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Review article

Towards a systematic use of effect biomarkers in population and occupational biomonitoring

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Abbreviations: ACGIH, American Conference of Governmental Industrial Hygienists; ADME, Adsorption, Distribution, Metabolism, Excretion; AOP, Adverse Outcome Pathways; AO, adverse outcome; AOP-KB, Adverse Outcome Pathway Knowledge Base; B-Pb, Blood Lead; BBLV, Binding Biological Limit Value; Biological, limit value; BOELV, Binding Occupational Exposure Limit Value; CAD, Chemical Agents Directive; CMD, Carcinogenic and Mutagenic Directive; DNEL, Derived No-Effect Level; DNT, Developmental Neurotoxicity; EC, European Commission; ECHA, European Chemicals Agency; EFSA, European Food Safety Authority; Effect, Biomarker Effect biomarkers are measurable biochemical, physiological, and behavioral effects or other alterations within an organism that depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease; EQ, Equivalent concentration, integrative response of an effect biomarker translated in an effect concentration of a reference compound; EGMAST, Extended Advisory Group on Molecular Screening and Toxicogenomics; HBM, Human Biomonitoring; HBM4EU, European Human Biomonitoring Initiative; IOELV, Indicative Occupational Exposure Limit Value; ISES, International Society for Exposure Science; ISO, International Organization for Standardization; IUCLID, International Uniform Chemical Information Database; MIE, Molecular Initiating Event; KE, Key Event; KER, Key Event Relationship; MDA, malondialdehyde; MoA, Mode of Action; OECD, Organization for Economic Co-operation and Development; OBL, Occupational Biomonitoring Level; OBEL, Occupational Biomonitoring Effect Level; OEL, Occupational Exposure Limit; OSH, Occupational Safety and Health; PBK modelling, Physiologically Based Kinetic modelling; PBD modelling, Physiologically Based Dynamic modelling; PoD, Points of Departure; PPE, Personal Protective Equipment; RAC, Risk Assessment Committee, European Chemicals Agency; REACH, Registration, Evaluation, Authorization and Restriction of Chemicals; RMM, Risk Management Measure; SCOEL, Scientific Committee on Occupational Exposure Limits; SEGs, Similar Exposure Groups; WHO, World Health Organization; WPEA, Working Party on Exposure Assessment; WPHA, Working Party on Hazard Assessment.

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ABSTRACT

Effect biomarkers can be used to elucidate relationships between exposure to environmental chemicals and their mixtures with associated health outcomes, but they are often underused, as underlying biological mechanisms are not understood. We aim to provide an overview of available effect biomarkers for monitoring chemical exposures in the general and occupational populations, and highlight their potential in monitoring humans exposed to chemical mixtures. We also discuss the role of the adverse outcome pathway (AOP) framework and physiologically based kinetic and dynamic (PBK/D) modelling to strengthen the understanding of the biological mechanism of effect biomarkers, and in particular for use in regulatory risk assessments. An interdisciplinary network of experts from the European chapter of the International Society for Exposure Science (ISES Europe) and the Organization for Economic Co-operation and Development (OECD) Occupational Biomonitoring activity of Working Parties of Hazard and Exposure Assessment group worked together to map the conventional framework of biomarkers and provided recommendations for their systematic use. We summarized the key aspects of this work here, and discussed these in three parts. Part I, we inventory available effect biomarkers and promising new biomarkers for the general population based on the H2020 Human Biomonitoring for Europe (HBM4EU) initiative. Part II, we provide an overview AOP and PBK/D modelling use that improved the selection and interpretation of effect biomarkers. Part III, we describe the collected expertise from the OECD Occupational Biomonitoring subtask effect biomarkers in prioritizing relevant mode of actions (MoAs) and suitable effect biomarkers. Furthermore, we propose a tiered risk assessment approach for occupational biomonitoring.

Several effect biomarkers, especially for use in occupational settings, are validated. They offer a direct assessment of the overall health risks associated with exposure to chemicals, chemical mixtures and their transformation products. Promising novel effect biomarkers are emerging for biomonitoring of the general population. Efforts are being dedicated to prioritizing molecular and biochemical effect biomarkers that can provide a causal link in exposure-health outcome associations. This mechanistic approach has great potential in improving human health risk assessment. New techniques such as *in silico* methods (e.g. QSAR, PBK/D modelling) as well as 'omics data will aid this process.

Our multidisciplinary review represents a starting point for enhancing the identification of effect biomarkers and their mechanistic pathways following the AOP framework. This may help in prioritizing the effect biomarker implementation as well as defining threshold limits for chemical mixtures in a more structured way. Several *ex vivo* biomarkers have been proposed to evaluate combined effects including genotoxicity and xeno-estrogenicity. There is a regulatory need to derive effect-based trigger values using the increasing mechanistic knowledge coming from the AOP framework to address adverse health effects due to exposure to chemical mixtures. Such a mechanistic strategy would reduce the fragmentation observed in different regulations. It could also stimulate a harmonized use of effect biomarkers in a more comparable way, in particular for risk assessments to chemical mixtures.

1. Introduction

Human biomonitoring (HBM) measures people's exposures to toxic substances in the environment and is a growing area in environmental and occupational health (Choi et al. 2015). HBM uses biomarkers to assess specific exposures and predict the risk of (adverse) health effects in individuals and populations (Ladeira and Viegas 2016). Biomarkers are chemicals, their metabolites, or products of an interaction between a chemical and some target molecule that is measured in the human body (WHO 2006). They are indicators of changes, or events, in biological systems that result from complex pathways of human exposure and play an important role in elucidating dose–effect relationships. Biomarkers are generally divided into three main categories: exposure, effect, and susceptibility (WHO 1993, NRC 2006). While exposure biomarkers reveals the concentration of a parent compound or its metabolites in human biospecimens, effect biomarkers are measurable biochemical, physiological, and behavioral effects or other alterations within an organism that depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease (WHO 1993, NRC 2006). This definition is generally accepted in Europe and used in the H2020 European Human Biomonitoring Initiative (HBM4EU) project., HBM4EU is a joint effort of 30 countries, including the European Economic Area (EEA) and the European Commission involving European Food Safety Agency (EFSA) and European Chemicals Agency (ECHA) (HBM4EU et al., 2017). Effect biomarkers often reflect subclinical changes before the onset of disease. They may also suggest effects from exposures to chemical mixtures or aggregate exposure (i.e. exposure to the same chemical from multiple exposure routes) through different sources (Bopp et al. 2018, Santonen et al. 2019). For example, an elevated frequency of micronuclei in human peripheral blood lymphocytes was predictive for cancer risk (Bonassi et al. 2007). In an occupational setting, the frequency of micronuclei correlated with exposures to hexavalent chromium (Annangi et al. 2016). Effect biomarkers provide a link between internal exposure and preferably early health effects. Consequently, they range from early biological changes (e.g. enzyme induction responses) to altered structure and function (Ladeira and Viegas 2016). Effect biomarkers can help in identifying early effects in humans at low doses, establish dose–response relationships, explore mechanisms and increase the biological plausibility of epidemiological associations. In addition, effect biomarkers can improve the risk assessment of specific chemical families as well as exposure to chemical mixtures (HBM4EU et al., 2017). A chemical mixture is defined as an exposure to multiple chemicals via a single or multiple sources and exposure routes, that may or may not be identifiable and that may contribute to a joint toxicity in a target population (Bopp et al. 2018). Susceptibility biomarkers reflect intrinsic or acquired proneness of an organism to respond to specific chemical substances. Inter-individual biological differences may cause some individuals to be more susceptible to environmentally induced diseases (DeBord et al. 2015). For example, polymorphisms of relevant xenobiotic metabolizing enzymes (e.g. cytochrome P450 enzymes) are used as susceptibility biomarkers (Bi et al. 2016, Kim et al. 2016).

The discovery of new effect biomarkers following exposures to single chemicals and chemical mixtures will increase the weight of evidence in exposure-health outcome associations in epidemiological studies as well as experimental exposure studies with human volunteers (Fernández et al. 2019a, 2019c). Although, several effect biomarkers have been introduced in a number of environmental health studies, only a few have been considered to be sufficiently validated. Moreover, effect biomarkers have been used to a lesser extent than exposure biomarkers,

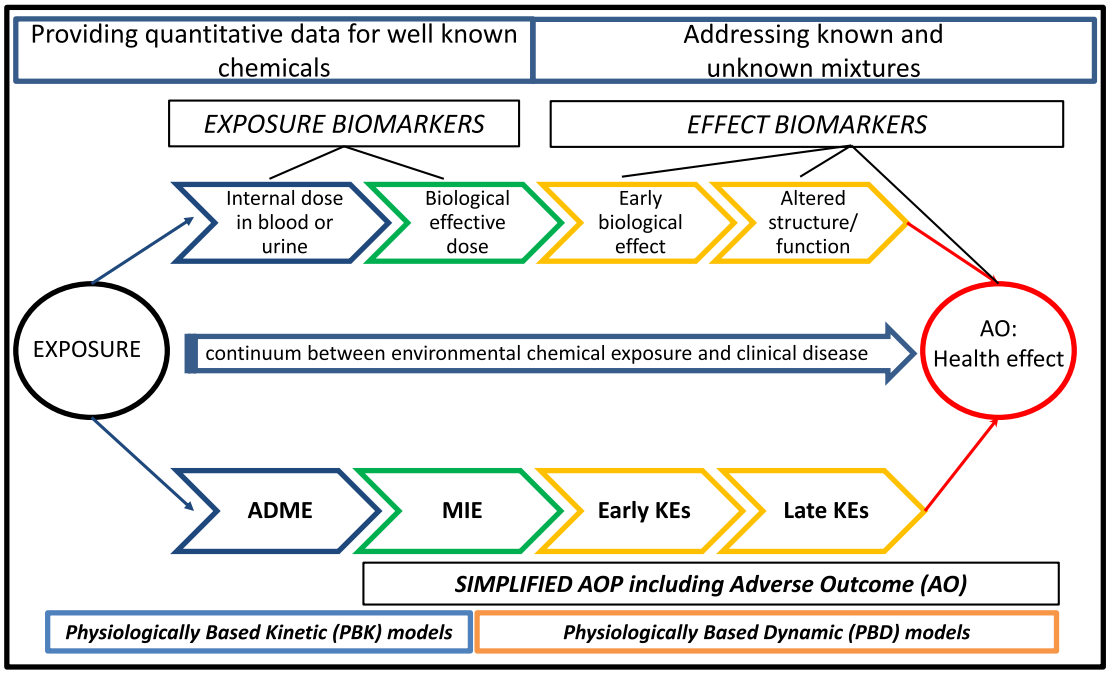

Fig. 1. Integrative conceptual framework from exposure to the adverse outcome, including the roles of exposure and effect biomarkers, Adverse Outcome Pathways (AOP), and physiologically based kinetic and dynamic (PBK/D) models. ADME: adsorption, distribution, metabolism, excretion. MIE: Molecular Initiating Event. KEs: Key Events.

