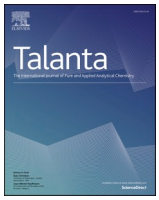
Talanta 279 (2024) 126616

Contents lists available at ScienceDirect

Talanta

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



Analytical challenges and opportunities in the study of endocrine disrupting chemicals within an exposomics framework

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ARTICLE INFO

Handling Editor: A Campiglia

Keywords:
Exposomics
Endocrine disrupting chemicals
HRMS
Data processing
Biomarkers
Metabolomics

ABSTRACT

Exposomics aims to measure human exposures throughout the lifespan and the changes they produce in the human body. Exposome-scale studies have significant potential to understand the interplay of environmental factors with complex multifactorial diseases widespread in our society and whose origin remain unclear. In this framework, the study of the chemical exposome aims to cover all chemical exposures and their effects in human health but, today, this goal still seems unfeasible or at least very challenging, which makes the exposome for now only a concept. Furthermore, the study of the chemical exposome faces several methodological challenges such as moving from specific targeted methodologies towards high-throughput multitargeted and non-targeted approaches, guaranteeing the availability and quality of biological samples to obtain quality analytical data, standardization of applied analytical methodologies, as well as the statistical assignment of increasingly complex datasets, or the identification of (un)known analytes.

This review discusses the various steps involved in applying the exposome concept from an analytical perspective. It provides an overview of the wide variety of existing analytical methods and instruments, highlighting their complementarity to develop combined analytical strategies to advance towards the chemical exposome characterization. In addition, this review focuses on endocrine disrupting chemicals (EDCs) to show how studying even a minor part of the chemical exposome represents a great challenge. Analytical strategies applied in an exposomics context have shown great potential to elucidate the role of EDCs in health outcomes. However, translating innovative methods into etiological research and chemical risk assessment will require a multidisciplinary effort. Unlike other review articles focused on exposomics, this review offers a holistic view from the perspective of analytical chemistry and discuss the entire analytical workflow to finally obtain valuable results.

1. Introduction

Humans are exposed to a large number of agents that can be harmful to health throughout life. Environmental exposures, defined as all non-genetic exposures that interact with living organisms, represent a high percentage of them. Environmental exposures include internal (agents such as metabolism, hormones, gut microflora, inflammation, lipid peroxidation, oxidative stress, etc.), general external (related to individual life conditions such as climate, social capital, or living areas) and specific external exposures [1]. Chemical contaminants are included in this last group, being most of them synthetic compounds used in a wide range of industrial processes or non-intentionally released to the environment as by-products. There are more than 350000 compounds and mixtures registered in chemical inventories, and about 69000 chemicals compounds currently in commerce; however, not all of them have been registered at high-volume production that could result in human exposure. Also, some of them are claimed to be confidential or they are poorly described [2]. For example, 2683 chemicals were registered in

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https://doi.org/10.1016/j.talanta.2024.126616
Received 6 May 2024; Received in revised form 11 July 2024; Accepted 23 July 2024
Available online 24 July 2024

REACH at productions of >100 tons/year during 2018. Therefore, some of these compounds can reach human bodies and cause changes in the normal functioning, such as the case of endocrine disrupting chemicals (EDCs) [3].

EDCs comprise a wide range of highly heterogeneous and persistent environmental pollutants extensively used in various aspects of human life, that affect the endocrine system by different action modes. Consequently, may be involved in a wide variety of hormone-dependent diseases (Table 1). They can bind to hormone receptors and mimic their actions, through the antagonist action blocking the receptors and either inhibiting their action or interfering with hormone synthesis, transport, metabolism and/or elimination; therefore, decreasing the concentration of natural hormones [4]. Even when the exposure is produced at trace levels (ng/L), they can produce severe health effects such as impaired reproductive function, like cryptorchidism or a reduction of semen quality in men, and polycystic ovarian syndrome, endometriosis (EM) or uterine fibroids among women, all contributing to global infertility epidemics. In addition, some EDCs are involved in metabolic disorders

Table 1
Analytical methodologies applied for the determination of EDCs in environmental and biological samples, and their most important health effects.

Compound	Health effect	Environmental analysis		Biological analysis				Refs.
		Extraction	Analysis	Preferable matrix	Biomarkers of exposure	Extraction	Analysis	
Low molecular weight phthalates (DBP, DEP)	Reproduction disease affection (EM), associated with diabetes and cardiovascular diseases, metabolism disorders, oxidative stress, preterm birth and reduce semen quality	LLE, SLE, SPE and microextraction	GC-FID, GC-MS or LC-MS	Urine	Glucuronic acid metabolites	SPE or direct injection after deconjugation with β-glucuronidase	LC-MS	[6,7,11,12]
High molecular weight phthalates (DINCHs and DINPs)					Glucuronic acid metabolites of oxidized phthalates			
Bisphenols	Produce metabolism disorders and produce men and women reproductive problems (semen quality, ovarian diseases, EM, …)	LLE, SLE, SPE and microextraction	GC-MS or LC-MS	Urine, plasma and serum	Parent compounds, sulphate and glucuronide conjugates	Deproteinization and SPE or direct injection	LC-MS or GC-MS	[8,13,14]
Parabens	Induce the proliferation of cancerous cells, and estrogenic effects	LLE, SLE and SPE	LC-UV and LC-MS	Urine or saliva	Parent compounds, PHBA, and glucuronic acid, sulfuric acid or glycine conjugates of PHBA	Enzymatic digestion, direct injection, LLE or DLLME	LC-MS or GC-MS	[14–17]
OCCs (PCB, OCP and TCDD)	Promotion of EM, proliferation of cancerous cells, neurotoxicity, estrogenic and androgenic effects, reproductive diseases	Soxhlet, LLE, SLE, SPE, SPME	GC-MS	Plasma and serum	Parent compounds, $MeSO_2$-PCBs and OH-PCBs conjugates	Deproteinization, enzymatic digestion and SPE	GC-MS	[18–24]
BFRs (PBDE, PBB)	Endocrine disrupter with carcinogenic properties, reproductivity problems (cryptorchidism)	Soxhlet, SLE and SPE	GC-MS or LC-MS	Plasma and serum	Parent compounds or metabolites in urine	Deproteinization, LLE and SPE	GC-MS	[25–28]
PFAS	Related with metabolism disorders as hypercholesterolemia or diabetes, EM, immunosuppression, reduced birthweight, and reduced semen quality	LLE and SPE	LC-MS	Plasma and serum	Parent compounds or precursors as FTOHs, FOSAs and FOSEs	Deproteinization, enzymatic digestion and SPE	LC-MS	[5,6,18,29,30]
OPCs (OPEs, OPFRs and OPPs)	Toxic and with influence over lipids metabolism, produce neuro-deficiencies and neurotoxicity in babies and reduce semen quality	LLE, SLE, SPE and microextraction	GC-MS or LC-MS	Urine or plasma and serum	Parent compounds in plasma and serum or metabolites in urine (OPP metabolites (DAPs) and HO-OPEs)	Deproteinization, enzymatic digestion and SPE	LC-MS	[31–34]
PAHs	Mutagenic, carcinogenic and estrogenic disrupting properties	LLE, SLE, SPE and microextraction	GC-MS or LC-MS	Urine	Parent compounds and hydroxylated metabolites, PAH-DNA adducts, methoxyphenols, and levoglucosan	Enzymatic digestion, LLE, SPE and microextractions	LC-MS	[35–38]
Metals (Co and Cd)	Associated with metabolism and cardiovascular disorders (osteoporosis, hypertension, diabetes)	–	ICP-MS	Urine or hair	–	Microwave assisted acid digestion	ICP-MS	[36,39–41]

Abbreviations: BFR: Brominated flame retardant; DAP: Dialkylphosphate; DBP: Dibutyl phthalate; DEP: Diethyl phthalate; DINCH: 1,2-cyclohexane dicarboxylic acid diisononyl ester; DINP: Di-isononyl phthalate; DLLME: Dispersive liquid-liquid microextraction; EM: Endometriosis; FID: Flame ionization detector; FOSA: Perfluoroalkyl sulfonamides; FOSE: Perfluoroalkyl sulfonamidoethanols; FTOH: Fluorotelomer alcohols; GC: Gas chromatography; ICP: Inductively coupled plasma; LC: Liquid chromatography; LLE: Liquid-liquid extraction; $MeSO_2$-PCB: Methylsulfonyl-PCB; MS: Mass spectrometry; OCC: Organochlorinated compounds; OCP: Organochlorinated pesticides; OH-OPE: Hydroxylated organophosphate ester; OH-PCB: Hydroxylated polychlorinated biphenyl; OPC: Organophosphate compounds; OPE: Organophosphate ester; OPFR: Organophosphate flame retardant; OPP: Organophosphate pesticides; PAH: Polycyclic aromatic hydrocarbon; PBB: polybrominated benzenes; PBDE: Polybrominated diphenyl ethers; PCB: Polychlorinated biphenyl; PFAS: Perfluoroalkyl substances; PHBA: P-hydroxy benzoic acid; SLE: Solid-liquid extraction; SPE: Solid-phase extraction; SPME: Solid-phase microextraction; TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin; UV: Ultraviolet.

such as obesity, diabetes, hypercholesterolemia or even cardiovascular diseases, in addition to neurodevelopmental disorders or cancer [5–9]. All of that highlights the need of effectively control these substances in environmental and human matrices.

According to the Endocrine Disruptor Knowledge Base (EDKB), about 800 chemicals have been identified or suspected as EDCs. Nevertheless, only some families have been thoroughly studied. Some of these chemical families of EDCs, as well as their health effects and the analytical methodologies proposed for their determination in environmental and biological samples are detailed in Table 1. Those include legacy chemicals identified as persistent organic pollutants (POPs), currently banned or strongly regulated, such as chlorinated [i.e., organochlorinated pesticides (OCPs) and polychlorinated biphenyl (PCBs)], brominated [brominated flame retardants (BFRs)] and fluorinated [perfluoroalkyl substances (PFAS)] compounds, as well as non-intentionally produced chemicals like dioxins and polycyclic aromatic hydrocarbons (PAHs), formed during the incomplete combustion of organic material. Furthermore, a list of more emerging chemicals such as phthalates [from polyvinyl chloride (PVC) resins, medical devices, drug coating and personal-care products], bisphenols (plastic and can containers, dental sealants), parabens (used as preservatives in food and cosmetics) or organophosphate esters (OPEs) have been identified as EDCs, in addition to inorganic chemicals (e.g., cobalt or cadmium) [4, 10].

Environmental epidemiology has contributed substantially to characterize the role of EDCs in a long list of human diseases, especially since the implantation of biomarkers of exposure in the 1990s, boosted by the technological developments in mass spectrometry (MS) [42]. Since then, the development of analytical methods and applications in exposure assessment has been massively growing each year, increasing in terms of coverage (e.g., number of compounds) and/or sensitivity, as shown by the national human biomonitoring (HBM) programs. These advances have not always been reflected in epidemiological research, where the number of compounds examined on health outcomes has been limited to individual compounds from a few congeners from the same family [43]. However, humans are exposed to complex chemical mixtures that can jointly interplay (even interact) along the pathogenic pathways, and it is necessary a broader study of EDCs exposure taking into account real life exposures. In fact, although the exposure to individual compounds may not raise health concerns, co-exposure to multiple chemical agents at low doses can cause health problems, even after years of contact with these chemicals [44,45]. Unfortunately, the interrelations between exposures in exposome-cohort studies nowadays are poorly studied [46]. In addition, health disorders may also occur by a combination of genetic-exposure contributions, making it necessary to study both [9].

In this ongoing struggle to improve the knowledge about the link between chemical exposures and diseases, studies applying the exposome concept, or exposomics, could be the ideal approach to address this goal. Exposome is a term coined by Wild in 2005 [47], and it refers to all the influences that an individual is subjected to during its lifespan, including chemical, biological, and psycho-social agents, and it depends on several factors such as diet, personal habits, habitats, etc. The term has been redefined over the years to include in its definition the biological response induced by these environmental exposures [48]. The exposome encompasses a wide range of exposures, among which the study of the chemical exposome addresses the influence on health of exposure to chemical mixtures throughout human lifespan. Therefore, this framework has a great potential to support the identification of complex exposure-disease relationships involving mixtures of EDCs. In this sense, exposomics has been growing as a novel discipline with the aim of holistically providing comprehensive information on biomarkers of exogenous chemical exposures and their internal biological response, rather than just providing a list of targeted compounds as traditionally done in hypothesis-driven studies [1,43]. The underlying concept has been shaped since the 1980s, when molecular epidemiology was born as a novel approach to gain insight into causal relationships in observational studies, nowadays accommodated to the high-throughput capabilities of omics platforms [49,50].

