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Implementation of effect biomarkers in human biomonitoring studies: A systematic approach synergizing toxicological and epidemiological knowledge

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ABSTRACT

Human biomonitoring (HBM) studies have highlighted widespread daily exposure to environmental chemicals. Some of these are suspected to contribute to adverse health outcomes such as reproductive, neurological, and metabolic disorders, among other developmental and chronic impairments. One of the objectives of the H2020 European Human Biomonitoring Initiative (HBM4EU) was the development of informative effect biomarkers for application in a more systematic and harmonized way in large-scale European HBM studies. The inclusion of

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Environmental chemicals
Occupational chemicals

effect biomarkers would complement exposure data with mechanistically-based information on early and late adverse effects. For this purpose, a stepwise strategy was developed to identify and implement a panel of validated effect biomarkers in European HBM studies.

This work offers an overview of the complete procedure followed, from comprehensive literature search strategies, selection of criteria for effect biomarkers and their classification and prioritization, based on toxicological data and adverse outcomes, to pilot studies for their analytical, physiological, and epidemiological validation. We present the example of one study that demonstrated the mediating role of the effect biomarker status of brain-derived neurotrophic factor BDNF in the longitudinal association between infant bisphenol A (BPA) exposure and behavioral function in adolescence.

A panel of effect biomarkers has been implemented in the HBM4EU Aligned Studies as main outcomes, including traditional oxidative stress, reproductive, and thyroid hormone biomarkers. Novel biomarkers of effect, such as DNA methylation status of BDNF and kisspeptin (KISS) genes were also evaluated as molecular markers of neurological and reproductive health, respectively. A panel of effect biomarkers has also been applied in HBM4EU occupational studies, such as micronucleus analysis in lymphocytes and reticulocytes, whole blood comet assay, and malondialdehyde, 8-oxo-2′-deoxyguanosine and untargeted metabolomic profile in urine, to investigate, for example, biological changes in response to hexavalent chromium Cr(VI) exposure.

The use of effect biomarkers in HBM4EU has demonstrated their ability to detect early biological effects of chemical exposure and to identify subgroups that are at higher risk. The roadmap developed in HBM4EU confirms the utility of effect biomarkers, and support one of the main objectives of HBM research, which is to link exposure biomarkers to mechanistically validated effect and susceptibility biomarkers in order to better understand the public health implications of human exposure to environmental chemicals.

1. Biomarkers: A paradigm shift in environmental health

Health impairments associated with human exposure to certain environmental chemicals are of major public health concern (Schug et al., 2016; Zare-Jeddi et al., 2021). Toxicological and epidemiological studies have implemented biological markers, so-called biomarkers, to evaluate environmental chemical exposure and subsequent adverse health effects. This approach represents an improvement over classical toxicological methods, which were not sufficiently sensitive to identify intermediate events between exposure and clinical disease. Biomarkers have led to a revolution in epidemiology (Schisterman and Albert, 2012) by strengthening the evidence of causality between chemical exposure and the risk of adverse effects, especially at an early stage before disease onset (DeCaprio, 1997). Biomarkers of effect may therefore play a pivotal role in disease prevention.

In a pioneering step, the U.S. National Research Council (NRC) provided a biomarker-based knowledge framework ('black box' in Fig. 1.1) designed to identify gaps in knowledge around the cascade of events along the continuum between chemical exposure and effect, *i.e.,* from (i) internal dose to (ii) biologically effective dose, (iii) early biological effect, and (iv) altered structure/function (Fig. 1.2). These events are divided between those referred to biomarkers of exposure [(i) and (ii)] and those referred to biomarkers of effect [(iii) and (iv)]. Thus, exposure biomarkers measure the absorbed ("internal") and active doses in the putative target organ/tissue ("biologically effective dose"), whereas effect biomarkers are related to molecular and/or cellular alterations along temporal and mechanistic pathways. In this way, environmental chemical exposure can be connected to a potential alteration in human health and, eventually, a disease (NRC, 2006).

Nevertheless, these assignments are not exclusive, given that the distinction between events can sometimes be unclear. For example, depending on the study design, DNA adducts may be considered biomarkers of exposure or effect (Castaño-Vinyals et al., 2004). Consequently, these events should be conceptualized as a continuum rather than a series of isolated events (Grandjean, 1995). Furthermore, in this complex scenario, susceptibility biomarkers would be useful to identify individuals with a particular intrinsic (genetic/epigenetic) predisposition to xenobiotic-induced toxicity (Mustieles et al., 2020a). The HBM4EU effect biomarker working group proposed a systematic and multilevel integration of different streams of evidence within this conceptual framework after observing a parallelism between the sequence of events at toxicological level with the adverse outcome pathway (AOP)

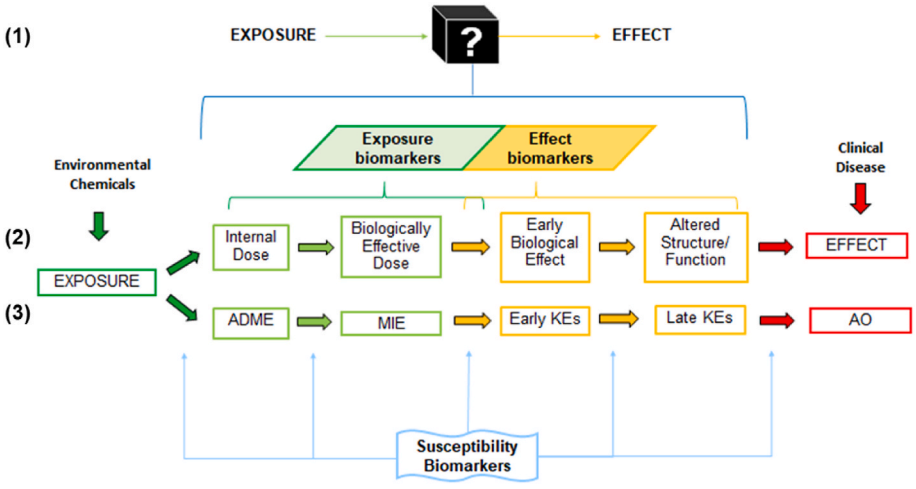

Fig. 1. Epidemiological 'Black Box' according to the classic model linking exposure with disease (1), the NRC biomarker paradigm (2), and the Adverse Outcome Pathway (AOP) (3). Fig. 1.2 and 1.3 show the equivalence between the biomarker paradigm and its parallelism with the AOP. Figure adapted from (Mustieles et al., 2020a) with some modifications. ADME: Absorption, Distribution, Metabolism and Excretion; MIE: Molecular initiating event; KE: Key event; AO: Adverse outcome.

