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SCIENTIFIC REPORT

Conceptual basis for the development of guidance for the use of biomarkers of effect in regulatory risk assessment of chemicals

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts

Abstract

This Scientific Report was carried out in the context of the self-task mandate (M-2023-00097) of the EFSA's Scientific Committee on 'Guidance on the use of biomarkers of effect in regulatory risk assessment of chemicals'. In the first phase, the project on biomarkers of effect started with a feasibility study (EFSA-Q-2024-00128), with the intention to look closer at definitions and descriptions of biomarkers of effect, as well as to explore several concepts related to the context of application and other scientific principles to be further considered for its development. In addition, relevant activities, initiatives and knowledge in this area were collected and analysed within a complementary mapping study. The outcome of this phase aimed to create a structured basis for future guidance, to identify challenges and to recommend a way forward for its development. The recommendations refer especially to terminologies, the scope of the guidance and several scientific and technical aspects of the selection and interpretation of biomarkers of effect that need to be addressed in future guidance. Moreover, further recommendation refers to the collaborative process to be established with other regulatory organisations that should support the harmonisation and reduce divergencies in the application of methodologies across organisations or sectors.

KEYWORDS

adverse effect, adverse outcome pathways, biomarkers, biomarkers of effect, risk assessment

CONTENTS

SUMMARY

This Scientific Report represents the first output of the self-task mandate (M-2023-00097) of EFSA's Scientific Committee aimed to develop guidance on the use of biomarkers of effect in regulatory risk assessment of chemicals. The aim of this report (EFSA-Q-2024-00128) was to gather relevant information to further lay the basis for defining the criteria or conditions for the use of biomarkers of effect for the risk assessment of chemicals. As such, the report compiles relevant information laying the groundwork for subsequent steps of the guidance development, e.g. it covers definitions and descriptions of biomarkers of effect, as well as other scientific principles to be further considered in this process. The study has been complemented by a mapping study that collected and analysed examples of biomarkers of effect, as well as relevant projects, publications and databases/tools that can be further used to support the overall project. Finally, the report includes the outcomes of the engagement and collaboration activities performed during the project implementation.

The methodology designed for the report followed the overall goal of the project in creating a structured basis for future guidance, identifying challenges and recommending a way forward for guidance development. The main steps for developing the report included the definition of the context and scope, the definition and description of biomarkers, implications of the use of biomarkers of effect in risk assessment, and completed conclusions and recommendations for the next phase of the project.

Regarding its context, despite the broad fields of application of biomarkers, this document focuses mainly on adverse effects, the risk assessment context and biomarkers of effect following chemical exposures for hazard assessment. These were completed with background information and definitions of other types of biomarkers or applications that could be useful in defining the risk assessment context of use.

The multitude of uses and types of biomarkers generates also a challenge related to the use of terminologies in this area. Therefore, in addition to the generic definition of biomarkers of effect, the report investigated different terms related to biomarkers of effect, used to describe the intermediate and final effects, and the relationship between them. The link to the terminology used with the adverse outcome pathways (AOP) approach was also established.

Further, several characteristics applicable to biomarkers and that establish their validity and/or qualify them for a specific context of use, are described, completed with specific considerations regarding the biomarkers of effect used in the risk assessment context. For the latter, several principles useful for the development of future guidance were identified and discussed. These refer to the main analytical and biological characteristics of the biomarkers of effect, the validity and the selection criteria to be established for the risk assessment use, the link to the AOPs, etc. Furthermore, the report includes a set of representative examples of biomarkers of effect and their description based on an analysis performed by EFSA's Scientific Opinions as well as received via a survey or identified in the scientific literature.

These aspects and concepts are completed by a discussion regarding the possible implications of the use of biomarkers of effect in risk assessment, providing an overview of different aspects and challenges which will be useful for the next phase of the project.

Finally, the report acknowledges the great potential of the use of biomarkers of effect in risk assessment, but also the remaining challenges that need to be addressed and considered within the guidance development, a process implemented ideally within an international co-creation mechanism.

The Annexes of the report include a template proposed for the description of biomarkers of effect with representative examples (Annex 1), the outcome of the mapping study and the inventory with examples of resources generated within the study (Annex 2 and Annex 3), and the outcomes of the collaboration and engagement activities including the survey (Annex 4), the stakeholder workshop (Annex 5) and the public consultation (Annex 6).

1 | INTRODUCTION

1.1 | Background and Terms of Reference as provided by the requestor

Background

Biomarkers *'include almost any measurement reflecting an interaction between a biological system and an environmental agent, which may be chemical, physical or biological'* (WHO/IPCS, 1993). Biomarkers are largely used in clinical medicine, diagnosis, therapeutics, occupational biomonitoring etc., as biological observations to monitor and predict clinically relevant endpoints at an early stage, when organ damage is preventable or less severe and hence appropriate interventions can be planned (Gupta, 2014; WHO/IPCS, 1993).

Biomarker of effect is defined as *'a measurable biochemical, physiological, behavioural or other alteration within an organism that, depending upon the magnitude, can be recognised as associated with an established or possible health impairment or disease'* (WHO/IPCS, 1993). These may consist of molecular biomarkers of effect that indicate an early biological response resulting from exposure to a chemical but not necessarily representing adversity, or that may be used as a predictor for the development of a disease (Rodríguez-Carrillo et al., 2023). It is generally agreed that despite several benefits, the use of biomarkers of effect in risk assessment is limited due to the e.g. lack of validation of most biomarkers of (intermediate) effect (WHO/IPCS, 2001) or the absence of a general guidance on how to integrate and use biomarkers of effect in risk assessment.

Within EFSA remit and generally in chemical risk assessment there are well established procedures for establishing a Health Based Guidance Value (HBGV) that is based on the identification of a suitable reference point (RP) and the application of uncertainty factors (UFs). Usually, a RP is a benchmark dose lower confidence limit (BMDL) or a no observed adverse effect level (NOAEL) based on the observation of adversity (EFSA Scientific Committee, 2021, 2022). Furthermore, an identified Reference Point may be used for the derivation of a margin of exposure (MoE) which is used in the risk assessment of substances which are genotoxic and carcinogenic (EFSA Scientific Committee, 2012), or for which uncertainty does not allow for establishing an HBGV.

However, the difficulty arises when there is no clear evidence of adversity or overt toxicity, as represented by a disease, histopathology or traditional clinical chemistry markers indicative of organ toxicity. This is often the case when the assessment is based on human data and here the risk assessor may need to consider other types of evidence.

EFSA's Scientific Committee (SC) adopted a Statement for establishing Health Based Guidance Values (HBGVs) (EFSA Scientific Committee, 2021), in which the need for early markers of biological changes that precede cellular and tissue architectural and functional damage in the absence of overt toxicity was emphasised. Moreover, it was identified that there is a need to consider sensitive biomarkers of effect in risk assessment more widely across the different sectors within the remit of EFSA, together with a need to harmonise their use across EFSA's Scientific Panels.

For this, a specific guidance is needed to support the risk assessors in applying a harmonised approach regarding the use of biomarkers of effects which are intermediate events in the toxicological pathway leading to apical adverse effects.

Objectives

The goal of the project is to develop a guidance document on the use of biomarkers of effect in regulatory risk assessment to derive Reference Points (RPs) for establishing Health Based Guidance Values (HBGVs) or margins of exposure (MoEs).

In order to achieve this, several challenges need to be addressed, e.g. the harmonisation of terminology and definitions, identification and description of biomarkers, establishing scientific criteria for the selection, validation and correct interpretation of biomarkers of effect data in the risk assessment of chemicals, clarification regarding the type and level of evidence required regarding the causal association between an intermediate event and the adverse outcome, establishing uncertainty factors following an uncertainty analysis, incorporation of existing knowledge and frameworks, e.g. adverse outcome pathways (AOPs), new approach methodologies (NAMs). Establishing a clear scope of the guidance and its applicability (e.g. type of chemicals addressed, combined exposure to multiple chemicals) is also part of the objectives.

Within this project, a dialogue will be established with scientists and organisations from EU and non-EU institutions to learn from existing experiences, to gather information on the approaches taken so far, to collect views and recommendations on possible ways to work in this area. The objective is to collaborate in the development of the approach and co-create an internationally harmonised and agreed guidance on the use of biomarkers of effect in regulatory risk assessment of chemicals. The establishment of such a mechanism for international cooperation is within the scope of this project.

To facilitate the implementation of the project, two phases are planned, as detailed below. The objectives for the two phases refer to:

Phase 1 (feasibility):

- Objective 1. Establishing definitions and descriptors for biomarkers of effect and the overall scope of the guidance.
- Objective 2. Reviewing and mapping the activities, initiatives, knowledge, approaches relevant to the project goal.

Phase 2 (guidance development):

- Objective 3. Developing the methodology, including a stepwise approach, criteria and workflows for the use of biomarkers of effect to derive Reference Points.
- Objective 4. Demonstrating the applicability of the established methodology within case studies.
- Objective 5. Developing a guidance on the use of biomarkers of effect in regulatory risk assessment of chemicals.

Terms of reference

1. In Phase 1, the project will deliver a feasibility study including:

   - Definition and descriptors for biomarkers of effect to be used in this context;
   - A review of literature and mapping of activities, initiatives, knowledge, approaches relevant to the project goal (this task may be outsourced);
   - Definition of the scope and the overall aim of the guidance;
   - Conclusions and recommendations regarding the feasibility of such guidance.

   A public consultation will be organised during the implementation of Phase 1 to collect feedback on the outcomes of the study.
   Timeline: the outcome of this activity should be delivered within 12 months after the establishment of the Working Group of the Scientific Committee.

2. The implementation of Phase 2 will follow the outcome and addresses the recommendations of the first phase of the project.

   The aim is to:

- develop a methodological approach (e.g. stepwise approach, criteria, workflows) for the use of biomarkers of effect to derive Reference Points;
- test the methodological approach within case studies;
- develop a guidance on the use of biomarkers of effect in regulatory risk assessment of chemicals.

Public consultation is foreseen at key steps of the project to ensure gathering feedback from all different stakeholders.
Timeline: the final plan and timelines will depend on the conclusion of Phase 1 and the scenario taken. Provisional evaluations indicate the need of around 24 months after the completion of task 1.

1.2 | Interpretation of the Terms of Reference

This mandate is intended to establish the conceptual framework for biomarkers, and especially biomarkers of effect, to assess their applicability in hazard identification and characterisation during the process of risk assessment, and eventually develop a guidance in this direction. To achieve these goals, the mandate has been divided in two phases.