Chemical exposures can be measured directly from exposure sources (i.e., external exposome, including environmental and food samples) or in body matrices (i.e., internal exposome related to the analysis of biological samples) (Table 1), either in their original form (xenobiotic substances) or as their metabolites (biomarkers of exposure). In turn, biomarkers of effect represent organism's metabolites whose concentration levels are altered as an internal response to a health disorder or disease [51]. Interestingly, biomarkers of effect may help to elucidate molecular changes that link chemical exposures and environmental diseases. Some examples of biomarkers of effect of EDCs are summarized in Table 2, which describes the analytical methodologies to measure them. Information on both types of biomarkers (i.e., biomarkers of exposure and effect) contributes to the ultimate goal of exposomics, which is to provide a link between external exposures and adverse health effects. Some biomarkers of effect are lipids or small molecules that can be determined by comprehensively measuring the internal metabolome with non-selective analytical methods in exploratory studies [52] or using targeted methods if prior knowledge is available. This is the case of the sphingosine:sphinganine ratio, one of the first discovered biomarkers of effect, driven by its established capacity to inhibit the ceramide synthase of fumonisin B1 [53].

The recent development of high-performance analytical instruments and approaches has been key in the growing number of studies that are contributing to the development of the exposome concept. In this regard, although nuclear magnetic resonance (NMR) has been applied for this purpose, especially in metabolomics studies to determine biomarkers of effect [62,63], mass spectrometry (MS) is the predominant technique used in exposomics. This is due to the fact that it is a sensitive analytical technique, allowing the determination of both, targeted and non-targeted compounds. In addition, it does not require laborious sample preparations or extensive optimization of mass spectrometer parameters. It also provides relevant chemical information about molecules, such as accurate mass, isotope intensities, and isotopic patterns, for compounds with a wide range of physicochemical properties, ultimately dependent on whether MS is coupled to gas chromatography (GC) or liquid chromatography (LC) [64].

Within this framework, some authors have already published studies focusing on the study of exposome to discover the link between environmental exposures and health outcomes [3,65–67]. However, they do not provide a holistic overview of all the steps involved in applying the exposome concept from an analytical point of view and most of them presented a scarcely discussed data processing and statistical analysis workflow, which is an essential step to simplify the information obtained and establish the link between exposures and health outcomes.

Thus, the aim of this review is to overview and illustrate the potential of current analytical approaches to address EDCs in chemical exposome-health studies, including both epidemiological and toxicological studies, and exploring all aspects of analytical chemistry to be considered: from study design, through sample collection and treatment, to data acquisition, analysis and interpretation. Fig. 1 illustrates the main analytical and data treatment steps necessary to apply the exposome concept, with the ultimate goal of discovering the link between chemical exposures and health outcomes. It also includes some key aspects to obtain quality results and summarizes the content of this review. Due to the vastness of the contaminants to which humans are exposed, this review focuses on analytical methodologies applied to the exploration of EDCs in an exposomics framework. References were selected by searching in different databases (SCOPUS, PubMed, and Web of Science), using the following keywords: (Environmental samples OR biological samples) AND (endocrine disrupting chemicals OR endogenous metabolites) AND (exposomics OR metabolomics OR biomonitoring) AND (liquid chromatography OR gas chromatography OR mass spectrometry). Due to the high number of resulting studies, those studies that contained a greater number of citations (sorting by "cites by highest") or with greater

Table 2
Examples in which the exposome concept have been applied to the study of EDCs and their effects on health.

Compound	Matrix	Extraction	Analysis	Statistical analysis	Annotation and pathway analysis tools	Metabolic pathway altered	Ref.
BPA	Urine and serum	*BPA: Deconjugation and LLE with ethyl acetate *Endogenous metabolites: Deproteinization with MeCN	*BPA: HPLC-fluorescence (275 and 300 nm) *Endogenous metabolites: UHPLC-QTOF-ESI-MS	Normalization, log transformation, and pareto scaling. PCA, PLS-DA and ASCA	Searching in METLIN, KEGG and Metaboanalyst	Steroidogenesis pathway and amino acid metabolism	[54]
	Urine	*BPA: Deconjugation and LLE with MTBE and TEA *Endogenous metabolites: Deproteinization with MeCN	*BPA: GC-EI-MS *Endogenous metabolites: UHPLC-QTOF-ESI-MS (full scan)	UV scaling. OPLS-DA, Student's t-test and Pearson's correlation	Searching in HMDB and KEGG	Fatty acid elongation and sphingolipid metabolism	[55]
Metals and PAHs	Urine	*PAHs and biomarkers: Deconjugation and SPE with C_{18} cartridges *Endogenous metabolites: Deconjugation, LLE with MeOH and derivatization with BSTFA (1 % TMS)	*Metals: ICP-MS *PAHs: HPLC-fluorescence (281 and 388 nm) *Oxidative stress biomarkers: HPLC-QqQ-ESI-MS/MS (MRM) *Endogenous metabolites: GCxGC-TOF-EI-MS (full scan)	Normalization, log transformation, and autoscaling. PLS-DA, Student's t-test, Pearson's correlation, Wilcoxon-Mann-Whitney and ANCOVA	Searching in NIST, HMDB, KEGG and ChEBI	Tryptophan, phenylalanine, glycine, serine and threonine metabolism	[56]
Phthalates	Urine	*Phthalates: Deconjugation and SPE with HLB cartridges *Endogenous metabolites: Dilution and centrifugation	*Phthalates: HPLC-QqQ-ESI-MS/MS (MRM) *Endogenous metabolites: HPLC-QTOF-ESI-MS (full scan and DDA)	Pareto scaling. PCA, PLS-DA, Mann-Whitney and Spearman correlation	Data Analysis 4.0 for possible formulas, searching in HMDB, METLIN and Massbank, and Mass Frontier to simulate fragmentation	Fatty acid oxidation and prostaglandin, tryptophan and phenylalanine metabolism	[57]
	Urine and plasma	*Phthalates: Online-SPE *Endogenous metabolites: LLE with chloroform:MeOH (2:1, *v/v*) (lipids) and LLE with MeCN:MeOH (1:1, *v/v*)	*Phthalates: HPLC-QqQ-ESI-MS/MS (MRM) *Endogenous metabolites: HPLC-QqQ-ESI-MS/MS (SRM)	Log transformation. Spearman correlation, PCA and regression models	MetaboAnalyst, Impala, KEGG and HMDB for pathways	Lipid, steroid and nucleic acid metabolism	[58]
PFAS	Plasma	Deproteinization with MeCN	HPLC-Q-Orbitrap-ESI-MS (full scan)	Log transformation. PCA, Spearman correlation and linear regression models fitting	LUCIDus R package	Lipid and amino acids pathways and glucose homeostasis	[59]
	Plasma	*PFAS: Dilution *Endogenous metabolites: Deproteinization with methanol	*PFAS: UHPLC-QqQ-ESI-MS/MS (MRM) *Endogenous metabolites: UHPLC-QTOF-ESI-MS/MS (full scan and DDA)	Peak detection and alignment, log transformation and ANOVA-type normalization	Searching in HMDB and MetaboAnalyst for pathways analysis	Lipid metabolism	[60]
	Serum	*PFAS: Ion-pair LLE with water, tetrabutylammonium hydrogen sulphate, Na_2CO_3 and MTBE *Endogenous metabolites: Deproteinization with MeOH	*PFAS: HPLC-QTrap-ESI-MS/MS (MRM) *Endogenous metabolites: HPLC-Orbitrap-ESI-MS/MS (full scan and DDA)	Normalization and Pareto scaling. PCA, PLS-DA, Wilcoxon-Mann-Whitney, Spearman and Partial correlations analysis	Pathways Searching in HMDB	Lipid metabolism, xenobiotic detoxifying, anti-oxidation and nitric oxide	[61]
OCCs, PCBs and PFAS	Serum	*OCCs and PCBs: LLE with pentane *PFAS: SPE with HLB and graphitized carbon cartridges *Endogenous metabolites: AbsoluteIDQ™ kit for lipids	*PCBs: GC-HRMS-Double sector (full scan) *OCCs: GC-QqQ-EI-MS/MS *PFAS: HPLC-ESI-MS/MS *Endogenous metabolites: LC-QTrap-ESI-MS/MS and FIA-QTrap-ESI-MS/MS (for lipids)	Log transformation, scaling, and normalization * POPs-EM: PCA, LVC, BKMR, ENET and MLR * Metabolome-EM: PCA, ENET, OPLS-DA and MLR *Biomarkers of exposure-effect link: sPLS and COMDIM *Multiblock: LUCID	Targeted approach with 630 endogenous metabolites	Dysregulation of bile acid homeostasis and lipase activity. Association with endometriosis development	[18]

Abbreviations: AN(C)OVA: Analysis of (co) variance; ASCA: Analysis of variance-simultaneous component analysis; BKMR: Bayesian kernel machine regression; BPA: Bisphenol A; BSTFA: N,O-Bis (trimethylsilyl)-trifluoroacetamide; COMDIM: Common dimension; DDA: Data dependent analysis; EI: Electronic impact; EM: Endometriosis; ENET: Elastic-net regression; ESI: Electrospray ionization; FIA: Flow injection analysis; GC: Gas chromatography; HLB: Hydrophilic lipophilic balance; HRMS: High resolution mass spectrometry; ICP: Inductively coupled plasma; LLE: Liquid-liquid extraction; LUCID: Latent unknown clustering with integrated data; LVC: Latent variable clustering; MeCN: Acetonitrile; MEOH: Methanol; MLR: Multivariate logistic regression; MRM: Multiple reaction monitoring; MS: Mass spectrometry; MTBE: Methyl tert-butyl ether; OCC: Organochlorinated compounds; PAH: Polycyclic aromatic hydrocarbon; PCA: Principal component analysis; PCB:

Polychlorinated biphenyl; PFAS: Perfluoroalkyl substances; POP: Persistent organic pollutants; (O)PLS-DA: (Orthogonal) Partial least-squares discriminant analysis; QqQ: Triple quadrupole; (Q)TOF: (Quadrupole) Time of flight; QTrap: Quadrupole-ion trap; SPE: Solid-phase extraction; sPLS: sparse partial least-squares; SRM: Selected reaction monitoring; TEA: Trimethylamine; TMS: Trimethylsilane; (U)HPLC: (Ultra) High-performance liquid chromatography; UV: Unit variance.

