Mutation Research - Genetic Toxicology and Environmental Mutagenesis 896 (2024) 503768

Contents lists available at ScienceDirect

Mutation Research - Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox

The use of effect biomarkers in chemical mixtures risk assessment – Are they still important?

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ARTICLE INFO

Keywords:
Genotoxicity
Micronucleus
Chemical mixtures
Adverse outcome pathways
New approach methodologies
Human risk assessment

ABSTRACT

Human epidemiological studies with biomarkers of effect play an invaluable role in identifying health effects with chemical exposures and in disease prevention. Effect biomarkers that measure genetic damage are potent tools to address the carcinogenic and/or mutagenic potential of chemical exposures, increasing confidence in regulatory risk assessment decision-making processes. The micronucleus (MN) test is recognized as one of the most successful and reliable assays to assess genotoxic events, which are associated with exposures that may cause cancer. To move towards the next generation risk assessment is crucial to establish bridges between standard approaches, new approach methodologies (NAMs) and tools for increase the mechanistically-based biological plausibility in human studies, such as the adverse outcome pathways (AOPs) framework. This paper aims to highlight the still active role of MN as biomarker of effect in the evolution and applicability of new methods and approaches in human risk assessment, with the positive consequence, that the new methods provide a deeper knowledge of the mechanistically-based biology of these endpoints.

1. Introduction

Health risk assessment of exposure to chemicals, and subsequent regulatory measures are generally based upon data from studies on individual substances [1–4] and one chemical at a time approach [5]. However, humans are simultaneously exposed to a wide range of chemical agents, which potentially possess a number of similar or different toxic effects [6–8]. As outlined by the United States Environmental Protection Agency (US EPA), that the research strategy for 2000 and beyond would emphasized on chemical mixtures [9–11], by the European Commission Communication on the combination effects of chemicals [4], and also The Organization for Economic Co-operation and Development (OECD) considerations for assessing the risk of combined exposure to multiple chemicals document [12], there are several open issues to address in chemical mixtures research, such as a lack of understanding of real co-exposures, combined toxicity, interactions, chemicals' modes of action, and criteria for grouping chemicals [2]. As a proposal to fill the gaps, the European Food Safety Authority (EFSA) had published a guidance document on scientific criteria for grouping chemicals into assessment groups for human risk assessment of combined exposure to multiple chemicals [13]. The framework proposes, when available, the use of mechanistic information on toxicity through a structured weight of evidence (gold standard); and when data do not support determination of mode of action (MoA), grouping can be done based on toxicokinetic data and/or common adverse outcomes [13]. In addition, a roadmap for action on risk assessment of combined exposure to multiple chemicals was addressed [14].

A pragmatic approach for human risk assessment of chemical mixtures was summarized by Boberg et al. [5]. In brief, grouping chemicals were suggested for mixture risk assessment based on integrated *in vivo* and *in vitro* data, read-across, as well the use of new approach methodologies (NAMs) as quantitative structure activity relationship (QSAR) models and other computational approaches to predict the toxicity of chemical mixtures [reviewed in [15]. In the same line, a methodology for health risk assessment of combined exposures to multiple chemicals anchored in the adverse outcome pathways (AOPs) concept is widely considered in the context of exposome research [16–19].

As shown by the large majority of the human biomonitoring studies, populations from distinct geographic areas are exposed to a large number of chemicals in their lives, with these exposures generally occurring at intermittent and inconsistent doses [20], instead of a consistent rate and dose magnitude such as observed in arsenic in

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https://doi.org/10.1016/j.mrgentox.2024.503768
Received 21 February 2024; Received in revised form 29 April 2024; Accepted 13 May 2024
Available online 16 May 2024

drinking water and radon exposure. However, the dynamics of chemicals interaction is not linear, that can be shown, for instance, by non-monotonic doses responses [21] and by the possibility that single compounds cannot have an effect in comparison on a mixture [22]. It has been shown by experiments that chemicals may act jointly and the combined toxicity may be higher than the toxicity of each of the single components on its own [8,11]. Therefore, the interest from the scientific community in evaluating and, if possible, quantifying multiple adverse health effects caused by numerous combinations of different substances with toxic properties have increased [23]. The fact that exposure is dynamic and has different doses of the various compounds [24] makes the whole problem around mixtures extremely difficult to evaluate and predict its consequences. The use of a more flexible approach allows deployment of new tools, such as high-throughput assays and advanced sequencing approaches, in ways that permit integration of new knowledge on both genomic endpoints and the potential health consequences of genomic interactions [25].