- In **Phase 1** (*current report*; EFSA-Q-2024-00128), essential knowledge will be gathered to shape and support the conceptual framework for biomarkers of effect; this includes exploring the diverse range of existing biomarkers and understanding the various fields of knowledge where they are applied. Therefore, the study conducted during this phase will be descriptive in nature, as its purpose is to compile all relevant information laying the groundwork for subsequent steps.
- In **Phase 2** (*to be implemented after and based on Phase 1 outcome*; EFSA-Q-2023-00583), guidance on how biomarkers of effect can be used and applied for risk assessment will be developed, if deemed appropriate. In the best scenario, harmonised criteria will be developed to assist the process of calculating RPs to further establish HBGVs or MoEs. It is noted that diverse EFSA sectors have already made use of biomarkers of effect for risk assessment (see Annex 1) and bearing in mind that this is a growing scientific area of translational research, the SC deemed it appropriate to address if and how biomarkers of effect can be used for risk assessment and, if so, to develop cross-cutting guidance to harmonise their use across EFSA sectors and beyond, to avoid potential divergences and to ensure consistency in the risk assessment approaches used.

To achieve this, several **challenges** need to be addressed, e.g.:

– Harmonisation of terminology and definitions (*to be addressed in Phase 1)*.
– Identification and description of biomarkers (*to be addressed in Phase 1 and further developed in Phase 2*).

- Establishment of scientific criteria for the selection, validation and correct interpretation of biomarkers of effect in the risk assessment of chemicals (*to be partially addressed in Phase 1*, e.g. *characteristics of biomarkers, while the development of criteria will be part of the guidance development in Phase 2*).
- Clarification regarding the type and level of evidence required regarding the association between an intermediate event, used as a biomarker of effect and the adverse outcome (*to be addressed in Phase 2*).
- Establishment of UFs following an uncertainty analysis (*to be addressed in Phase 2*).
- Incorporation of existing knowledge and frameworks (e.g. AOPs, NAMs) (*to be addressed in Phase 1 and 2*).
- What change in the benchmark response (BMR) of a biomarker of effect would be considered relevant for hazard characterisation? Information on the benchmark response for a biomarker that would be considered relevant for the hazard characterisation (*to be addressed in Phase 2*).

2 | METHODOLOGY

The methodology designed and implemented for this report was aligned with the terms of reference (ToR) and the objectives of Phase 1 of the mandate. Therefore, its steps aimed to create a structured basis for future guidance, identify challenges and recommend a way forward for the guidance development.

Scientific developments in the field of biomarkers are addressed, including references on the use of biomarkers in different contexts (e.g. drug development, medical/clinical settings, occupational health, general health surveillance, nutrition assessment, risk assessment).

The development of the current report included the following iterative steps:

- **Define the context and scope of the report**

In this step, the context of this mandate was discussed together with the main aspects that are included or excluded from the scope of the report.

The outcome of this step supported and created the basis for the discussions on the scope of the current report, but also for future guidance.

- **Definition and description of biomarkers**

In this step, a descriptive approach was used to address the definitions as well as the description of biomarkers, including references to different areas of application and examples.

The definition section includes references to biomarkers in general, subtypes of biomarkers, applications of biomarkers and finally definitions of biomarkers of effect and related terms.

For the description of biomarkers of effect, several aspects were addressed, e.g. the characteristics (that establish the validity of biomarkers of effect and their selection for the use in risk assessment of chemicals), and descriptors for biomarkers completed with examples (extracted mainly from published EFSA's Scientific Opinions or reports).

The outcome of this step includes:

- clarifications regarding definitions of biomarkers of effect and related terms;
- a set of characteristics of biomarkers of effect (e.g. that could further support the selection and the validation of biomarkers);
- a template proposed for the description of biomarkers of effect, with representative examples (Annex 1);
- a set of challenges or issues that should be further addressed in the next steps of the project.

- **Mapping of relevant resources**

This step refers to the identification, collection and analysis of relevant publications, projects, databases and tools. It was implemented in parallel with the previous two steps, including, e.g.:

- the selection and review of relevant scientific publications;
- the analysis of existing projects and initiatives in this area;
- the identification of relevant databases and tools.

The outcome of this step (besides the information provided for the core report) is represented by an inventory of resources with three subsections (publications, projects and databases/tools) and the detailed methodology with the summary analysis of the data collected (Annex 2 **and** Annex 3).

- **Consultation with stakeholders**

In this step, different collaboration and engagement activities were undertaken, including launching of a survey as well as bilateral information exchange, a stakeholder workshop and public consultation of the report.

The results of these activities were used to complement the development of the current report and are summarised in **Annexes 4, 5 and 6**.

- **Draft the conclusions and recommendations**

In this part, the conclusions and recommendations were drafted, following the implementation of the steps described above. These should eventually support further information exchange with other organisations and in co-designing future guidance.

3 | CONTEXT AND SCOPE

EFSA contributes to the safety of the EU food chain by providing scientific advice to risk managers (EFSA, 2021). In this context, EFSA's major tasks are to provide advice aimed at ensuring the safety of food and feed beyond a reasonable doubt, to assess the scientific substantiation of health claims made on food and to provide advice to ensure a sufficient intake of nutrients. Conclusions on dietary reference values (DRVs) for nutrients and health claims are predominantly based on human studies. For advice on safety, studies in humans may also be used to reach conclusions, but such studies may not always be available or even possible. For this reason, animal studies, as well as in vitro and in silico data, may be used.

In toxicological risk assessment, the setting of HBGVs (see **Appendix A**) has classically been done based on in vivo animal studies using adverse outcomes indicating overt toxicity (i.e. apical endpoints). Such studies are usually not able to predict the health consequences of exceeding these HBGVs, i.e. in terms of the proportion of the population that will experience adverse consequences of exposure to the food, or the severity thereof. Evolving science is switching to the use of biomarkers of effect that may inform in advance on the potential occurrence of apical endpoints before overt toxicity manifests. These may be intermediate endpoints in animal (e.g. rodent) studies, as well as biomarkers of effect in humans, in vitro or in silico studies. Such biomarkers of effect can potentially be used to establish HBGVs. The development of biomarkers of effect is further stimulated by the societal urge to reduce, and ultimately phase out, studies in experimental animals and to replace these with NAMs.

It is of note that **the same biomarker may be of value for safety as well as benefit assessments**, depending on, for example, the direction of the change or the population of interest. The validity of biomarkers of effect for the assessment of health benefits requires specific considerations, and fit-for-purpose guidance in this area (e.g. health claims) already exists.[1] For this reason, **the scope of the current document is on the use of biomarkers to assess adverse effects, while beneficial effects are out of the scope of this report**. The potential duality of a biomarker of effect must, however, be considered when interpreting a change (direction and magnitude – see Section 5) and its significance and relevance in terms of safety for the target population for the assessment (Figure 1).

[1]The EFSA General scientific guidance for stakeholders on health claim applications (https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2021.6553) provides fit-for-purpose guidance on the characterisation of beneficial effects and the endpoints that can be used for their assessment in vivo in humans in the context of function claims and reduction of disease risk claims, including the requirements for risk factors that can be used alone (i.e. in the absence of data on disease endpoints) for the scientific substantiation of such claims. EFSA has also developed specific guidance on different subject areas. For further information please visit: https://www.efsa.europa.eu/en/applications/nutrition/regulationsandguidance.

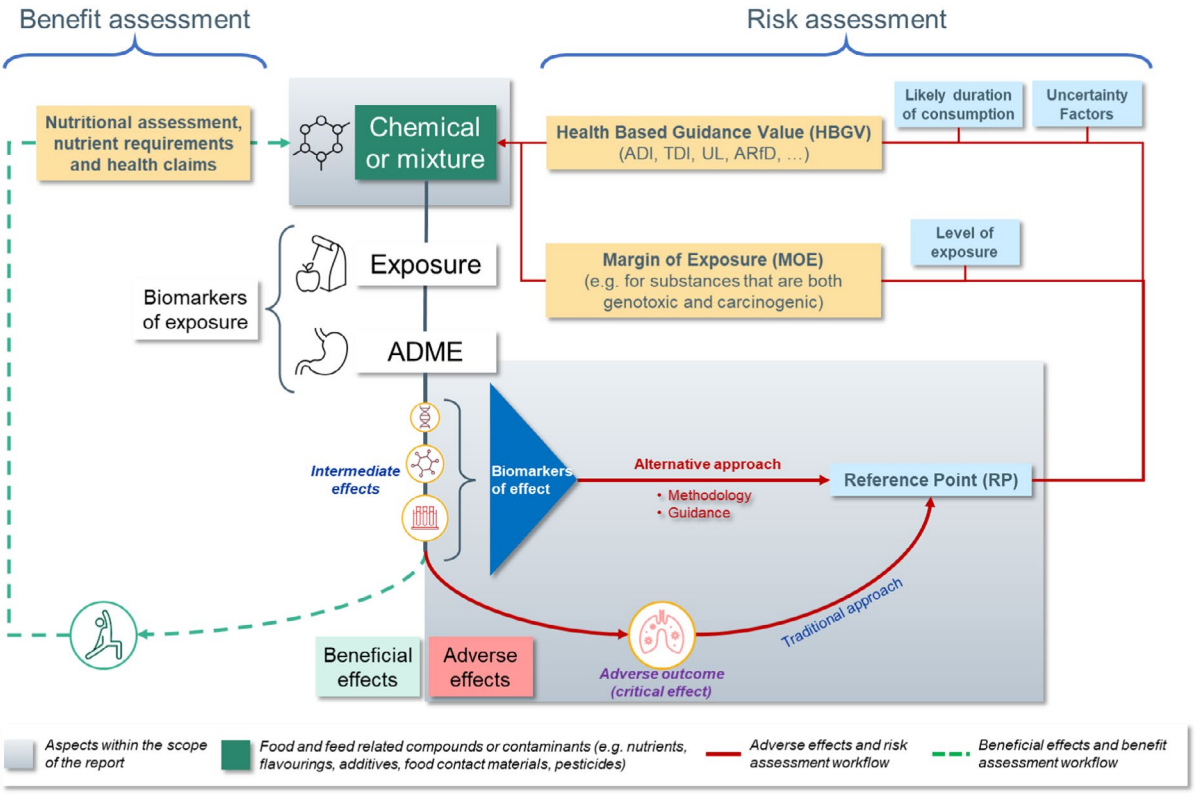

FIGURE 1 Schematic representation of biomarkers of effect within the risk and benefit assessment contexts and the focus of the current report (represented by a dark background).

Despite the broad fields of application of biomarkers, this document refers mainly to (a) adverse effects (not to beneficial effects); (b) risk assessment context (not to clinical, occupational, drug development, etc.); and (c) biomarkers of effect following chemical exposures for hazard assessment,[2] instead of diagnosis or early detection of a disease or to assess the clinical prognosis of a medical intervention.

A change in biomarkers of effect may be evident at earlier time points than when the apical effect emerges. For all these reasons, **a clear understanding of the mechanistic role of the biomarker in the chain of events ultimately leading to an adverse effect and causality is important**, although this information may be incomplete or unknown. To achieve these, more clarity and scientific consensus is needed regarding different aspects, as described below.

In addition, it should be noted that **a single biomarker of effect may not be sufficient in the** *EFSA evaluations*, but information from diverse biomarkers (i.e. a panel of biomarkers) related to or preceding the same apical outcome may be necessary for final conclusions.