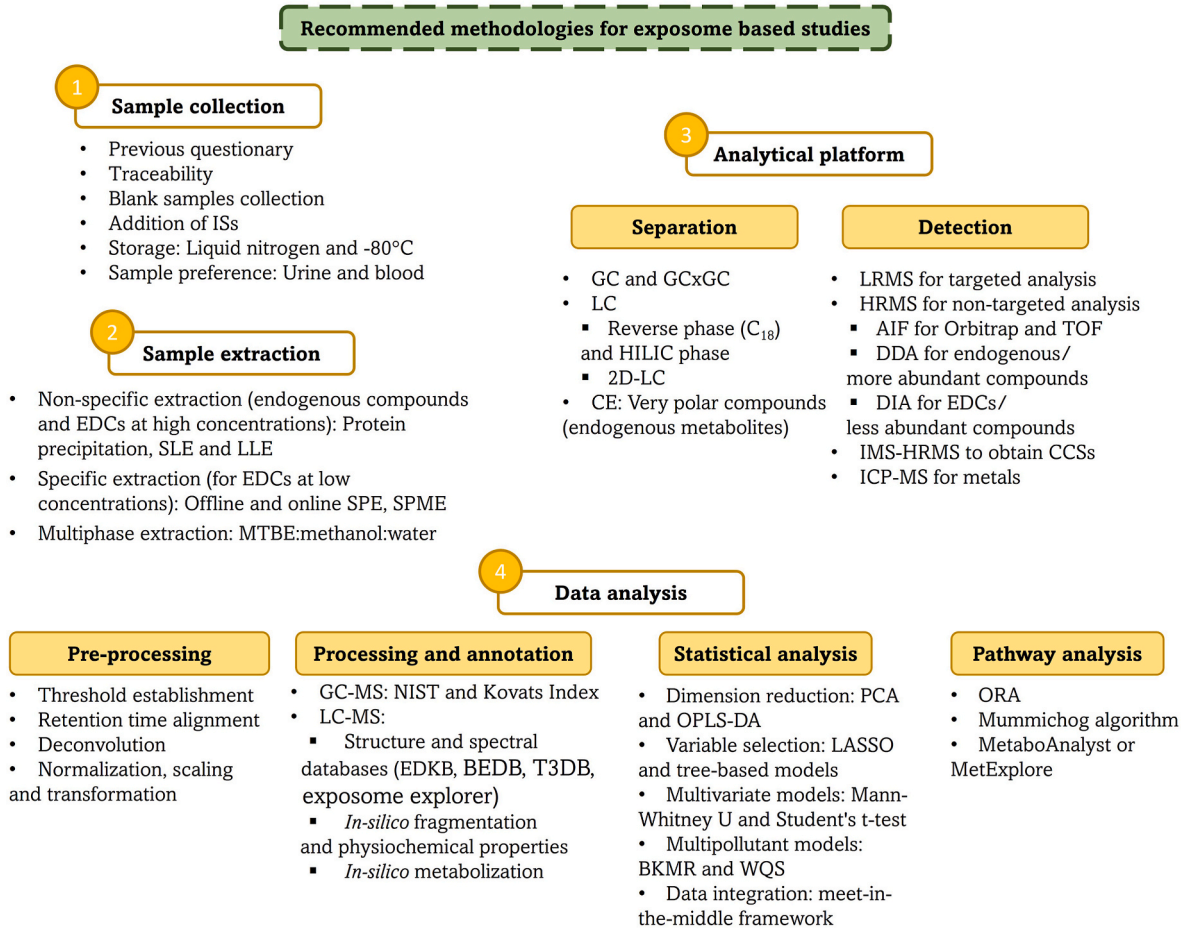

Fig. 1. Summary of the analytical steps involved in exposome research.

relevance (sorting by "relevance") were finally selected. Additional references have been also included to address specific aspects of this study.

2. Exposome-based study designs

Studies examining the exposome can generally be addressed by two main approaches: top-down and bottom-up. The "top-down" approach aims to simultaneously study exogenous and endogenous chemicals by measuring them in biospecimens. Thus, it is possible to generate hypotheses about exposure-disease and exposure-response relationships [68]. However, finding the relationship between exposure and biological effects is often a complex task, since it may be too difficult to characterize each of the exposures, especially considering the wide concentration range at which we can find each of the pollutants. On the other hand, the "bottom-up" approach is a prospective strategy in which chemical exposures are first characterized in exposure sources following targeted hypothesis [69], and generally their health consequences are later evaluated through a toxicological study. This methodology has the advantage that it is possible to select a method for chemical exposures and another for endogenous compounds, which overcomes the difficulty of analyzing compounds with a wide range of physicochemical properties and concentrations. Thereby, bottom-up approaches have traditionally been followed in exposome-focused studies [70] (Table 2).

Although the study of the exposome can be tackled by following a bottom-up approach with samples from different exposure sources, it may be unrealistic and overambitious as sources and levels of exposure change over time, the number of exposure sources may be endless, as well as it is difficult to consider all the sources of exposure to which an individual is subject. Furthermore, this approach can miss endogenous exposures [71,72]. In contrast, the top-down approach based on biomonitoring seems more logical to establish the link between chemical exposures and health outcomes, and its implementation is the trend in exposomics [71]. However, the top-down approach does not capture the exposure timing, route and source; thus, it limits the ability to implement actions to reduce chemical exposures [68,71]. In a top-down approach, it is necessary the use of generic extraction and analysis techniques and this implies some problems such as the loss of information for the less abundant compounds (usually xenobiotic compounds) or the impossibility to find the ideal analytical methodology to perform it. It is also complex to find the connection exposure-disease, being necessary the definition of potential exposures between all the detected ones.

Recently, a third approach has emerged to integrate the objectives of finding the cause of an environmental disease (top-down) and the relation of a chemical exposure with a health outcome (bottom-up), the so-called "meet-in-the-middle" (MITM) approach [73]. This approach consists in measuring intermediate biomarkers (often with an agnostic investigation by omics tools) and relating them retrospectively to measurements of external exposure, and prospectively to the health

disturbances [74].

In this framework, the exposome is still considered a concept. Hence, it is necessary to establish a large variety of study settings and frameworks articulated with the ambitions of exposome objectives. It is noteworthy that chemical exposome has not been the focus of major European or international exposome projects [46] although recent investments, such as the made by Human Biomonitoring Initiative of the European Union, are going in that direction. On the other hand, several epidemiological studies have advanced towards this concept by extending the number of chemicals under study and/or integrating endogenous biomarkers (omics profiling).

As the description of the term exposome explains, studies evaluating the exposome must include the influence of exposure to chemical agents over a human lifetime. Thus, to achieve better exposure coverage over long periods of time, Exposome-Wide Association Studies (ExWASs) have been developed, which involve the study of the exposome following a top-down approach. ExWASs are focused on the analysis of large data sets at different stages of life with the aim to overcome some of the remaining challenges in exposomics such as cofounding effects of various exposures, the performing time of the study, or the cross-sectional designs of the experiments [5]. In addition, in ExWASs it must be considered that exposures fluctuate considerably depending on different parameters such as the sample collection window, time of study performance, or sample size. In this context, to perform ExWAS, it is necessary to determine exposures at different life stages since the exposure profile could be different at each one of them. In this sense, early life stages such as gametogenesis and early fetal development are the most critical life stages in studies whose objective is to understand the link between chemical exposures and health perturbations, especially for those involving EDCs [4]. This is due to the fact that at this age the body is more sensitive to perturbations produced by contaminants and, particularly, to hormone-like activity compounds such as EDCs [75], although the health effects may not become apparent until adulthood. Low exposure doses that might not cause any problems in adult stages can produce important effects in these early stages. Indeed, 90 % of childhood cancers are believed to be caused by environmental exposures [76]. Therefore, some recent projects as CHEAR, HELIX or EXPOsOMICS aim to characterize the exposome during critical periods of life such as early life [77–79].

However, ExWASs may be too difficult to carry out either because of sample bioaccessibility for continuous sampling (e.g., sampling of fetus or neonate individuals), the long times to be operationalized or because of individual changes of exposures with respect to another (e.g., changes in socioeconomic activity of some individuals in a study with a large list of people). For that, studies should at least include multiple measures across days at different time periods rather than performing short-term studies with a unique sample collection/analysis, or measurements at particular life stages, such as gestation, childhood, puberty, and reproductive age, as recommended by Rappaport and Smith [70]. To achieve these goals, simple-to-take and non-invasive biospecimens such as urine, saliva, hair or breast milk are preferred [80–82]. Furthermore, although there is a general analytical workflow to follow due to the recent developments in analytical instrumentation and data processing approaches (Fig. 1), the lack of standardized methodologies remains one of the biggest weaknesses to transform exposomics approaches for the application of a concept into a real methodology [68].

Within this scenario, interdisciplinary research strategies and complementary sciences are involved to reach the main goal of exposomics, which is to characterize both, exposures and exposure-induced effects (i.e., molecular and cellular disturbances); therefore, linking the causes and effects of a wide variety of environmental diseases. Metabolomics, which includes both glycomics and lipidomics, is one of these sciences involved in the identification of disease biomarkers and their correlation with disease phenotypes. The importance of metabolomics science in exposomics is reflected in the study by Sun et al. [3], in which the most common exposure to EDCs is linked to the metabolic pathways disturbed by them (Fig. 2). As metabolomics, several omics sciences are relevant in exposomics, such as genomics, epigenomics, transcriptomics, or proteomics [45]. In fact, the combination of exposomics with genome-wide association studies (GWASs) may help to better understand the relationship between a disease and the environmental-genetic interaction [83]. Indeed, some diseases such as obesity or neurodevelopmental disorders have been proposed to occur due to a combination of genetic susceptibility, exposure to EDCs and microbiota, rather than being caused by an individual contribution [9].

3. Sample collection and preparation

3.1. Sample selection

One of the critical steps in exposure studies is the matrix selection, either an environmental and/or a biological sample, depending on whether the study comprises the external and/or the internal exposome, respectively. Environmental samples must be selected in accordance with the exposure environment, for example, dust from work, indoor air, environmental samples close to residential zones, most commonly consumed foods, etc., so there is a high variance respect the sample to select (Fig. 3). The use of silicone wristbands has been proposed as an effective method to passively control individual environmental exposure for long periods of time and has already been used to assess the occupational exposure to PAHs in firefighters [84,85]. However, this methodology has the disadvantage that only volatile/semi-volatile compounds (i.e., dermal or inhalation exposure routes) and with affinity for a particular silicone are considered [85].

With respect to biological samples, there are several matrices that can be picked in EDCs studies. However, as biological matrices are extremely complex and EDCs are normally found at trace concentrations, the matrix selection may produce the impossibility of measuring certain compounds. The physico-chemical characteristics of the EDCs will also influence the selection of the target matrix. In urine, only polar contaminants are excreted in their original form, as well as endogenous polar compounds [87], while most EDCs are therefore excreted as phase 1 and phase 2 metabolites (i.e., glucuronide or sulphate conjugates). More persistent and hydrophobic chemicals are excreted in feces, if not absorbed [87], or stored in fat or blood [88]. In contrast, metals are normally biomonitoring in urine and hair [41]. Furthermore, as previously mentioned, the aim of studies applying the exposome concept is to measure exposure throughout life at critical moments. Therefore, to develop studies following this methodology, continuous sampling is required instead of conducting isolated and occasional sampling. Thus, non-invasive/non-embarrassing sample collection is always preferred, allowing greater continuous analysis. For these reasons, the most commonly selected matrices for exposome-like studies are urine, feces, and blood (serum and plasma) (Fig. 4).

Urine is easier to obtain than blood and is considered a minimally invasive sample. Besides, it is a high aqueous content matrix (>95 %), allowing direct analysis or with a minimal sample preparation. In this sense, it is preferable to collect morning urine because the concentration of metabolites of interest is higher at this time of day [89]. On the other hand, blood more faithfully reflects exposure to a greater number of chemical exposures, so it seems to be the best alternative to perform toxicological studies involving EDCs. Blood contains those chemical substances that are transported from tissues and deposition sites and with which it is in equilibrium concentration [87,90], so it will better reflect the exposure level of EDCs. Despite all its advantages, blood is considered an invasive matrix. Thus, new alternatives have recently been developed to collect this type of sample. This is the case of dried blood spot (DBS) sampling [91]. New devices have been developed to collect DBSs with high reproducibility (variations of less than 5 %) [92]. Its main disadvantage is that compounds can only be detected if they are found in a sufficient concentration [32], although recent advances in analytical techniques have made this technique increasingly used, for

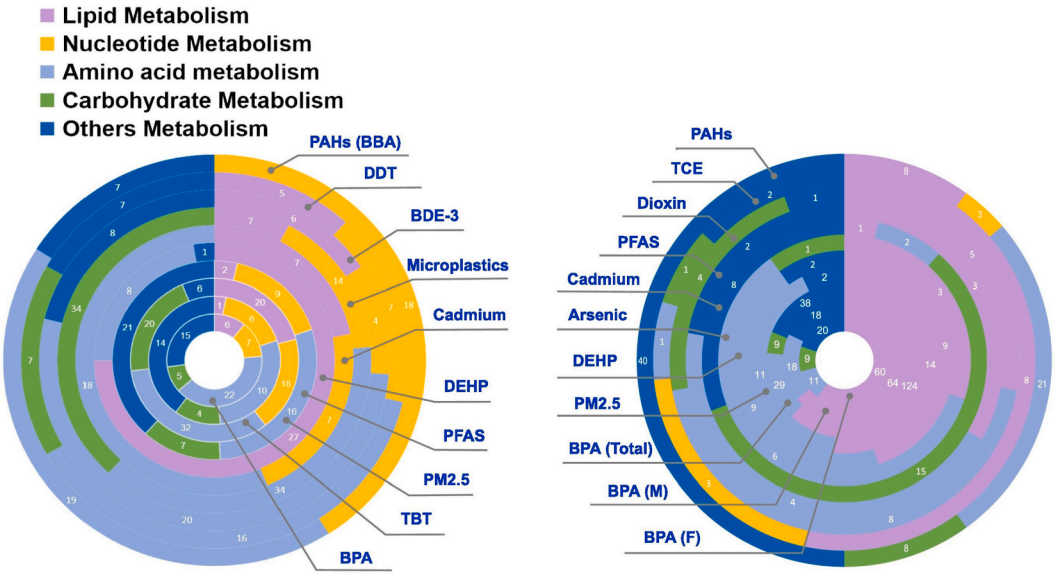

Fig. 2. Disrupted metabolic pathways due to exposure to EDCs in animals (left) and humans (right), indicating the number of biomarkers identified to assure these disruptions. Obtained from Sun et al. [3].