2. Effect biomarkers

Human exposure to chemicals in the environment will almost always be to complex mixtures of chemicals from different sources, among which are food, water, air, consumer products, and other media and sources [2,26,27]. Exposures may contain genotoxic compounds and/or compounds with capacity to modify genotoxic agents responses [28,29]. These genotoxic events can be related to early biologic effects and/or altered structure/function which constitute the biomarkers of effect.

Effect biomarkers can provide a link between internal exposure and biological alterations, by helping in identify early effects in humans at low doses, establish dose-response relationships, explore mechanisms and establish plausible AOPs obtained from epidemiological observations. In addition, can improve the risk assessment of specific chemical families as well as exposure to chemical mixtures. The main challenge is to link effect biomarkers to specific exposures (i.e., sources, pathways and routes) and early health effects (i.e., the key events along the pathway from exposure to disease) [19,30].

In general population studies, effect biomarkers can help in supporting regulatory decisions by increasing the weight of evidence for a given chemical family such as providing information on potential MoA, assessing dose-response relationships, detection of subclinical effects, and evaluation of potential mediators between exposure biomarkers and health outcomes [19,31]. The mechanistic association between effect biomarkers and health outcomes can be systematically explored by making use of information collected in AOPs [17]. AOPs represent biologically plausible and empirically supported links between a series of key events, which are measurable downstream changes at molecular, cellular, tissue organ, individual, and population level, from a molecular initiating event to an adverse outcome [17,18,31]. When effect biomarkers coincide with key events depicted in AOPs, its use can synergize and align toxicological and epidemiological knowledge, contributing to a better understanding of the biological fingerprint generated by exposure to chemicals [32]. Lastly, biomarkers offer the opportunity to provide scientific confirmation of proposed exposure-disease pathways *in vivo* in human populations.

3. Genotoxicity assessment challenge

Genotoxicity information has been extensively used for safety risk assessment of chemicals together with general toxicity, carcinogenicity, reproductive toxicity, and other endpoints. Genotoxicity *in vitro* is probably the most frequently studied effect of complex mixtures and many studies have been performed on the genotoxic potential of complex environmental mixtures [33,34] and reviewed in [35].

Genotoxic substances alter the structure, information content, or segregation of DNA in different ways. DNA reactive substances primarily cause DNA damage by forming covalent DNA adducts and by cross-linking (i.e., intra-strand DNA crosslinks, and DNA-protein crosslinks). In contrast, non-DNA reactive genotoxic substances give rise to DNA damage through indirect mechanisms, for example by the generation of reactive oxygen species (ROS), or by interfering with cellular components involved in maintaining genomic stability, chromosome integrity, or functioning of the mitotic spindle [36].

Although the fair amount of information on binary and complex mixtures assayed for genotoxic effects *in vitro* and *in silico*, limited information is available about how combined actions or interactions between chemical in mixtures may affect the net genotoxic outcome, in particular *in vivo*, in which parameters as bioavailability, metabolism, DNA binding, DNA repair, may have pronounced influence on the ultimate genotoxicity of a complex mixture.

Traditionally, genetic toxicology testing has only been used for hazard identification and the screening of compounds in a simple binary approach (positive/negative). However, the limitations of this approach, alongside the realization, that much more information can be gained from genetic toxicity dose-response data are increasingly recognized [37].

There are several factors to consider for understand the possible genotoxic effects which a chemical mixture can offer, namely if the constituents of the mixture (i) cause the same critical effect; (ii) act on the same molecular target at the same target issue, and (iii) act by the same mode of action and may share a common toxic intermediate [29]. Critical issues to be addressed in this topic are the co-exposure (concurrent exposure) in which more than one chemical is able to interact with a defined target in a specific target tissue during a particular time of frame interest [29], and individual genetic susceptibility factors. This latter, by affecting absorption, distribution, metabolism and excretion (ADME) of a xenobiotic or influencing toxicodynamics related to differences in receptor binding, other critical molecule, variations in response after an initial molecular event, and others [38–41].