concept (Fig. 1.3). AOPs are pragmatic constructs from toxicological studies that organize the sequential chain of causally linked events triggered by a endogenous or exogenous chemical leading to an adverse health effect (Escher et al., 2017; Louro et al., 2019; Tollefsen et al., 2014). When effect biomarkers coincide with key events depicted in AOPs, the utilization of AOPs can synergize and align toxicological and epidemiological knowledge. This can contribute to a better understanding of the biological fingerprint generated by exposure to environmental chemicals.

The implementation of effect biomarkers has not yet been fully developed in the context of human biomonitoring programs (HBMs) or occupational studies. It is therefore necessary to pay particular attention to the detailed definition of their properties. For example, among many other aspects, the biological meaning of the proposed effect biomarker should be validated at analytical, toxicological, and physiological levels before its use in these studies. In addition, it is necessary to take into account the type of biomarker (novel or classical), the matrix in which it is determined, and the measurement time (Rodriguez-Carrillo, 2022), among others. All these previous screening steps would improve the interpretation of exposure-effect relationships and would fulfill the final goal of the NRC: "*the ultimate objective of biomonitoring is to link information on exposures, susceptibility, and effects to understand the public health implications of exposure to environmental chemicals*" (NRC, 2006).

1.1. Biomarkers of adverse effects

Effect biomarkers are associated with a physiological system rather than with exposure to a given xenobiotic. They are therefore defined as a biochemical, physiological, behavioral, or other quantifiable alteration in an organism that, depending on its magnitude, may be associated with an established or potential health impairment or disease (Baken et al., 2019; Mustieles et al., 2020; Zare-Jeddi et al., 2021). Consequently, biomarkers of effect can, *a priori*, predict adverse effects at different levels of biological organization, from molecules, cells, or tissues to organs or systems (e.g., testes, brain, kidney function). They can also be associated with a wide range of environmental chemicals (bisphenols, perfluoroalkyl substances [PFAS], phthalates, heavy metals such as arsenic or the pesticide chlorpyrifos, among many others), providing the link between internal exposure and its early and late health effects (Mustieles et al., 2020; Zare-Jeddi et al., 2021).

Effect biomarkers or key events that occur early in the interaction of a xenobiotic with a biological target could be considered "early biomarkers of effect". Examples would include epigenetic markers, enzyme induction/inhibition, DNA adducts, DNA strand breaks, chromosomal aberrations (changes in chromosome number [gains/losses] or structure [deletions, inversions, and exchanges]), sister chromatid exchange, or the number of mutations in the micronuclei assay, using this last parameter as genotoxicity endpoint (Tsitsimpikou et al., 2013; Weinhouse et al., 2015). For their part, effect biomarkers measuring functional or structural changes in affected tissues or systems ("altered structure/function") or actual clinical disease are known as "late biomarkers of effect" (e.g., elevated serum glucose and lipid levels relevant to metabolic disease). Consequently, a wide variety of biomarkers can be selected, ranging from biochemical biomarkers, such as metabolic parameters and hormone levels, to other markers that provide quantitative information about the human body, including magnetic resonance imaging (MRI), behavioral tests, or anogenital distance (Rodriguez-Carrillo, 2022; Ventura et al., 2021; Zare-Jeddi et al., 2021). Ultimately, effect biomarkers may enhance the identification of early effects caused by chemical stressors in humans, establish exposure-effect relationships to identify possible modes of action, and increase the biological plausibility of established epidemiological associations. They could therefore improve the risk assessment of a given chemical and/or complex chemical mixture (Baken et al., 2019; Rodríguez-Carrillo et al., 2021).

1.2. Biomarkers of combined effect or biological activity

People are generally exposed to mixtures of multiples chemicals *via* single or multiple sources and routes of exposure (Bopp et al., 2018). Effect biomarkers also have the potential to map biological effects resulting from exposure to mixtures of environmental chemicals, which can be chemically isolated and identified from the chosen human matrix (urine, blood/serum, milk, or placenta) or from *ad hoc* mixtures of chemical compounds. In this context, an effect biomarker can also be considered as an integrated biological activity measurable by *in vitro* assays, with the potential to assess the combined action of the mixture on endocrine receptors or other physiological targets. These biomarkers are also known as "combined internal exposure biomarkers", "combined biological activity biomarkers" or "*ex vivo* hormone activity biomarkers" (Rodríguez-Carrillo et al., 2021; Vinggaard et al., 2021). Their major advantage is their capacity to evaluate real-world chemical mixtures using different human biological matrices. The main limitation is that individual components of the mixture are often unknown, representing a barrier to regulatory acceptance. Notwithstanding, new analytical methods allow the isolation of specific chemical families (for example, PFAS) to assess their combined effect using *in vivo* and/or *in vitro* assays, thereby increasing their regulatory uptake (Bjerregaard-Olesen et al., 2019). However, this approach does not take account of differences in the potency of individual components of the mixture, which must be characterized separately.

Some epidemiological studies have already used these biomarkers to assess the effects of complex mixtures of persistent organic pollutants on diverse human health outcomes, such as the development of breast cancer in adults or the risk of cryptorchidism and hypospadias in newborns. These studies showed that the estrogenic or antiandrogenic effect of chemical mixtures isolated from human biological matrices were linked to an increased risk of breast cancer or male urogenital malformations; these results therefore complement the one-chemical-at-a-time classical approach to exposure-health associations followed in epidemiological studies (Arrebola et al., 2015; Bonefeld-Jorgensen et al., 2011; Ibarluzea et al., 2004; Pastor-Barriuso et al., 2016; Vinggaard et al., 2021; Wielsøe et al., 2018).