The aim of this Scientific Report is to gather the relevant information to further lay the basis for defining the criteria or conditions for use of biomarkers of effect for risk assessment of chemicals. Such criteria could be laid down in a future guidance.

4 | DEFINITION AND APPLICATION DOMAINS OF BIOMARKERS

4.1 | Generic definition of biomarkers

Biomarkers are indicators signalling an event or condition in a biological system or sample and giving a measure of exposure, effect or susceptibility and include almost any measurement reflecting an interaction between a biological system and an environmental agent, which may be chemical, physical or biological (OECD, 2012; WHO/IPCS, 1993). Several other definitions or adaptation exists (see Table B.1, **Appendix B**).

The term biomarker is the abbreviation of a biological marker. A wide variety of terms have been used to describe the concept of biomarker. These include, for example, biological markers, surrogate markers, surrogate endpoints, surrogate response variables, intermediate endpoints, intermediate markers, biomarker endpoints, intermediate marker endpoints and bioindicators (Aronson, 2022; Strimbu & Tavel, 2010). In addition, in the National Cancer Institute Thesaurus included in the BioPortal ontology (NCI, n.d.), biomarker is defined as *'a characteristic that can be objectively measured and serves as an indicator for normal biologic processes, pathogenic processes, state of health or disease, the risk for disease development and/or*

[2]Chemicals in this context refer to food- and feed-related compounds or contaminants (e.g. nutrients, flavourings, additives, food contact materials, pesticides).

prognosis, or responsiveness to a particular therapeutic intervention' and mentions the following synonym terms: signature molecule, biological marker, molecular marker and marker.

The term 'biomarker' has the advantage of replacing all these terms, thus avoiding misunderstandings and contributing to the harmonisation of the underlying concept.

In practice, biomarkers (*except for biomarkers of exposure and susceptibility*) refer to any biological observation that substitutes for, and ideally predicts, a biologically relevant endpoint or intermediate outcome that is more difficult to observe (Aronson, 2022). That observation provides an objective indication of normal or abnormal biological processes or states, which can be measured accurately and reproducibly. Therefore, anything measurable that helps in the prediction or identification of a disease can serve as a biomarker, including metabolites, changes in biological structure or processes or a characteristic feature. Biomarkers offer the opportunity to provide scientific confirmation of proposed exposure-disease pathways in vivo in human populations (Ladeira & Viegas, 2016).

Understanding the relationship between measurable biological processes and clinical outcomes is vital for a better understanding of normal, healthy physiology. Biomarkers (restricting its concept to biomarkers of effect) could only serve as true replacements for clinically relevant endpoints if the normal physiology of a biological process, the pathophysiology of that process in the disease state, and the effects of an intervention on these processes are entirely understood. Studies using biomarkers should ultimately measure clinical outcomes, at least for retrospective analysis of biomarker correlation success. However, continual re-evaluation of the relationship between surrogate endpoints and true clinical endpoints is warranted (Lesko & Atkinson, 2001; Strimbu & Tavel, 2010).

As such, a biomarker refers to a defined characteristic that is measured as an indicator of (FDA/NIH, 2001):

- Normal biological processes.
- Pathogenic processes.
- Responses to an exposure or intervention (including pharmacological response to a therapeutic intervention and the biological response to chemical exposures).

Biomarkers are largely used in drug development, clinical settings, occupational settings, general population health surveillance, nutritional or risk assessment (*see details in* ***Section 4.3***), as biological observations to monitor and predict clinically relevant endpoints at an early stage, when organ damage is not yet evident and preventable, or less severe and hence appropriate interventions can be planned. As such, biomarkers allow new ways of understanding disease processes and the ways in which medicines work to counteract disease. Biomarkers may take the form of cellular characteristics, metabolites (e.g. sugars, lipids, hormones), molecular variations or physical features (e.g. measured clinical signs) (Aronson & Ferner, 2017; Califf, 2018; OECD, 2011, 2022).

Biomarkers may encompass '*molecular, histologic, radiographic or physiologic characteristics*' (Califf, 2018). Also, it is understood that the definition depends on the context of use, reflecting the complex associations between biological measurements and models of disease at the subcellular, cellular, organ, biological system and intact organism levels (Califf, 2018).

Despite the above-mentioned text, it is noted that the concept of biomarker often used in the scientific literature is more suited for biomarkers of effect.

4.2 | Subtypes of biomarkers

Several subtypes of biomarkers have been defined based on their application (Figure 2 **and** Table B.2, **Appendix B**), while a single biomarker may meet multiple criteria for different uses. However, a clear distinction between these subtypes of biomarkers may be difficult as there is no perfect classification, and they can overlap (e.g. the **response biomarkers and biomarkers of effect** that are more relevant for the scope of this report). Thus, while definitions may overlap, they also have clear distinguishing features that specify uses (Cagney et al., 2018; Califf, 2018; FDA/NIH, 2016; Morgan, 1997; Rodríguez-Carrillo et al., 2023).

The concept of biomarkers (i.e. biomarkers of effect) is evolving dynamically, as it changes over time. Recently, a definition for **multimodal biomarker** has been proposed (NASEM, 2023) as '*a defined characteristic or characteristics that includes features based on two or more measurements evaluated through an algorithm as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions and environmental exposures*'.

As shown in Table B.2 (**Appendix B**), a generally accepted classification of biomarkers, which is also relevant for the **application in risk assessment**, divides them into three main categories: biomarkers of exposure, effect and susceptibility. As such, biomarkers fulfil a role in the continuum from exposure to effect and they can be used to inform (early) effects and to study the effects of an exposure or intervention (Viegas et al., 2020).

4.3 | Application domains of biomarkers

The most common fields of application of biomarkers are listed below (see also Figure 2 and Table B.3 (**Appendix B**)) and include:

- Drug development: Biomarkers can help to identify potential therapeutic targets related to molecular pathways of disease and they can provide critical information on efficacy and safety of drugs.
- Medical/clinical settings: Biomarkers can be used to diagnose, monitor or predict the outcome of a disease or a treatment in a clinical setting.
- Occupational health: Biomarkers (of exposure) are used to assess exposure by all routes and to complement information obtained by workplace environmental monitoring. Biomarkers can be also used as early predictors of clinical disease in occupational health and thus can advance occupational health risk assessment.
- Health surveillance of the general population: Biomarkers and biomonitoring data can be for exposure and risk assessment of general population, help in identifying potential health risks and in developing effective public health policies.
- Nutrition: Biomarkers can be used to assess the intake of foods and nutrients (i.e. biomarkers of intake) and to assess an individual's ability to meet physiological requirements for a particular nutrient (i.e. biomarkers of nutritional status).
- Risk assessment: Biomarkers of effect can be used as measurable indicators of biological responses, providing information on the effects of exposure to chemicals, and they can bridge the gap between exposure and health outcomes.

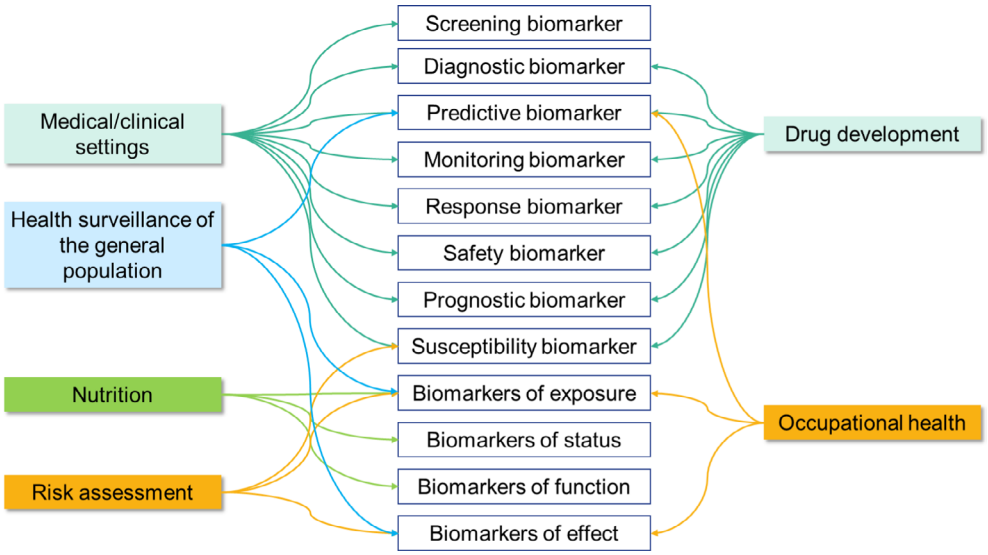

FIGURE 2 Examples of biomarkers subtypes and their primary application.

4.4 | Definition of biomarkers of effect

Biomarker of effect (also known as *effect biomarker*) is defined as a '*measurable biochemical, physiologic or other alteration within an organism that, depending on magnitude, can be recognised as an established or potential health impairment or disease*' (WHO/IPCS, 1993). In addition, the core definitions (Table B.4, **Appendix B**), several other terms identified and used in connection with the biomarkers of effect are defined and mapped (Figure 3 and Table B.5, **Appendix B**).

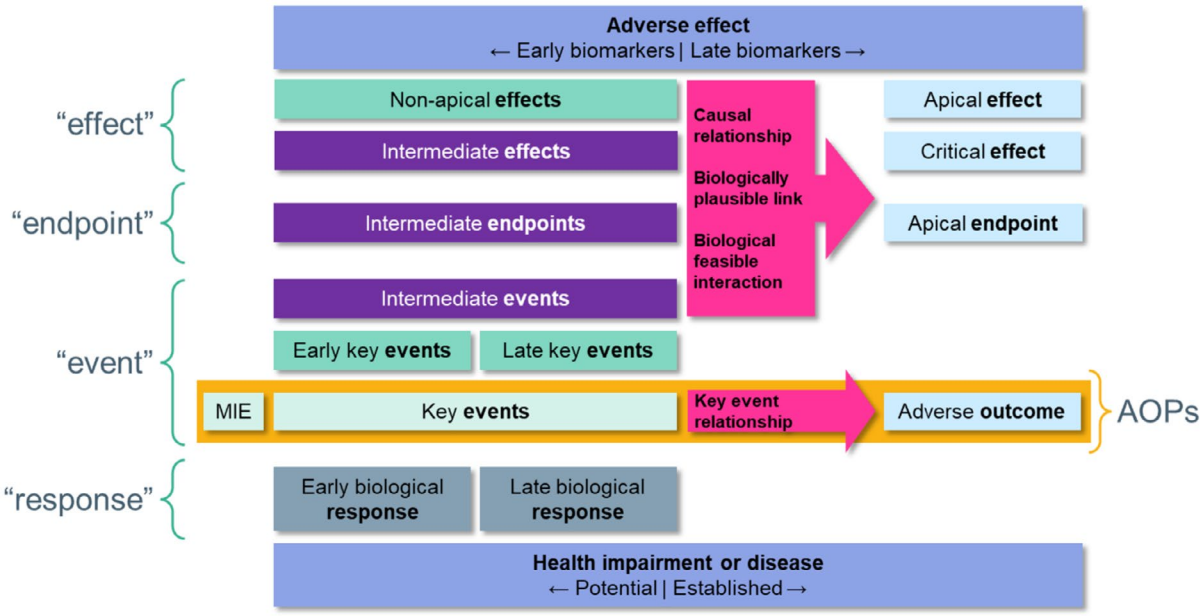

FIGURE 3 Mapping of different terms related to biomarkers of effect, used to describe the intermediate and final effects, and the relationship between them (definitions are provided in Table B.5, Appendix B).