Many test systems for different endpoints have been developed, improved, and used in practice. The battery strategy, combining several test systems to detect as many genotoxic chemicals as possible, was implemented because no single test is capable of detecting all relevant genotoxic agents with different mechanisms of genetic damage [42], and different batteries of genotoxicity tests are recommended depending on the use to which data will be put.

Short-term bioassays for genotoxic effects with OECD guidelines established – *in vitro* and/or *in vivo* - have been widely used as screening tools in complex mixtures toxicological assessment, since they are rapid, inexpensive and sensitive indicators of potential induction of genomic instability, specifically, genetic damage. Commonly studied endpoints include DNA damage (e.g., strand breaks, DNA adduct, and DNA recombination), gene point mutations, chromosomal aberrations, micronuclei and aneuploidy [12,43].

4. Classical effect biomarkers - Micronuclei

Although there are several available techniques for genotoxicity assessment, cytokinesis-blocked micronucleus (CBMN) assay is the most popular version of micronuclei (MN) evaluation used in human peripheral blood lymphocytes for biomonitoring, mainly due to its simplicity, sensitivity, and versatility to measure DNA strand breakage or chromosomal loss, which can be recognized as potential hallmarks of mutagenicity and carcinogenicity [44–51].

MN originate from chromosome fragments or whole chromosomes that lag behind anaphase during nuclear division and are not included in the main nucleus [52–55], and it is considered to be caused by DNA damage or genomic instability [44,55–58]. The evidence of its popularity is based in biomonitoring studies, systematic reviews and meta-analysis which have been conducted [35,59–65,35,59,61–63,65–67] in human populations, and its proposal to the clinical practice is currently being considered [68].

CBMN assay has showing its potential by continually evolved by

using of molecular probes that enable deeper understanding of the mechanistic origin of MN and their consequences, for instance the application of pan-centromeric probes to determine the origin of the MN from acentric chromosome fragment or whole chromosome; p53, gH2AX, ATM, Rad51, Smad7 probes to measure the expression of DNA damage response (DDR) proteins in MN and determine how they differ between different cells and type of genotoxin insult, and others [69]. Recent findings observed that transient sequestration of chromosomes in MN disrupts chromatin organization, leading to heritable epigenetic dysregulation long after the chromosome is reintegrated into the primary nucleus [70]. Knowledge on the molecular mechanisms by which *in vivo* exposure to exogenous chemical genotoxins in humans induces MN and other nuclear anomalies in lymphocytes *in vivo* and *ex vivo* after nuclear division *in vitro* is well reviewed in [71].

In occupational exposure human biomonitoring studies, the MoA/endpoints proposed to be addressed as major priority were carcinogenicity, which included cancer biomarkers for genotoxicity and oxidative stress [19]. CBMN assay and Micronucleus test (in erythrocyte, peripheral blood lymphocytes and buccal mucosa cells) were considered the potential effect biomarkers covering carcinogenicity, including genotoxicity [19].

Exposure scenarios simulating real life is a complex issue since exposure to multiple chemicals may lead to a web of interactions with a wide array of underlying mechanisms that ultimately may result in diverse health outcomes [72], mostly due to gene-environment interactions [73]. Therefore, not only environmental and/or occupational exposure are important outcomes, but also factors linked to the individual's lifestyle habits, such as smoking, drinking, body mass index and others [74], and individual genetic makeup, introduce variability and difficulty in interpreting and assessing human health outcomes from exposure to mixtures of toxic compounds [73].

An example of an human biomonitoring study which aimed to translate real scenario exposure to *in vitro* approach was developed in two phases: (1) occupational exposure assessment to solvent mixture of styrene and xylene in workers in a polymer-producing chemical facility through exposure assessment and biomonitoring (CBMN assay and comet assay) campaigns; (2) *in vitro* exposure of human peripheral blood cells with the concentrations found in the working environment (in phase 1) using both biomarkers as in the first phase of the study with the aim to better understand the MoA of this mixture, and how it can be responsible for the observed results [75]. In other words, toxicological and AOP data determine the physiological validity of using a set of effect biomarkers for chemicals sharing the same MoA.

Among the diverse effect biomarkers available, some allow a direct interpretation in terms of risk at an individual level, such as blood pressure or hormone levels with validated reference values. Others provide information that can be interpreted only at the population level such as genotoxicity biomarkers or novel biomarkers for which reference levels are not available [19].