2. Selection criteria: Effect biomarkers

The aforementioned framework for biomarkers of effect formed the basis for their search, identification, preliminary validation, testing, and implementation within the European project (HBM4EU). Biomarkers of effect selected for application in HBM studies should meet the following criteria:

a) **Non/Low-invasive but predictive:** they should be measured, mainly in peripheral blood, serum, or urine matrices, and correlated with the physiological response in the target tissue (Mustieles et al., 2020a; Vineis et al., 2013).
b) **Sensitive:** the biomarker of effect must change in response to the exposure to environmental chemical compounds to a degree that allows the alterations caused to be detected. The biomarker should also allow reliable measurement of biological changes, providing an accurate, precise, reproducible, interpretable, and predictive measurement of the health outcome with which they were correlated.
c) **Population variability and discriminative power**: there should be sufficient population variability and measurement range to enable the discrimination of the health status of individuals (healthy vs. unhealthy) and the characterization of inter-individual variability and to ensure that sensitive populations are identified and adequately addressed in the assessments.
d) The **analytical performance** of the biomarker should:
   - **Provide specific and sensitive measurements,** free of potential interferences, detecting as many samples as possible (if the effect

biomarker is poorly "expressed or concentrated" in the matrix, it is more difficult to measure).
- **Be reproducible**, showing an adequate intra- and inter-assay coefficient of variation to ensure the reproducibility of measurements.

e) Analytical and clinical validation studies must show that the effect biomarker is appropriate for its intended use.

Within the HBM4EU Initiative, health effects elicited by exposure to the prioritized chemical families were also selected, and biomarker data on exposure to pollutants of particular interest were combined with biomarkers of effect. A stepwise strategy (identification, prioritization, and validation) was also followed before the implementation of effect biomarkers in HBM4EU-aligned studies (Gilles et al., 2022, 2021). The main process is detailed below; please see the HBM4EU project website https://www.hbm4eu.eu/for more information on deliverables and additional deliverables: D14.1, D14.2, D14.3, D14.4, AD14.4, AD14.6, D14.7, and D14.8 (Fernandez et al., 2019a; Fernandez et al., 2019b; Fernandez et al., 2021; Mustieles et al., 2018a, 2018c, 2019, 2020b; Olea et al., 2017; Rodríguez-Carrillo et al., 2020).

3. Process & implementation of effect biomarkers in HBM studies

The entire process is graphically summarized in Fig. 2 and briefly explained below.

3.1. Literature searches and inventory of effect biomarkers

Fourteen comprehensive literature searches were conducted in PubMed and Scopus databases, each covering one of the HBM4EU prioritized chemical substance groups (Ougier et al., 2021). Relevant health outcomes (reproduction, neurodevelopment, cancer, allergies, immune system, cardiovascular diseases, obesity, and oxidative stress) and prioritized chemical substance groups (bisphenols, cadmium, chromium IV, flame retardants, polycyclic aromatic hydrocarbons, per-/poly-fluorinated compounds, phthalates, acrylamide, arsenic, lead, mercury, mycotoxins, pesticides, and benzophenones) were chosen as key search terms. Boolean terms e.g., 'AND', 'OR', were combined with key terms to complete the search strategy. Successively, the following PubMed filters a) Full-text articles and b) articles published in no more than 10 years, were used to gain precision. The screening for potential relevant studies consisted on two stages: abstract screening and full-text screening. In both stages, the following selection criteria were: a) articles including effect biomarkers measurement, b) original research articles, and c) epidemiological or toxicological studies, preferably the first case. At least two scientists were involved at each stage to ensure minimal loss of information. Thus, epidemiological and/or toxicological studies that used effect biomarkers to assess the possible impact of relevant chemical compounds were selected. Detailed information of each literature search can be found in some of the articles published in HBM4EU (Bajard et al., 2021; Baken et al., 2019; Boesen et al., 2020; Gundacker et al., 2021; Mustieles et al., 2020a, 2023).

In the analysis of relevant articles, predefined selection criteria were used to select potential effect biomarkers with added value for HBM studies. This process was developed in combination with the information provided by toxicological data, preferentially organized following the AOP framework (visit AOP wiki webpage) (Mustieles et al., 2020a), allowing their classification according to their biological plausibility. A panel of relevant and toxicologically supported biomarkers of effect was established (Table 1), classified according to their previous use in epidemiological and clinical settings as follows:

- **Classical/clinical effect biomarkers**: these are markers with sufficient knowledge regarding their physiological roles and extensively used and/or with available reference levels in clinical and epidemiological settings, including reproductive hormones, thyroid hormones, biomarkers of glucose metabolism, and biomarkers of