The WHO definition mentioned above was used in guidance documents (e.g. in EFSA Scientific Committee, 2017; EFSA NDA Panel, 2022) mentioning that the biological relevance of biomarkers depends on its relation to the mode of action and the linkage with the adverse effect or the relevant AOP. The definition has been further adapted by other projects (e.g. EU Horizon 2020 HBM4EU (Rodríguez-Carrillo et al., 2023)), where the term 'effect biomarker' was defined as '*a biochemical, physiological, behavioural, or other quantifiable alteration in an organism that, depending on its magnitude, may be associated with an established or potential health impairment or disease*'.

As biomarkers of effect indicate an individual's biological response to a chemical exposure or drug treatment, or to an external stressor, they are also called 'response biomarker' or pharmacodynamic biomarkers. This response can be of a molecular, functional or morphological nature (FDA/NIH, 2016).

Moreover, the distinction between **early and late biomarkers of effect** usually depends on the timeline of disease onset and progression, spanning from the preclinical to clinical stages. Early biomarkers of effect manifest during the initial phases of a disease or clinical condition, reflecting molecular or cellular abnormalities in the pathophysiological pathway leading to the overt disease. These early changes can thus serve as predictors for clinical outcomes. In acute diseases, such as myocardial infarction or acetaminophen poisoning, biomarkers may become elevated due to cell death in the target organ. Although the timeframe for this elevation is very short - typically just a few hours - both early and late biomarkers of effect can still be distinguished. Conversely, in chronic and degenerative diseases, identifying early biomarkers of effect before disease symptoms emerge is crucial, as early interventions during the pre-symptomatic stage can be more effective than later interventions.

Within the AOP framework, early biomarkers of effect correspond to upstream key events (KEs) (i.e. those closer to the molecular initiating event), whereas late biomarkers of effect correspond to downstream key effects (i.e. those closer to the adverse outcome). These late biomarkers are closer to the apical adverse outcome and, therefore, they represent higher levels of biological organisation (e.g. organ or individual). Unlike early biomarkers, they take longer to become altered and show significant changes only in the advanced stages.

5 | DESCRIPTION OF BIOMARKERS OF EFFECT

In this section, several characteristics generally applicable to biomarkers that establish their validity and/or qualify them for a specific context of use are described. Furthermore, specific considerations regarding the biomarkers of effect used in the risk assessment context are also included.

5.1 | General characteristics of biomarkers

Generally, the biomarker description includes the name, the source/matrix, the measurable characteristic(s) and the analytical method used to measure the biomarker (FDA, 2021).

- The **validation of biomarkers** as early predictors of clinical disease can enhance health risk assessment (Bonassi et al., 2001). Therefore, a **valid biomarker** is defined as '*a biomarker that is measured in an analytical test system with well*

established performance characteristics and for which there is an established scientific framework or body of evidence that elucidates the physiologic, toxicologic, pharmacologic or clinical significance of the test results' (FDA, 2005; Kraus et al., 2011). As such, the classification and validation of biomarkers are context specific and the criteria for validation will vary depending on the intended use of the biomarker (FDA, 2005; Kraus et al., 2011).

- Modifiers of biomarkers of effect are factors like genetic variations, environmental exposures, lifestyles and other diseases or conditions that can influence the measurement or interpretation of these biomarkers. These modifiers should be accounted for during the validation process to ensure that biomarkers accurately and reliably reflect biological effects under the conditions mentioned above.
- Also, the approach on **qualification of biomarkers** (defined by FDA within the 'Biomarker Qualification Program'[3,4]) means that the biomarker has undergone a **formal regulatory process** to ensure that it has a specific interpretation and application in therapy development and the marketing review process, within the stated context of use (example of qualified biomarkers is available[5]).

In a **clinical context**, an 'ideal biomarker' should meet the following universal characteristics (Bennett & Devarajan, 2011; FDA/NIH, 2016; Verma et al., 2011), e.g.:

- To be non-invasive, easily measured, inexpensive and produce rapid results.
- To be collected from easily accessible biological samples, such as blood or urine, using minimally invasive techniques.
- To have high sensitivity, allow early detection and ensure no overlap in values between diseased patients and healthy controls.
- To have high specificity, being increased or reduced specifically in the diseased samples and unaffected by comorbid conditions.
- To vary rapidly in response to treatment.
- To aid in risk stratification and possess prognostic value in terms of real outcomes.
- To be biologically plausible and provide insight into the underlying disease mechanism.

Ideally, the biomarker would be **specific for a particular disease/organ dysfunction** and **able to differentiate between physiological and pathophysiological states**. However, **very few biomarkers meet all these characteristics**. Analytical methods must be reproducible, easy to perform and applicable to many samples. Sampling procedures must be ethically acceptable. Other factors, such as age, gender and ethnicity, may be of relevance depending on the purpose of use of the biomarkers.

5.2 | Characteristics of biomarkers of effect relevant for the use in risk assessment of chemicals

For the application in a **risk assessment**, a similar approach and analytical and biological characteristics (and criteria) can be used for the selection and validation of the biomarker of effect (or the set of biomarkers) to be used in the assessment (WHO/IPCS, 2001). In this context of use, a few **principles** need to be mentioned, as useful for the development of future guidance, e.g.:

- Biomarkers of effect provide information for a measurable biological effect, but they do not necessarily discriminate between adverse and non-adverse effects (e.g. this may depend on the direction and magnitude of change), while their biological relevance depends on their relation to mode of action (MOA) of an adverse effect or an AOP (Blaauboer, 2012; EFSA Scientific Committee, 2017).
- Biomarkers of effect may predict adverse effects at different levels of biological organisation, e.g. at molecular, cellular, tissue, organ or system level, providing the link between exposure to a xenobiotic and its early and late health effects (*adapted from* Rodríguez-Carrillo et al., 2023).
- The selection of the biomarker should be based on the understanding of the causal relationship (biologically plausible link) with the adverse outcome/apical effect. This requires (i) a qualitative understanding of the mechanism that links the biomarker to an adverse outcome, e.g. the AOP or MOA of the chemical, and (ii) a quantitative understanding of the relationship between the biomarker and an adverse outcome (i.e. whether there is a threshold in the magnitude of change in the biomarker necessary to trigger the adverse outcome, and also whether a greater magnitude of change in the biomarker is associated with a higher incidence of the adverse outcome). This should further inform us of the UFs used in HBGV derivation. As such, the AOP framework may represent a tool that can be used in integrating biomarkers of effect in risk assessment (OECD, n.d.; Baken et al., 2019; Lee et al., 2015; Sinitsyn et al., 2022; Zare Jeddi et al., 2021).
    - In an AOP, a KE is necessary for an adverse apical outcome to occur, but it is not sufficient on its own. If a KE is absent, the adverse effect will not manifest. However, the mere presence of a KE may not be enough to trigger the outcome,

[3]https://toolkit.ncats.nih.gov/module/discovery/developing-translational-research-tools/biomarkers/.
[4]https://www.fda.gov/drugs/drug-development-tool-ddt-qualification-programs/biomarker-qualification-program.
[5]https://www.fda.gov/drugs/biomarker-qualification-program/list-qualified-biomarkers.

as the magnitude and duration of the change associated with that KE could be insufficient. The dose and duration of exposure required to activate an intermediate KE are assumed to increase along the causal pathway of an AOP. Consequently, the magnitude and/or duration of exposure needed for a downstream KE must be greater than that required for an early KE. The duration of exposure thus becomes crucial, as sustained exposure is often necessary to trigger downstream KEs and eventually the adverse outcome, particularly in long-lasting diseases.
    - Early KEs occur shortly after exposure to a chemical. They are often close to the initial interaction with a biological target and serve as indicators of initial biological response. However, their impact on the apical adverse outcome may be limited if they do not lead to subsequent downstream effects. In contrast, downstream KEs occur later in the AOP, following early KEs, and are more directly linked to the final adverse outcome.
- Biomarkers of effect can be specific or non-specific (see **Appendix C**), depending on whether they indicate a biological effect of exposure to a particular chemical, or reflect the total, integrated effect from exposure to a mixture of chemicals, respectively (Viegas et al., 2020). Also, in relation to the AOPs, many KEs may be common across AOPs, and therefore less specific.
- In a clinical setting, an ideal biomarker of effect should reflect early, reversible changes in an organism and can complement other indicators to refine epidemiological studies and risk assessments (Ladeira & Viegas, 2016). However, in other contexts of use, e.g. in risk assessment of chemicals when biomarkers of effect are used for establishing the link with an apical adverse outcome, the 'reversibility' does not represent an ideal characteristic, while they should still reflect an early change in the organism (i.e. non-reversible early biomarkers of effect).

Criteria to ensure that the selected biomarkers of effect are relevant, reliable and informative for use in risk assessments should be considered and developed as part of future guidance in this area. To support this task, different **analytical characteristics** (e.g. quantifiability, accessibility, robustness/reproducibility, non-invasiveness, cost–effectiveness) as well as **biological characteristics** (e.g. predictivity, plausibility, sensitivity, specificity, translatability, human relevance) were compiled and are described in **Table C.1**, **Appendix C**.

In addition, Hill's criteria (that have been used in environmental health, toxicology, pharmacology, epidemiology and medicine) may serve as valuable tools for investigating causality, e.g. in assessing the biomarker-clinical endpoint relationship (IOM US, 2010). While all of Hill's criteria (see **Appendix C**) provide a robust framework for causal inference, some of them (e.g. temporality, biological gradient, plausibility, consistency, specificity and strength of the association) are particularly crucial in ensuring that a biomarker of effect is not only associated with an adverse effect or clinical outcome, but is also likely to be causally linked, even if not all criteria are met.

No single biomarker is likely to have all the characteristics necessary for a robust understanding of response, therefore **combinations of multiple biomarkers** will enable improved prediction. Although a biomarker can provide predictive information based solely on the association between its magnitude of change and organ toxicity or other outcomes, biomarkers will have greater value if they are closely related to the pathogenic mechanism leading to the apical effect/adverse outcome under consideration (IOM US, 2009).