thus constituting an understudied field. The main challenge in HBM studies is to link effect biomarkers to specific exposures (i.e. sources, pathways and routes) and early health effects (i.e. the key events along the pathway from exposure to disease). Effect biomarkers represent physiological processes where upon many different chemical families affect pathways and organs similarly. Thus, chemicals targeting metabolic disorders for instance could be mapped with a set of validated effect biomarkers covering the main metabolic pathways. One way to fill the current gap and determine the group of chemicals that can converge into a particular disease is to integrate mechanistic and toxicologic knowledge using new approaches that make best use of all available experimental and epidemiological knowledge such as adverse outcome pathway (AOP) framework and physiologically-based kinetic and dynamic (PBK/D) modelling. With the influx of new data, there is a need to create a framework to organize and use the chemical hazard, kinetics and exposure information to improve risk assessment processes. This is also consistent with the 21st century paradigm indicating the shift in human health risk assessment from identification of apical endpoints of toxicity to understanding the mechanisms of toxicity (Lanzoni et al. 2019). To reflect this shift, we present here an updated conventional conceptual pathway representing the continuum between environmental chemical exposures and clinical diseases (NRC 2006). Fig. 1 illustrates this integrative conceptual framework identifying the major steps in the continuum from exposure to chemicals to a gradual disruption of biological functions (including early signals and already altered structure and function) until clinical onset of diseases. The key aspect supporting this framework is the implementation of a given effect biomarker for a given chemical family that must be based on toxicological data showing that the chemical family under study is able to alter a particular physiological system and its related molecular and biochemical pathways. In other words, toxicological and AOP data determine the physiological validity of using a set of effect biomarkers for chemicals sharing the same Mode of Action (MoA) (Ankley et al. 2010). AOPs are useful in supporting chemical risk assessment because they provide mechanistic reasoning supporting the association. Additionally, PBK/D models can support, by means of mathematical simulations, the interpretation of both exposure and effect biomarkers. One example of an integrative conceptual framework from exposure to the adverse outcome was recently published by (Mustieles et al. 2020). They demonstrated how a previously published AOP on estrous cycle disruption helped prioritize hormonal biomarkers and better interpret biomarker data. Furthermore, they also described how to merge different AOPs in the AOP wiki to create an AOP network for a novel biomarker of altered neurodevelopment (BDNF-brain-derived neurotrophic factor), and link this biomarker to chemical exposure.

Among the diverse effect biomarkers available, some allow a direct interpretation in terms of risk at an individual level, such as blood pressure or hormone levels with validated reference values. Others provide information that can only be interpreted at the population level such as genotoxicity biomarkers or novel biomarkers for which reference levels are not available. In these cases, the data obtained is compared with the data from a population with a background exposure as a reference, which is typical for occupational studies, or with a matched non-exposed population in general population studies. At the group level, the data may aid in identifying groups at increased risk, but in this situation, individual data should be communicated to participants of the HBM study within the context of the group results. Conversely, data on effect biomarkers such as blood pressure or hormone levels may be communicated individually to provide relevant inputs for health surveillance, in addition to risk assessment.

A major challenge in effect biomarker discovery and validation for health outcome assessment, is the understanding of the complex biological mechanisms involved in disease pathogenesis. The use of effect biomarkers in regulatory risk assessment of chemicals could drive the research forward, and thus the evidence for strengthen an exposure–health outcome relationship. It would also improve the interpretation of effect biomarker data, advance the field of effect biomarkers for the general population and workers, and identify validated and suitable effect biomarkers.

Here, we aim to provide an overview of available effect biomarkers

Table 1
Inventory of effect biomarkers reported in the literature in human biomonitoring and epidemiologic studies, related to exposure to specific chemicals (Mustieles et al. 2018, Fernández et al. 2019a, 2019c).

Type of Effect Biomarker	Related health outcomes	Effect Biomarker and other indicators	Used for Chemicals	Strengths/ Limitations
Anthropometric biomarkers	Obesity, metabolic and reproductive diseases	Birth Weight, Birth Length and Head circumference, Body Mass Index (BMI), Body fat mass, Anogenital Distance (AGD)	Bisphenols; Perfluorooctanesulfonic acid (PFOS), Perfluorooctanoic acid (PFOA); Acrylamide	Easy to assess, reliable and predictive of cardio-metabolic outcomes. AGD constitute a marker of androgen balance in both rodents and humans.
Cardiovascular biomarkers	Cardiovascular events including ischemic heart disease, atherosclerosis and stroke	Blood pressure (systolic, diastolic, and pulse pressure), 1st-, 2nd-and 3rd-min Heart Rate Recovery (HRR), carotid intima media thickness (cIMT), and electrocardiographic (ECG) parameters (QT interval, JT interval, PR interval, QRS duration, and QT dispersion)	Bisphenols; Inorganic arsenic	Specific of heart variables. However, with the exception of blood pressure, are not easy to implement in large HBM studies.
Serum lipids	Atherosclerosis and Metabolic Syndrome	Low density lipoprotein (LDL), high density lipoprotein (HDL), total cholesterol (TC) and triglycerides (TG)], related to obesity and cardio-metabolic diseases; Adipokines [Leptin and Adiponectin][a], as biomarkers of adipose tissue function.	Bisphenols; PAHs; PFOS, PFOA; Brominated Flame Retardants; Inorganic arsenic; Mycotoxins	There are reference and cut-off values for these biomarkers. Although serum lipids may be useful in adult and elder populations, they may not be enough sensitive for children (with the exception of adipokines).
Glucose homeostasis	Type 2 diabetes mellitus	Fasting blood glucose (FBG), fasting insulin levels and glycated hemoglobin (HbA1c) and the homeostatic model assessment (HOMA)[b]	Bisphenols; Phtalates; Brominated Flame Retardants; Organophosphate flame retardants; Acrylamide; Inorganic arsenic	The relationship between fasting glucose and insulin levels calculated through the HOMA index, constitutes a validated biomarker of β-pancreatic cell function and insulin resistance.
Hepatic biomarkers	Fatty liver disease, hepatic injury	Liver enzymes including alanine transaminase (ALT) and aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), serum bilirubin, prothrombin time (PT), the international normalized ratio (INR), and albumin; Cyp17A1, Cyp19A1, Cyp1A1, Cyp2E1, Cyp2J2.	Bisphenols; PFOS, PFOA; Brominated Flame Retardants; Mycotoxins	Reference levels exist. These markers are not always specific for the liver, and other tissues can contribute to altered levels. May not be sufficiently sensitive biomarkers in young and healthy populations.
Renal function	Renal injury, including kidney tubular and glomerular damage	Serum creatinine; high molecular weight proteins such as urinary β2-microglobulin (B2-MG), α1-microglobulin, retinol-binding protein, albumin, transferrin, IgG	Brominated Flame Retardants; Cadmium	Urinary biomarkers such as B2-MG, NAG and KIM-1 are validated markers of tubular damage, that can be complemented with other markers of glomerular damage such as urinary albumin. They are evaluated in urine, constituting the most easily accessible biospecimen.
		Urinary B2-MG; NAG (N-acetyl-beta-(D)-glucosaminidase activity); ALAD (delta-aminolevulinic acid dehydratase); albumin; KIM-1 (kidney injury molecule-1); NGAL (Neutrophil gelatinase-associated lipocalin); protein carbonyls; metallothioneins	Hexavalent chromium	
Glucocorticoid hormones	Metabolic syndrome, immune system and psychological stress	Hypothalamic corticotrophin-releasing factor (CRF), corticosterone (CORT), decreased hippocampal 11-hydroxysteroid dehydrogenase Type 1 (11-HSD 1), subcellular glucocorticoid receptor (GR)	Inorganic arsenic	Exist reference levels. Repeated cortisol measurements in saliva is feasible. It is an understudied biomarker in relation to chemical exposures.
Reproductive hormones	Depending on age: pregnancy outcomes, puberty, fertility, metabolic disease and neurodevelopment.	Luteinizing hormone (LH), follicle stimulating hormone (FSH), Total testosterone (TT), estradiol (E2), sex hormone binding-globulin (SHBG)	Bisphenols; Phtalates; Brominated Flame Retardants; Organophosphate flame retardants; Acrylamide	Reference levels exist. Ratios among related hormones provide information on enzymatic activity and on feedback loops. Diurnal and seasonal variations. Requires the consideration of sex and developmental period.
Sperm quality	Fertility problems	Sperm quality parameters including counts, concentration, motility and morphology.	Hexavalent chromium	Semen constitutes a non-invasive sample that can provide both *in situ* exposure and effect data, specific to the male reproductive system.
Thyroid homeostasis	Depending on age: Neurodevelopment, adverse pregnancy outcomes and metabolic disease	Thyroid stimulating hormone (TSH), triiodothyronine (T3), thyroxine (T4), anti-thyroperoxidase (TPO) antibodies.	Bisphenols; Phtalates; PFOS, PFOA; Brominated Flame Retardants; Organophosphate flame retardants; Inorganic arsenic; UV-filters	Reference levels exist. Even subclinical dysfunction of thyroid homeostasis during pregnancy may affect offspring neurodevelopment.
Cancer biomarkers	Carcinogenesis	Plasma carcinoembryonic antigen: NSE (neuron specific enolase); SCC (squamous cell carcinoma antigen);	Hexavalent chromium	Although cancer biomarkers may provide useful information, the inherent complexity of tumors

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Table 1 (*continued*)