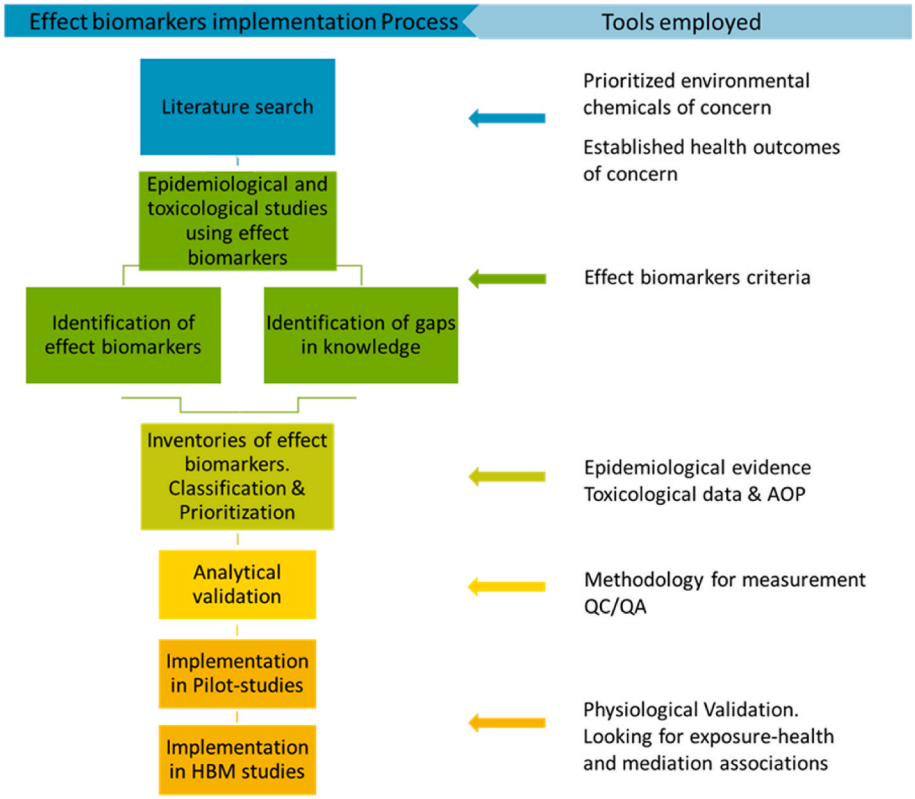

Fig. 2. Full procedure developed in HBM4EU for the search, identification, prioritization, validation, and implementation of effect biomarkers in HBM studies.

Table 1
Available effect biomarkers considered for their implementation in HBM studies.

Effect Biomarkers		
Classical (studied)	Classical (less studied)	Novel
Reproductive Hormones: Estradiol (E_2), total testosterone (TT), follicle stimulating hormone (FSH), luteinizing hormone (LH), sex hormone-binding globulin (SHBG). **Sperm quality:** Sperm counts, concentration, motility and morphology	**HP-Adrenal Axis, psychological stress:** Cortisol, hypothalamic corticotrophin-releasing factor (CRF), corticosterone (CORT), hippocampal 11-hydroxysteroid dehydrogenase type 1 (11-HSD 1), subcellular glucocorticoid receptor (GR)	**Reproductive Molecular:** Kisspeptins
Thyroid Hormones: thyroxine (T4), triiodo thyronine (T3), thyroid stimulating hormone (TSH)	**Adipokines:** Leptin, adiponectin	**Gene expression of nuclear receptors**: Peroxisome proliferator-activated receptor gamma (PPARγ), estrogen receptor (ER), androgen receptor (AR), aryl hydrocarbon receptor (AhR).
Glucose metabolism: Insulin (INS), glucose (GLU), homeostatic model assessment HOMA, Fasting blood glucose (FBG), fasting insulin levels, glycated hemoglobin (HbA1c)	**Inflammatory markers**: Tumor necrotic factor (TNF-α), immunoglobulin E (IgE), interleukins 1, 6 (IL1, 6), Leukotriene B4 (LTB4), Protein complement 3, 3a, 4 (C3, C3a, C4). Chemokines, C-reactive protein (CRP), neutrophil count.	**Neurological Molecular:** Brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), transcription factor Sp4 (Sp4).
Serum lipids: Total cholesterol (CHO), high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides (TRY).	**Liver enzymes:** Aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT), bilirubin, albumin, gamma-glutamyl transferase (GGT), serum bilirubin, prothrombin time (PT), international normalized ratio (INR)	**Epigenetic markers:** DNA methylation/ demethylation (5-methylcytosine, 5-hydroxy-methylcytosine, DNA-methyltransferases, ten-eleven translocations genes), histones modifications, methylated arginines, dimethyl arginine; 7-nAchR expression, MAPK expression, protein expression PSD-95, SYP, miRNA-219, CaMKII; H3K18ac, H3K9me2, H3K36me3, GR mRNA, H-Ras protein, Raf-1 protein, ERK expression, global DNA methylation from leukocytes
Cardiovascular: Electrocardiographic (ECG) parameters (QT interval, JT interval, PR interval, QRS duration, and QT dispersion). Blood pressure: systolic, diastolic, and pulse blood pressure. 1st-, 2nd-and 3rd-min Heart Rate Recovery (HRR), carotid intima media thickness (cIMT).	**Renal function:** N-acetyl-β-D glucosaminidase (NAG), Kim-1: kidney injury molecule-1 (Kim-1), urinary creatinine, β2-microglobulin (B2-MG), α1-microglobulin, retinol-binding protein, albumin, transferrin, IgG, urinary B2-MG, Neutrophil gelatinase-associated lipocalin (NGAL), protein carbonyls.	
Genotoxic markers: Chromosomal aberrations, sister chromatid exchange, micronucleus test	**Oxidative stress & DNA damage markers:** Urinary 8-hydroxy-2′ -deoxyguanosine (8-OHdG) Lipid peroxidation: 8-iso-prostane, alkaline comet assay, malondialdehyde (MDA) **Antioxidant defense:** Glutathione peroxidase (GPx), selenium, and glutathione (GSH)	**Cancer markers:** Plasma carcinoembryonic antigen: neuron specific enolase (NSE), squamous cell carcinoma antigen (SCC), cytokeratin fragment antigen 21–1 (CYFRA21-1), cancer antigen 72–4) (CA72-4), α-fetoprotein (AFP), 3-nitrotyro- sine, prostate-specific antigen, high sensitive C reactive protein, Clara cell secretory protein (CC16), surfactant protein D (SP-D), plasma total homocysteine
Anthropometric indices: Body mass index (BMI), body fat mass, height, weight, waist circumference, hip circumference, waist to height ratio, waist to hip ratio, birth weight, birth length, head circumference, anogenital distance (AGT). Fetal biometry [biparietal diameter (BPD), femur length (FL), abdominal circumference (AC) and estimated fetal weight (EFW)].		
Neurophysiological domains: Child Behavior Check List (CBCL), Strengths and difficulties questionnaire (SDQ), Behavior Assessment System for Children (BASC), Behavior Rating (BRIEF-P), Wechsler Intelligence Scale for Children (WISC), Social Behavior (Skills Improvement Rating scale), (WISC-IV: Standard Progressive matrices test		

HP-Adrenal Axis: Hypothalamic pituitary adrenal axis.

genotoxicity such as oxidative DNA damage, protein and DNA adducts, frequency of micronuclei in human lymphocytes, single and double DNA strand breaks, and chromosomal alterations (Annangi et al., 2016; Balachandar et al., 2010; Fenech et al., 2011; Junaid et al., 2016).