The **selection of biomarkers** of effect will depend on the specific assessment question, the exposure of interest, the adverse health outcomes being investigated and the context of use/application (e.g. diagnostics, drug development, occupational biomonitoring, risk assessment) and will be based on several interlinked characteristics (criteria) with different impact/weight in the validation process. As such, if biomarkers are to be used properly, there needs to be an understanding of:

- the context of use;
- their analytical and biological characteristics;
- their interpretation in that context.

These aspects on the **validity and selection** of biomarkers should eventually be integrated into the risk assessment process, together with **weight-of-evidence** considerations (e.g. consistency with other existing information) and **uncertainty analysis** (known uncertainties, quantifiable uncertainties, to which level uncertainties impact conclusion, etc.).

In a risk assessment context, in addition to the characteristics mentioned above and following the selection process of the biomarkers of effects, several aspects to support the description of biomarkers of effect are needed (the 'descriptors'). For the compilation of such descriptors, a set of representative examples of biomarkers of effect were analysed from published EFSA Scientific Opinions and are compiled in Annex 1.

6 | IMPLICATIONS OF THE USE OF BIOMARKERS OF EFFECT IN RISK ASSESSMENT

As mentioned above, biomarkers of effect provide an assessment of how chemicals impact physiological processes and serve as indicators of potential adverse health effects. Extensive data have been generated regarding intermediate endpoints, which occur after exposure but before the onset of overt damage. These intermediate endpoints, commonly referred to as biomarkers of effect, are expected to capture early modifications that precede progressive functional or structural damage at the molecular, cellular and tissue levels. Consequently, biomarkers of effect should identify early

events that may also predict later responses. Therefore, biomarkers should exhibit a high degree of correlation with the later outcomes with which they are associated or causally linked. However, it should be noted that changes occurring in target tissues or cells may not necessarily be reflected by corresponding biochemical changes in peripheral, accessible media. While early damage can sometimes be repaired and subsequent dysfunction compensated for, it can also set off a cascade of events eventually leading to clinical disease. Biomarkers of effect can therefore enhance risk assessment (WHO/IPCS, 1993):

- providing early indications of adverse effects before clinical symptoms manifest,
- supporting dose–response modelling,
- identifying susceptible populations, and
- informing regulatory decisions and setting exposure limits.

The development of biomarkers of effect has primarily relied on epidemiological, clinical and experimental strategies. Epidemiological studies play a crucial role in identifying biomarkers associated with adverse health outcomes, as well as recognising the importance of using biomarkers of effect as evidence for or against causality for the disease endpoint of concern, especially where there is limited evidence on the endpoint (EFSA Scientific Committee, 2024). This approach is particularly effective for common, multifactorial health outcomes when the measured biomarker is readily accessible and cost-effective. Most biomarkers of effect have been identified using a pathophysiological approach, often starting from clinical outcomes and tracing back changes that precede disease manifestation. When these biomarkers are subsequently employed in epidemiological studies, their interpretation can vary due to differences in methodological context.

The experimental approach involves several steps (WHO/IPCS, 2001):

- Use in vitro studies, preferably over animal experiments, to identify the mechanism of toxic action of chemicals.
- Conduct comparative studies to assess whether candidate biomarkers behave differently in the target tissue and in readily accessible biological samples.
- Conduct epidemiological investigations to assess the sensitivity of potential biomarkers to toxic chemicals in real-world scenarios.

In most cases, the occurrence of a molecular interaction is directly linked to the dose of a chemical. However, exposure to low doses might not result in any noticeable effects due to the existence of a threshold level of effect. If this threshold is not exceeded, protective mechanisms come into play, effectively concealing the adverse effects. For instance, the induction of metallothionein or stress proteins can mitigate the impact of chemical exposure. Beyond considering dose–response relationships, it is also crucial to understand the temporal dynamics of biomarker responses. In other words, how biomarkers change over time after exposure to a chemical. This knowledge is essential for assessing toxicity accurately and designing effective interventions (Hagger et al., 2006).

The interpretation of early biological effects as warning signals requires mechanistic studies to investigate the underlying mechanisms behind early biological effects, and follow-up investigations to confirm the existence of an increased risk for long-term outcomes.

Notwithstanding the above, it is imperative to distinguish between correlation and causation. The selection of relevant biomarkers requires careful consideration as opting for inappropriate biomarkers can lead to resource inefficiencies, erroneous conclusions and misguided decisions in public health policy. For instance, setting inappropriate reference values or guidelines can have far-reaching consequences.

Biomarkers of effect serve not only for individual-level screening but also for identifying at-risk groups in the general population. Detecting shifts in sensitive biomarkers contributes to the identification of risk groups; however, determining the threshold triggering action (e.g. by risk assessors, risk managers) remains a topic of ongoing debate. Several challenges arise when defining biological limits or health-based criteria, including the inherent uncertainties in scientific conclusions. This is why translating evidence into practical standards for regulation and risk management is inherently complex.

Currently, the risk assessment is still relying on animal testing data and is based primarily on clinical criteria and most of the HBGVs are based on the critical effects represented by morphological or clinical observations of apical adverse outcomes. The shift to next generation risk assessment (NGRA) (Dent et al., 2021) and use of NAMs implies a risk assessment process that may be primarily based on mechanistic data, e.g. early biomarkers of (adverse) effects, instead of the observations of the apical adverse outcome.

In this context, a crucial aspect is the availability of data in a standardised format to facilitate their exchange, comparability and usability in regulatory decision-making. The OECD harmonised template (OHT) 201[6] is designed exactly for this purpose, i.e. to report 'non-apical intermediate effects/mechanistic information' derived from NAMs (Carnesecchi et al., 2023). An ongoing project at EFSA (OC/EFSA/iDATA/2022/02)[7] is exploring the possibility of populating the EFSA Chemical Hazard Database (OpenFoodTox)[8] with mechanistic data that are used and reported in EFSA Scientific Opinions,

[6]OECD Harmonised Template 201 https://www.oecd.org/ehs/templates/harmonised-templates-intermediate-effects.htm.
[7]https://etendering.ted.europa.eu/cft/cft-display.html?cftId=11248.
[8]https://zenodo.org/doi/10.5281/zenodo.780543.

using the OHT 201. Such a repository would facilitate retrospective analysis of the available data and provide insight into the relevance and usability of certain biomarkers of effect for establishing HBGVs.

The diverse classes of biomarkers of effect play a crucial role in predicting specific disease outcomes and hold significant potential for early detection of preclinical effects caused by chemicals. However, to fully realise this potential, extensive validation studies are necessary aimed at establishing the reliability of biomarkers and facilitating their translation into practical chemical risk assessment. To achieve this, focused and well-designed studies must be conducted to link chemical exposure to the response of specific biomarkers, or a suite of biomarkers, related to health outcomes. The central question is whether the biomarker response is a transient event with no significant health implications or if it serves as an early indicator of adverse health events, such as target organ toxicity, birth defects or cancer. While these interpretive studies can be challenging, they are feasible with sufficient resources (Fowler, 2012).

Once relevant biomarkers of effect and criteria have been established, this information becomes a valuable resource for formal risk assessment. Biomarkers can provide valuable insights to understand complex processes that are otherwise challenging to assess due to the associated complexities. While fully validated biomarkers are desirable, such validation is often incomplete. Testing the validity of assumptions generated by hypothesis-generating studies is inherently difficult and often relies on converging evidence from multiple sources. In cases where epidemiological investigations are not feasible, animal experiments can provide valuable insights, although the intimate nature of biomarkers as surrogate measurements poses challenges. In summary, striking the right balance between clinical criteria and early biomarkers is essential for effective risk assessment (WHO/IPCS, 2001).

Biomarkers of effect, such as they are, used for HBM serve the same goal as biomarkers used in the more experimental settings, including those that are being developed for NAMs. Some, but not all, may be similar for both settings, but all share the same criteria for being able to aid in the prediction of an adverse outcome of certain exposures. Obviously their nature has an impact on how to validate, measure and interpret the outcomes of measuring these biomarkers.

Both NAMs and HBM utilise and generate biomarkers of effect, but they offer distinct advantages and face different challenges. NAMs, such as in vitro and in silico models, allow precise control over experimental conditions, providing detailed mechanistic insights into how chemicals affect biological pathways. They can measure or simulate early molecular effects in a controlled setting, making it easier to identify and measure biomarkers that may be difficult to assess in humans. Measuring early molecular biomarkers of effect in humans may be challenging due to ethical constraints and the invasiveness of sample collection. HBM can track changes over time, providing valuable longitudinal data that can inform risk assessment and public health interventions. In more experimental settings, including those being developed for NAMs, some biomarkers may be similar for both NAMs and HBM, but all share the characteristic of aiding in the prediction of an adverse outcome of certain exposures. The nature of these biomarkers impacts how they are validated, measured and interpreted. When assessing mixture effects and deriving HBGVs, NAMs may be able to identify specific interactions and mechanisms, while HBM offers evidence of real human exposure and health outcomes. Integrating both approaches may lead to more accurate and comprehensive risk assessments, leveraging the strengths of each method to address their respective limitations.

The validation of biomarkers of effect identified in NAMs versus those identified in humans (i.e. HBM) involves distinct processes and characteristics due to the different contexts in which these biomarkers are discovered and utilised. For biomarkers identified in NAMs, the validation process focuses on ensuring reliability and reproducibility within the specific non-animal system. This involves technical characterisation to assess precision, accuracy, sensitivity and specificity, as well as ensuring biological relevance to reflect processes that can be extrapolated to human biology. Additionally, regulatory acceptance is crucial, often requiring comparison with traditional animal data to establish credibility. In contrast, validation in humans involves a more complex and rigorous process, including biological validation to demonstrate association with health outcomes or disease states, and providing epidemiological evidence through large-scale population studies. Challenges in NAMs include ensuring that biomarkers are predictive of human biological responses, requiring robust cross-validation with human data and continuous refinement of the methodologies. In human studies, variability in genetic, environmental and lifestyle factors can complicate the validation process, making it challenging to ensure that biomarkers are universally applicable across diverse populations.

Overall, biomarkers hold immense promise for risk assessment, but their successful translation requires concerted efforts to overcome validation and interpretation issues. These challenges need to be addressed so that these potentially extremely valuable biomarkers of effect can reach their full potential as predictive tools for public health (Fowler, 2012) and risk assessment.

The projects on AOPs and biomarkers of effect conducted by the OECD within the Working Party on Exposure Assessment (WPEA) and the Working Party on Hazard Assessment (WPHA) recommend performing a targeted risk assessment interpretation. This interpretation focuses on several bottom-up key aspects, particularly for occupational settings: exposure, elevated exposure, potential health effects and health effects for every kind of biomarker of effect (see Annex 5 and Annex 6).

7 | CONCLUSIONS AND RECOMMENDATION

The report addresses several aspects to be used as the basis for future guidance, acknowledging the great potential of the use of biomarkers of effect in risk assessment. This could possibly impact the NGRA methodologies and, more broadly, on the improvement in public health. While the initiation of this project and discussions on this topic were considered timely, several questions remain to be addressed and clarified further in the guidance.