Type of Effect Biomarker	Related health outcomes	Effect Biomarker and other indicators	Used for Chemicals	Strengths/ Limitations
		CYFRA21-1 (cytokeratin fragment antigen 21–1); CA72-4 (cancer antigen 72–4); AFP (α-fetoprotein); 3-nitrotyrosine; prostate-specific antigen; high sensitive C reactive protein, CC16 (Clara cell secretory protein), SP-D (surfactant protein D), TNF-α (tumor necrosis factor-α); Plasma total homocysteine		makes their prediction more difficult, and it is preferable to use a set of related markers.
Genotoxicity biomarkers	Carcinogenesis and teratogenesis	Chromosomal aberrations, sister chromatid exchange, micronucleous test	PAHs; Hexavalent chromium; Acrylamide; Mycotoxins	Some of these biomarkers, such as the micronucleous test, have been prospectively linked to cancer. While frequently used in occupational settings, their feasibility in population studies should be further studied.
Oxidative stress	Many different outcomes including cancer, cardio-metabolic diseases and adverse pregnancy outcomes	DNA damage: Urinary 8-hydroxy-2′ -deoxyguanosine (8-OHdG) Lipid peroxidation: 8-isoprostane	Hexavalent chromium; Inorganic arsenic; PAHs; Bisphenols; Phthalates; PFOA; Acrylamide; UV-filters	Normally assessed in urine, which is preferred over serum. They are predictive of diverse chronic diseases including metabolic syndrome, cardiovascular disease and cancer. As a limitation, it is not specific of a defined health outcome. Given that this type of biomarkers capture disruptions at different levels of biological organization, they have been shown useful in mediation analyses between exposure biomarkers and health endpoints.
		Antioxidant defense: Glutathione peroxidase (GPx), selenium, and glutathione (GSH)	Cadmium; Acrylamide; Inorganic arsenic	
Epigenetics and gene expression biomarkers	Depends on window of exposure and the molecular targets investigated (from neurodevelopment, growth, metabolism and reproduction to cancer).	Gene expression and methylation: p-MAPK expression, DNA methylation levels and histone methylation levels in oocytes	Acrylamide	DNA methylation is more stable over time compared to gene expression or circulating protein levels, which are subjected to short-term variations. DNA methylation regions must be carefully selected, mainly the promoter regions, so the status of DNA methylation is related to its gene expression. As a limitation, the predictive potential of epigenetic biomarkers for a given disease is unknown. Notwithstanding, recent findings are supporting their suitability.
		Arsenic methylation capacity; serum BDNF, serotonin receptor 5B gene expression; Protein expression PSD-95, SYP; miRNA-219, CaMKII; H3K18ac, H3K9me2, H3K36me3; GR mRNA, H-Ras protein, Raf-1 protein, ERK expression; DNA methylation/ demethylation (5-methylcytosine, 5-hydroxymethylcytosine, DNA-methyltransferases, ten-eleven translocations genes); Methylated arginines, dimethyl arginine; 7-nAchR expression	Inorganic arsenic; Lead	
Immunology/ allergy biomarkers	Immunotoxicity, predisposition to infections, allergy, asthma, autoimmune diseases	IgE; Protein complement (C3, 3a, 4), TNFα; Cytokine production; LTB4 (leukotriene B4); Lymphocyte subsets characterisation Inflammation biomarkers: chemokines, C-reactive protein (CRP), neutrophil count.	Phthalates; PFOS, PFOA; Brominated Flame Retardants; Hexavalent chromium; Inorganic arsenic Phthalates; Bisphenols;	As an advantage, immune cells can be isolated from blood and studied *in vitro* or at a molecular level. Additional research is needed to further explore the most sensitive immune biomarkers in relation to environmental chemicals.
Neuropsychological biomarkers	Behavioral function (anxiety, depression, attention deficit and hyperactivity, etc.) Cognitive functioning (Intelligence quotient, working memory, vocabulary, etc.).	CBCL: Child Behavior Check-List (6–18 years); SDQ: Strengths and Difficulties Questionnaire; BASC-2: Behavior Assessment System for Children ; BASC-2: Behavior Assessment System for Children; BRIEF-P: Behavior Rating	Bisphenols; Organophosphate flame retardants; Hexavalent chromium; Lead	Molecular and biochemical markers of brain function are especially important, given that children are evaluated using psychological tests, in most cases completed by fathers or teachers.

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Table 1 (*continued*)

Type of Effect Biomarker	Related health outcomes	Effect Biomarker and other indicators	Used for Chemicals	Strengths/ Limitations
	Psychiatric and neurodegenerative diseases	Inventory of Executive Function-Preschool; WISC: Wechsler Intelligence Scale for Children; Social Behavior (Skills Improvement Rating scale) WISC-IV; Standard Progressive matrices test[c]		Neurotrophins like BDNF constitute promising effect biomarkers of brain function currently investigated in different fields and HBM4EU project. BDNF counts with the support of a recently developed AOP network (Mustieles et al., 2020)
		Hypothalamic-Pituitary-Adrenal (HPA) axis [Corticotropin releasing hormone	Brominated Flame Retardants; Bisphenols; PFOS, PFOA	
Neuropsychological biomarkers Systemic/signaling	Behavioral function (anxiety, depression, attention deficit and hyperactivity, etc.)	(CRH) – Adrenocorticotropin hormone (ACTH) – Cortisol]	Serum brain derived neurotrophic factor (BDNF); blood BDNF DNA methylation	Bisphenols; Lead; PFOS, PFOA
	Cognitive functioning (Intelligence quotient, working memory, vocabulary, etc.).	Molecular and biochemical markers of brain function are especially important, given that children are evaluated using psychological tests, in most cases completed by fathers or teachers.	Cholinesterase activity	Inorganic arsenic; Pesticides
	Psychiatric and neurodegenerative diseases		Neurotransmitters and hormones: Epinephrine, dopamine, norepinephrine, mitochondrial monoamine oxidase;	Inorganic arsenic
	Depends on window of exposure and the molecular targets investigated (from neurodevelopment, growth, metabolism and reproduction to cancer).	Neurotrophins like BDNF constitute promising effect biomarkers of brain function currently investigated in different fields and HBM4EU project. BDNF counts with the support of a recently developed AOP network (Mustieles et al., 2020)	Corticosterone receptor (CR)	
ESR1, ESR2, Faz, NR3C1	Brominated Flame Retardants	Signaling biomarkers can shed light on potential MoAs. As a limitation, their predictive potential for a given disease is unknown.		
		Ideally, signaling biomarkers should be complemented with more predictive biochemical biomarkers to better investigate a given adverse outcome.		
		Rac1, Cdc42 expression; NGF, GAP-43 mRNA; NR2A, PSD-95, p-CaMKII, SynGAP, p-ERK1/2 activity; mRNA tight junction (TJ) proteins, PI3K-Akt-mTOR signaling pathway	Inorganic arsenic	

[a] Note that for the scientific substantiation of health claims on foods, a number of outcome variables allow for claims on cardiovascular health to be made such as beneficial changes in the blood lipid profile, arterial blood pressure, elastic properties of the arteries, endothelial function, plasma homocysteine concentrations, platelet aggregation and venous blood flow.

[b] Note that for the scientific substantiation of health claims on foods, biomarkers in relation to blood glucose and related biomarkers have been described in EFSA guidelines.

[c] Although neuropsychological tests could fit the effect biomarker definition, they are normally used as surrogate endpoint of brain function, that is, treated as a health outcome. The complementation of tests with measurable molecular/biochemical biomarkers of brain function is recommended.

that can be used to monitorchemical exposures in the general and occupational populations and highlight their potential in monitoring populations exposed to chemical mixtures. We also discuss the role of the AOP framework and PBK/D modelling to strengthen the understanding of the biological mechanism of effect biomarkers, and in particular promote regulatory acceptance of effect biomarkers in risk assessment frameworks. We summarized the key aspects and discussed these in three parts.

Part I, we inventory available effect biomarkers and promising new biomarkers for the general population based on the H2020 Human Biomonitoring for Europe (HBM4EU) initiative.

Part II, we provide an overview AOP framework and PBK/D modelling use that improved the selection and interpretation of effect biomarkers.

Part III, we describe the collected expertise from the OECD Occupational Biomonitoring subtask effect biomarkers in prioritizing relevant MoAs and suitable effect biomarkers. As a starting point, we propose to use existing AOP knowledge for derivation of cross-regulatory usable effect-based trigger values to address known and unknown mixtures encountered in the occupational setting. MoA specific exposure biomarkers should be used in setting preliminary health-based threshold values. We provide a prioritization of suitable effect biomarkers for regulatory use from the perspective of the European Chapter of the International Society for Exposure Science (ISES Europe) and OECD Occupational Biomonitoring working groups.

The combination of suitable effect biomarker with future effect-based trigger values will lead to an improved and systematical use of effect biomarker, which in turn will reduce chemical exposures and related health effects in human populations.

2. Methods

This is a joint effort of the ISES Europe network and OECD Occupational Biomonitoring activity of Working Parties of Hazard (WPHA) and Exposure Assessment (WPEA) groups. These working groups constitutes of an interdisciplinary network of experts in different regulatory and research roles. In addition, some of the experts are already contributing to HBM4EU projects, as well as in other national HBM programs. The main aim for these working groups is to promote the use of effect biomarkers in regulatory risk assessment frameworks for chemical and chemical mixtures to strengthen decision-making processes.

The present work follows the updated framework (Fig. 1), with a separate focus for the general population and the occupational population. Effect biomarker profiles differ by exposure ranges (Mustieles et al. 2020), and consequently, will differ between the general population

(low exposures expected) and workers (high exposures expected). Therefore, while recognizing the overlaps in the general concept, we addressed the use of effect biomarkers in risk assessment for the general population separately from the occupational population.

In part I, we summarized the most important existing effect biomarkers used in epidemiologic studies based on preliminary (not curated) data collected and reported in the HBM4EU project for the general population (Mustieles et al. 2018, Fernández et al. 2019a, 2019c). Under the HBM4EU project a series of comprehensive structured literature searches were performed to determine the most relevant effect biomarkers, as well as the chemical families more frequently associated with these biomarkers (HBM4EU et al., 2017). We presented our summary as an inventory of effect biomarkers previously reported in the literature for different chemical families. We emphasized the effect biomarkers' advantages and limitations and discussed current progress and challenges in the utility of effect biomarkers as a useful HBM screening tool as well as their use for chemical mixtures in the general population. Each type of effect biomarker represents a different physiological system, evaluation and discussion, and a comprehensive discussion for each chemical family is outside the scope of this work. Indeed, detailed discussions on available effect biomarkers are under evaluation in the HBM4EU project and a follow-up survey in the OECD Occupational Biomonitoring activity.

In Part II, we discuss the incorporation of AOP and PBK/D modeling into effect biomarker frameworks addressing the complexity of effect biomarker utility.

In Part III, we provide a prioritization of relevant MoAs and a recommendation of effect biomarkers in the occupational settings. We discuss general needs and recommendations collected from the OECD Occupational Biomonitoring activity of WPHA and WPEA within its subtask "effect biomarkers". Subsequently, we suggest new paradigms and measurement strategies for 21st century HBM providing examples to improve chemical risk assessment. We summarize future work focus such as developing regulatory accepted effect-based trigger values, prioritizing MoAs in different regulations, characterizing suitable effect biomarkers and developing assessment schemes and tiered approaches.

3. Results and discussion

3.1. Part I. Available effect biomarkers for the general population

An overview of available effect biomarkers is given in Table 1. These effect biomarkers were collected in the HBM4EU project, which aims to identify validated biomarkers reflecting specific exposures in different matrices and quantitatively linking exposures and adverse outcomes in human population studies (Fernández et al. 2019a, 2019c). We have added in Supplementary Material (Table S1) a summary of the HBM4EU project work on identifying potential effect biomarkers in human biomonitoring studies published in the scientific literature and their proposed types of effect biomarkers for specific chemicals (Mustieles et al., 2018, Fernández et al., 2019b). In our current work, we aim to provide an integrative overview, and do not discuss details of specific effect biomarkers but briefly mention some general limitations and advantages of the effect biomarkers listed in the Table 1. We provide in supplementary information a checklist for effect biomarkers (Baken et al. 2019, Mustieles et al. 2020).