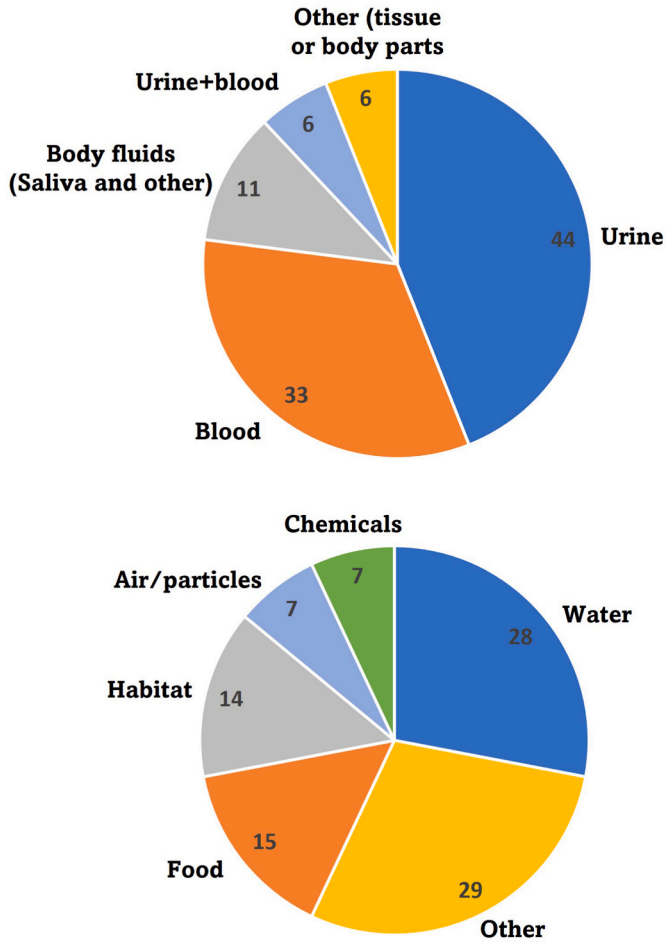

Fig. 3. Percentage of studies involving biological or environmental matrices in metabolome-oriented and external source-oriented studies respectively. Adapted from Benedikt et al. [86].

example for the measurement of PFAS or mycotoxins [93,94].

The investigation of other biological matrices such as breath, sweat, cells, saliva, hair, nails or breast milk has also been proposed for epidemiologic studies [86,95]. The success of selecting these matrices in exposome-related studies will depend on the objective of the study itself, since the selected matrix must include a wide range of endogenous and exogenous compounds. For example, samples such as breath or saliva are only useful for the identification of those metabolites or contaminants that are capable to pass through the blood-saliva barrier [96], or those found directly in the mouth, such as the PAHs in smokers [97]. Besides, the EDCs levels of these matrices are highly influenced by food consumption. In addition, it is not possible to collect samples such as sweat or certain kind of cells from newborns or toddlers [87]. On the other hand, hair and nails are non-invasive matrices which have been employed to evaluate the long-term exposure to EDCs [98]. They are simple to extract although they need to be washed prior extraction protocol to remove potential interference coming from the environment surrounding the specimen. Breast milk is non-invasive matrix and easy to operate matrix, very interesting since it reflects the exposure in the newborn, highly influenced by EDCs endocrine effects [99]. Moreover, it is a non-invasive matrix and easy to operate.

In addition to the mentioned samples, interstitial fluid (ISF) has emerged as an effective biomonitoring matrix since it has a composition similar to plasma and can be collected with easy-to-use tools such as patches, although it has not been directly applied for the analysis of EDCs. Despite that, Niedzwiecki et al. compared both matrices and they found that of a list of 7044 compounds, including exogenous and endogenous compounds, 5583 were detected simultaneously in plasma and ISF [82]. Another advantage is that ISF contains compounds produced in near tissues, so it provides information of sample local environment.

Finally, it could be mentioned other alternative matrices which could be only employed for particular epidemiological studies, although they imply a more difficult and invasive sampling. These are the cases of peritoneal fluids [100–102], follicular fluid [62,103] or endometrium and endometrial fluid [104], which have been widely used to study reproductive diseases such as EM, or cord blood, placenta, and teeth, that have been employed to establish early life exposure [105–109].

Thus, in conclusion, the preferred methodology is to use blood (or DBS when possible) and urine for continuous biomonitoring over time, considering also recently employed and minimally invasive matrices

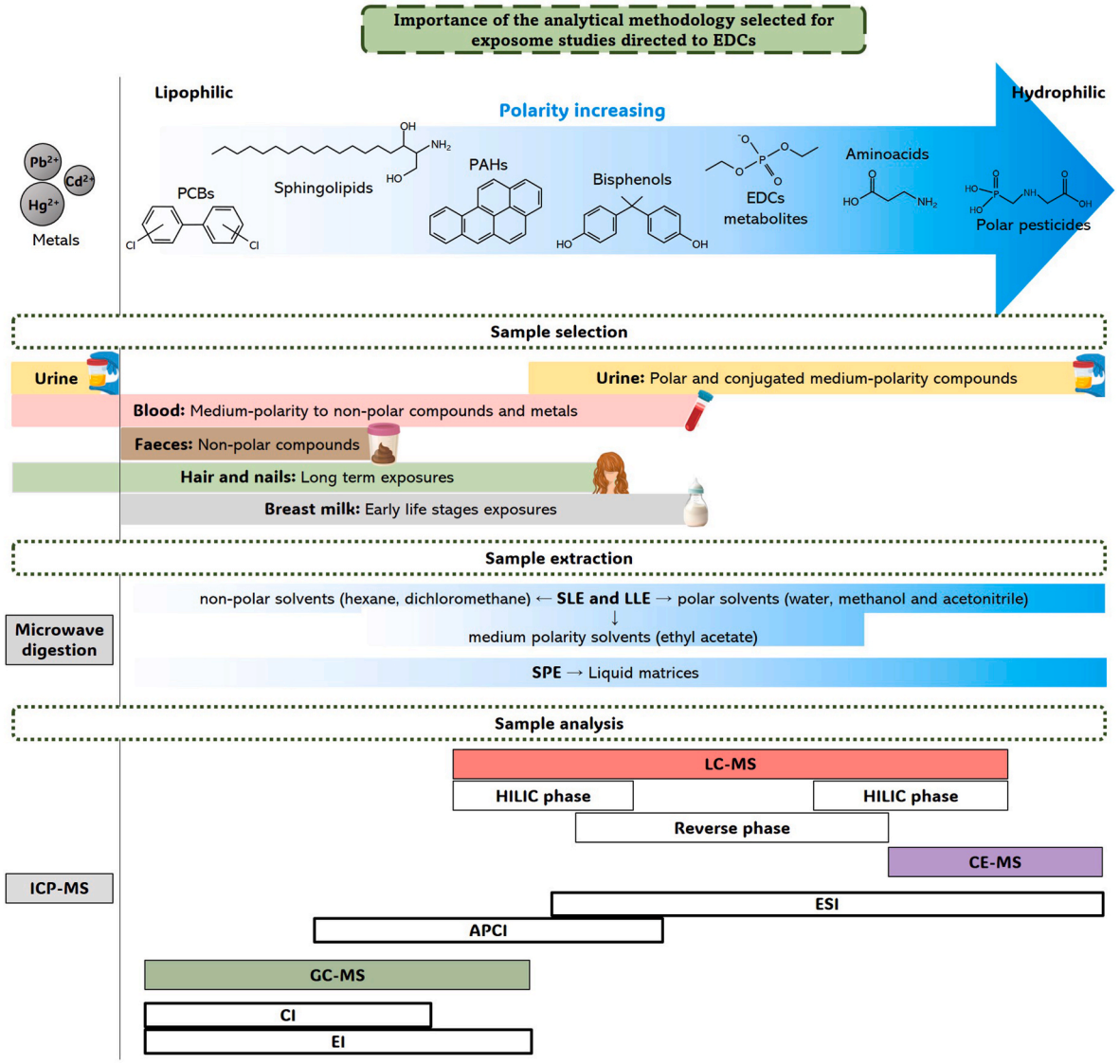

Fig 4. Analytical complementarity in EDCs exposure studies during sample selection, extraction and analysis based on the physicochemical properties of the analytes.

such as silicone wristbands or ISF patches, and sparingly more invasive matrices for specific objectives when there is no other possibility.

3.2. Key aspects in sample collection

Once the matrices to be evaluated have been selected, it is necessary to take into account some points prior to sample collection. For example, for the collection of biological samples, it is very relevant to include a questionnaire on the subjects who will participate in the research. When carrying out non-targeted analysis, this step is critical because annotation step would be easier if information about the exposures is known. In addition to general information such as age, ethnicity, sex, or body mass index (BMI), other aspects must be considered such as employment and work environment, smoking, exercise habits, nutrition, or alcohol intake [89]. There is not a standardized questionnaire for this type of studies, but for diseases in which EDCs directly affect the pathogenesis of it, special items should be included such as: *1)* eating and drinking habits, since food containers are composed of substances suspected of being EDCs that can leach of them (e.g., bisphenols are used in plastics and coatings of metal food containers [110]); *2)* the frequency of use of personal care products, which can contain compounds such as phthalates or parabens [111]; *3)* whether the patients are using a hormonal treatment, since EDCs affect hormonal balance, or if they have undergone some medical treatment, being some compounds as dibutyl phthalate (DBP) employed for the coating of some medicines [111].

Other important aspects must be taken into account during sample collection. Sample information is important to control its traceability, so a brief description with information should be included together with the samples. This could include, but is not limited to sample location, sampling instrumentation, collection time, or patient's status (if it is a biological sample). This step is important when performing large epidemiological studies, especially when they involve the collection of a high number of samples over time and, normally handled by different people in the different research steps (e.g., those who take the samples and others who analyze them) or for studies involving samples already kept in sample banks, as is often the case in ExWASs [112], because sample information could affect the reliability of the results.

Proper selection of collection containers is also significant when non-targeted analysis needs to be performed. For the collection of blood-based samples, if plasma is required anticoagulants are needed in the blood-collection tube, while only centrifugation is needed when serum is the selected matrix. Although it is not clear what anticoagulant is better

to perform metabolomics analysis, K^+-EDTA or Na-heparin plasma are preferred over others such as Li^+-heparin, which may increase the matrix effect [113]. The selection of the containers must consider the material from which they are made since some containers may contain several EDCs such as phthalates or bisphenols, and also a large list of both intentionally added substances and non-intentionally added substances, being most of them unknown and at unknown concentration levels [114–116]. Besides, the use of plastics with Cd-based softeners should be avoided for blood collection because Cd is considered an accumulative metal with endocrine health implications and, therefore, it cannot be measured when using these tubes [40,87,89]. Nonetheless, the collection of a blank sample helps to minimize these problems. In this regard, it is highly recommended to collect blank samples at the same time as real samples to eliminate the background signals after analysis and to evaluate possible contamination during sample collection or transport. In the case of biological samples for which blank samples are not available, artificial matrices may be collected in the same way and time as real samples. Likewise, the addition of internal standards (ISs) with comparable properties to those of the expected compounds (xenobiotics or endogenous compounds) could be included right after sample collection to evaluate the loss or degradation of compounds prior the extraction. In non-targeted studies it could be difficult to choose the adequate internal standard (IS) so more than one of different chemical families could be included (e.g., plasticizers).