Following the completion of Phase 1 of the project, a set of conclusions and recommendations for the implementation of Phase 2 of the project (guidance development) were compiled:

A. The report addressed **definitions and terminologies** extensively, creating a solid basis for the future harmonisation within the guidance. It is further recommended to use the already established terminology of OECD in this area, to achieve the best possible harmonisation in the description and interpretation of biomarkers of effect across organisations, sectors and regulatory frameworks. This can be accomplished by establishing a platform for dialogue across regulatory agencies and international organisations, as well as by exploiting the existing knowledge and frameworks.
B. Regarding its **scope** and how specific or broad the guidance should be, the conclusion is that due to its complexity, starting with a general guidance would be recommended, e.g.:

- The scope of the guidance should be broad enough to cover various sectors and regulatory frameworks (e.g. risk assessment of chemicals, pharmaceuticals, food, including mixtures). The starting point of its development should be the common principles/similarities across different sectors.
- The general guidance can then be adapted to specific sectors, accounting for the specific context of use (CoU), regulatory framework and integration with other available approaches.
- Some of the steps in the guidance (e.g. criteria for the selection and validation of biomarkers of effect, calculation of RPs) can be general and applicable to all different frameworks, whereas other steps (e.g. CoU, uncertainty analysis, establishment of risk assessment parameters) can be sector specific. When needed, this will require prior consideration of the regulatory boundaries that may impede the harmonisation of criteria.
- The guidance should be applicable to existing but also to potential future biomarkers of effect (e.g. molecular biomarkers based on 'omics studies).

C. There are several **scientific and technical aspects** that need to be addressed in future guidance, especially related to the characteristics of biomarkers of effect and their interpretation in risk assessment contexts. The recommendation for the next Phase, as part of the guidance development, is to focus on defining the critical aspects related to the selection and validation of biomarkers of effects, e.g.:

- Minimum requirements for the biomarker(s) of effect to be selected and considered further in the risk assessment process.
- Biological criteria for assessing the relevance of selected biomarker(s) of effect to hazard identification and characterisation.
- Analytical criteria for assessing the reliability of biomarker(s) of effect.

Within the process of selecting and validating biomarkers of effect, several other aspects should be considered, e.g.:

- Criteria to differentiate between adaptive mechanisms and irreversible adverse effects.
- Aspects related to dose–response that are relevant for the hazard characterisation and calculation of RPs.
- The use of molecular/'omics biomarkers.
- Further exploitation of the AOP framework and knowledge, both qualitative and quantitative AOPs.
- Integration of NAMs for the biomarkers of effect measurements and assessment.
- The use of biomarkers of effect for the hazard assessment of chemical mixtures.
- Defining adequate UFs to be applied to RPs based on biomarkers of effect to establish HBGVs.
- Relevance of biomarkers for individuals or specific population subgroups.

As there is a large variability among biomarkers of effect, e.g. each with its own potential, uncertainties and limitations, it is essential to discuss and demonstrate representative examples or use cases of these biomarkers in risk assessment. This should be done in parallel with guidance development.

D. The guidance should be the result of a **co-creation process;** therefore, the collaboration between regulatory organisations is essential for creating a more impactful guidance, with a wider acceptance and implementation (*aspects addressed also in the stakeholder workshop, see* Annex 5). For the development of the guidance, the implementation of a feasible collaborative mechanism that accommodates different levels of participation is recommended, in parallel with a continuous information/knowledge exchange between initiatives. This approach should further support the harmonisation and reduce divergencies in the interpretation and application of methodologies across organisations, sectors or regulatory frameworks, contributing to a robust, scientifically excellent, transparent and strategic risk assessment process.

ABBREVIATIONS

ADI	acceptable daily intake
ADME	absorption, distribution, metabolism, excretion
AO	adverse outcome
AOP	adverse outcome pathway
BMD	benchmark dose
BMDL	Benchmark Dose Lower Confidence Limit
COU	context of use
DOI	digital object identifier
DRV	dietary reference value
HBGV	Health-Based Guidance Value
HBM	human biomonitoring
KE	key event
KER	key event relationship
LOAEL	lowest observed adverse effect level
MIE	molecular initiating event
MOE	margin of exposure
MOA	mode of action
NAM	new approach methodology
NGRA	next generation risk assessment
NOEL	no observable effect level
NOAEL	no observed adverse effect level
PoD	point of departure
PFAS	perfluoroalkyl substances
QSARs	quantitative structure–activity relationships
RP	reference point
TDI	tolerable daily intake
TK/TD	toxicokinetic/toxicodynamic
TWI	tolerable weekly intake
UF	uncertainty factor
UL	tolerable upper intake level

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

Generic methodology for establishing HBGVs within EFSA remit

In the 'Statement on the Derivation of Health-Based Guidance Values (HBGVs) for regulated products that are also nutrients' (EFSA Scientific Committee, 2021) the HBGV is defined as *'a science-based recommendation for the maximum (oral) exposure to a substance that is not expected to result in an appreciable health risk, taking into account current safety data, uncertainties in these data, and the likely duration of consumption*.' Furthermore, in EFSA Scientific Committee (2024) HBGV is defined as '*a numerical value derived by dividing a point of departure (a no-observed-adverse-effect level, benchmark dose or benchmark dose lower confidence limit) by a composite uncertainty factor to determine a level that can be ingested over a defined time period (e.g. lifetime or 24 h) without appreciable health risk*.'

The process for the establishment of the HBGV includes the selection of a dose that can be used as a starting point for risk assessment known as the 'reference point' (RP), also named 'point of departure' (PoD), followed by the selection of uncertainty factors (UFs) or safety factors, which are applied to the RP to ensure a sufficient level of protection for humans.

The conventional approach consists of assessing the dose–response relationships for the adverse effects and identifying and calculating an RP (i.e. a no observed adverse effect level (NOAEL), a lowest observed adverse effect level (LOAEL) or the lower confidence limit of the calculated identified benchmark dose (BMDL)) when applicable, based on the most sensitive endpoint relevant for humans.

HBGVs includes e.g. *Tolerable Upper Intake Levels (ULs)* for nutrients,[9] Acceptable Daily Intake (ADI) for regulated chemicals, Tolerable Daily/Weekly Intake (TDI/TWI) for other substances in food (e.g. contaminants).

HBGVs, can be established for compounds for which thresholded mechanisms of toxicity can reasonably be expected based on the available data, and for which the safety-related data are relatively complete. Alternatively, when available data are not sufficient to establish an HBGV (i.e. there is high uncertainty), the margin of exposure (MoE) approach[10] (i.e. consideration of the margin between the RP and the estimated exposure), or the setting of a safe level of intake (i.e. highest level of chronic intake where there is confidence in absence of adverse health effects) for nutrients, can be used to conclude on the safety of chemicals (EFSA NDA Panel, 2022; EFSA Scientific Committee, 2005, 2012; EFSA Scientific Committee, 2021; EFSA Scientific Committee, 2022; Tarazona et al., 2024; WHO/IPCS, 2009).

[9]'UL for nutrients are included in the definition of both DRVs and HBGVs' (EFSA SC, 2024).

[10]The margin of exposure (MOE) is the ratio between a defined point on the dose–response curve for the adverse effect and the human intake, and therefore it makes no implicit assumptions about a 'safe' intake (EFSA SC, 2005)

APPENDIX B

Collection of definitions relevant to the mandate

TABLE B.1 Evolution in time of the generic definition of biomarkers (NRC, 1989a, 1989b; WHO/IPCS, 1993; WHO/IPCS, 2001; FDA/NIH, 2016).

Year	Definition
1989	US National Research Council provided a broad definition of the term biomarkers that included 'almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction'.
1993	WHO defined a biomarker as 'any measurement reflecting an interaction between a biological system and an environmental agent, which may be chemical, physical or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction'.
1998	The Biomarkers Definitions Working Group of the National Institutes of Health (NIH) defined a biomarker as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.'
2001	WHO defined a biomarker as 'any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease'.
2016	US Food and Drug Administration (FDA) and the NIH established biomarker definitions as part of their joint Biomarkers, EndpointS and other Tools (BEST) resource. A biomarker was considered as a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or therapeutic interventions.

TABLE B.2 Subtypes of biomarkers grouped by their application.

Subtype of biomarker	Definition
Use in medical/clinical settings or drug development *(adapted from* FDA/NIH, 2016; Bodaghi et al., 2023)	
Screening biomarker	Biomarker used to screen for subclinical diseases when definite or easily observable symptoms are not presented yet.
Diagnostic biomarker	Biomarker used to detect or confirm the presence of a disease or condition of interest or to identify individuals with a subtype of the disease.
Predictive biomarker	Biomarker used to identify individuals who are more likely than similar individuals without the biomarker to experience a favourable or unfavourable effect from exposure to a medical product or an environmental agent.
Monitoring biomarker	Biomarker measured repeatedly for assessing status of a disease or medical condition or for evidence of exposure to (or effect of) a medical product or an environmental agent.
Response biomarker	Biomarker used to show that a biological response, potentially beneficial or harmful, has occurred in an individual who has been exposed to a medical product or an environmental agent.
Safety biomarker	Biomarker measured before or after exposure to a medical product or an environmental agent to indicate the likelihood, presence, or extent of toxicity as an adverse effect. For many therapies, monitoring for hepatic, renal or cardiovascular toxicity is critical to assuring that a given therapy can be safely sustained (Califf, 2018).
Prognostic biomarker	Biomarker used to identify the likelihood of a clinical event, disease recurrence or progression in patients who have the disease or medical condition of interest.
Susceptibility/risk biomarker	Biomarker that indicates the potential for developing a disease or medical condition in an individual who does not currently have a clinically apparent disease or the medical condition.
Surrogate biomarker	Surrogate biomarkers (also known as surrogate endpoints) serve as an indicator of, or a substitute for a clinical outcome of a disease, and may help to monitor a therapeutic intervention. Such biomarkers may have a strong correlation with clinical endpoints (e.g. serum creatinine to monitor renal function and risk of kidney disease) or a weaker correlation (e.g. elevated serum LDL-cholesterol levels and increased risk of coronary heart disease).
Use in nutritional assessment *(adapted from Gibson, 2024)*	
Nutritional biomarkers	Defined as biological characteristics that can be objectively measured and evaluated as indicators of normal biological or pathogenic processes, or as responses to nutrition interventions. They can be classified as: • biomarkers of exposure • biomarkers of status • biomarkers of function (biochemical and physiological or behavioural). Biomarkers of status measure a nutrient (or its metabolites) in biological fluids (e.g. blood, urine) or tissues; these ideally reflect total body nutrient content or the status of the tissue store most sensitive to nutrient depletion.