HBM4EU developed relevant criteria for prioritizing effect biomarkers and considered the following chemicals: Bisphenols, Phthalates, Polycyclic Aromatic Hydrocarbons (PAHs), Perfluorinated Compounds (PFOS, PFOA), Brominated Flame Retardants, Organophosphate retardants, Metals (Cadmium, Lead, Mercury, hexavalent Chromium), inorganic Arsenic, Acrylamide, Pesticides, Mycotoxins and UV-filters (Table 1) (Mustieles et al. 2018, Fernández et al. 2019a, 2019c). Two scientific literature reviews; BPA and phthalates, conducted in the HBM4EU project have recently been published (Mustieles et al., 2020; Baken et al., 2019). Specific reviews for other chemical families in relation to effect biomarkers are currently under preparation in the HBM4EU project. In addition, the HBM4EU project assessed different *ex vivo* bioassays for their potential use as effect biomarkers for addressing exposures to chemical mixtures (Rodríguez-Carrillo et al., Submitted). The conclusion was that chemical mixtures can be extracted and identified from human samples and their biological activity quantified using different cell-based tools (Fernández et al. 2019a, 2019c).

Some of the effect biomarkers have been validated (e.g. HOMA-IR, Kidney Injury Molecule-1 (KIM-1), micronucleus test, endogenous hormones) including some that are predictive of specific health outcomes. Of note, KIM-1 is not only validated, but also qualified by the FDA biomarker qualification program[1]. Others constitute promising candidates under active study such as BDNF in the case of neurodevelopment, and epigenetic omics biomarkers. Effect biomarkers are usually not specific to environmental chemicals. Thus, the selection of the best effect biomarkers to map the potential impact of specific chemical families on human health should be based on toxicological knowledge (Mustieles et al. 2020). While some of the effect biomarkers are validated and/or qualified scientifically, none of the mentioned effect biomarkers are currently used in the regulatory risk assessment of the general population. While few effect biomarkers (e.g. cholinesterase inhibition) are currently used in regulatory occupational biomonitoring. We aim to pave the way for more systematic use and regulatory acceptance of effect biomarkers in risk assessment of chemicals.

In general population studies, effect biomarkers can help in making regulatory decisions by increasing the weight of evidence for a given chemical family such as providing information on potential MoAs, assessing dose–response relationships, detection of subclinical effects, and evaluation of potential mediators between exposure biomarkers and health outcomes (Ferguson et al. 2017, Louro et al. 2019, Mustieles and Arrebola 2020). Overall, mechanistically-based effect biomarkers improve the understanding of correlative and causal relationships in observational and HBM studies.

Biomarkers in the area of food and nutrition have been validated by EFSA to substantiate health claims (EU Regulation 1924/2006) such as "beneficial to human health" (EU. European Parliament 2006), and six guidance documents identifying these biomarkers have been published. These biomarkers are related to functions of the nervous system including psychological functions (EFSA Panel on Dietetic Products, 2012a, 2012b, 2012c), muscle function and physical performance (EFSA Panel on Nutrition, Allergens et al. 2018), bone, joints, skin and oral health (EFSA Panel on Dietetic Products, 2012a, 2012b, 2012c), appetite ratings, weight management, and blood glucose concentrations (EFSA Panel on Dietetic Products, 2012a, 2012b, 2012c), the immune system, the gastrointestinal tract and defense against pathogenic microorganisms (EFSA Panel on Dietetic Products and Allergies 2016), and antioxidants, oxidative damage and cardiovascular health (EFSA Panel on Nutrition, Allergens et al. 2018).

Accompanied by the challenges arising from the terminological and epistemological complexity of biomarker science, there are also practical challenges in generating, tracking, and aggregating the evidence for a biomarker used in the general population studies. Several of these challenges are discussed in the following sections.

3.1.1. Technical Feasibility: The pre-analytical and analytical factors

The feasibility of an effect biomarker will rely on the possibility of measuring it in an accessible biological matrix (e.g., blood, urine, or exfoliated cells), analytical cost for a large number of individuals, and technical viability. Technical issues associated with the development and pre-analytical validation of effect biomarkers are among others the lack of standardization of biological media (e.g. the selection of blood fraction, i.e. whole blood, plasma, or serum), analytical method, and

[1] https://www.fda.gov/drugs/cder-biomarker-qualification-program/list-qualified-biomarkers

storage of samples. Furthermore, study design and execution may introduce bias and contribute to quantification errors in both laboratory and human studies. Altogether, key elements that are needed to move an effect biomarker from promising to successful in exposure assessment and health surveillance studies encompass appropriate study designs with ethical considerations, standardized and validated analytical methods along with sophisticated statistical analyses for interpreting results.

It is important to indicate whether the existing analytical methods are sufficiently robust and sensitive (e.g. by geometric mean and standard deviations) to characterize (a) background levels in the population, and (b) levels at which biological effects occur. Meanwhile, an available analytical method might be sensitive in the laboratory but not in samples from human populations. For instance, micronuclei frequency is more sensitive in the laboratory than in the general population studies (Hayashi 2016). Therefore, effect biomarkers validated in *in vitro* methods must be tested in populations as part of a proof of concept.

To address biological complexity and discover new effect biomarkers, omics technologies have great potential as omics profiling enable a comprehensive characterization of a biological sample within a single analysis (Quezada et al. 2017). It has the advantage of being applicable for a large number of individuals and generating new biomarkers for hazard identification and risk assessment. For instance, high-throughput transcriptomic (HTTr) can be used as a bottom up approach to identify chemical-induced changes (gene expression biomarkers). These include oestrogen receptor α (ERα) and androgen receptor (AR) biomarkers for endocrine disrupting chemicals (EDCs). ERα and AR accurately identify both agonists (94–97%) and antagonists (93–98%) in microarray data derived from human breast or prostate cancer cell lines (Corton et al. 2019). The rise of genomics owing to the sequencing of the human genome has been closely followed by analogous technologies that can be used to characterize downstream biological events such as RNA expression (transcriptomics), proteins (proteomics) and metabolites (metabolomics) in cells, tissues or body fluids (Wild 2012). Epigenetic signatures are potential candidates to link exposure to phenotype alterations. Epigenetic biomarkers can incorporate information on environmental and lifestyle effects on health and disease, and monitor the effect from applied therapies (García-Giménez et al., 2017; Jeremias et al., 2020). Epigenetic studies provide information on modifications of gene expressions rather than alteration in the underlying DNA sequence itself. They characterize the methylome- and microRNA profiles, and chromatin regulation such as nucleosome occupancy, DNA accessibility, transcription factor binding, and post-translational histone modifications (Wild, 2012; Jeremias et al., 2020). Epigenetic biomarkers can be useful to predict and inform on health-related outcomes in later life from a specific early life exposure, but epigenetic endpoints are currently not yet incorporated into risk assessments.

The metabolomics is the youngest of the omics technologies and still suffer from technological (e.g., sensitivity) and methodological (e.g., annotation) issues but hold great promises since it allows to profile, during the same analyses, endogenous (effect biomarkers) and exogenous (exposure biomarkers) molecules In addition, the advent of high-throughput screening (HTS) with reporter gene assays and *in silico* models that allow the rapid evaluation of hundreds to thousands of compounds has been instrumental for the shift toward *in vitro* methods in toxicity testing and risk assessment (Escher et al. 2019). Reporter gene assays which are mechanism of action related, less variable, accurate, precise, and labor-saving are becoming more and more recognized and adopted in the quality control (Wang et al. 2020). The reporter gene assays are useful for identifying endocrine disruptors from the multitude of chemicals commonly in use (Kojima et al. 2004).

In silico models can also offer opportunities using the chemical structure to simulate the exposure level and the (adverse) effects elicited by a chemical substance (Benfenati 2016). Regarding the possible use of *in silico* tools for the adverse effects, there are a variety of models available providing predicted values for properties such as binding to thyroid, estrogenic, androgen receptors, or property values closer to the apical effect, such as carcinogenicity, reproduction toxicity, and developmental toxicity (see Appendix A) (Judson et al. 2018).

The emphasis is now to align various exposure platforms (e.g., *in vitro*, *in vivo*, and epidemiological) to support quantitative dose–response relationship knowledge regarding chemicals and chemical mixtures.

3.1.2. Validation of effect biomarkers

To establish the credibility and effectiveness of an effect biomarker, it must be validated both analytically and physiologically. Analytical validation should follow recommendations such as those described in the ISO 17025/2017 scheme (ISO/IEC 2017) or a similar system. Only by having adequate control over the analytical method, is it possible to produce reliable, retrievable and repeatable results. Only then can the results be compared to one another. It may seem obvious that when there is a drift in results over time this may have consequences for interpretation of data and for the validity of conclusions being drawn from the results obtained, such as the (apparent) absence (or presence) of effects. Accuracy and precision are also indispensable characteristics for the analytical methods.

Other key aspects of validation include establishing detection limits for the effect biomarker and an acceptable coefficient of variation. Physiological validation should follow the scientific justification of the effect biomarker and its response to changes in exposure. Measuring a biomarker based only on analytical feasibility or just repeating previous work is not a prudent way forward. A hallmark example of this is the quantification of thiobarbituric acid reactive substances (TBARS) or malondialdehyde (MDA) as a biomarker of oxidative stress. The technical approach is easy, swift and cheap, but the biological/physiological validity is poor (Griffiths et al. 2002, EFSA Panel on Nutrition, Allergens et al. 2018). In food and nutrition/health claims, several effect biomarkers were considered as not reliable *in vivo* such as biomarkers of lipid peroxidation (in addition to TBARS and MDA, also high-density lipoprotein (HDL)-associated paraoxonases, conjugated dienes, breath hydrocarbons, autoantibodies against low-density lipoprotein (LDL) particles and *ex vivo* LDL resistance to oxidation). It was noted that MDA concentrations in blood or tissue can only be used as supportive evidence (i.e. in addition to measurements of F2-isoprostanes and/or *in vivo* LDL oxidation) if appropriate analytical methods are used (e.g. by chromatography, viz not by colorimetry) (EFSA Panel on Nutrition, Allergens et al. 2018).

Another aspect of physiological validation is the (lesser known) concept of “kinetics of biomarkers” or “ADME” (absorption, distribution, metabolism and excretion). It is well-known that biomarkers of exposure are influenced by the pattern of uptake and excretion of the target substance (or its parent compound or metabolite). For biomarkers of exposure, a thorough knowledge of ADME characteristics is pivotal for identification of an appropriate sampling scheme. For instance, an exposure biomarker that has a very short half-life (plasma, blood) requires precise sampling times, or can better be sampled through excretion into the urine. An example of this is artificial sweeteners that can be correctly measured in 24-h urine samples (Logue et al. 2016, Logue et al. 2017, Logue et al. 2020). Effect biomarkers show similar variation over time and may even require an adequate timeframe for revealing an effect. That is the case of mutation fixation that requires the doubling of cells before being expressed and may disappear if the damaged cells are

removed due to cell turnover. Likewise, it is recognized that blood cholesterol markers need several months in order to react to an intervention, *viz.* measuring blood cholesterol values after a few days of intervention is useless. A review of the concept of kinetics of biomarkers is provided by Verhagen et al (Verhagen et al. 2004).

Physiological validation also entails the assessment of whether the effect biomarker reflects changes at the individual or at the population level. If the latter case applies, the population subgroups (i.e., age, sex, and ethnicity) for which there are an evidence that the biomarker actually reflects effect changes have to be documented.