5. Combined effect biomarkers

Human molecular epidemiology studies are highly relevant as they provide insight into the true range of human exposures avoiding the uncertainty associated with extrapolation across species. The study from Rodríguez-Carrillo et al. [76] in human placentas regarding relevant endocrine activity and signaling, examined the effects of "real-world" mixtures exposure by using biomarkers of combined activity/effect/internal exposure, where bioassays tested the combined biological effect of chemical fractions isolated from human samples. The advantage of this promising approach is that describe and discuss the biological effect measured, taking in account the bioactive chemicals as well plausible interactions among them which may lead to synergistic or antagonistic mixture effects, also showed in the Bjerregaard-Olesen et al. [77] study. Also, the study from Vinggaard et al. [78] showed a new and integrative experimental approach, namely by using the Effect-Directed Analysis (EDA), to elucidate the link between human chemical exposure and health effects. Taken together, these novel biomarkers show that the *ex vivo* bioassays open the possibility to evaluate the combined effects of complex chemical mixtures in human samples, and efforts are needed to integrate these approaches in risk assessment.

6. Next generation risk assessment

Next generation risk assessment is an approach used for regulatory purposes that has the potential of reducing the use of animal testing that poses several issues related to ethics, relevance to human health, costs and efficacy. This paradigm shift in toxicity testing from *in vivo* (animal-based) approaches towards NAMs that most rely on *in vitro* (molecule and cell-based) and *in silico* (computational) methods [79,80]. Simultaneously, epidemiological and biomonitoring studies are crucial to identify hazards potentially associated with chemical exposures in humans, and to have a strong evidence-based mechanistic knowledge linking molecular perturbations and adverse outcomes, AOPs can be an instrumental tool [79].

AOPs define a series of measurable biological changes that can be expected to occur if the perturbation is sufficiently severe (i.e., in terms of potency, duration, frequency) to drive the pathway all the way to an adverse outcome considered relevant to risk assessment or regulatory decision-making [81]. They also identify current knowledge gaps which, if filled, could further improve predictive utility.

The current suite of standardize OECD test guideline methods for genotoxicity assessment provides only very limited information on the substance MoA. Thus, complementing existing standard methods, such as biomarkers of effect delivered by CBMN assay and MN test, with assays targeting at the underlying mechanisms is an important step towards the development of MoA-based quantitative models [82]. The study from Baken et al. [18], provided solid mechanistic support for causal associations between phthalate exposure and reproductive outcomes reported in epidemiological studies by using an combination of biomarkers of effects retrieved by systematic literature search from human observational studies as well as knowledge in existing AOPs to which phthalates were listed. This approach allowed to show an association between the majority of the biomarkers of reproductive effects and phthalate exposure, supported by mechanistic information described in existing AOP; and also to identify novel biomarkers of effect which may result in adverse reproductive outcomes [18].

The development of AOPs linking genotoxic key events to adverse health outcomes may also create new opportunities for the use of NAMs and the further development of integrated approaches to genotoxicity testing and assessment.

NAMs are defined as any technology, methodology, approach, or combination that can provide information on chemical hazard and risk assessment without the use of animals, including *in silico, in chemico, in vitro*, and *ex vivo* approaches [10,83]. NAMs are gaining traction as a systematic approach to support informed decision making in chemical risk assessment. They intend to modernize traditional toxicology testing strategies by addressing the current limitations with conventional assays with the aim to accelerate the pace of hazard assessment and reduce reliance on animal tests that are time-consuming and resource intensive [84].

As it was previously addressed, the *in vitro* MN test is an important tool for evaluating chemicals' potential to cause chromosomal damage, being MN formation sensitive to both clastogenic and aneugenic activity. There are many researches which combined MN scoring and panels of DNA damage response-type biomarkers in order to supplement the *in vitro* MN endpoint with biomarkers that comment on genotoxicants' mode of action, improving substantially the specificity of the assay [85]. The MEGA-Screen assay is a image analysis system that combines MN scoring with kinetochore labeling, ɣH2AX and cell cycle analysis; the iScreen assay is a confocal image analysis approach that combines MN scoring with ɣH2AX, MPM2, phospho-histone H3 (p-H3), centromere