Finally, after sample collection, storage conditions need to be evaluated to assess an optimal sample condition until the extraction is performed. Samples must be stored at controlled temperature and dark conditions, due to the possible degradation of some compounds, especially for long-term studies. This step could be less problematic for the measurement of most of EDCs (being enough in most cases to freeze at −20 °C) with respect to the determination of endogenous compounds. However, as endogenous compounds are more unstable at high temperatures, it is preferred the use of liquid nitrogen and to storage the samples at −80 °C to ensure the stability of both, exogenous and endogenous compounds [87,117]. Furthermore, in exposome-related studies, a large number of samples need to be storage at adequate conditions, so the storage capacity of the laboratory is highly relevant.

3.3. Sample preparation

Once the sample is collected, the next question to answer is whether any extraction step is necessary. As EDCs are normally found at low or trace concentration in environmental or biological samples respectively, extraction is needed. For environmental and food samples, simple extraction techniques have been applied, such as solid-phase extraction (SPE) or solid-phase microextraction (SPME), for the analysis of phthalates [12], bisphenol A (BPA) and alkylphenols [13], parabens [17], or chlorinated compounds [20], in liquid matrices (i.e., water or drinks). When the selected matrix is solid (i.e., dust, soil, food, cosmetic, etc.) the predominant methodology is the applying of any variant of solid-liquid extractions (SLE) such as QuEChERS, ultrasound assisted extraction (USAE) or microwave assisted extraction (MAE), among others, in some cases employing SPE protocols to achieve lower detection limits [13,17,19]. Most relevant articles about EDC extraction in environmental samples are disposed in Table 1.

As it happened with environmental samples, sample treatment of biological samples will depend on their nature. For solid biological samples such as endometrium or feces, lyophilization and/or grinding (e.g., via tissue lysers, freezer mills) could be employed to assure the sample homogeneity prior the extraction stage [67]. For saliva or blood samples centrifugation is performed prior to the sample storage, in the case of blood to obtain plasma or serum samples which are normally used to determine exogenous or endogenous compounds. Besides, if urine is selected as the preferred biomonitoring matrix, sample normalization is mandatory, since water content could greatly vary depending on several factors. Although urinary creatinine is the most used technique to achieve sample normalization, gravimetric normalization seems to be a technique with more robust results [113].

After this step, it should be noted that most contaminants, including EDCs, are often found forming conjugates, especially in urine samples (i.e., phase I and II metabolites) [118–120]. Thus, they could be determined directly or after a deconjugation step (e.g., with β-glucuronidase for β-glucuronide conjugates or aryl sulfatase for sulphate conjugates [88]). In general, the analysis of conjugated forms is more challenging because conjugates have a higher polarity than unconjugated molecules, although their direct analysis may provide a better insight into the compounds present in the samples since, unlike free forms, conjugated molecules cannot be found ubiquitously in any sample due to external contamination (e.g., container contamination), minimizing sources of contamination. In addition, exhaustive sample preparation with the inclusion of additional steps may lead to greater method variability and introduce an external background from the preparation materials [67]. In any case, the addition of conjugated ISs prior to the deconjugation step is recommended to evaluate deconjugation performance. Furthermore, the removal of matrix components such as proteins (e.g., protein precipitation) and lipids (e.g., sorbents such as enhanced matrix removal (EMR) lipids [121] or freeze filtration [122]) is recommended when xenobiotic compounds are determined individually to extend the life of analytical instruments and to reduce matrix effects [52]. Protein precipitation for plasma samples seems to be better performed by acetonitrile or acetone [123], while methanol is a more efficient solvent for the deproteinization of serum samples [124].

Following pretreatment steps, different extraction approaches could be followed depending on the goal of the study and the analytical strategies to be followed. One of the complexities of exposomics lies in its broad ambitions which endorses the evaluation of chemical exposures and metabolic changes produced by them. This implies the determination of a wide range of compounds at very different concentration levels, being the determination of biomarkers of exposure and effect usually carried out independently and applying different analytical methods.

On one hand, for the analysis of EDCs (i.e., biomarkers of exposure), extraction methods focused on the removal of matrix compounds are needed since compounds are found at low concentrations. Moreover, biological matrices are complex matrices with a high number of compounds, which can lead to high matrix effect and high ion suppression in the ionization source and, therefore, resulting in a competition with each other for ionization efficiency. Table 1 includes the most commonly used methods in sample extraction for the determination of the most common EDCs in biological samples. In summary, the most employed methodologies include non-selective extractions such as liquid-liquid extraction (LLE) or SLE using different solvents, although the most employed are ethyl acetate, due to its excellent solubility [125], and hexane, very useful for samples with a high lipid content [126,127]. If more polar compounds are needed to be extracted, polar solvent such as water, methanol or acetonitrile can be also employed (Fig. 4). As an alternative to LLE, salting-out liquid-liquid extraction (SALLE) is a more effective methodology, consuming shorter amount of sample and solvents with lower extraction times [128,129], which has been already employed to determine a wide range of compounds such as BPA, mycotoxins or pesticides, among others. On the other hand, if the proposed matrix is solid, SLE methods are the preferred and they have been applied for the determination of BPA in placenta [130], and phthalates and BPA in hair [131]. For the extraction of metals, microwave assisted acid digestion is the preferred methodology [41]. More selective methods can be applied when preconcentration is needed. In this matter, SPE has been used for the analysis of EDCs, mostly employing silica gel-based sorbents (C_8 and C_{18}) [14], or polymer-based sorbents (e.g. HLB cartridges) for a wide range of compounds including bisphenols, parabens, triclosan or organochlorine compounds [132]. Microextraction techniques have emerged as an interesting alternative, especially for those studies that involve biological samples for which the

available amount is minimal. As example, dispersive liquid-liquid microextraction was utilized to determine 17 EDCs (including bisphenols, parabens, benzophenones and triclocarban) in saliva [16], or 14 EDCs (including bisphenols, parabens, benzophenones) in urine [15], with adequate method performances.

On the other hand, if the compounds of interest are endogenous compounds, extraction methods focused on a particular family of compounds are rarely applied and, instead, broad-spectrum extraction methods are usually carried out (Table 2). In the case of samples with high water content as urine, it may be possible to analyze them after applying a simple sample preparation such as deproteinization, dilution or centrifugation [54,57], being LLE the most widely used approach [56,58]. Sample dilution also eases the sample normalization, minimizing signal variability by the reduction of detectable compounds [66]. Although less common, extraction of pre-set compounds can also be carried out if they are known to be altered by chemical exposure, such as lipids that have been demonstrated to be altered by exposure to EDCs. In this case, lipid extraction for subsequent determination can be accomplished using extraction kits such as AbsoluteIDQ™ kit, including different acylcarnitines, phosphatidylcholines, and sphingomyelins [100]. Finally, if a more exhaustive extraction method is needed, SPE is the most common one. It should be taken into consideration that, as SPE protocols normally involve an evaporation step after extraction, recomposition solvents must be carefully selected to guarantee a correct dissolution of all compounds of interest. This can be achieved by using a combination of solvents with different polarities and the application of ultrasonically assisted dissolution.

Although the approach of performing two individual extraction methods for xenobiotics and endogenous compounds seems to be the most consistent one and, actually it is the most employed one, most biological samples are collected at low amounts and sometimes it is not possible to perform it [113]. Nevertheless, the application of a simple extraction method to extract both biomarkers of exposure and effect might not be adequate in many cases in which interest compounds have not-encompassable concentration ranges. For these cases, there is a third possibility, which is the extraction of both exogenous and endogenous compounds simultaneously with a multiphase extraction. There are different possibilities such as the application of the Folch or Bligh-Dyer methods, which involve the extraction with chloroform:methanol:water mixtures. Originally, these methods have been developed for the extraction of lipids, which are extracted in the chloroform phase. As lipids remain in this phase, methanol and water phases can be recovered for the analysis of more polar compounds [133]. Similarly, there are multiple variants to avoid the use of chloroform, which is considered a high toxic solvent, such as performing the extraction with methyl tert-butyl ether (MTBE) or dimethyl carbonate (DMC) [133,134].

Finally, in large cohort studies, extraction methods as simple as possible are recommended due to the large number of samples to be treated. Thereby, automated extraction methods constitute a more efficient, accurate and scalable alternative. Another advantage of this approach is that sample requirement decreases. Some automated methods have been already employed, as for example, an online-SPE method which employed 100 μL of plasma and used a turboflow C18 system for the analysis of hydroxylated PCBs [135], or the study published by Stubleski et al., who optimized a SPE optimized methodology where a SPE 96-well plate was employed for the determination of 23 POPs using only 150 μL of serum or plasma [136].

4. Analytical methodology

4.1. Nuclear magnetic resonance

The continuous improvements in analytical techniques have promoted an exponential increase in studies addressing the exposome concept. In this regard, proton-NMR (^{1}H NMR) instruments have been extensively employed in metabolomics studies [137–139]. Some advantages of NMR compared to other analytical techniques are that it is a non-destructive technique which allows the evaluation of a complex mixture, minimizing the sample handling, all of that with a high elucidation power. Furthermore, as most of the biological samples investigated in exposomics are liquid, no sample preparation is required other than dilution with a deuterated solvent [139,140]. Despite these advantages, the high number of signals resulting from the analysis of biological samples, for example in the case of lipids, could affect compound identification due to signal overlapping. There are some alternatives to solve that, such as the use of pulses only in a region that does not contain the highest analyte signals (double pulsed field gradient spin echo, DPFGPE), or by suppression of certain selected strong lipid signals (NOESYGPPS) [141]. However, these instruments have lower sensitivity than MS instruments, being this the main reason why NMR is mainly used for studies in which sensitivity is not a restricting factor (i.e., metabolomics) [62,63,103].

4.2. Mass spectrometry

In order to have a higher compound coverage including exogenous and endogenous compounds, MS seems to be the only approach to perform analysis at such low concentrations needed in exposomics [142]. The simplest analytical approach is the injection of the samples directly in the MS system. It is a rapid and effective strategy; however, it is not recommended for non-targeted analysis of complex matrices because of the high matrix effect produced in the ionization source and because the annotation step becomes very difficult (i.e., without chromatographic separation isobaric compounds cannot be distinguished). Thus, although it is a promising strategy, it has been scarcely used in exposomics approaches [143]. Sample dilution can be performed to improve reliable results for non-complex matrices as urine [144].

MS presents several advantages over other detection techniques such as its robustness, selectivity, sensitivity, and confirmation capacity, in addition to the possibility of an easier quantification. Low-resolution mass spectrometry (LRMS) is more employed for the determination of expected compounds, as contaminants or metabolites, in combination with extraction methods for a specific compound/compound's family (Table 2). On the other hand, HRMS seems to be more interesting for cross-sectional studies or broad studies in which non-targeted analysis need to be applied. In addition to the advantages explained for general MS instruments, HRMS allows retrospective analysis with accurate mass measurements, isotope intensities and isotopic patterns, and with a higher sensitivity than LRMS instruments in full scan mode, all of them achieving an easier compound identification in non-targeted approaches, even when analytical standards are not available [145]. HRMS comprises (Q)-Orbitrap, (Q)-time of flight (TOF) and Fourier transform-ion cyclotron resonance-mass spectrometer (FT-ICR-MS) analyzers, although the last one has been scarcely employed for the measurement of the exposome. Regarding the differences between (Q)-Orbitrap and (Q)-TOF analyzers, while (Q)-Orbitrap instruments have a higher mass-resolving power and mass accuracy than TOF instruments and generally exhibit a higher sensitivity when full scan mode is employed, (Q)-TOF analyzers maintain the scanning rate independently of the resolving power [146].

Regarding the ionization mode, while for LC electrospray ionization (ESI) is the predominant one, atmospheric pressure chemical ionization (APCI) can be employed for some applications, especially for less polar compounds that have a poor ionization under ESI. For GC-MS, electron impact (EI) ionization source is the preferred. While ESI source is considered a 'soft ionization' technique that allows the obtention of biomolecules without significant structural disruptions (normally the charged molecular ion), EI employs high-energy electrons causing extensive fragmentation of the analysed molecules, which may complicate the interpretation of mass spectra, especially for larger molecules, if appropriate annotation tools are not employed. If a softer ionization is needed when GC-MS is employed, for example in

compound elucidation studies, chemical ionization (CI) can be applied, improving the ionization of low polarity compounds and obtaining in most cases the molecular ions.