TABLE B.2 (Continued)

Subtype of biomarker	Definition
Use in risk assessment and/or occupational health *(adapted from* NRC, 1989a, 1989b*;* WHO/IPCS, 1993*;* WHO, 2011*;* Gupta, 2014*)*	
Biomarkers of exposure	An exogenous substance or its metabolite(s) or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism. These biomarkers are measured in biological samples taken from an organism (e.g. cotinine in blood or urine for tobacco smoke). Therefore, biomarkers of exposure indicate internal dose, or the amount of chemical exposure that has resulted in absorption into the body. The use of a panel of biomarkers of exposure in biomonitoring studies would allow a better understanding of the aetiology of chronic diseases (Neveu et al., 2017*)*.
Biomarkers of effect (also known as *effect biomarkers*)	A measurable biochemical, physiologic or other alteration within an organism that, depending on the magnitude, can be recognised as an established or potential health impairment or disease.
Biomarkers of susceptibility	An indicator of an inherent or acquired limitation of an organism's ability to respond to the challenge of exposure to a specific xenobiotic substance. Biomarkers of susceptibility are natural or acquired characteristics that may make certain individuals more sensitive to a specific disease or to respond to exposure to a specific chemical. Biomarkers of susceptibility are therefore natural indicators that predict individual differences in response to a drug or chemical before exposure. The susceptibility of certain subsets of individuals to a particular chemical is determined by genetically based factors (e.g. polymorphic variations in the coding sequences of genes) as well as environmental influences, health status (e.g. underlying diseases), nutritional status, lifestyles, life stages and polymedication, which may affect an individual's susceptibility to chemical exposure (Chen et al., 2014).

TABLE B.3 Applications of biomarkers.

Application	Description
Drug development	Biomarkers play a key role in the initial phases of drug discovery and development, as they can help identify potential therapeutic targets related to molecular pathways of disease. Biomarkers are also vital in the later stages of drug development as they provide critical information on efficacy and safety throughout drug development. As biomarkers typically quantify physiological states or therapeutic responses, selecting values in decision rules, such as 'cut-off points' becomes crucial and challenging, as different values can lead to varying perspectives (IOM US, 2009). As such, biomarkers play an increasingly important role in all phases of drug development, including regulatory review. However, only a few of these biomarkers will become established well enough to serve in regulatory decision-making as surrogate endpoints, thereby substituting traditional clinical endpoints. Combinations of biomarkers probably will be needed to provide a more complete characterisation of the spectrum of pharmacologic response (Lesko & Atkinson, 2001).
Medical/clinical settings	Biomarkers that are used to diagnose, monitor or predict the outcome of a disease or a treatment in a clinical setting are called clinical biomarkers. All biomarkers can be considered clinical biomarkers when used for a clinical application (Bodaghi et al., 2023). There are different categories of biomarkers that can be used in the clinical setting, including: a. screening biomarkers, used to screen for subclinical diseases when definite or easily observable symptoms are not presented yet; b. diagnostic biomarkers, which help in the diagnosis of a disease, by confirming the presence of that disease; c. prognostics biomarkers, which contribute to predicting the future progression of an individual's disease, the potential disease recurrence, and the outcome after an intervention (e.g. to assess response to pharmacological treatments). Depending on the applied technique, they can also be categorised as: i. imaging biomarkers, which consist of a feature of an image (e.g. X-ray, CT or functional MRI) relevant to a patient's diagnosis and guiding clinical practice, and ii. molecular biomarkers, which can be measured in biological samples, such as blood, urine or cerebrospinal fluid.
Occupational health	Biomarkers of exposure in occupational health practice are used to assess exposure by all routes and to complement information obtained by workplace environmental monitoring. They are frequently used as a better substitute for environmental monitoring, because they provide information at the individual level (Ladeira & Viegas, 2016). The measurement of chemicals, or their metabolites, in human biological samples is referred to as biomonitoring. These measurements can provide information on the levels of exposure to environmental chemicals and their potential health effects by comparing them with an appropriate reference. Biomonitoring has been a longstanding practice in occupational health as it integrates exposure from all routes, helps identify unintentional and unexpected exposures, and assesses the effectiveness of existing risk management measures. If validated, biomarkers of effect can be used as early predictors of clinical disease in occupational health and thus can advance occupational health risk assessment and trigger new effective disease prevention actions. As such, these biomarkers may assist in elucidating the causal relationship between chemical exposure and health outcomes and can serve as a 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 (Zare Jeddi et al., 2021).

(Continues)

TABLE B.3 (Continued)

Application	Description
Health surveillance of the general population	Biomonitoring data can be a powerful tool for health surveillance and exposure and risk assessment of the general population. The use of biomonitoring data can help identify potential health risks and develop effective public health policies to reduce exposure to harmful chemicals (Viegas et al., 2020). The correct interpretation of a biomarker requires clearly defined standards of reference. Reference values are the values of an analyte in a reference population that is usually formed by a group of healthy individuals. There are several human biomonitoring programmes and initiatives underway worldwide (e.g. NHANES,[11] HBM4EU[12]). The National Health and Nutrition Examination Survey (NHANES) is a programme of studies conducted by the Centers for Disease Control and Prevention (CDC) to assess the health and nutritional status of adults and children in the United States. NHANES includes a biomonitoring component that measures the levels of environmental chemicals in the blood and urine of participants. The data collected from NHANES have been used to identify trends in exposure to environmental chemicals and to inform public health policies. The European Human Biomonitoring Initiative (HBM4EU) established a European platform and collected human biomonitoring (HBM) data to understand human exposure to chemicals and resulting health impacts and communicates with policymakers to ensure that the results are exploited in the design of new chemicals policies and the evaluation of existing measures. HBM4EU generated scientific evidence on the causal links between human exposure to chemicals and negative health outcomes and adapted chemical risk assessment methodologies to use human biomonitoring data and account for the contribution of multiple external exposure pathways to the total chemical body burden. Following the HBM4EU initiative, the Partnership for the Assessment of Risks in Chemicals (PARC)[13] continues to advance HBM in the EU. PARC harmonises national and regional HBM studies across Europe, like HBM4EU, and focuses on collecting data from various age groups to assess exposure levels to different environmental contaminants. This continuity ensures that the valuable insights gained from HBM4EU are expanded and refined under PARC, contributing to informed policymaking and public health protection.
Nutrition:	Biomarkers have been developed in nutrition epidemiology to assess the intake of foods and nutrients (i.e. biomarkers of intake), and to assess an individual's current ability to meet physiological requirements for a particular nutrient (i.e. biomarkers of nutritional status). Biomarkers of intake and status have been used to monitor adherence to a dietary intervention, or to study the relationship between diet and disease in human studies. Recovery biomarkers provide information on the absolute intake of nutrients and are largely unaffected by other factors. They can be used for the validation of dietary assessment methods (based on self-reported food consumption coupled with food composition data to estimate nutrient intakes), but are currently available only for energy and a few nutrients (e.g. nitrogen, sodium, potassium), and their use in epidemiology is limited. Biochemical measurements of nutrient concentrations in blood and other tissues (i.e. integrated markers of nutritional status) depend not only on the intake of the nutrient, but also on homeostatic mechanisms. They are also affected by individual determinants of ADME, which in turn may depend on genetic, lifestyle, and pathophysiological factors. The criteria to be met by biomarkers to be useful in assessing dietary intake in epidemiological studies include sensitivity to intake, time integration (representing long-term intake), analytical accuracy, specificity and ability to control for relevant determinants of the biomarker concentration besides nutrient intake. The use of dietary assessment methods vs. biomarkers of intake or status in relation to health outcomes depends on the availability of such markers for specific nutrients, on the biomarker that mediates adverse health effects (nutrient intake vs. nutrient status), and on whether the biomarker could also be affected by pathological processes implicated in the aetiology of the disease being investigated (Van Dam & Hunter, 2012).
Risk assessment	Biomarkers of effect, as measurable indicators of biological responses, provide valuable insights into the effects of these exposures as they bridge the gap between exposure and health outcomes. Their integration into risk assessment frameworks ensures a more comprehensive understanding of chemical toxicity and aids in safeguarding public health. Additionally, biomarkers of effect enable regulators to make informed decisions, improve health, and enhance our understanding of the intricate relationship between health and disease (WHO/IPCS, 2001).

TABLE B.4 Evolution in time of the definition of biomarkers of effect (NRC, 1987; NRC, 1989a, 1989b; WHO/IPCS, 1993).

Year	Definition
1987	US National Research Council indicates that a biological marker of an effect or response can be any change that is qualitatively or quantitatively predictive of health impairment or potential impairment resulting from exposure.
1989	US National Research Council updated the definition, including that a biologic marker of an effect or response can be any change that is qualitatively or quantitatively indicative of health impairment or potential impairment (disease process) associated with exposure.
1993	WHO defines biomarkers of effect as a measurable biochemical, physiological, behavioural or other alteration within an organism that, depending upon the magnitude, can be recognised as associated with an established or possible health impairment or disease.

[11] https://www.cdc.gov/nchs/nhanes/index.htm.
[12] https://cordis.europa.eu/project/id/733032.
[13] https://www.eu-parc.eu/.

TABLE B.5 Terminology related to biomarkers of effect.