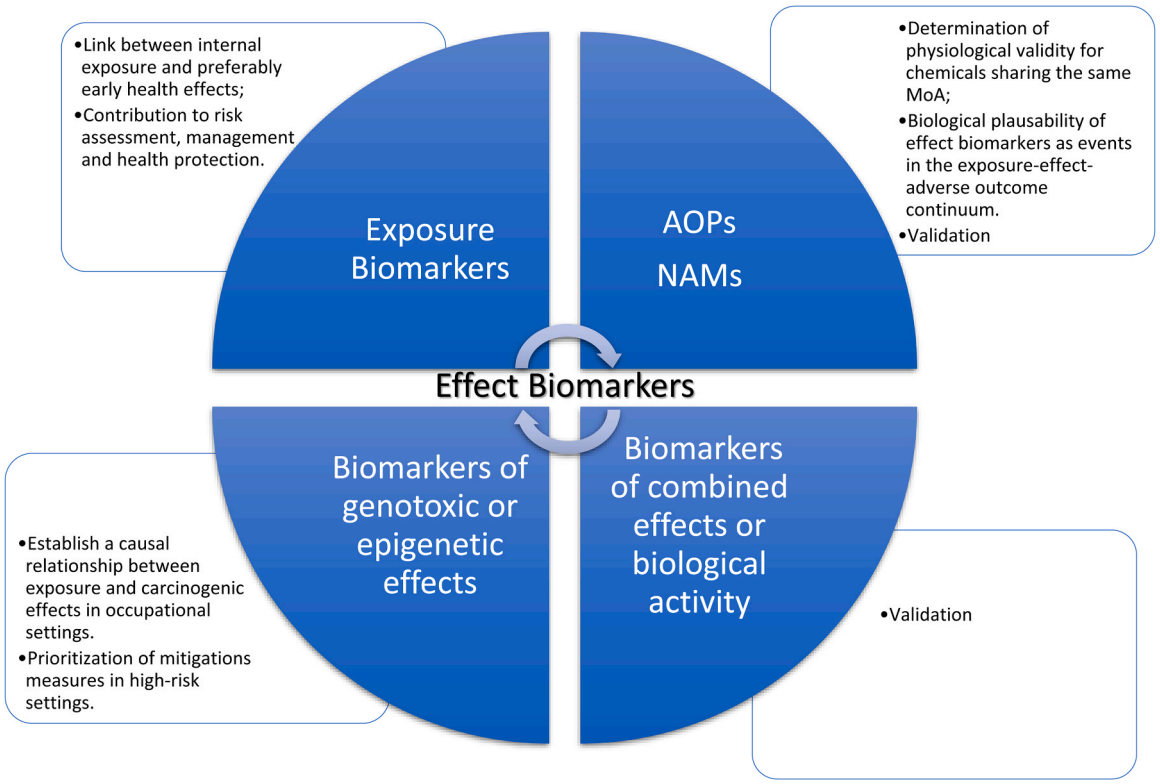

Figure 1. Relevance and versatility of classical effect biomarkers in filling the knowledge gaps and leverage novel biomarkers research. AOP – Adverse Outcome Pathway; NAMs – New Approach Methodology.

protein A (CENPA) and cell analysis [85]. Another approach is ToxTracker assay, which showed improved sensitivity and specificity for the detection of known *in vivo* genotoxicants when compared to an existing "standard battery" of tests, such as the results from MN. Briefly, the ToxTracker uses unique gene expression biomarkers to evaluate a variety of cellular responses related to toxicological stress, providing information to assess the magnitude of the detected hazard as well as the substance's mode of action [86]

The study from Wills et al. [37] showed cross-systems genotoxic potency correlations for chemical compounds between the DNA-damage ToxTracker endpoints and from *in vivo* MN assay, as the latter could be considered a "gold standard" for the sensitive, human-relevant detection of chromosomal damage.

The *in vitro* MicroFlow® assay applies an automated scoring of MN frequency to identify chromosomal damage induced by chemicals, and this approach has been thoroughly validated and is included in the Test Guideline 487 as an option for MN scoring [51]. The MultiFlow® DNA damage assay uses several multiplexed biomarker responses to further classify genotoxic activity based on the mode of action, including the measurement of ɣH2AX (indicative of double strand-breaks), phosphorylation of histone H3 (identification of mitotic cells); nuclear p53 localization (identification of DNA damage responses); and the frequency of 8n DNA content (polyploidization detection). By using MultiFlow® it is possible to classify genotoxic agents into two main modes of action: clastogenicity or aneugenicity [84]. The study from Bryce et al. (2018) used the *in vitro* MultiFlow® DNA damage assay of 86 chemicals, selected based upon responses in genetic damage assays conducted under the Tox21 program, including the MN endpoints [87].