Furthermore, ion mobility spectrometry (IMS) can be integrated in MS instruments. This technique is based on the mobility of the ions when they pass through an electric field in a carrier buffer gas, providing molecular and conformational information about them. It provides a collision cross section (CCS) value characteristic for each compound, which could be used as an extra annotation parameter in the identification step [147]. Ion mobility-mass spectrometry (IM-MS) is extremely useful for the differentiation between molecules with the same mass-to-charge ratio (m/z) but different structural shape, as commonly happens with human metabolome. Besides, IM-MS has demonstrated to allow the determination of compounds at low concentration levels in complex samples by reducing detector suppression produced by high concentrated compounds [148]. There are several IMS technologies which include drift tube IMS (DTIMS), traveling wave IMS (TWIMS), trapped IMS (TIMS), field asymmetric IMS (FAIMS) or differential mobility analyzers (DMA), each one with their advantages and disadvantages, already deeply reviewed [52,148]. For this reason, the appropriate IMS technique will depend on the purpose of the research. However, as a general appreciation, TIMS, DTIMS and TWIMS allow the simultaneous analysis of all ions of a sample, being recommended for the analysis of metabolome samples, whereas FAIMS and DMA devices scanned specific ions or classes, so they should be used when there is some information about the compounds of interest [148].

Whatever analytical technique is selected, it is recommended injecting the samples randomly to assure representative results and using a quality control along the batch to monitor the stability of the system. Quality controls can be composed by a pool of all the analysed samples, and including one or more ISs.

4.3. Chromatographic platforms

Although MS can be applied alone, chromatographic techniques are generally coupled to it to reduce data complexity and matrix effect and to determine a wider range of compounds. The most widely used chromatographic techniques are LC and GC, which are selected based on the physicochemical properties of the compounds of interest. Volatile/semi-volatile and non-polar EDCs such as organochlorinated compounds (OCCs) or BFRs or endogenous lipophilic compounds are better determined by GC [21,26,27], whereas thermolabile, polar to medium polarity and non-volatile EDCs such as PFAS or OPEs, as well as most of endogenous metabolites are preferably determined by LC [6,30,52] (Table 1). There are also other families of compounds that consist of LC/GC-amenable compounds such as phthalates, bisphenols, parabens, PAHs or lipids [16,37,52], while other contaminants, such as metals can only be determined by special techniques such as inductively coupled plasma (ICP)-MS (Fig. 4). Derivatization is possible to have a wider range of compounds which in their natural form are not correctly analysed by some of the mentioned techniques. However, derivatization normally involve time-consuming and non-green methods, as well as the application of temperature with the risk of compound degradation, making them less applicable in exposome-related studies.

GC-MS has been less used for the determination of both biomarkers of exposure and effect, because its usefulness is more limited than LC-MS, being restricted to volatile/semi-volatile compounds if sample derivatization is not carried out. Notwithstanding, there are some examples, as the study carried out by Chen et al. [56] in which, after LLE extraction with a derivatization step and a comprehensive two-dimensional GC (GC × GC)-MS method is applied to improve peak resolution. GC × GC seems to be a promising analytical tool since it provides an enhanced separation, normally using a nonpolar and a semipolar column, expanding the range of analyzable compounds [113]. In addition, for some samples not thoroughly investigated in exposomics, such as gas phase breath samples, GC-MS is the predominant technique [149]. However, GC-MS has been mainly used for the determination of biomarkers of exposure, as for example for the determination of PCBs in hair [150], polybrominated diphenyl ethers (PBDEs) in maternal serum and placenta [151], organochlorine pesticides (OCPs) in serum [132], or a mixture of EDCs including PCBs, OCPs, dioxins and PBDEs in plasma and serum samples [136].

In LC separations, the most adopted mode is reverse phase, being carbon-based columns the most widely used. Depending on the functional group linked to the silica phase (e.g., C_{18}, C_8, phenyl, phenylhexyl, etc.), the separation of a wide range of compounds is possible, from polar to medium polarity compounds. The choice of the chromatographic column depends on the purpose of the study. C_{18} columns are the most employed for both EDCs determination and metabolomics. When reverse phase columns are selected, the mobile phases are composed of methanol or acetonitrile in combination with water. The use of buffered mobile phases is highly recommended to avoid variation along the batch analysis, achieving a good reproducibility even between batches. These buffered solutions are normally prepared with some acids such as acetic or formic acid together with their salts (i.e., ammonium acetate or ammonium formate, respectively). The addition of acids could also improve the ionization of some compounds in both positive and negative ionization [152]. However, there are some situations where the use of C_{18} columns may be restricted. For example, in lipodomics studies the excessive amounts of lipids could limit the method performance and column life [52]. Besides, the analysis of compounds with the lowest polarities is problematic when using reverse phase columns, being retained in the column and appearing as ghost peaks in the next injection. To solve that, flushing the column with an organic solvent such as acetone or isopropanol could be programmed after the LC separation, which reduces carryover between injections [67].

Despite special reagents (i.e., ion-pairing agents) can be used for better analysis of samples containing highly polar compounds or a high content of lipids (i.e., lipidomics approaches), this may cause a high ion suppression and MS source contamination. The use of normal phase columns could be another alternative. However, this last option often requires the use of non-environmentally friendly solvents as mobile phases, such as hexane or chloroform, as well as acetonitrile or methanol, which also provides some disadvantages such as poor ionization [52]. To overcome these problems, hydrophilic interaction liquid chromatography (HILIC) has been increasingly used for the analysis of both, very polar compounds and samples with high lipid content. In the case of lipidomics, HILIC has proven to be highly useful, especially for lipid compounds that contain hydrophilic functionalities in their molecules, such as phospholipids, sphingolipids, or glycerolipids, among others, separating these compounds based on these funcionalities [153]. Furthermore, as HILIC-based columns are composed of a hydrophilic stationary phase that involves the use of an organic-rich mobile phase, it means that HILIC columns can be used in combination with reverse phase columns such as C_{18} columns, increasing not only lipids coverage, but also allowing the determination of biomarkers of exposure and effect with very varied hydrophilicity under similar chromatographic conditions. The combination of C_{18} and HILIC columns can be done by a dual injection [104], although this methodology implies the need for larger sample volumes and longer analysis times. Other strategy is the use of a two-dimensional (2D)-LC system using a trap between chromatographic platforms (for example a C_8 trap column combined with a C_{18} and an HILIC column) [154]. Thus, a comprehensive separation can be done with a single analysis making use of some of these methodologies.

When interest compounds are those with a very polar nature, the use of capillary electrophoresis (CE) is a promising alternative. This technique is very useful for the separation of highly polar or charged compounds such as a lot of endogenous metabolites not covered by HILIC columns [155]. Due to the high number of compounds to be profiled (xenobiotics and metabolome), the combination of different analytical platforms is of special interest to cover as much compounds as possible, as it is shown in Fig. 4. In this sense, as CE could be coupled to MS

analyzers in the same way as GC and LC, the combination of these techniques may help to the determination of compounds with a wide range of polarities, especially in studies involving small amounts of samples [156].

Finally, it is necessary to mention that LC has also been coupled with other detection techniques different to MS, such as the fluorescence detector, used in the studies by Khan et al. [54] and Chen et al. [56]. Nonetheless, this detection has been employed mainly for the determination of biomarkers of exposure presenting native fluorescence (i.e., BPA and PAHs, respectively), so its application is restricted to targeted analysis of interest EDCs (Table 2). Thus, as it is indicated in Fig. 1, the smartest strategy to assure a total exposome coverage is a combination of both GC-MS and LC-MS platforms, using C_{18} and HILIC columns, and including other specific techniques such as ICP-MS or CE when needed.

5. Data-acquisition

5.1. Targeted approaches

In targeted methods, pre-selected molecules of interest are measured, being very difficult their application when limited or not information is known before the sample analysis. Overall, targeted MS methods are useful for the quantitation of specific molecules in complex biological samples, as they provide high sensitivity and specificity. These methods are widely used in clinical research, drug metabolism, and toxicology studies. LRMS is usually applied in targeted methods since it provides better robustness, selectivity, sensitivity, and confirmation capacity than HRMS instruments and they are normally cheaper. However, the application of multi-targeted HRMS methods has increased thanks to the development of new and more sensitive instruments which enable the performance of MS and MS/MS of thousands of compounds in a single analysis with all the benefits of accurate mass measurements [76,136,157].

Triple quadrupole (QqQ) and quadrupole-ion trap (QTrap) are the analyzers most used when LRMS is applied, employing multiple reaction monitoring (MRM) in its variant dynamic MRM (dMRM) as working mode, together with selected reaction monitoring (SRM) MS/MS modes (Table 2). In this sense, dMRM consists of the application of MRM to a narrow retention time window (RTW), which could allow the quantification of a larger list of compounds in one analysis. When HRMS instruments are used, there are various possibilities [158]. On the one hand, "all-ion fragmentation (AIF)" (Orbitrap) or 'all ion MS/MS' (TOF) working mode can be applied right after the full-scan mode and they consist of the fragmentation of all ions entering collision cell. However, although precursor and product ions can be linked following this approach, it is a challenging task due to the high number of ions fragmented in the collision cell. Besides, this high number of ions could reduce the sensitivity and selectivity on the MS^2 spectra in complex matrices such as biological samples. On the other hand, in recent HRMS instruments (Q-Orbitrap and Q-TOF analyzers) there is a similar working mode than dMRM, which is called "data dependent analysis" (DDA). In this working mode, after the full scan step, the mass analyzer evaluates the ions detected and decides which ions are going to be fragmented based on a predefined m/z list. Thus, when a m/z from the list is detected in the scan, this ion is isolated and fragmented. This is the preferred approach when HRMS is used in multi-targeted methods.

Targeted methods are involved in an important part of EDCs studies, as they are essential to perform robust statistics on epidemiological scale studies, which made possible to discover the link between exposures and health outcomes. Indeed, most studies determining different EDCs families make use of this approach. Some examples (Table 2) are the determination of phthalates in urine by QqQ analyser (MRM mode) [56, 57], the determination of PFAS in plasma and serum by QqQ and QTrap analysers (MRM mode) respectively [60,61], or the analysis of pyrethroids metabolites in urine by QqQ [159]. In addition to all these examples for a reduced number of selected EDCs, some authors have employed targeted analysis for the determination of EDCs and the metabolic response to them. In the study published by Zhou et al. [58], a LRMS targeted method is used to determine a large list of polar and nonpolar metabolites in urine and plasma of pregnant women to study the effects of phthalates in the metabolome. Furthermore, Matta et al. [18] have also applied a targeted approach to evaluate the effect of OCCs, PCBs and PFAS over a list of 630 endogenous metabolites from 26 biochemical classes. Positive associations for endometriosis risk were founds for some OCCs (*trans*-nonachlor) and PCBs (PCB 114). As another example, González-Domínguez et al. have monitored and quantified more than 1000 compounds, comprising endogenous metabolites, food-related and environmental pollutants, in urine and plasma samples [160].

5.2. Non-targeted approaches

Targeted methods are widely used in exposome-based studies since they provide quantitative data with a high sensitivity and a lower matrix influence over results. However, the need of having sample information in advance makes this approach insufficient to carry out comprehensive research. Thus, as a complement to targeted methods, non-targeted approaches have been increasingly used. Non-targeted methods are used for the complete analysis of complex samples, such as environmental or biological matrices, to identify new biomarkers or potential contaminants following a non-hypothesis driven approach. These methods are typically applied as exploratory proposals to create lists of target analytes further studied in-depth with targeted methods, or to perform screening analysis of human metabolome to assess the effects of exposures previously determined by targeted methods [157].

