exposure misclassification to what would be observed in studies with a perfect assessment of all exposures. We will obtain the input information for the simulations from existing

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data in exposome studies and from existing HBM mixture data. We will expand and combine two former simulation studies from our group dealing 1) with the identification of the effect of exposures on health in an exposome context but without exposure misclassification by Agier et al. (2016) and 2) with the efficiency of the within-subject biospecimens pooling approach to reduce bias due to exposure misclassification developed in a single-exposure context by Perrier et al (2016). More specifically:

1. We will simulate realistic exposome data in a fictitious population; the correlation structure between the individual exposures will be based on realistic exposome data (Agier et al 2016). We will assume that exposures are assessed with biospecimens, with classical type error, the amount of error varying between compounds, some having low and other high within subject variability, using realistic data on the variability of chemical urinary concentrations during pregnancy. When results of 15.1 come available later in 2019, we will assess whether alternative patterns in correlation structure need to be assessed.
2. We will assume that a small number of exposures impact the health outcome and
3. Compare the efficiency of an approach in which a single biospecimen is collected in each subject to assess exposures, with an approach in which an increasing number of biospecimens are being collected and pooled. The efficiency of each approach will be characterized by the sensitivity and specificity to detect compounds really affecting health, as well as bias in dose-response relations, and compared to what would be observed if the true exposure levels had been available.
4. In a further stage, this simulation will be extended to consider the case of “synergy” (e.g., supra-additive effects) between exposures, again expanding a previous simulation study in an exposome context ignoring exposure misclassification (Barrera-Gomez et al. 2017).
5. We will also simulate the effect of classical error on the correlation structure and patterns in individual mixture data, in relation to the task 15.1 activities.

3.5.4 Link with other HBM4EU work packages

Pillar 3: WP 13 (exposure health relationships, AOP); results will be shared with WP5 (impact of classical error on exposure-response functions and meaning for risk assessment) and WP8 Design of future surveys.

3.5.5 Indication of time lines and work phases within HBM4EU

Time line is depicted in the Gantt chart at the end of this document (best seen on A3 format paper). Development of the data simulation plan will start in the second half of 2018, followed by selection and definition of the input data to the simulation. The actual simulation and analysis will start early 2019, with a first report expected August 2019. Based on results from the analysis of existing HBM mixture data, simulation of bias in correlation structures in mixture data will be performed with expected reporting in April 2020.

3.5.6 Indication of team / partners

Inserm will lead the case study; ISGlobal and RIVM will provide information about correlation patterns in existing HBM mixture data.