- **Classical/clinical effect biomarkers that are less well studied:** those with previous knowledge on their physiological mechanism of action but with limited epidemiologic data, such as adrenal hormones, adipokines, inflammatory proteins, or urinary 8-hydroxy-2-deoxyguanosine (8-OHdG), among others.
- **Novel effect biomarkers:** those little (or rarely) used in clinical or epidemiological settings, such as kisspeptin, brain-derived neurotrophic factor (BDNF), gene expression of nuclear receptor, or epigenetic markers.

An **inventory of currently available effect biomarkers and**

promising new biomarkers that can be used to assess the health impact of chemical exposure in the general population was first developed (Fernandez et al., 2019b; Mustieles et al., 2018b, 2019; 2023) in accordance with human developmental windows selected for HBM4EU Aligned Studies (*i.e.,* childhood, adolescence, and adulthood). Effect biomarkers were prioritized according to: i) the aforementioned inventory, ii) gaps in knowledge identified during the comprehensive literature searches, iii) their toxicological relevance provided by AOP information, and iv) the sample availability and adequacy of the Aligned Studies. Selected new effect biomarkers include BDNF as a marker for neurodevelopmental disorders and kisspeptin for reproductive disorders (Gundacker et al., 2021; Mustieles et al., 2020a) (Table 1).

We proposed investigation of the complete exposure-effect continuum from molecular through clinical to health outcome levels within the HBM4EU project to strengthen the weight of evidence for novel biomarkers. For example, promoter methylation and/or histone marks of the BDNF and kisspeptin gene measured in peripheral blood mononuclear cells (PBMC), were hypothesized to be representative of gene expression levels of the selected genes in both PBMCs and relevant target tissues. Thus, protein levels and DNA methylation patterns of the gene encoding BDNF and kisspeptin were set as main targets for physiological and analytical validation analyses. The information provided by these novel effect biomarkers was complemented with clinical biomarkers prioritized according to their implication in BDNF and kisspeptin signaling cascades (potential AOPs) and the epidemiological evidence (Mustieles et al., 2020a) as follows: thyroid hormones, reproductive hormones, glucose metabolism, serum lipids, and oxidative stress markers.

Some nuclear receptors (androgen receptor-AR-, estrogen-related receptor -ERα, ERβ, ERRγ-, peroxisome proliferator-activated receptor -PPARα, PPARγ-, glucocorticoid receptor -GR-, and thyroid receptor -TR-activation) potentially affected by multiple environmental chemicals and related to various adverse health effects were also considered as promising targets for the development of novel effect biomarkers. Nuclear receptor activity can be assessed *via* transcription factor activity, receptor gene expression, epigenetic modification of the gene promoter region, receptor binding, and transcription of downstream signaling cascade components (Binder et al., 2018; Pawlik-Pachucka et al., 2018).

3.2. Importance of the biological matrices selected for effect biomarker measurement

Human biomonitoring of chemicals measurements provides an estimate of the amount of a chemical absorbed into the body from all pathways of exposure. Importantly, biological samples in HBM should be easily accessible under routine conditions and without health risk for the individual. For these reasons, blood and urine samples are the most widely accepted matrices for evaluating environmental chemicals in occupational and environmental toxicology (Gil and Hernández, 2015). Other non-invasive samples (saliva, hair, dried blood) are increasingly being used for human biomonitoring of chemicals. The measurement of a xenobiotic or its metabolite in a target matrix yields information on the amount of xenobiotic metabolized by the organism and on the magnitude and the time of exposure. A similar concept can be applied to effect biomarkers, given that their biological meaning depends on the matrix chosen, making this choice a crucial decision (Rodriguez-Carrillo, 2022).

The characteristics of the most suitable matrix for measuring biomarkers of effect should be based on its feasibility, invasiveness, volume availability, storage and processing requirements, and on the predictive value of the effect biomarkers measured (Zare-Jeddi et al., 2021). Although some effect biomarkers can be assessed in urine, blood represents a more suitable matrix, as it allows measurement of numerous biomarkers (genetic polymorphisms, genetic and epigenetic changes, and protein levels) in a suitable biological environment (Steffensen et al., 2020). While the analysis of gene expression changes in response to chemical exposure is regularly applied to study chemical toxicity, the analysis of fragile RNA transcripts requires specific conditions for the collection, handling, and storage of blood samples. Sample storage requirements for epigenetic marker analysis are less stringent, and these markers may also be more stable than circulating protein levels. However, harmonized protocols for sample processing, preservation, and storage need to be developed to allow the application of biomarkers of effect under the highest quality standards.

3.3. Analytical validation

Effect biomarker measurements between different laboratories have to be systematically quality assured, it has done in the HBM4EU exposure biomarker ICI/EQUAS comparisons, and, the analytical methodology for effect biomarker assessment must be validated to ensure that it provides reliable and fit-for-purpose results. In addition, the validation of proposed effect biomarkers must be sensitive, reliable, reproducible, and affordable. Accordingly, stringent analytical and quality control (QA/QC) protocols were designed, and in some cases, centralizing biomarker effect measurements were performed in a single laboratory, to avoid potential inter-laboratory heterogeneities, as detailed elsewhere (Fernandez et al., 2021; Rodríguez-Carrillo et al., 2020; Olivas-Martinez et al., 2023). For example, maximum thresholds were established for inter- and intra-variation coefficients (5 and 15%, respectively) in the measurements of protein levels by enzyme-linked immunosorbent assay (ELISA). Moreover, all samples were measured in duplicate and in different assays to reduce assay variability (Rodriguez-Carrillo, 2022; Rodríguez-Carrillo et al., 2020). Further, DNA methylation patterns of BDNF and kisspeptin genes were studied with the gold-standard method, bisulfite pyrosequencing analysis, applying the strict quality controls used in molecular biology (Mustieles et al., 2022).