Although non-targeted could be performed with LRMS instruments, the lower sensitivity using full scan compared to HRMS instruments have made the last one's indispensable for non-targeted studies. In GC-HRMS methods, MS analyzers work in a full scan mode and, due to electronic impact (EI) is usually employed, no further fragmentation steps are required since it applies a high ionization energy [18,56]. In LC-HRMS methods, (Q)-Orbitrap and (Q)-TOF analyzers are mostly employed. When using Orbitrap and TOF analyzers, a full scan acquisition is performed with the possibility of total ion fragmentation to acquire more sample information, as described in section 5.1 for targeted methods (i.e., AIF or all 'ion MS/MS' working modes). In addition to AIF or 'all ion MS/MS' working modes, Q-Orbitrap and Q-TOF analyzers offer two other possibilities to perform MS/MS experiments [158]. The predominant one is the DDA, previously described in section 5.1 [57,60,61]. In DDA experiments, besides the inclusion of a fragmentations list, ion fragmentation can be carried out based on an intensity threshold defined by the user. When a precursor ion is detected above this value, it is isolated and fragmented with different preselected collision energies in the collision cell. This acquisition mode is useful when the characterization of the sample is required. The ions with higher intensities, normally the most abundant ones, are fragmented first and global information about them is provided. However, although DDA is the preferred data acquisition approach in exposomics, it exhibits some disadvantages. First, if it works with a m/z list, it can only be applied in targeted methods. On the contrary, if it works with an intensity threshold, it might be insufficient to detect compounds at lower concentrations that are generally the most important ones since they correspond to biomarkers of exposure. Furthermore, when the analysed samples are too complex, the number of scans is sometimes low due to the high number of precursor ions isolated and fragmented, reducing the sensitivity.

For these reasons, data independent acquisition (DIA) represents an effective alternative to DDA, especially for the determination of those compounds at very low concentrations [52,67]. In DIA experiments, a selected group of precursor ions are filtered and fragmented in the collision cell. The selection of precursor ions is made based on a m/z window established by the operator (e.g., each m/z 50 units). Thus, by

doing ion fragmentation in a narrow window, sensitivity and selectivity are better than when AIF is performed and without a limiting threshold as for DDA. The handicap of this working mode compared to DDA is that the link between precursor and product ion is broken; thus, it is more difficult to assure that fragment come from a precursor ion or from more than one simultaneously. However, as discussed in section 6.2, there is some available software to solve this problem (for both, AIF and DIA modes). Besides, some HRMS analyzers, such as the last commercialized Q-Orbitrap, have the possibility to perform both fragmentation modes simultaneously (i.e., DIA and DDA) although this entails a lower sensitivity, especially for the fragment ions, due to the reduction of scans [161].

For all these reasons, there is no single strategy to apply for data acquisition in exposomics, but rather the most appropriate one must be selected based on the results sought. In summary, targeted methods are aimed to evaluate chemical exposures and confirming non-targeted results. On the other hand, non-targeted methods operating in DDA mode should be applied if the goal is to determinate endogenous/more abundant compounds, whereas DIA experiments are intended for the determination of exogenous/less abundant compounds. Regardless of all this, it is necessary to mention that whatever the selected method it is recommended to use the aforementioned ISs to account for extraction losses, matrix effects and instrumental signal variability and to employ rutinary internal calibration standards to achieve a correct mass accuracy.

6. Data analysis

Whereas data processing can be a simple task in targeted analysis, non-targeted analysis performed by HRMS instruments provide a high amount of information that normally requires high level of knowledge in data processing to obtain reliable results. For that, there are a series of steps to follow to achieve valuable information and to get to the ultimate purpose of non-targeted analysis, which is the identification of compounds of interest [157].

6.1. Data pre-processing

Pre-processing is made to eliminate variances and reduce data complexity, improving the subsequent steps and enhancing significant signals of interest. There are several reviews that describes in detail the pre-processing steps [162–164], so only a summary is presented here.

6.1.1. Intensity threshold establishment

First, it is necessary to stablish an intensity threshold for peak detection before the data treatment. This is not an easy task, since setting a too low threshold involves that a lot of peaks will be detected. This effect is especially relevant in LC-MS data, where the ionization source produces a high number of molecular ions, including chemical and random noises and mobile phases as a source of interferences [162]. In this sense, the inclusion of a reactive blank and an instrumental blank in the sample batch is mandatory to subtract the background signals in the real samples. Contrary, when a too high threshold is selected, valuable information is likely to be lost, particularly in the case of EDCs exposure which is expected to be at low concentration levels relative to endogenous metabolites [165]. Thus, a compromise between these two effects must be agreed.

6.1.2. Retention time alignment

This step is performed to remove shifts for a given signal between different samples along the batch, in order to assure a correct reproducible information along it. Although retention times should not vary greatly if all the samples are run in the same batch and all the above considerations are taken into account (e.g., buffered mobile phases, column temperature setting, etc.), some variation is expected. Time alignment is normally performed by different available software as XCMS [60]. Another possibility is to use some compounds, which produce multiple fragment ions, as landmarks to generate pseudo-mass spectra for a coarse time-shift correction [166]. In this line, it could be interesting that, since the use of ISs has already been recommended for sample collection, extraction, and analysis steps, these ISs could be used as a reference for the retention time alignment.

6.1.3. Data deconvolution

Although chromatographic peaks have been previously filtered by establishing an intensity threshold, some of them could be overlapped, requiring a deconvolution step. It is important to choose the parameters to perform data deconvolution to avoid false positives or negatives. This issue is important for GC-MS data, for which there are statistical programs to perform an automatic deconvolution (DRS, AnalyzerPro, ChromaTOF®, etc.) [162]. Deconvolution is also relevant for LC-MS data when AIF or DIA modes are employed, since product ions could come from more than one precursor ion. There are specific deconvolution software platforms for LC-MS DIA data, for example MS-DIAL, DIA-Umpire, RAMClustR, or R-MetaboList [167].

6.1.4. Data normalization, scaling and transformation

Finally, normalization is of great importance to reduce data variability due to methodological or instrumental errors and to obtain a better comparison among samples. Normalization could be done by using the appropriate ISs, QCs or surrogates. Each one has its advantages and disadvantages. Specifically, ISs normalization accounts for variations in sample preparation and instrument response and can provide an accurate quantification. However, the lack of ISs of similar characteristics than the monitored compounds is an important handicap, especially when the compounds are not known a priori. QCs as pooled samples can be employed for reducing batch effects, although differences from the reference sample may overshadow biological variations [163].

As alternative, Pareto scaling and log transformation could reduce instrument variabilities throughout the batch, providing a balance between small and large variances by limiting the impact of high-abundance ions and, thus, biological variations are maintained. However, the interpretation of the data can be challenging, having some limitations such as zero values for log transformation and the assumption of normal distribution and difficulties when variances are too high for Pareto scaling [163] (Table 2).

Nowadays, most available software such as MZmine, XCMS, OpenMS, MetAlign, MetSign, Workflow4Metabolomics, MSFACTs or MetaboliteDetector, as well as data treatment software from commercial brands, perform all these pre-processing steps automatically or by indicating some parameters and some of them are free to use. The different software for each MS analyzer is detailed in the review of Pourchet et al. [67].

6.2. Data-processing and annotation

Once data pre-processing is done, the next objective is to identify as many compounds of interest as possible. For GC-MS data, the pre-processed data can be directly exported for compound search to the NIST library (or AMDIS, in NIST library), which provides experimental spectra for both, precursor and fragment ions. When non-targeted analysis is performed, there are some tools to improve compound identification as Kovats Index (KI). This index works by comparison between the experimental KI for each tentative compound and the KI of a commercial mixture (n-alkane mixture). If the difference between them is ±20 units, it could be said that tentative identification is reliable [168].

For LC-MS data, this task is more difficult since there are not libraries as complete as NIST. Furthermore, fragmentation patterns are less reproducible in LC-MS, which involves soft ionization. Thus, great differences between experimental and library data can be observed,

depending on the ionization voltages, the kind of mass spectrometer employed, variations in retention times (more common in LC than in GC since due to different parameters, such as the mobile phases, the modifiers added to them and the wide range of columns), and the higher matrix effect observed in comparison to GC-MS. The lack of a standardized chromatographic methodology makes more complex the use of retention times indexed in databases. The smartest strategy in this case is the use of different databases to achieve a complete compound annotation (Table 3).

Compound annotation can be achieved at different levels of confidence. Schymanski et al. have defined some possible levels of confidence and the parameters that must be met to reach each level [169]. Thus, five confidence levels are established. Level 5 is the lowest confidence level, implying a compound annotation with only the exact mass of the parent ion. On the other hand, level 1 is the highest confidence level and is only achieved if the exact mass of the parent ion, the retention time, and the ion fragments of the suspected compound match the chemical standard. In this sense, databases include different useful information that contributes to increase the confidence level of an annotated compound. Some of these data are retention times obtained with standardized experimental conditions, isotopic patterns, exact mass, and possible ion fragments, as well as CCS values if IMS is employed. All this information is compiled from mining bibliography, general databases or international agencies.

There are not concrete databases for the annotation of all the compounds in a matrix, and even less in non-targeted analysis. While general databases as Chemspider or Pubchem comprise more than 100,000,000 of compounds, but with little information on MS spectral data, others only provide information on the chemical formula, chemical properties, medical information or biological properties. Among all these databases, some of the most interesting for exposomics are: Blood Exposome Database (although it only includes compounds detected in blood), Exposome Explorer, Toxic Exposome Database (T3DB) and Endocrine Disruptor Knowledge Base (Table 3). There are other databases that include MS and MS^2 spectral information, including different ionization modes, sources and energies, and predicted or experimental chromatographic information. Besides, some of them have spectral information for HRMS, much more valuable for this kind of studies, and even CCS data, implementing a new molecular parameter for identification. Finally, mention that Meijer et al., under the guidelines of the European Human Biomonitoring for Europe (HBM4EU) initiative [170], have recently developed a database for chemicals of emerging concern (CECs) with more than 70,000 parent substances and more than 300,000 known or modelled metabolites [171].

When information on the compounds of potential interest is not included in databases or when the retrieved results for them offer too many possibilities, which normally occurs in non-targeted studies, it is not possible to reach a high confidence level. In these cases, is easy to make errors such as false negatives and false positives, respectively [157]. There are some tools to increase the confidence level in annotation such as in-silico fragmentation software platforms. Starting from a putative annotated structure, these programs perform many possible ion fragmentations based on preset parameters, in order to compare the reproducible fragmentation patterns obtained with the experimental MS/MS to find matches. There are several fragmentation programs including both, payment programs (e.g., MassFrontier from Thermo Scientific) or open-source software packages (e.g., CFM-ID or MetFrag) [157,162,172]. Furthermore, there are also programs that provide theoretical properties such as environmental fate, toxicity, absorption, distribution, metabolism and excretion (ADME) properties and, most important in this case, physicochemical properties. Among these physicochemical properties are the octanol-water partitioning coefficient (log P) and theoretical LC retention times, making possible their comparison with the experimental values. Examples of them are the Toxicity Estimation Software Tool (T.E.S.T.) Version 5.1.1 of the Environmental Protection Agency (EPA) [173] or the OPEn (q)saR App (OPERA) [174].

Table 3
Most employed databases for compound annotation.

Database	Compounds	Source	Information
Chemspider	General database (>100,000,000 compounds)	Various sources	Structures and some properties
Pubchem	General database (>100,000,000 compounds)	Various sources	Structures and some properties
CompTox Chemicals Dashboard	Endogenous compounds and environmental chemicals (>900,000 compounds)	US EPA DSSTox and databases	Structures and some properties
BEDB	Compounds of exposome research interest (>65,000 compounds)	Data mining from PubMed and PubChem	Structures, molecular biology/biochemistry data and exposure data
ChEBI	Small chemical compounds (<60,000 molecules)	Different databases	Structures and some properties
LIPID MAPS	Biologically relevant lipids (>47,000 compounds)	Various sources and databases	Structures and some properties
KEGG	Endogenous compounds (>18,000 compounds)	Literature	Structures, molecular biology/biochemistry data and metabolic pathways
Exposome Explorer	Biomarkers of exposure to environmental factors (>1200 compounds)	Literature	Information about nature of biomarkers, populations affected and analytical methods used
EDKB	Computational predictive endocrine disrupter compounds (>800 compounds)	US FDA	Information about suspicious endocrine compounds
T3DB	3678 toxins (pollutants, pesticides, drugs, and food toxins)	Databases, government documents and literature	Link between toxin and toxin target proteins, chemical properties, toxicity values, molecular and cellular interactions, and medical information
NIST 20	General database (>1,250,000 compounds)	Standard reference data	Only for GC-MS LRMS and HRMS spectral information
METLIN	Endogenous compounds and environmental chemicals (>860,000 compounds)	Literature and US EPA DSSTox	MS and MS^2 spectral information with different ionization modes and energies
Meijer et al. database	CECs and simulated metabolites (>375,000 compounds)	Literature, US EPA DSSTox and databases	–
HMDB	Endogenous compounds and environmental chemicals (>200,000 compounds)	Literature and real data from laboratories	LC-MS and GC-MS spectral data with different ionization modes and energies, including chromatographic data, some MS^2 and CCS data
Metabolomics workbench	Endogenous compounds (>160,000 compounds)	Literature	RTs, MS data and some properties
Mass bank	Endogenous compounds and environmental	Public and private	LC-MS and GC-MS spectral data with

(continued on next page)

Table 3 (*continued*)

Database	Compounds	Source	Information
	chemicals (>90,000 compounds)	organizations and databases	different ionization modes and energies
Golm Metabolome Database	Endogenous compounds (>26,500 compounds)	Literature and reference standards	Only for GC-MS RTs and MS spectra
mzCloud	Endogenous compounds and environmental chemicals (>20,000 compounds)	Public and private organizations	Only for LC-MS LRMS and HRMS and MS^2 spectral information with different ionization modes and energies and chromatographic data

Abbreviations: BEDB: Blood exposome database; CCS: Collision cross section; CEC: Chemicals of emerging concern; ChEBI: Chemical entities of biological interest; DSSTox: Distributed Structure-Searchable Toxicity; EDKB: Endocrine disruptor knowledge base; EPA: Environmental protection agency; FDA: Food and drug administration; GC: Gas chromatography; HMDB: Human metabolome database; HRMS: High-resolution mass spectrometry; KEGG: Kyoto encyclopedia of genes and genomes; LRMS: Low-resolution mass spectrometry; MS: Mass spectrometry; NIST: National Institute of Standards and Technology; RT: Retention time; T3DB: Toxic exposome database.