In summary, the physiological validation of effect biomarkers requires some key aspects to be considered such as the dose–response relationship and the time-response as well as the robustness and reproducibility. Ideally, *in vitro* experiments with varying exposure levels demonstrating the differential impact on the effect biomarker would be required. Examination of the timeframe of response of the biomarker would indicate suitable sampling times for the biological samples. An ideal effect biomarker should be feasible, accurate, precise and robust, i.e. give consistent results across a range of populations and ethnic groups. Sex and age specific values/cut-offs should be determined, if appropriate, and potential interactions with combined exposures should be documented.

3.1.3. Using effect biomarkers for tracking complex mixtures of chemicals

Regulatory guidelines for risk assessment of chemical mixtures recommend MoA of the individual chemical to predict dose–response characteristics of the mixture (Borgert et al., 2004). However, exposure to chemical mixtures is rather challenging for risk assessment and considers: (i) dose or concentration addition for similar MoA chemicals; (ii) response addition for dissimilar MoA chemicals; and (iii) interactions between chemicals in the mixture. The term interaction includes all forms of joint actions that deviate from either dose or response addition. In exposure scenarios, chemical mixtures are rarely composed of either only similar or only dissimilar MoA substances. Therefore, recent guidelines for assessment of chemical mixtures have emphasized the necessity to incorporate interaction concepts and methods to evaluate the possible influence of such interactions on the overall joint toxicity of chemical mixtures (Kienzler et al. 2014).

The European Commission (EC) published a communication in 2012 stating that there is a need to better understand human and environmental exposures to mixtures. There is a lack of a systematic, comprehensive and integrated assessment of chemical mixture effects taking into account routes of exposure in different exposure scenarios. We support using both monitoring and modelling approaches (EC 2012). Following this statement, several efforts have been made to improve risk assessment of specific chemical mixtures under the EU regulatory framework (Kienzler et al. 2016, Committee et al. 2019, More et al. 2019, EFSA, 2020a, 2020b). Moreover, a workshop with representatives from the EU-funded research projects (EDC-MixRisk, EuroMix, HBM4EU, SOLUTIONS, and EU-ToxRisk), Commission services and EU agencies concluded that there is a need to identify a range of approaches to tackle the complexity of mixture effects (Drakvik et al. 2020).

Exposure to chemical mixtures (e.g. for substances exhibiting similar MoAs) may lead to adverse health effects even at exposures below a chemical's threshold level. Additionally, exposure to multiple chemicals significantly above their respective threshold level, toxicokinetics and/or toxicodynamic interactions may occur, resulting in new toxic effects usually not observed for single exposures because of potentiation or synergism. Nevertheless, such interactions are difficult to predict. These assessments should be based on data from measuring and simulating relevant mixtures in models and by measuring a wide array of molecular biomarkers of target organ toxicity and nonspecific biomarkers of toxic response (oxidative stress, DNA damage, etc.) in different body fluids (Hernández et al. 2019).

The use of effect biomarkers in studies of complex exposures could help to identify both the active components of the mixtures/combined exposure as well as the consequences of specific mixture exposures (Silins and Högberg 2011).

in silico models can also help in evaluating effects from exposures to chemical mixtures (Sauer et al. 2020). Whereas the MoAs are not known for some chemicals in the mixture, one possibility is to read across, which can be used to cluster substances following the concentration addition concept. *In silico* models and read across tools for MoA often refer to collections of structural alerts, which can be used to identify families of substances with the same MoA. The identification of these families should involve multiple perspectives, based not only the chemical similarity, but also on the toxicological, toxicokinetics and physico-chemical properties. Examples of the available software tools for the use of *in silico* models and read across comprise the OECD QSAR Toolbox[2], VEGA[3], and AMBIT[4]. Furthermore, other models can be useful to assess possible interactions, for instance using a program that predicts if a substance inhibits the activity of an enzyme involved in the metabolism of a second substance.

3.1.4. Examples using bioassays to characterize the combined effect of real-world chemical mixtures: Endocrine disrupting chemicals

One of the main challenges when evaluating the effects of exposure biomarkers for endocrine disrupting chemicals (EDCs) is that there are many known and unknown compounds in a complex mixture. EDCs are important reproductive toxicants. Xenoestrogenicity/Antiandrogenicity due to exposure to mixtures have been associated with hormone dependent cancers in humans such as breast cancer, cryptorchidea/hypospadias, birth weight and neurodevelopmental disorders (Vilahur et al. 2013, Vilahur et al. 2014, Arrebola et al. 2015, Pastor-Barriuso et al. 2016). *Ex vivo* bioassays can evaluate the combined effect of complex chemical mixtures in human samples.

Within the PROTECTED project in random samples originating from the Norwegian HUMIS biobank of human milk estrogenic (using ERα-CALUX) and anti-androgenic activities (using AR CALUX bioassays) have been detected in response to the presence of anthropogenic polar EDCs without direct interferences from natural sex hormones (Collet et al. 2020).

Several different occupational exposure profiles have been also linked to estrogenic and androgenic activities in blood of men (Brouwers et al. 2011). Elevated androgenic levels were found in smokers and heavy drinkers and in men occupationally exposed to disinfectants or welding/soldering fumes. Occupational exposure to pesticides, disinfectants, and exhaust fumes seemed to be associated with increased plasma estrogenic levels.

Leukemia incidence has increased in recent decades among European children, suggesting that early-life environmental exposures play an important role in disease development. There is also increasing evidence that some kind of cancer risks are linked with EDCs interfering with growth and sexual hormone system. This evidence is supported by estrogenic and androgenic activities (ER and AR CALUX) in ~ 750 blood samples from mother-child cohorts from five different European countries within the NewGeneris project. The examples show that with existing HTS technics relevant information for health protection can be generated (Judson et al. 2018). And endocrine disrupting MoAs that may contribute to carcinogen-induced leukemia and require further research (Merlo et al. 2014).

However, one of the challenges using bioassays is that, when the composition of the mixture is unknown, its inclusion in risk assessment is difficult. Therefore, it is important to isolate specific chemical families and test their combined activity. For example, mixtures of PFAS were isolated from serum obtained from 702 pregnant women (Bjerregaard-

[2] https://www.oecd.org/chemicalsafety/risk-assessment/oecd-qsar-toolbox.htm

[3] https://www.vegahub.eu/

[4] http://ambit.sourceforge.net/

Olesen et al. 2015), and showed that the PFAS mixture exerted a higher xeno-estrogenicity, which was associated with reduced fetal growth suggesting an estrogenic MoA on this outcome (Bjerregaard-Olesen et al. 2019). This opens the possibility to evaluate the combined effects of chemical families and mixtures in human populations, and efforts are needed to integrate this approaches in risk assessment.

Subsequently, comprehensive characterization of exposures and effects in an integrated fashion will facilitate inferences for regulatory risk assessment (Fan et al. 2019, Jarabek and Hines 2019).

3.2. Part II. Approaches to handle complexity of effect biomarker utility

3.2.1. Adverse outcome pathway (AOP) and its role in revealing effect biomarkers

One of the important sources of information for potential biomarkers identification and interpretation could be the molecular initiation events (MIEs) and key events (KEs), especially those common KEs, described in the existing framework of Adverse Outcome Pathways (AOP; OECD, 2018). Currently, available AOPs can be retrieved from the AOP knowledge base (AOP-KB), which was developed under the OECD umbrella. The AOP-KB is an open-data repository of chemical induced toxicity pathways as part of the OECD AOP Development Effort by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EGMAST). The Adverse Outcome Pathway Knowledge Base (AOP-KB)[5] is the main entry point. It consists of two main modules: the AOP-Wiki[6] and the Effectopedia[7]. The AOP-Wiki is designed to support formal, qualitative AOP development following the guidance provided in the OECD Users' Handbook (OECD, 2018). Qualitative AOP provides a scientifically credible basis to link apical hazards of regulatory concern to specific pathway perturbation or altered biological processes (often measured using *in vitro* assays). However, for many regulatory purposes the exposure data are required (dose, duration, frequency etc.) under which an adverse outcome (AO) will be observed. This information is captured in Effectopedia, where data on dose- and time-response are introduced. This information can be used to derive quantitative response-to-response relationships between KEs (quantitative AOPs) resulting in an AO. Therefore, regulators seeking mechanistic information to support the chemical safety evaluation process can consult the AO-KE information. The mechanistic information facilitates the identification of effect biomarkers that then can be further validated prior to their implementation for various regulatory purposes (Sachana 2019).

Furthermore, reliable effect biomarkers can be interpreted and used better, when there is a sufficient understanding of the AOP or mode of action (MoA) of the chemical, and a causal relationship of biological events linking the marker and the AO. This can be achieved by developing AOP or even better AOP networks, for a specific AO/disease through sharing of KEs and key event relationships (KERs). However, the molecular initiating event (MIE) in an AOP can be initiated by a variety of chemicals, while a related MoA can be specific for a chemical. Therefore, from the AOP a MoA can be developed by including chemical-specific information and a prediction of the relationship between the chemical concentrations at the site of the MIE, causing the MIE perturbation, which would then trigger a cascade of key events resulting in AO. AOP describes a sequence of key events (KEs) beginning with the initial interaction of a chemical with a molecule in a target cell or tissue (MIE) progressing through causally linked KEs at different biological organization (cell, tissue, organ, organism), resulting in an AO (Bal-Price et al. 2017, Corton 2019). KEs must be empirically observable and should be reproducibly and quantitatively measurable using different test systems. Early KEs (molecular or cellular events) can serve as a basis to develop mechanistically based, reliable and predictive effect biomarkers for adverse outcome/disease, that are easily measured using *in vitro* assays. AOPs developed according to the OECD template should be submitted to the OECD AOP -Wiki where they are publicly available and can serve as a reliable source of information when identifying potential effect biomarkers. Each individual AOP should be considered as a building block within a larger AOP network that represents more comprehensively the complexity of biological processes involved in an adverse outcome (Bal-Price et al. 2017). Moreover, MIE of a single AOP is likely to be triggered by a limited number of compounds and probably belonging to the same class. Therefore, a development of an AOP network by assembling individual AOPs, interconnected through common KEs is a more reliable approach as shown in the case of neurotoxicity (Spinu et al. 2019).

The identified CKEs in the AOP network can serve as reliable anchors for identification of effect biomarker in *in vitro* assays for single chemicals or mixtures. A threshold value defined for MIEs (or KEs) can directly inform risk assessment for regulatory purposes. In environmental risk assessments the terminology effect-based trigger values is used (Escher et al. 2018, Brion et al. 2019), but for occupational assessments the terminology Occupational Biomonitoring Effect Level (OBEL) was proposed this year in the Occupational Biomonitoring activity of OECD Working Parties on Exposure and Hazard Assessments (WPEA/WPHA) (2nd Webinar on the 3rd of April 2020). An easy translation of identified mixture effects by existing biomonitoring knowledge can be envisaged. Occupational Biomonitoring Levels (OBL) are linked to adverse and accepted Point of Departures (PoD) in risk assessments. Therefore, if a mixture response of an effect biomarker exceeds an OBL as an equivalent concentration (e.g. lead (Pb) can be used as trigger equivalence substance for many neurotoxic effects), an identification for an adverse risk potential is given and can trigger further steps. This approach needs to be further discussed and developed to bridge exposure and effect biomarkers in the future (see chapter 3.3).