After prioritization, selection, and analytical validation, the next step was physiological validation of the effect biomarkers for epidemiological purposes, prior to their implementation in HBM4EU aligned studies. Pilot studies were designed for this purpose by taking advantage of available information and biological samples from existing European cohorts.

3.4. Toxicological-epidemiological validation

An example of the integrative framework followed in the HBM4EU (from exposure to adverse outcome) is the implementation of BDNF measurement, a novel biomarker of neurological function that could *a priori* fill some of the knowledge gaps between exposure to chemicals and altered neurodevelopment (Mustieles et al., 2020a), used alongside other classical biomarkers identified in additional post-hoc searches in the AOP-Wiki (https://aopwiki.org/) [e.g., using ''reduced BDNF levels'' (KE 381, https://aopwiki.org/events/381) or ''reduced BDNF expression'' (KE 1329, https://aopwiki.org/events/1329) as search terms] (Martens et al., 2022). Each key event selected in the AOP network was queried in all available *in vivo* toxicology studies. A longitudinal pilot study was then designed to assess the physiological validity of BDNF in the impact of bisphenol A (BPA) on the neurobehavioral function of adolescent males belonging to the INMA-(*Environment and Childhood*)-Granada birth cohort (Mustieles et al., 2022), with the aim of ascertaining:

a) The longitudinal association of childhood BPA (9–11 years) with behavioral function during adolescence (15–17 years).
b) The longitudinal association between childhood BPA (9–11 years) and BDNF as a novel effect biomarker during adolescence (15–17 years).
c) The cross-sectional association between BDNF effect biomarkers and adolescents' behavioral function.

d) The mediation role of BDNF in the potential BPA-behavior association.

Results obtained showed that higher urinary BPA concentrations at 9–11 years were associated with more thought problems among adolescent males at 15–17 yrs (Fig. 3A brown-color bars). as well as with increased BDNF gene DNA methylation, especially at CpG6 (Fig. 3A blue-color bars). Moreover, higher DNA methylation at CpG6 was cross-sectionally associated with thought problems (Fig. 3A green-color bars). On the contrary, neither urinary nor serum BDNF protein levels were associated with BPA concentrations or thought problems. BDNF methylation at CpG6 mediated up to 34% of the BPA-thought problems association (Fig. 3B). It was therefore concluded that the DNA methylation of the BDNF gene may be a valuable biomarker of adverse neurological effect that allows identification of some of the major steps in the continuum from exposure to BPA to neurological function (Mustieles et al., 2022). Although one limitation of this study was the assessment of BPA in a spot urine sample, the expected bias would be towards null findings, meaning that we would be underestimating rather than overestimating effects (Mustieles and Fernández, 2020c).

3.5. HBM-occupational studies

In the framework of the HBM4EU project, the effects of exposure to chemicals in several occupational settings was also monitored through the characterization of effect biomarkers, following harmonized protocols for data gathering and sampling together with exposure biomarker analysis (Santonen et al., 2019, 2022). One example is the multi-center cross-sectional study involving workers from several activities with potential exposure to hexavalent chromium [Cr(VI)], an occupational carcinogen that causes lung, nasal, and sinus cancer and other severe adverse effects in men (Baszuk et al., 2021; Humphries et al., 2016; Santonen et al., 2022). Workers are mainly exposed by inhalation during occupational activities such as welding, Cr(VI) electroplating, and other surface treatments. Although ambient CrVI exposure can be supposed at the specific working conditions and workplaces, exposure was measured in exhaled breath condensate and urine samples (Leese et al., 2023) (Leese et al., 2023). The study aimed to increase knowledge on the use of both exposure and effect biomarkers, following harmonized protocols for data gathering, sampling, and exposure biomarker analyses (Fernandez et al., 2019a, Santonen et al., 2022, 2023).

The potential relationship between urinary Cr levels, early biological effects, and health outcomes was explored by characterizing conventional and novel effect biomarkers in blood and urine samples collected from 399 workers and 203 controls. Classical biomarkers included the alkaline comet assay in leukocytes, the cytokinesis-block micronucleus assay in peripheral blood lymphocytes and reticulocytes, urinary oxidative stress markers (malondialdehyde-MDA, and 8-oxo-2′-deoxyguanosine-8OH-dG), global DNA methylation from leukocytes, and metabolomic markers in urine matrices. Potential relationships were explored between exposure and effect biomarkers and among the different effect biomarkers studied (Tavares et al., 2022; Viegas et al., 2022).

Genotoxicity biomarkers in blood cells revealed that workers exposed to Cr(VI) had a significantly higher level of DNA and chromosomal damage in comparison to non-exposed participants (controls selected outside the industrial setting). Genotoxicity biomarkers also evidenced that controls recruited within the industry (e.g., office workers) had much higher levels of genotoxic damage than those in controls recruited from outside. Oxidative stress biomarkers (8-oxodG levels) measured in pre-shift urine samples were also significantly higher in on-site *versus* off-site controls. These findings highlight the possibility of a bystander effect in office workers at companies where Cr (VI) is used (Tavares et al., 2022).

A statistically significant decrease in DNA methylation was also observed in Cr-exposed workers compared to controls, suggesting a dysregulation of gene expression. In summary, the characterization of classical effect biomarkers in the chromates study allowed the identification of diverse biological effects in workers exposed to Cr(VI) concentrations below the current occupational exposure limit in Europe, evidencing the contribution of these biomarkers to knowledge on the relationship between exposure and health effects. Importantly, these biomarkers also highlighted that even unexposed workers (e.g., office staff) may be at risk of developing long-term effects and should be better protected (Kozłowska et al., 2022).