Finally, for the identification of biomarkers of exposure, there is some software that allows in-silico metabolization. For example, the open-source software tool BioTransformer, which simulates the formation of phase I (CYP450) transformation for a particular compound [171], or MassChemSite, which simulates organic and inorganic reactions of a parent molecule to elucidate its metabolites [175]. Besides, there are special software packages for the annotation of low concentration compounds, as the recently presented by Guo et al. [176]. This is an important issue because in exposomics compounds at lower concentrations are generally the most interesting for linking health outcomes from chemical exposures. With the aforementioned methodology, 2.3-fold more compounds were identified regarding conventional peak picking.

Although all the presented information could be useful for a first step annotation, final and unequivocal identification should be done by comparison with analytical standards. In some cases, this is not possible as there are not commercially available standards for all the annotated compounds and the acquisition of a high number of them is expensive. The use of a standard from the same family or a very similar one to the annotated compound can improve the confidence level. To do this, it should show similar retention time and fragmentation pattern to those of the unknown compound. This could be even used for quantification purposes if identical analytical standards are not available [177]. But even in the case that identified compound has not any family-related standard available, if the controls done during experimental design are appropriate, it is possible to use relative areas to perform statistical analysis [157].

6.3. Statistical analysis

The omics fields applied in exposome-related studies face with analytical challenges derived from the high-dimensionality and complexity of data generated, hence multiplicity. The challenge of multiplicity adds to common statistical challenges in environmental epidemiology of EDCs, such as strong measurement error, subtle associations or dealing with non-lineal exposure-outcome associations and exposure-exposure interactions. The integration of omics (e.g., metabolomics) layers demands additional steps to identify functional links on the continuum exposure-metabolite-outcome. Common workflows involving datasets with more than 10–50 variables rapidly demands techniques to select a subset of them shrinking the rest (e.g., variable selection methods), or aggregate the data of original variables in lower dimensional mathematical constructs or latent variables (e.g., dimension reduction).

6.3.1. Dimension reduction techniques

They are widely extended in metabolomics research, including principal component analysis (PCA) and partial least squares (PLS) regression (Table 2). PCA is an unsupervised method that is performed to assess an overview of two or more group of samples without considering the outcome of interest. It is made by reducing data dimensionality through orthogonal transformations called principal components (PCs). Thus, it has been applied to determine group variabilities and to identify possible patterns of exposure [18,54,57]. This technique forces orthogonality between components, imposing a rigid structure. For this reason, it is not appropriated for non-negative data, such as exposome data, being more appropriated to evaluate the general trend in the data and capture the total variance of it [178]. After PCA, PLS is typically used to distinguish the overall differences among datasets and explain the features that make them different. It has two main variations called PLS-discriminant analysis (PLS-DA) [54,56,57,61] and orthogonal PLS-DA (OPLS-DA). In PLS-DA, a dataset is categorical, while in OPLS-DA data are separated into predictive and uncorrelated (orthogonal) information. The variant with the best results for the determination of differential metabolites between groups is OPLS-DA [18,55,179].

6.3.2. Variable selection techniques

Commonly used in metabolomics or chemical exposomics, they include least absolute shrinkage and selection operator (LASSO) and elastic-net regression. In both cases, regression models introduce a penalization parameter to shrink some of the coefficients to zero to improve interpretability [180]. Tree-based models are a family of supervised methods that can deal with high-dimensionality and collinearity with large applications in metabolomics. The Random Forest (RF) model builds multiple decision trees averaging the trees decision rules to establish the partition of the data space. The gradient boosted decision trees (GBDT), contrary to RF, builds a series of decision trees, sequentially, each model depending on the previous one (e.g., "boosting"), improving the predictive/classification capacity along the process.

6.3.3. Univariate and bivariate methods

These methods include bivariate correlation analysis (Pearson or Spearman) and Mann-Whitney U or Student's t-test (used to compare groups), which are mainly used for exploratory purposes because they do not take into account the variability of confounding variables such as age or body mass index [56,57]. Hence, multivariate regression models are used to establish the associations between chemicals or metabolites with health outcomes accounting for the covariation of individual variables but also experimental variables (batch, cohort, etc.).

6.3.4. Multipollutant models

This approach involves a large family of algorithms and strategies specifically developed to overcome the specific constraints of biomarker exposures data. During the last years, one the most used multipollutant models is the Bayesian Kernel Machine Regression (BKMR) that allows to assess either overall effect of the mixture or the individual effect of chemicals, to conduct variable selection, accommodating non-linear associations and interactions [181]. Weighted-quantile sum regression (WQS) is another multipollutant method based on generalized linear regression of weighted individual exposures.

6.3.5. Data integration strategies

They are a key step in exposome-health studies, involving endogenous data. Different statistical strategies have been developed to integrate endogenous biomarker data on the link between EDCs and disease, including the MITM framework. A first proposal focused on

metabolomics data integration conceived a sequential strategy to first identify biomarkers of exposure and metabolomic biomarkers linked with the disease, and second, the intersecting intermediate biomarkers as potential causal markers [182]. Since then, a list of different strategies based on MITM principles have been developed and applied in epidemiological studies. Mediation analysis formally compute the weight of direct effects of exposures on the outcomes and indirect effects mediated by the intermediate (omics) variables with two multivariate regression equations. The extension towards complex scenarios with multiple mediators (e.g., chain of metabolites within a pathway) can be accommodated with high-dimensional mediation models [183].

7. Pathway analysis

One of the fundamental goals of exposome research is deciphering the biological mechanisms underlying the links between exposures and diseases from complex data generated with high-throughput omics analytical platforms. The principle of pathway analysis is mapping the findings on dysregulated metabolites or molecular pathways into structured and curated networks that represents biologically anchored processes [184]. Pathway analysis have been developed in different omics disciplines. Over-representation analysis (ORA), functional class scoring (FCS), and topology-based methods are commonly used pathway analysis methods; however, the application in epidemiological research is basically limited to the first one otherwise referred as enrichment analysis [185]. To perform ORA, three essential inputs are required: a collection of pathways (e.g., obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) or Reactome Pathway database [186,187]), a list of metabolites of interest (generated by the experimental setting), and a background or reference set of compounds. The prediction of pathway activity from raw mass spectra has been developed as a bioinformatics solution to by-pass the complex metabolite-specific annotation process in non-target analysis, with the *mummichog* algorithm [188]. This algorithm has been widely used for instance in non-targeted analysis applied to the observational study linking PFAS and health (6 out of 11 studies included in a recent scoping review), contributing the most to functional analysis [30]. Open access applications such as MetaboAnalyst or MetExplore can assist the overall process of mapping and pathway analysis [189,190]. A major complexity arising pathway analysis in observational studies is the interpretation of metabolic dysregulations in a highly dynamic, metabolically rich and unspecific matrix such as blood using single time point samples. In addition, a list of parameters can affect the results and interpretation of pathway analysis including pathway database choice, organism-specificity, and database updates, prompting to their misuse as discussed elsewhere [191]. Some recommendations to conduct proper and transparent pathway analysis include the specification of realistic background sets, using organism-specific pathways sets, using multiple pathway databases and using multiple-testing correction to select metabolites and pathways, besides applying a minimal reporting criterion [185].

8. Conclusions and future perspectives

This review presents the most used analytical strategies to address the different pieces that make up the puzzle of chemical exposome studies, from experimental design and sample collection to data analysis and interpretation, and focusing on studies related to exposure to EDCs. In this framework, it could be said that the preferential matrices selected for exposomics are urine and blood, making the selection of alternative matrices, such as breast milk, umbilical cord blood or placenta, interesting to study the exposures at relevant periods of life. Samples are subsequently treated by simple extraction methods such as LLE and analysed by GC and LC (with reverse and HILIC phase columns) coupled to MS, applying both targeted and non-targeted methods.

In addition, nowadays great efforts are being focused on developing efficient tools for data processing and analysis, especially for those large data sets generated in non-targeted methods, since it is the key to find a good correlation between biomarkers of exposure and effect. Sophisticated statistical analysis is already used to differentiate between group of samples and determinate features of interest, but new methodologies involving machine learning statistical analysis could help gain insight into human exposures and their relationship to high-incidence of environmental diseases. In this case, feature annotation for compound identification can be considered a challenge and one of the bottlenecks of exposomics, but the development of open access databases, in combination with simulation software to generate important information such as ion fragmentation patterns, is contributing to overcome this drawback. In this sense, a unified exposome database is needed to increase the confidence level of the annotated compounds and efforts such as those made by HBM4EU are moving in this direction.

After data analysis, whether targeted or non-targeted methods are applied, pathway analysis and data interpretation are required to successfully unveil the mode of action of chemical exposures that lead to the development of environmental diseases in population. Therefore, the experience and knowledge of toxicologists and epidemiologists is highly relevant and that is why they are leading the growth of this research field. However, it is unrealistic to think that these studies can be carried out without an integrated and multidisciplinary approach.

The findings of this review provide valuable information on the study of EDCs within an exposome framework, highlighting the most relevant analytical aspects for turning this concept into a real methodology. However, several avenues for further research and exploration emerge from these results. Thus, it is necessary to explore new advances in sampling to achieve greater population coverage and for longer periods of study. Some techniques, such as *in-vivo* SPME (e.g., needle trap device technology, which has already been used to evaluate human exposure to smoke [192]) or microsampling techniques such as dried plasma spot, capillary microsampling or volumetric absorptive microsampling may be the ideal alternatives [193]. Among all the challenges involved in moving from the exposome concept to a real applied science, there is a need to harmonize extraction methods that allow the extraction of a wide range of compounds, such as multiphase methods (e.g., MTBE extraction), for including the simultaneous extraction of endogenous metabolites and chemical exposures. Furthermore, new advances in analytical tools such as CE-HRMS or IMS could be implemented to have greater coverage of compounds or to have another parameter in the annotation step, respectively.

Funding sources

This work was supported by Andalousian Government (Project ref. PROYEXCEL_00195).

CRediT authorship contribution statement

Jesús Marín-Sáez: Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **Maykel Hernández-Mesa:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Germán Cano-Sancho:** Supervision, Methodology. **Ana M. García-Campaña:** Writing – review & editing, Resources, Project administration, Funding acquisition.

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

Data will be made available on request.

Acknowledgments

This work was supported by Andalousian Government (Project ref. PROYEXCEL_00195). JMS thanks the University of Almería for the "Convocatoria de Recualificación del Sistema Universitario Español-Margarita Salas" postdoc grant under the "Plan de Recuperación Transformación" program funded by the Spanish Ministry of Universities with European Union's NextGenerationEU funds. MHM gratefully acknowledges the grant IJC2019-040989-I funded by MCIN/AEI/10.13039/501100011033.

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