Recently such an approach was used for the evaluation of developmental neurotoxicity (DNT) effects induced by a combined exposure to mixtures of different classes of chemicals (Pistollato et al. 2020). Effect biomarkers in *in vitro* assays were used to evaluate synaptogenesis, neurite outgrowth, and brain derived neurotrophic factor (BDNF) protein levels. BDNF levels were identified as CKEs in the AOP network relevant to impairment of learning and memory in children (recently the most prevalent AO) (see Fig. 1 in Pistollato et al.) (Pistollato et al. 2020). Scientific validation of 16 DNT *in vitro* assays and their readiness for diverse regulatory applications have recently been evaluated. Thirteen semi-quantitative criteria were used for this evaluation referring to the characterization of diverse test systems, exposure schemes, main measured endpoints, cytotoxicity, test methods controls, data evaluation, reproducibility, test benchmarks (sensitivity, specificity and acceptance criteria), prediction model, applicability domains and definition of a hit (Bal-Price et al. 2018). Inclusion of this battery of *in vitro* test methods in the OECD Guidance Document for *in vitro* DNT testing is planned. It is currently under development in collaboration with EFSA and US EPA (Sachana et al. 2019).

Sensitivity of the proposed *in vitro* assays is evaluated by comparing neurotoxic effects that are observed at the concentrations below acceptable population blood levels. For example in occupationally exposed adults, subtle or nonspecific neurocognitive effects have been reported at blood lead levels as low as 20–30 μg/dL (Mantere et al. 1984, Schwartz et al. 2001), with overt encephalopathy, seizures, and peripheral neuropathy generally occurring at much higher levels (e.g., higher than 100–200 μg/dL) (CDC, 2017). Lead is also an important neurodevelopmental toxicant since it crosses the human placenta and accumulates in fetal tissue during gestation (Gundacker and Hengstschläger 2012). Although, no blood lead level for children is considered safe, most governmental agencies, including the US Environmental Protection Agency and Centers for Disease Control and Prevention (2012), have concluded that there is sufficient evidence for adverse health effects in children and adults at blood lead level below 5

[5] https://aopkb.oecd.org/index.html
[6] https://aopwiki.org/
[7] https://www.effectopedia.org/

μg/dL. This level was derived from the upper 2.5% distribution of blood lead levels among U.S. children ages 1–5 years. Indeed, several research groups observed impaired cognitive functions at levels as low as 5 μg/dL (Chiodo et al. 2007, Bellinger 2008, Jusko et al. 2008). Furthermore, sensitive *in vitro* effect biomarkers e.g., synaptogenesis quantification, can identify the toxic effects from lead exposures as low as 0.2 μg/dL (Pistollato et al. 2020). This demonstrates that the *in vitro* assays have sufficient sensitivity to identify neurotoxic effects.

3.2.2. Application of physiologically based kinetic modelling for exploring effect biomarkers of mixtures

Physiologically Based Kinetic and dynamic (PBK/D) models can assist in evaluating the appropriateness of effect biomarkers utility. PBK/D models are built using biologically relevant compartments representing organs of the body interlinked by blood circulation. The models are driven by mass-balance ordinary differential equations (ODEs) to describe the ADME processes of a chemical as a function of the physiochemical (tissue:blood partition coefficient), biochemical (metabolic rate constant, like Vmax and Km), and physiological (body weight and tissue volume) characteristics with link to dynamic endpoints. Internal concentrations of chemicals and relevant metabolites and/or biological changes can be estimated with PBK/D models which are not feasible in humans (Phillips et al. 2014). When co-exposure to multiple chemicals is considered, changes in the rates of ADME processes should be taken into account. For instance, the distribution rate may differ due to competitive binding sites to protein, reduction of metabolic rate (inhibition) may be the result of two or more chemicals competing for the same enzyme (Tan et al. 2011). As reported in Tan et al. 2011 a mechanistic model for chemical mixtures should take into account three major elements: (1) The interaction among individual chemicals in the mixture at the level of kinetics and dynamics; (2) Quantitative descriptions of both temporal (i.e., concurrent or sequential exposures) and dose relationships among individual chemicals; and (3) Each chemical's mode of action. PBK/D models for single chemical or a mixture of chemicals can provide insight into the selection of biomarkers of effects to strengthen the correlation between exposure and endpoint/adverse outcome. Table S2 in the Supplementary Material expands the work published by Desalegn et al. (2019), who identified 35 types of PBK/D models developed in the last three decades for mixtures. In addition, the pathways of effect, effect biomarkers and endpoints for several classes of chemicals and with different complexity of mixtures were mapped. Among these studies, PBK/D models were built to simulate the kinetics of the alkenylbenzene class (Estragole, Safrole, Methyleugenol), which are known to be hepatocarcinogenic in rodent species (Desalegn et al. 2019). Not only the kinetics were investigated, but also a translation of results into the likelihood of formation of DNA adducts (Paini et al. 2010, Martati et al. 2014). By expanding the PBK model to PBD model to predict *in vivo* DNA adduct formation in liver, it was shown how an early effect biomarker, can provide a step closer to the ultimate toxic effect (genotoxicity) (Paini et al. 2010). Other examples of biomarkers and PBK/D models for VOCs, Pesticides, Aldehydes and PCB are summarized in the Supplementary Material Table S2. These studies are also described in Tan et al. (2011) showing the potential of using effect biomarkers with PBK/D models. Overall, the proper use of PBK models can help in understanding the uncertainties that arise from biokinetic interactions and disposition of chemical mixtures and to identify data gaps to derive health-based guidance values (RfD and/or ADI). These models can be developed to describe the MoA and tissue responses of a chemical, but also incorporate information on the human inter-individual variability and incorporate complex elements (like fetus compartment). By linking the kinetics to the associated dynamic health risks, the exposures to complex chemical mixtures can be assessed due to identification and quantification of biomarkers of effect. PBK/D models can enhance the interpretation of effect biomarkers by providing insight into quantitatively connecting external exposure to the internal concentration at the organ site linked to known key events (Phillips et al. 2014).

PBK/D models have several advantages but also some limitations. To be able to interpret and simulate an effect biomarker the model must have a higher degree of complexity including, for instance, metabolism or including other dynamic processes underling the chemical MoA. PBK/D models have the potential to extrapolate outside the range of constraining data but within quantifiable limits of uncertainty for complex mixtures (Jasper et al., 2016). Here expert judgment come in accounting for between model simplicity and complexity, and biological plausibility. PBK/D models can also help in understanding how specific an effect biomarker is to a specific chemical within a mixture, like a "specific fingerprint". Finally, it is known that the kinetic interactions due to mixture exposures can significantly complicate the interpretation of HBM data. However, PBK/D models can help to interpret HBM, providing the understanding of chemical exposures and biomarkers of effects in human populations. In addition, application of the PBK/D models accounts for ADME processes and helps in establishing the degree of correlation between the biomarker and the metric of interest and, finally, they can facilitate the discovery of new biomarkers (Phillips et al. 2014).

3.3. Part III. Use of effect biomarkers in occupational biomonitoring

The OECD Occupational Biomonitoring subtask effect biomarkers is an *Ad hoc* expert group proposed for OECD WPEA and OECD WPHA in April 2019 and launched in September 2019 as a joint proposal from biomonitoring experts from four countries. This group now has support from more than 60 experts representing 40 different institutes and aims to provide a biomonitoring guidance for effect biomarkers. This group has produced a set of priority mode of actions, recommendations of usable effect biomarkers, and a suitability checklist for characterizing effect biomarkers. The following sections describe the status, preliminary conclusions, and knowledge collected by this group. The current use of biomarkers was intensively discussed within ISES Europe activities and have been described in a recent publication (Viegas et al., 2020).

3.3.1. Rationale for implementing the use of effect biomarkers in occupational biomonitoring

The knowledge regarding co-exposures to chemicals is quite limited for many work situations. This is mainly because it is difficult to identify all the compounds and their fluctuations during the wide diversity of job tasks performed during a workday or production period. In addition, little information is available regarding the effectiveness of the risk management measures (RMM) in place, particularly for personal protective equipment.

Numerous studies have shown a significant association between occupational exposures to chemicals and various diseases including chronic diseases such as cancer (Prüss-Ustün et al. 2011, Purdue et al. 2015). Despite this knowledge, only very few chemicals at work are measured which is often due to cost constraints and analytical limitations. The economic cost of cancer has been estimated to 396′000 Euro per case in an ECHA study (Ščasný and Zvěřinová 2014). For comparison, the value of a statistical life was estimated at 6.2 million Swiss francs or 5.5 million Euros (OECD 2012). Moreover, occupational diseases not only reduce life expectancy but can also lower productivity because, besides mortality, morbidity is also responsible for high economic costs to society. An overview of economic costs and effects of policies aiming at reducing work-related diseases and injuries can be found in Appendix B, Fig. B1.

Workers are exposed to a large number of chemicals, products and formulations as well as physical and biological agents; however, only a few of these are characterized in terms of health risks. For example, an exposure scenario in the REACH regulation estimating the probability and intensity of exposure for a relevant work activity is only required if the chemical is put on the market at 10 tons per year and carries a hazard

Table 2
Proposed priority mode of actions /endpoints to be addressed by occupational biomonitoring.

No	Mode of actions /endpoints	Abbreviation	Available methods as OECD guideline, DIN EN ISO standards or others
1	Carcinogenicity (including cancer biomarkers for genotoxicity and oxidative stress)	C including genotoxicity	yes
2	Mutagenicity	M	yes
3	Reproduction toxicity	R	yes
4	Endocrine disruption	ED	yes
5	Neurotoxicity (including acetylcholine esterase inhibition)	NT	yes
6	Developmental Neurotoxicity	DNT	OECD GD on *in vitro* DNT assays is under development
7	Developmental Toxicity	DT	yes
8	Respiratory toxicity (including methemoglobin binding)	ResT	yes

classification (ECHA. 2016). The toxicological data for many chemicals is insufficient or inconclusive for a hazard classification. The law requires ECHA to check at least 5% of the registration dossiers for compliance. In a majority of the dossiers that ECHA checks for compliance, important safety information on chemicals are omitted. ECHA then requests this information to complete the registration dossier. Moreover, some industrial processes can change often and workers need to adapt to these changing working conditions. These changes will likely result in different occupational exposure scenarios implying different chemical substances at variable levels and durations. Few regulatorydemanded exposure scenarios can only try to reflect a part of this ever-changing reality. Consequently, there exists a large knowledge gap and uncertainty in the current substance-based risk assessment EU approach, where no options to change it midterm exists. To address these challenges in the future, we recommend using suitable effect biomarkers. Integrating effect biomarkers into HBM programs as occupational risk assessment and management tools, can directly quantify the effects of the occupational exposure from single chemicals and chemical mixtures. These HBM programs have an important role in occupational health interventions since it provides information about the group of workers with higher risk that should be targeted for RMM. We provide here examples of successfully developed biomarkers, their application, and explore their potential use in occupational risk management.