In addition, non-targeted metabolomics methodology was applied for the quantitative analysis of low molecular weight metabolites to

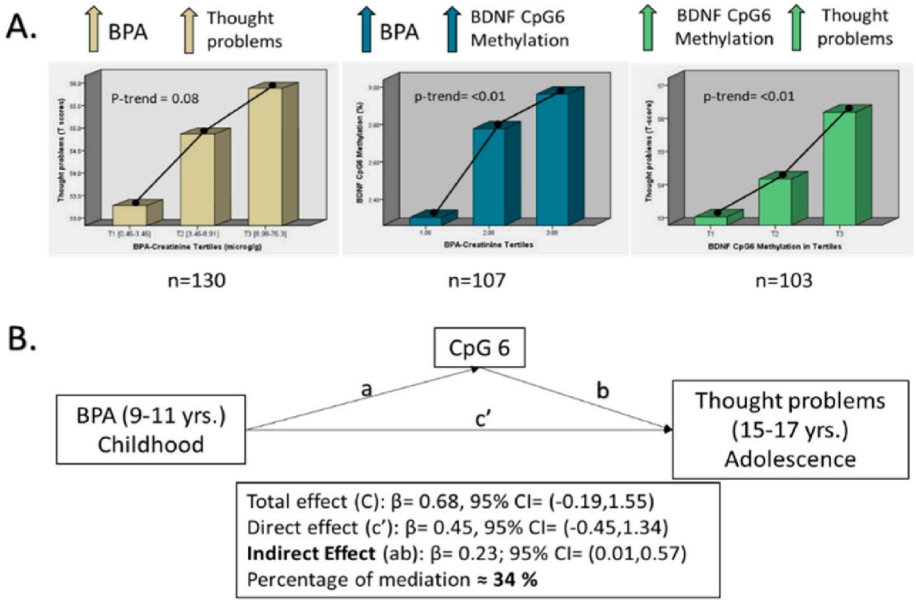

Fig. 3. Adapted from Mustieles et al. (2022). (A) Longitudinal association between childhood BPA with thought problems, and with BDNF gene DNA methylation at CpG6. Cross-sectional and dose-dependent association between higher BDNF gene DNA methylation at CpG6 and thought problems. (B) Mediation analysis showing that BDNF gene DNA methylation at CpG6 mediated up to 34% of the association between childhood BPA and thought problems.

improve understanding of the metabolic, physiological, and pathological mechanisms of Cr(VI) toxicity. The largest number of putatively annotated metabolites belonged to pathways of amino acids, especially tryptophan and tyrosine metabolism, fatty acids, and arachidonic acid metabolism, as well as carbohydrate metabolites and conjugates, vitamins, hormones, nicotine, and oxidative phosphorylation. Significant differences in metabolite abundance were detected among subgroups of workers exposed to Cr(VI). Findings also suggested that changes in the urinary metabolite abundance of exposed groups could be due to work-related factors other than Cr(VI) exposure. Further targeted metabolomics studies are needed to better understand the modifications observed and to explore the suitability of urinary metabolites as early indicators of adverse effects associated with Cr(VI) exposure (Kozłowska et al., 2022; Viegas et al., 2022).

3.6. HBM4EU-aligned studies

HBM4EU concluded with the implementation of several clinical and novel effect biomarkers associated with the prioritized chemical families according to human developmental windows selected for Aligned Studies, *i.e.*, childhood, adolescence, and adulthood (Gilles et al., 2021, 2022). Measurements of clinical effect biomarkers were centralized in the same laboratory to reduce the possibilities of heterogeneity due to inter-laboratory differences. Participants were recruited between 2014 and 2021 from 11 to 12 primary sampling units geographically distributed across Europe. Urine and blood samples were collected in groups of children (3137 aged 6–12 years) and adolescents (3117 aged 12–18 years), and adults (3522 aged 20–39 years). Sociodemographic, lifestyle, health status, environmental, and dietary information was collected using questionnaires. All data analyses are currently in progress and only very preliminary results are available. Some examples of ongoing work using this approach are described below.

The HBM4EU Aligned group of children included analyses of biomarkers of exposure such as phthalates, HEXAMOLL® DINCH, and flame retardants as well as others from the second list of HBM4EU priority substances (Gilles et al., 2021). Information on neurodevelopmental status (specifically behavioral function, assessed with the Child Behavior Check List, and cognitive function, assessed with the Wechsler's Intelligence Scale for Children) was also available for this population. Epigenetic biomarkers, such as DNA methylation and BDNF protein levels, were measured in blood and serum samples, respectively, alongside additional clinical markers (e.g., thyroid and reproductive hormone levels) to cover a broader toxicity pathway.

For the adolescent population, biomarkers of exposure to phthalates, HEXAMOLL® DINCH, and PFAS were measured in biological samples. Information on sexual maturation (Tanner stage scale) and metabolic function (lipid, glucose markers, and BMI) was also available for this population. The biomarkers of effect analyzed included DNA methylation (KISS1 gene) and kisspeptin protein levels (kisspeptin 54, kiss54) in blood and serum samples, respectively. Data analyses are ongoing, with some promising results (Rodriguez-Carrillo et al., 2023).

Information on health outcomes demonstrates the presence of tangible adverse effects and may indicate converging effects of different chemical families. Exposure-health outcome associations alone may not demonstrate a mechanistic or biological relation; however, when combined with molecular and clinical biomarkers, these endpoints may confirm the occurrence of disease pathways. In cross-sectional studies, analyzed exposures are assumed to be representative of an individual's lifestyle and environment, which is supported by reports of good correlations between the internal exposure of mothers and children in the same household (Den Hond et al., 2015). However, because many diseases have a long latency period, longitudinal studies are more appropriate for studying exposure-effect associations.