Studies in which this methodologies were applied, demonstrated that integrating several *in vitro* genotoxicity assays provides a more robust and accurate hazard assessment, with a limited number of irrelevant positives, and some of these assays were already incorporated into the GeneTox21 research program at Health Canada to establish an effective platform for genotoxicity assessment [84]. Specifically on regard of the ability to use NAMs for the assessment of human health effects on industrial chemicals and pesticides within the United States, Canada, and European Union regulatory frameworks it is summarize in [88].

In summary, the integration of the quantitative dose-response information along an AOP should allow more informed assessment of the likely effect sizes at low, human relevant exposures [82].

7. Conclusions and perspectives

Human epidemiological studies with biomarkers of effect play an invaluable role in identifying health effects associated with chemical exposures. There is an emphasis for the necessity to incorporate interactions concepts and methods to evaluate the possible influence of interactions on the overall joint toxicity of chemical mixtures assessment [7]. A major challenge in effect biomarker discovery and validation for health outcome assessment, is the understanding of the complex biological mechanisms involved in disease pathogenesis. The use of effect biomarkers in regulatory risk assessment of chemicals could drive the research forward, and thus the evidence for an exposure e-health outcome relationship [19]. This potential is summarized in Fig. 1, when it is found the relevance of effect biomarkers, in relation with (internal) exposure biomarkers, biomarkers of genotoxic or epigenetics effects, biomarkers of combined effects or biological activity in means of validation; and finally, with AOPs and NAMs in knowledge improvement of the MoA, other mechanistic aspects, and continuing validation in future new methodologies.

The assessment of the potential of genotoxicity is an essential step for a safe evaluation of hazardous substances, and it might be the most frequently studied effect of complex mixtures, and many of the above-mentioned test-systems have been used for screening potential genotoxic effect of complex environmental mixtures. However, a limitation of these biomarkers is the lack of specificity to particular chemical exposure, increasing uncertainty in the analysis of exposure-effect associations. Additionally, it is crucial to address that, not only the

genotoxicity outcome (positive/negative) for itself is important, but also its potency, dose and application, in what respect to health risk assessment [80,89,90].

The problem in simulating real life exposure scenarios is extremely complex, since the exposure to multiple chemicals can lead to a web of interactions and mechanisms which can result in diverse health outcomes [72]. A panel of novel bioassays is needed to provide a comprehensive assessment of "real world" chemical mixtures. Biomarkers of combined activity can enable the better characterization of signaling pathways through which mixtures could elicit adverse health outcomes in humans [76]. Although is important to correlate, when is possible, with validated (classical) methods in order to, in a first stage, increase the robustness of the biomarkers in development. At the present there is an effort by conducting systematic reviews and/or meta-analysis in order to better integrate the existing epidemiologic studies with the actual understanding of toxicodynamic processes from *in silico, in vitro* and *in vivo* in order to perform an adequate knowledge collection, treatment and integration of the data and results obtained from the different field [72].

To move towards the next generation risk assessment, is therefore crucial to establish bridges between NAMs, AOPs framework, and standard approaches, and to establish processes for increasing mechanistically-based biological plausibility in human studies [19,79, 88,91], and use that knowledge to contribute to the progress in refining assessment methods for multiple chemicals exposure scenarios. Micronucleus endpoint is a well establish standard method, recognized as a successful and reliable assay for genotoxic carcinogens and its applicability demonstrates that is still keeping up with novel biomarkers and new approaches in risk assessment.

CRediT authorship contribution statement

Carina Ladeira: Writing – review & editing, Writing – original draft, Investigation, Conceptualization.

Declaration of Competing Interest

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

Data availability

No data was used for the research described in the article.

Acknowledgments

The author thanks to H&TRC- Health & Technology Research Center-Escola Superior de Tecnologia da Saúde, Instituto Politécnico de Lisboa; FCT/MCTES national support through the UIDP/05608/2020 (https://doi.org/10.54499/UIDP/05608/2020), UIDB/05608/2020 (https://doi.org/10.54499/UIDB/05608/2020) and, IPL/2021/PLASCOGEN_ESTeSL.

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