3.3.2. Setting the priority mode of actions (MoA) and endpoints to be addressed

The OECD occupational biomonitoring subtask effect biomarker have prioritized eight relevant MoAs that should be considered when assessing occupational health. These MoAs are listed in random order in Table2 taking into account existing (CMR, ED) and needed classifications to cover important safety gaps for workers.

3.3.3. Recommendation of effect biomarkers and promising assays by experts

The experts in the OECD occupational biomonitoring subtask effect biomarker were asked: Which effect biomarkers should be used? Which are the promising assays to develop future effect biomarkers? This list of potential suitable effect biomarker was discussed, refined and concluded in several meetings and is provided in Table 3.

3.3.4. Examples using effect biomarkers for detection of genotoxic and carcinogenic substances

One of the main challenges of exposure biomarkers is that they are limited to assessable and analytically detectable compounds. Hexavalent chromium (Cr (VI)) is and remains an important occupational carcinogen. Exposures to Cr (VI) cause lung cancer in humans. The main limitation of urinary chromium detection is that it is not specific for Cr (VI) since it measures exposure to both Cr(III) and Cr(VI). Also, the lowest biological limit value (BLV) given for Cr(VI) (2.5 µg/L in France), which is close to background urinary Cr levels in populations with no known occupational exposure (e.g.in France 95th percentile in general population is 0.65 µg/L (ANSES 2017)). These circumstances illustrate the need to develop biomarkers specifically indicating Cr(VI) or effect biomarkers assessing overall genotoxic and carcinogenic risk, which are associated with exposures to the mixture. In the HBM4EU project, around 400 samples from workers exposed to chromium will be collected from eight European countries (Belgium, Finland, France, Italy, Poland, Portugal, the Netherlands and the United Kingdom (UK)). This study have been described in Santonen et al. (2019). The study compares exposure biomarkers with some selected effect biomarker for genotoxicity with micronuclei frequency, oxidative stress, and telomere length as well as an epigenetic biomarker. This study intends to the comparison of several markers of exposure and effect in a variety of exposure scenarios (Santonen et al. 2019). The results of the study will be reported within the reporting policy of the HBM4EU project, and a general report will be publicly accessible via the project website[8].

Additionally, a recent systematic review and *meta*-analysis of most recent literature on occupational exposure to styrene using the cytokinesis-block micronucleus (CBMN) assay identified a 34% overall increase of genomic instability and DNA damage in exposed workers as compared to unexposed controls, regardless of gender, age or smoking status. This result revealed convincing evidence linking exposure to styrene with micronuclei frequency and supports the use of the CBMN assay in monitoring genetic risk in workers (Costa et al. 2016).

3.3.5. Checklist to select appropriate effect biomarkers in occupational studies

A suitability checklist to characterize the recommended effect biomarkers was developed by the OECD biomonitoring effect biomarker subgroup. In principle, all the relevant MoAs (Table 2) can be covered by at least one effect biomarker associated with validated methods (see Table 3 with OECD and DIN EN ISO guidelines or standards). However, they are not necessarily suitable occupational effect biomarkers. Only some of these biomarkers are recommended (see Table 3) for further characterization. Neurotoxicity and respiratory toxicity are only included in the German occupational risk assessment. Effect biomarkers for reproduction and developmental toxicity are not easily feasible. One reason is that these effects can mainly be detected with long-term exposure experiments that can detect effect during a sensitive life stage. For effect biomarkers to be a suitable tool in occupational health risk management, they need to be reliable, robust, and provide an understanding of exposure and effect in the human body. Effect biomarkers need to be characterized in both internal and external validation processes. Validated test guidelines should be preferred, such as OECD test guidance or DIN EN ISO standard, to ensure analytical accuracy, reproducibility, and reliability. The suitability of these recommended biomarkers will be rated by effect biomarker experts, effect biomarker developers and applicants using a set of 13 questions (see Supplementary, Table S3) evaluated by a scoring system (HBM4EU et al., 2017). This should provide a more detailed understanding of the suitability of each of these biomarkers concerning validation, relevance, sensitivity, specificity, cost-efficiency, and robustness. The results of this survey are expected later this year.

[8] https://www.hbm4eu.eu/

Table 3
Potential suitable effect biomarkers to be characterized for occupational use or already applied in other contexts.

No	Covered MoA or endpoint	Name of the assay or effect biomarker	Biomarker categorization	Measured endpoint
1a	C including genotox	Mammalian Erythrocyte Micronucleus Test Peripheral blood lymphocyte micronucleus test	*ex vivo*	induction rate of micronuclei
1b	C including genotox	Buccal micronucleus assay	*ex vivo*	induction rate of micronuclei in buccal cells Micronuclei frequencies epithelial buccal cells
1c	C including genotox	Cytokinesis-block micronucleus assay (CBMN-Assay)	*ex vivo*	induction rate of micronuclei in peripheral blood lymphocytes
1d	C including genotox	Peripheral blood lymphocyte micronucleus test and Buccal mucosa micronucleus test	*ex vivo*	Micronuclei frequencies in lymphocytes and in epithelial buccal cells
1e	Oxidative stress level indicative for C and genotox	reduced/oxidized glutathione (GSH/GSSG) ratio	*ex vivo*	increase or decrease of the GSH/GSSG ratio
2a	M	The Ames Test/Bacterial Reverse Mutation Test	*in vitro*	measuring reverse mutation in bacterial cells
3	R	Reproductive Hormones - testosterone, estradiol, Luteinizing hormone, Follicle Stimulating Hormone	*ex vivo*	measuring levels in serum
4a	ED- ER receptor activation	ER CALUX	*in vitro*	measuring the receptor activation of the human estrogen receptor
4b	ED- AR receptor activation	AR CALUX	*in vitro*	measuring the receptor activation of the human androgen receptor
4c	ED- TR receptor activation	TR CALUX, anti-TR CALUX, TTR-TR CALUX, TTR-FITC assay, TPO assay	*in vitro*	measuring the receptor activation of the human thyroid receptor
4d	ED-steroidogenesis modulation	H295-R-steroidogenesis modulation assay	*in vitro*	measuring steroidgenesis modulation
5a	inhibition of acetyl-choline-esterase	acetylcholine-esterase-inhibition assay	biochemical/ biological	Measuring inhibition of acetyl-choline- esterase
5b	neuronal cytoskeleton integrity	neuroaxonal damage/ scaffolding proteins	biochemical/ biological	Neurofilament-light chain (NF-L) in serum
5c	neuronal cytoskeleton integrity	neuroaxonal damage/ scaffolding proteins	biochemical/ biological	Neurofilament light chain (NfL), neurofilament medium chain (NfM), neurofilament heavy chain (NfH), α-internexin and peripherin in serum/ blood
5d or 6a	Evaluation of key neurodevelopmental processes as Brain Derived Neurotrophic Factor (BDNF) (indicative of neuronal survival, development and synaptic plasticity)	Neurite outgrowth, synaptogenesis, glial proliferation, migration and neuronal electrical activity brain Derived Neurotrophic Factor (BDNF) isoforms IV and IX	biochemical/ biological/ morphological and functional *in vitro* assays *in vitro* and/or in blood sample	Measurements of length, number of neurites and branching points per neuron; expression for pre- and postsynaptic protein (e.g. co-localization of synaptophysin and PSD95); number of diverse neuronal and glial subtypes; measurements of cell migration distance; recording of neuronal electrical activity (spontaneous or evoked) using microelectrode array (MEA) Measurements of BDNF at protein and mRNA levels
6b	DNT	Thyroid stimulating hormone (TSH), triiodothyronine (T3), thyroxine (T4)	biochemical/ biological	Levels in serum during pregnancy
7a	The murine embryonic stem cell test (EST) – to assessembryo-toxicity (teratogenicity) potential of chemicals*	Sarcomeric myosin heavy chain and alpha-actinin proteins	*in vitro*	Quantitative expression of sarcomeric myosin heavy chain and alpha-actinin proteins in beating cardiomyocyte's as well as counting of contracting cardiomyocyte agglomerates. The morphological analysis of beating cardiomyocytes in embryoid body outgrowths compared to cytotoxic effects on murine ES cells and differentiated 3 T3 fibroblasts.
7b	Male-mediated developmental toxicity and mutagenicity*	dominant lethal and specific locus mutation tests: *in vivo*; DNA methylation: *in vitro*	Different biomarkers in battery	Battery of tests to identify germ cell mutations, such as dominant lethal and specific locus mutation tests, epigenetics (DNA methylation e. g. acrylamide, lead)
8a	Methemoglobin respiratory toxicity	Methemoglobin binding assay	*ex vivo* / biochemical	measurement of building of methemoglobin which is functional inactive

* Will be not characterized further due to expected sensitivity coverage under DNT.

Table 4
Preliminary list of a suitable reference compounds which can serve for sensitivity comparisons and as provisional Occupational Biomonitoring Effect Levels.

No	Proposed priority mode of actions /endpoints to be addressed by occupational biomonitoring	Proposed reference compound	Available accepted Occupational Biomonitoring Level which can serve as provisional Occupational Biomonitoring Effect Level
1	Carcinogenicity (including cancer biomarkers for genotoxicity and oxidative stress)	formaldehyde for genotoxicity and H_20_2 for oxidative stress	Other substance or derivation need for a provisional OBEL
2	Mutagenicity	4-nitro-ophenylenediamine or 2-nitrofluorene	Other substance or derivation need for a provisional OBEL
3	Reproduction toxicity	BPA or DEHP	Other substance or derivation need for a provisional OBEL
4	Endocrine disruption	17-β-estradiol for estrogenicity and 2-dihydrotestosteron for androgenicity	Other substance or derivation need for a provisional OBEL
5	Neurotoxicity (including acetylcholine esterase inhibition)	lead or Chlorpyriphos	BLV-ECHA/RAC = 150 microgramm/L in blood or BGW, TRGS 903 AChE inhibition erythrocytes 70%
6	Developmental Neurotoxicity	lead	BLV-ECHA/RAC = 150 microgramm/L blood
7	Developmental Toxicity	lead	BLV-ECHA/RAC = 150 microgramm/L blood
8	Respiratory toxicity (including methemoglobin binding)	Carbon monoxide	BGW, TRGS 903 CO-Hb: 5% blood

3.3.6. *A tiered- approach for interpretation of effect biomarker responses*

A tiered approach is proposed for using effect biomarkers to indicate exposure and health risks.