4. Lessons learnt

The systematic approach developed in the framework of the HBM4EU initiative on biomarkers of effect can be summarized in the following messages:

- HBM4EU has created a scientific body of information on biomarkers of effect that synergizes toxicological, analytical, and epidemiological viewpoints.
- The main basis and concepts are now settled, and the first systematic and harmonized implementation of effect biomarkers has been performed in HBM4EU aligned and occupational studies.
- Biomarkers of effect, when selected based on adverse outcome pathways and toxicological and clinical knowledge, can provide critical added value to HBM studies.
- When used in conjunction with exposure biomarkers in occupational and population settings, biomarkers of effect can make a major contribution to risk assessment and management, leading to improved worker and population health protection (Santonen et al., 2023).
- Biomarkers of the combined effect of real-life chemical mixtures should be further developed, based on chemical groups, human matrices, and AOP data.
- BDNF appeared as a promising neurological effect biomarker to identify neurotoxic chemicals and their mixtures, and it warrants further investigation. Nonetheless, research should be expanded to identify other molecular biomarkers that may also contribute to the prediction of early neurotoxic effects. The goal is to build up a battery of effect biomarkers with increased specificity and sensitivity.
- The implementation of biomarkers of early biological (genotoxic or epigenetic) effects, measured in peripheral blood cells, can help to establish a causal relationship between exposure and carcinogenic effects in occupational settings.
- The HBM4EU chromate study has shown that effect biomarkers are useful to identify occupational activities for which mitigation measures should be prioritized (high-risk settings).
- More work needs to be done in future projects such as the PARC (European Partnership for the Assessment of Risks from Chemicals) to increase the use of effect biomarker data in risk assessment. For example, by establishing a network of European laboratories under strict QA/QC protocols for the measurement of these biomarkers of effect within the PARC umbrella.

5. Strengths & limitations

The entire procedure to find, select, prioritize, validate, and implement effect biomarkers in HBM studies has important strengths but some limitations. First, although the literature search strategies followed a comprehensive approach, it is quite possible that they did not cover all relevant effect biomarkers. Notwithstanding, the fact that several independent literature searches were conducted (Bajard et al., 2021; Baken et al., 2019; Boesen et al., 2020; Gundacker et al., 2021; Mustieles et al., 2020a; Steffensen et al., 2020; Ventura et al., 2021; Vinggaard et al., 2021) minimizes the risk of missing relevant effect biomarkers in the overall inventory (Zare-Jeddi et al., 2021). Second, effect biomarkers are not specific to a particular chemical exposure, and this lack of specificity increases uncertainty in the analysis of exposure-effect associations. On the other hand, the toxicological and AOP-driven prioritization procedure allows validation of their utilization for specific chemical families. However, the scant availability of established AOPs limits the use of toxicological data to prioritize effect biomarkers for some health outcomes. Nonetheless, it remains possible to organize the toxicological data available to develop putative AOPs (e.g., pilot study with BPA exposure and BDNF), establishing bidirectional feedback between effect biomarkers and the AOP framework. Third, a targeted effect biomarker approach was followed, meaning that some adverse

effects could not be mapped, although the wide variety of effect biomarkers implemented may mitigate this issue. In this regard, high-throughput and non-targeted approaches could be used to complement the proposed targeted strategy, because they permit the analysis of multiple targets at different levels (genes, proteins, and metabolites) and the identification of potential effect-specific profiles, thereby yielding possible new information and hypotheses on gene networks, cell signaling pathways, and/or modes of action. Finally, it should be noted that some biomarkers of effect allow direct interpretation in terms of risk at individual level, with validated reference values (e.g., hormonal levels), whereas others provide information at population level (e.g., genotoxicity biomarkers), and no reference values are yet available for new biomarkers.

Among the strengths of this study, the careful stepwise strategy allowed the selection of potential biomarkers of effect in a systematic manner. In addition, the selection criteria enhanced the prioritization of effect biomarkers, while the pre-validation procedure, at analytical and epidemiological levels, increased the confidence and reproducibility of measurements. The feedback established between effect biomarkers and toxicological information organized in the AOP framework is one of the main strengths of this procedure, as it ensures the biological meaning of effect biomarkers as events in the exposure-effect-adverse outcome continuum. The classification of effect biomarkers and the creation of an inventory is another major strength, given that it establishes a framework and scientific corpus that will be useful for future HBM studies. The toxicology-driven hypothesis in the pilot study with BPA, BDNF, and behavioral function serves to illustrate how effect biomarkers can be validated for HBM purposes, although these results need to be replicated in studies with larger sample sizes. Another important example is the key role of biomarkers of effects in predicting potential carcinogenic and non-carcinogenic effects in occupational settings, serving as an early warning signal to improve safety protocols and mitigation measures in a timely manner and to identify population groups at increased risk.

6. Conclusion

The progressive strategy developed within HBM4EU has followed a reproducible, systematic, and innovative approach for identifying biomarkers of effect. Besides allowing its solid implementation in the HBM4EU project, it offers a useful point of departure for future HBMs and European Initiatives, including the European Partnership for the Assessment of Risks from Chemicals (PARC).

The work done under the HBM4EU initiative confirms the added value of including effect biomarkers in HBM, epidemiologic, and occupational studies, especially when selected and prioritized based on toxicological and clinical information. The advantages of implementing effect biomarkers together with exposure biomarkers are: i) the evaluation of potential adverse effects at an early and reversible stage (especially relevant for new chemicals/substitutes and for diseases with long-latency periods) and in cross-sectional studies; ii) the potential to align toxicological and epidemiological knowledge, thereby improving the biological plausibility of the associations found and increasing the likelihood of regulatory acceptance; iii) a higher degree of causal inference through mediation analyses in longitudinal studies; iv) the assessment of dose-response relationships; and even v) the identification of human groups at greater health risk, Further efforts are needed to promote the utilization of effect biomarker data as consolidated scientific evidence to support risk assessment and regulatory decision-making in the framework of EU chemical legislation and in occupational safety and health legislation.

Overall, this work establishes the grounds for achieving one of the main aims of HBM research, which is to link exposure biomarkers to mechanistically validated effect and susceptibility biomarkers in order to elucidate the public health implications of human exposure to environmental chemicals.

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