Term	Definitions
Adverse effect	Changes in the morphology, physiology, growth, development, reproduction or lifespan of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility *to other* influences (WHO/IPCS, 2004, 2009, WHO/IPCS, 2012). EFSA adapted this definition as follows: • 'An effect is considered 'adverse' when leading to a change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity to compensate for additional stress or an increase in susceptibility to other influences' (EFSA Scientific Committee, 2017). • 'Change in the morphology, physiology, growth, reproduction, development or lifespan of an organism that results in impairment of functional capacity to compensate for additional stress or increased susceptibility *to the harmful effects of other environmental* influences' (EFSA Scientific Committee, 2019).
Intermediate effect	The intermediate effect refers to key events (*see definition below*). In the context of OECD Harmonised Template 201,[14] on intermediate effects they are referred as 'non-apical observations obtained from methods such as in vitro testing or from other classes of methods (e.g. ex vivo or in silico methods) providing mechanistic information, i.e. effects on molecular, subcellular, cell, tissue or organ level that can be relevant to the hazard assessment'.
Apical effect	The apical effect refers to the apical endpoint (*see definition below*).
Critical effect	The adverse effect is seen at the lowest dose when a vulnerable population is exposed to a substance such as an environmental or food toxin. This can relate to humans as well as to other species such as animals, plants or microbes.[15]
Endpoint	The recorded observation coming from an *in chemico* method, an in vitro assay or an in vivo assay (OECD, 2012, 2017). Qualitative or quantitative expression of a specific factor with which a risk may be associated, as determined through an appropriate risk assessment (WHO/IPCS, 2009). An endpoint is a precisely defined variable intended to reflect an outcome of interest that is analysed using statistics to address a particular research question (Califf, 2018).
Apical endpoint	Apical endpoints are empirically verifiable outcomes of exposure, such as death, developmental anomalies, breeding behaviours, impaired reproduction, physical changes and alterations in the size and histopathology of organs, including clinical signs or pathologic states, that are indicative of a disease state (Krewski et al., 2011; OECD, 2012, 2017; Villeneuve & Garcia-Reyero, 2011).
Non-apical endpoint	Non-apical endpoints are defined as intermediate events or steps at a level of biological organisation below that of the apical endpoint (OECD, 2012, 2017).
Intermediate events	The intermediate event refers to a key event (*see definition below*). As such, intermediate events are key events between the molecular initiating event and the apical outcome that are toxicologically relevant to the apical outcome and experimentally quantifiable (OECD, 2012, 2017).
Adverse outcome pathway (AOP)	An AOP describes a sequence of events commencing with initial interaction(s) of a stressor with a biomolecule within an organism that causes a perturbation in its biology (i.e. molecular initiating event, MIE), which can progress through a dependent series of intermediate KEs and culminate in an adverse outcome (AO) considered relevant to risk assessment or regulatory decision-making. AOPs are typically represented sequentially, moving from one key event to another, as compensatory mechanisms and feedback loops are overcome (OECD, 2012, 2017, 2018a).
AOP network	An AOP network is defined as an assembly of two or more AOPs that share one or more KEs, including specialised KEs such as MIEs and adverse outcomes (Knapen et al., 2018).
Molecular initiating event (MIE)	A molecular initiating event is a specialised type of key event that represents the initial point of chemical interaction at the molecular level within the organism that results in a perturbation that starts the AOP (OECD, 2018a).
Key event (KE)	A key event is a change in biological or physiological state that is both measurable and essential to the progression of a defined biological perturbation leading to a specific adverse outcome (OECD, 2018a). When effect biomarkers coincide with key events depicted in AOPs, the utilisation of AOPs can synergise and align toxicological and epidemiological knowledge. This can contribute to a better understanding of the biological fingerprint generated by exposure to environmental chemicals (Rodríguez-Carrillo et al., 2023).
Key event relationship (KER)	A key event relationship is a scientifically based relationship that connects one key event to another, defines a causal and predictive relationship between the upstream and downstream event, and thereby facilitates inference or extrapolation of the state of the downstream key event from the known, measured, or predicted state of the upstream key event (OECD, 2018a). The **quantitative understanding** section of the KER description is intended to capture information that helps to illustrate how much change in the upstream KE, and/or for how long, is needed to elicit a detectable and defined change in the downstream KE.
Adverse outcome	An adverse outcome is a specialised type of key event that is generally accepted as being of regulatory significance based on correspondence to an established protection goal or equivalence to an apical endpoint in an accepted regulatory guideline toxicity test (OECD, 2018a).

(Continues)

[14] https://www.oecd.org/ehs/templates/harmonised-templates-intermediate-effects.htm.
[15] https://www.efsa.europa.eu/en/glossary/critical-effect.

TABLE B.5 (Continued)

Term	Definitions
Mode of action (MOA)	Mode of action is a biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data. An MOA describes key cytological and biochemical events – that is, those that are both measurable and necessary to the observed effect – in a logical framework (WHO/IPCS, 2009). A sequence of events, identified by research, which explains an observed effect (OECD, 2012, 2017).
Mechanism of action	The specific biochemical interaction through which a substance produces an effect on a living organism or in a biochemical system (WHO/IPCS, 2009). Mechanism of action for toxicity is the detailed molecular description of key events in the induction of cancer or other health endpoints. Mechanism of action represents a more detailed understanding and description of events than is meant by MOA (OECD, 2018b). The process by which a substance produces an effect on a living organism.[16]
Biomonitoring	The measurement of chemicals, or their metabolites, in human biological samples.

[16] https://www.efsa.europa.eu/en/glossary/mechanism-action.

APPENDIX C

Characteristics of biomarkers of effect

TABLE C.1 Characteristics defining the validity and value of biomarkers of effect in the context of risk assessment.

Characteristic	Description	Relevancy (high or low) for…	
		Data generator /developer	Risk assessor
Analytical characteristics			
Quantifiability	The biomarker of effect should be (easily) measurable and quantifiable via analytical methods.	*H*	*L*
Accessibility	Is measurable in accessible matrices.	*H*	*L*
Robustness/reproducibility	The measurement should demonstrate an adequate level of inter- and intra-laboratory accuracy and variability. The methods used to measure biomarkers must be accurate, precise, reproducible and reliable. Rigorous validation of analytical techniques is essential to ensure accurate results.	*H*	*H*
Non-invasiveness	Ideally the biomarker should be measured via non-invasive technique/minimally invasive methods.	*H*	*L*
Availability	The biomarkers' assays should be readily available	*H*	*L*
Cost–effectiveness	Ideally the biomarker should be measured using a non-expensive technique. High-cost assays may limit their practical use in large-scale studies.	*H*	*L*
Biological characteristics			
Specificity	Specificity whether the biomarker is associated with: • one or more adverse outcomes; • one or more organs or systems (biomarkers of effects on different systems/multifaceted effect); • one or more chemicals. Highly specific biomarker = low false-positive rate. High specificity minimises misclassification.	*H*	*H*
Sensitivity	Biomarkers should be sensitive enough to detect changes associated with exposure or disease. This means that the biomarker of effect must change in response to the exposure to environmental chemical compounds to a degree that allows the alterations caused to be detected. The biomarker should also allow reliable measurement of biological changes, providing an accurate, precise, reproducible, interpretable and predictive measurement of the health outcome with which they were correlated (Rodríguez-Carrillo et al., 2023). Sensitivity is related to variability, in that markers that are strictly physiologically controlled within a certain range (e.g. blood pressure (BP) or liver enzyme levels) are of more value because they do not vary within the normal healthy population, while other parameters, such as body mass index (BMI) are of less value because the numerical value is not necessarily a good indicator of health. Highly sensitive biomarker = low false-negative rate. High sensitivity ensures that even subtle effects are captured.	*H*	*H*

(Continues)

TABLE C.1 (Continued)

Characteristic	Description	Relevancy (high or low) for…	
		Data generator /developer	Risk assessor
Predictivity	The predictivity (or predictive capacity) refers to the property of a biomarker appearing early in the sequence of biological events of being indicative of an adverse outcome (early indication of an adverse outcome). **Predictive accuracy** measures the relationship between the prediction of the occurrence of the outcome and the real occurrence of the outcome (Burke, 2016). It should be noted that predictivity does not necessarily imply that the (apical) adverse outcome (e.g. a disease) will inevitably occur at a later point in time. If repeated exposure to chemicals ceases over several periods, the effects may be reversible, meaning that late key events and/or the final (adverse) outcome can be avoided. Thus, late key events or adverse outcomes will not always occur when previous (early) key events are affected. A higher dose and/or longer exposure time might be necessary to trigger late key events or the (apical) adverse outcome. The predictive capacity of a biomarker of effect can be influenced by different factors, e.g.:	*H*	*H*
	Individual and population variability (the response in different populations and conditions needs to be well documented): • Establishing **reference ranges** (normal values) for biomarkers in the target healthy (non-diseased) population is crucial, as well as using biomonitoring studies covering all age groups, sex and other determinants. • Deviations from these ranges can indicate exposure-related effects. • The levels of biomarkers of effect can fall within a normal or abnormal range. Abnormal values indicate deviations from what is expected in a healthy individual. These deviations can signal underlying health issues. Hence, biomarkers of effect cannot always be considered 'adverse' by themselves.	*H*	*H*
	Magnitude and duration of change: • **Threshold** in the magnitude of change: if there is a sufficiently established relationship between levels of a biomarker of effect and an adverse outcome, it may be possible to set a 'threshold level' which, once exceeded, would trigger further downstream events in the toxicity pathway eventually leading to the (apical) adverse outcome. Threshold values can directly inform risk assessment for regulatory purposes.	*H*	*H*
	Reversibility or (irreversibility) of the biomarker of effect and its 'point of no return': • When the change in the biomarker reaches a sufficient magnitude and duration that will inevitably trigger the adverse outcome regardless of whether exposure ceases or decreases at that time.	*H*	*H*
Plausibility	The plausibility provides insight into the underlying disease mechanism (Bennett & Devarajan, 2011) and refers to the credible mechanisms that connect the marker, the pathogenesis of the disease, and the MOA of the intervention (Aronson, 2005). The **biological plausible link** means the correlation between an activity (e.g. endocrine) and an adverse effect, based on biological processes, where the correlation is consistent with existing scientific knowledge.[17]	*H*	*H*
Translatability	The biomarker functions in both model systems and human condition.	*H*	*H*
Biological relevance	Biomarkers of effect should be biologically relevant to the specific exposure or health effect being studied. They should reflect the underlying biological processes related to the exposure or disease.	*H*	*H*

[17]Regulation No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on Classification, Labelling and Packaging of Substances and Mixtures, Amending and Repealing Directives 67/548/EEC and 1999/45/EC, and Amending Regulation (EC) No 1907/2006 (Text with EEA Relevance), (updated) 186 (2023). http://data.europa.eu/eli/reg/2008/1272/oj.

TABLE C.1 (Continued)

Characteristic	Description	Relevancy (high or low) for…	
		Data generator /developer	Risk assessor
Human relevance	Biomarkers of effects in animals do not necessarily reflect their relevance in humans.	*H*	*H*
Dose–response relationship	Ideally, biomarkers should exhibit a dose–response relationship. This means that their levels should change in proportion to the exposure dose or severity of the health effect. A clear dose–response relationship strengthens the evidence for causality.	*H*	*H*
Temporal stability	Biomarkers should remain stable over time (within reasonable limits) to allow accurate monitoring. If a biomarker fluctuates significantly due to external factors, it may not be suitable for long-term studies.	*H*	*H*
Biological half-life	Understanding the half-life of a biomarker helps to determine the appropriate sampling frequency. Short half-lives may require more frequent monitoring.	*H*	*L*
Validation in relevant populations	Biomarkers should be validated in populations such as the one being studied (e.g. age, sex, ethnicity).	*H*	*H*

In addition, Hills' criteria (IOM US, 2010) represent a useful tool for further developing criteria for biomarkers of effect. These refer to:

1. Strength (stronger links being more likely causal).
2. Consistency (consistent replication of findings).
3. Specificity (causation is supported if an exposure appears to cause only a specific effect).
4. Temporality (the cause preceding the effect).
5. Biological gradient (increasing dose associated with increased risk).
6. Plausibility (biological feasibility).
7. Coherence (no conflict in the data interpretation with generally known biological facts).
8. Experiment (removing exposure and lowering risk).
9. Analogy (*'appropriate comparison between weaker evidence of causation between an exposure and its effect and strong evidence of causality between another exposure and its similar effect'*).

ANNEXES

Annex 1. Template for the description of biomarkers of effect with representative examples
Annex 2. Mapping study report
Annex 3. Inventory of resources
Annex 4. Survey report
Annex 5. Stakeholder workshop report
Annex 6. Public consultation report

Annexes 1–6 can be found in the online version of this output (in the 'Supporting information' section): https://doi.org/10.2903/j.efsa.2024.9153