- Tier I: relevant MoAs/endpoint is or could be activated due to significant elevated effect biomarker levels in the exposed population compared to the general population level (baseline) or other evidence that can lead to adverse risks (see classifications in Table 2)
  → need to determine a provisional Occupational Biomonitoring Effect level (OBEL)
- Tier II: exceedance of a provisional Occupational Biomonitoring Effect level (OBEL)
  → Prioritization and improvement of Risk Management Measures (RMMs), further exposure assessments
- Tier III: exceedance of a refined OBEL
  → immediate RMM and health surveillance

To ensure that an effect biomarker can be used in an occupational health context, the response of the effect biomarker needs to be assessed and population or similar exposure groups specific cut-offs developed. The workplace response of the effect biomarker can be compared with the known responses in the general population. If there are no significant differences, occupational exposures can be excluded with a high probability. In case the effect biomarker response exceeds significantly the population level it is likely that the RMMs are insufficient or another unexpected exposure due to a change in the working conditions occurred. This result can trigger a derivation of a provisional Occupational Biomonitoring Effect level (OBEL) and later on improvements in the RMMs or further exposure assessments. This should be followed by the derivation of a refined health-based OBEL, which should consider the AOP knowledge to ensure that the workers are sufficiently protected. A procedure on how to conduct such a derivation needs to be elaborated, and preferably, within the framework of the ongoing OECD activity.

Challenges on data communication and report can be surpassed by applying the Similar Exposure Group (SEG) approach, fully described in the European norm EN 689/2020 2020 (Workplace Exposure—Measurement of Exposure by Inhalation to Chemical Agents—-Strategy for Testing Compliance with Occupational Exposure Limit Values). Additionally, the format developed by ECHA for exposure data reporting, with some adaptations, can also help reporting biomarkers of effect data[9]. An update proposal for the reporting of effect biomarker results is intended within the OECD activity.

3.3.7. *Need of health-related effect-based trigger values for selected suitable effect biomarker*

Exposure can be identified easily if the variability of the effect biomarker in exposed and unexposed (background) conditions are known. It becomes more challenging to assess health risks, but this is where effect biomarkers can be used. As a simple rule of thumb, the response of most effect biomarkers can be translated into equivalent concentration of a MoA specific reference substance. This equivalent concentration expresses the mixture effect in terms of an analytical comparable value (see Table 4).

In an optimal case, a valid biomonitoring exposure limit exist for the reference equivalent substance and can be compared directly. For setting provisional effect-based trigger values, an already existing and accepted Occupational Exposure Level can serve as the indicator for risks associated with exposure to chemical mixtures. Equivalent concentrations above an exposure limit value can be used to identify unacceptable health risks and trigger a follow up step. Therefore, for every relevant MoA, suitable reference substances should be provided, preferably with existing or easy to derive biomonitoring exposure limits.

4. Conclusions, outlook and identified needs

Effect biomarkers assist in elucidating the causal relationship between exposures and health outcomes in the general and occupational population. These type of biomarkers can serve as a powerful biomonitoring tool for assessing exposures to known and unknown chemical mixtures, interpreting exposure biomarker measurements, and bridging the exposure-health effects relationship gap in risk assessment. Despite such advantages, effect biomarkers are only marginally used in biomonitoring of human populations. This might be explained by the absence of health-based biological limit or guidance values in the general and specific populations (e.g., man vs. women, adult vs. children) as well as the absence of general guidance on how to use effect biomarkers in risk assessment frameworks. Additionally, effect biomarkers are required to be sensitive, specific, biologically relevant, feasible, practical (e.g. in terms of analytical complexity), and inexpensive for use in epidemiological, experimental, and mechanistic studies in relation to a given chemical compound of interest (Baken et al. 2019). The use of effect biomarkers in large studies depend also on the availability of high-quality, validated, high-throughput analytical methods. Such robust methods ensure that the biomarker data obtained are accurate and precise through careful quality control of all steps ranging from sample collection to analytical evaluation.

We showed with the AOP concept that effect biomarker can serve as

[9] available here: https://echa.europa.eu/documents/10162/22979809/tmpl_reporting_occupational_exp_data_du_en.xlsx/84ef3203-4294-75c8-3b79-9c024abc2bcd).

an early warning system in risk assessment. Further, identification of effect biomarkers and their mechanistic pathways following the AOP framework can definitively help to prioritize the implementation of related sets of effect biomarkers in a more structured way, as well as improve the interpretation of biomarker data. Given that several chemical families can influence an AOP, effect biomarkers integrate the convergent effects of chemical mixtures on a particular AO. We conclude that regulatory agencies can use AOPs to derive effect based trigger values to address adverse risk levels from chemical exposures, and then identify MoAs that relate exposure to response to set these limit values. Simulation tools such as PBK/D modeling can help in this regard. Several effect biomarkers are validated for relevant MoAs and offer a direct assessment of overall health risks associated with exposure to chemicals, their transformation products and chemical mixtures. An important step forward is a list of relevant MoAs generated by the regulatory agencies that can be implemented in an EU-wide human biomonitoring strategy. The EU HBM4EU project is currently comparing exposure biomarkers for chromium and some selected effect biomarkers for the general population and workers, but no regulatory use in population monitoring as of yet. The OECD Occupational Biomonitoring activity of the Working Parties of Hazard and Exposure Assessment has prioritized eight relevant MoAs. They recommend characterizing 20 more suitable effect biomarkers. We propose to use the existing AOP knowledge for derivation of cross-regulatory usable effect-based trigger values to be able to address exposures to known and unknown chemical mixtures. As a starting point, MoA specific exposure biomarkers can be used for further refinements. Inter-regulatory agency collaborations would not only reduce the fragmentation observed in different regulations, but also stimulate a harmonized use of effect biomarkers and development of new and the use of already established effect biomarkers. To this end, ISES Europe and OECD Occupational Biomonitoring working groups suggest developing a list of suitable effect biomarkers for regulatory use. Furthermore, a considerable amount of consistent data is necessary to quantitatively link results from different studies. Developing reporting standards would ensure reliability, reproducibility and efficiency of data collection, as well as transparency when interpreting findings, and relevance for evaluations, ultimately increasing their utility for informing regulatory risk assessment. Therefore, development of a reliable effect biomarkers' database, linked to exposure and susceptibility biomarkers, although challenging, is necessary for the best use of available data. The overarching direction is to advance the use of effect biomarkers in exposure science and implement these in ISES Europe's general HBM strategy roadmap. The link of existing suitable effect biomarker with future effect-based trigger values will lead to an improved and systematical use of effect biomarker.

Disclaimer

The publication was generated as a joint activity of European chapter of International Society for Exposure Science (ISES Europe) including experts of an OECD Occupational Biomonitoring activity effect-biomonitoring subtask. As the OECD work is in progress, any text that refers to opinions or recommendations about the OECD work is considered preliminary and as the opinions of the co-authors. The OECD expert group development of draft guidance documents is on-going and will be subject to review and endorsement under the processes of the OECD committee structure. This can lead to changes in the approaches and recommendations documented based on further OECD discussions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A

In silico Models simulating environmental exposures and/or human exposures exist and can estimate internal dose in an organism. Most of the efforts have been directed to model the internal dose of pharmaceuticals. There are also software programs applicable to animals, which are relevant for human health, when the animal or its products are used as food for humans.

Regarding the possible use of *in silico* tools for the adverse effects, there are hundreds of models available. Some platforms are commercial and require a payment, while others are freely available. Examples of freely available platforms are the OECD QSAR Toolbox[10], EPISuite[11], TEST[12], VEGA[13], the Danish QSAR Database[14], Toxtree[15], QSAR DB[16], and OCHEM[17].

The possibility to estimate exposure levels, and also effect biomarker levels for a large number of substances can facilitate the assessment of the overall scenarios to be investigated. There are several advantages offered by *in silico* models. They are fast, and the predictions can be obtained immediately. They can process a large number of substances; for instance, within the PROSIL projects[18] 6 million compounds have been processed for ten endpoints. As already mentioned, there are hundreds of tools which are free and easily accessible. They do not generate waste, do not use animals, chemical substances, solvents, and can be interrogated without the physical substance to be assessed, thus can be used also for hypothetical substances, before their preparation.

Another advantage is that the results from multiple tools can be easily wrapped, even though this may require some work. The EC project VERMEER[19] is producing a single platform integrating models for exposure and hazard, to facilitate the user.

Appendix B

See Fig. B1

[10] https://www.oecd.org/chemicalsafety/risk-assessment/oecd-qsar-toolbox.htm
[11] https://www.epa.gov/tsca-screening-tools/epi-suitetm-estimation-program-interface
[12] https://www.epa.gov/chemical-research/toxicity-estimation-software-tool-test
[13] https://www.vegahub.eu/
[14] http://qsar.food.dtu.dk/
[15] http://toxtree.sourceforge.net/
[16] https://qsardb.org/
[17] https://ochem.eu/home/show.do
[18] http://www.life-prosil.eu/
[19] https://www.life-vermeer.eu/

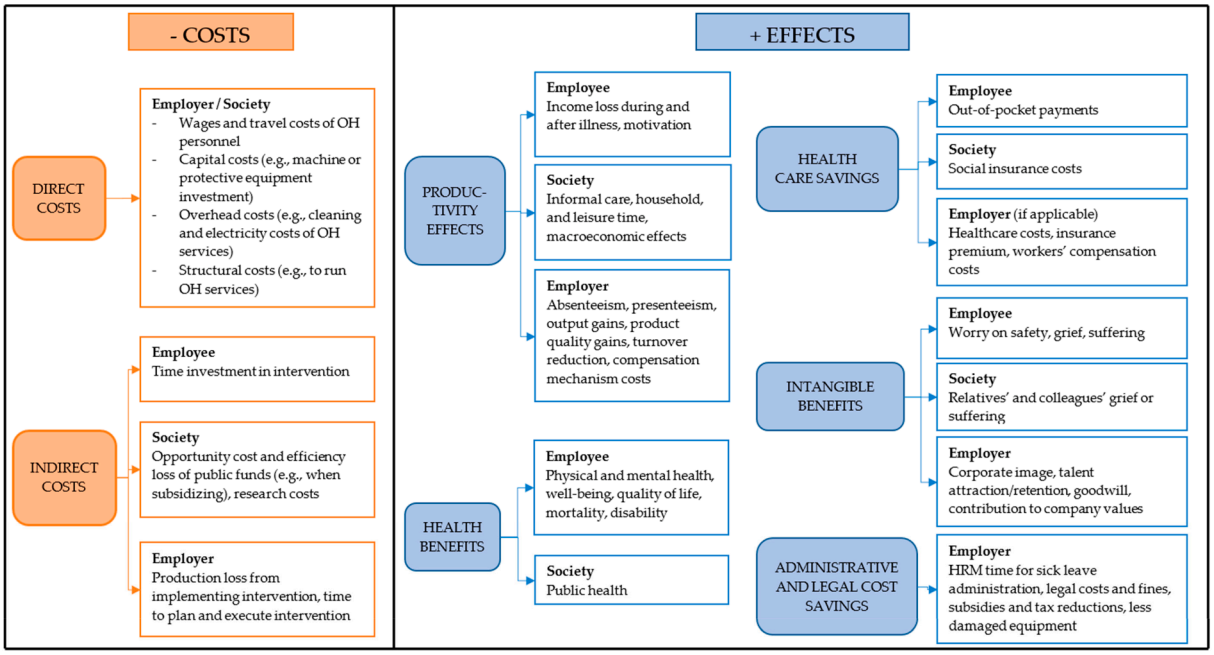

Fig. B1. Costs and effects (source: Jonas Steel et al. Int. J. Environ. Res. Public Health 2018).

Appendix C. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.106257.

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