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Biomarcadores de Efecto en Programas de Biomonitorización Humana

"Effect Biomarkers in Human Biomonitoring Programs"

Andrea Rodríguez Carrillo

FACULTAD DE MEDICINA

Departamento de Radiología y Medicina Física

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Andrea Rodríguez Carrillo

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BAJO LA DIRECCIÓN DE LOS DOCTORES/AS:

D. Nicolás Olea Serrano
Catedrático de Universidad
Departamento de Radiología y Medicina Física
Universidad de Granada

Dña. Mariana F. Fernández Cabrera
Catedrática de Universidad
Departamento de Radiología y Medicina Física
Universidad de Granada

Lo que hagas tu hoy, lo haré también,

Dime cómo es y aprenderé. Es razonable, mas no para mí.

Sé que hay tanto por aprender, parece claro más no es verdad

Si puedo ver mi propia imagen, descubriré cuan grande es mi futuro

Quiero saber, que me enseñes,

Quiero saber lo extraño que soy.

Dime más, que entienda,

Lo que es normal en lo extraño que soy

Phil Collins (Tarzán)

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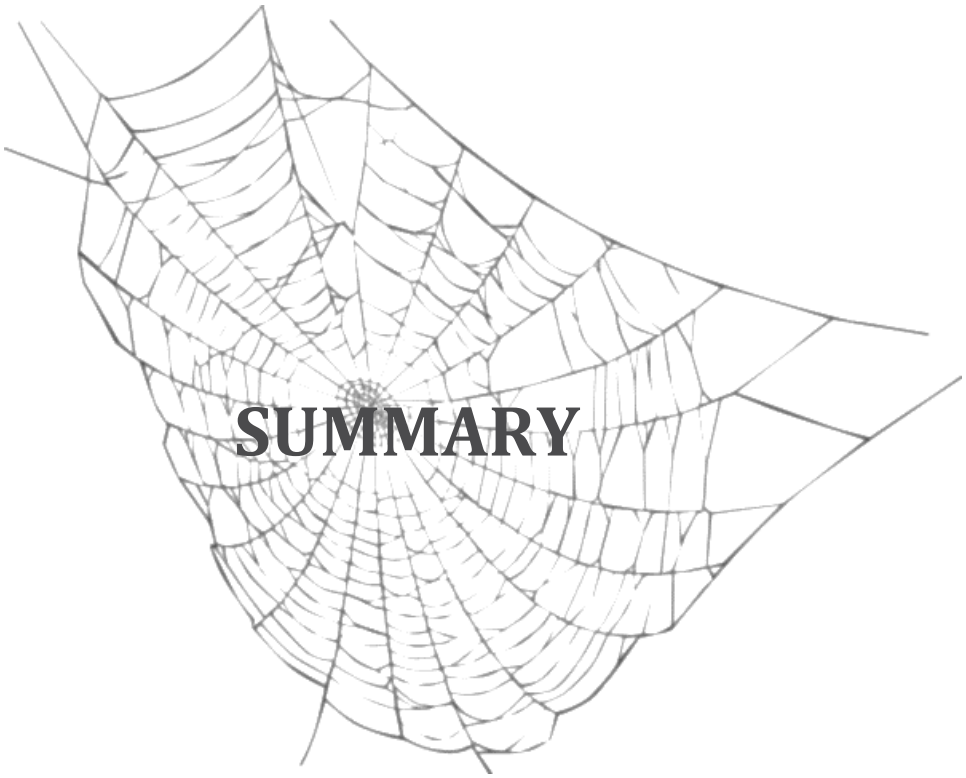


SUMMARY

Summary

Introduction

Humans are exposed to thousands of natural and man-made chemical substances. It is known that some of these chemicals could exert adverse effects on human health, however, the health impact over a lifetime associated with this exposure is not completely understood. The use of biomarkers of effect in Human biomonitoring (HBM) studies could help to understand the mechanisms of action by which environmental chemicals cause adverse human health outcomes.

Objective

The main purpose of the present doctoral thesis was to select, describe, test, and implement effect biomarkers for the assessment of exposure-health associations during critical windows of human development, related to cognitive and behavioral alterations. For this goal, the following specific objectives were proposed:

Objective 1: To identify and prioritize existing biomarkers of effect for BPA, metals, pesticides, and complex mixtures of chemicals.

Objective 2: To provide relevant mechanistic and adverse outcome pathway (AOP) information in order to cover knowledge gaps and better interpret effect biomarker data.

Objective 3: To investigate the effects of chemical exposure with cognitive domains, intelligence quotient (IQ), and behavioral function among Spanish adolescent males from the INMA-Granada cohort.

Materials and Methods

Boys from the INMA-Granada cohort were evaluated at three different periods of development: prenatal, childhood (9-11 years), and adolescence (15-17 years). Relevant chemical compounds as BPA, metals, pesticides, and mixtures of lipophilic compounds, were measured in human samples (spot urine and placenta samples). Several effect biomarkers were also assessed (Brain-derived neurotrophic factor (BDNF) protein levels and BDNF gene DNA methylation) in non-fasting serum, urine, and whole blood samples. Moreover, five in vitro/in vivo biomarkers of combined activity (for estrogenicity, anti-androgenicity, AhR, and anti-thyroid functioning) were used to quantify the combined biological activities exerted by the mixture of lipophilic compounds present in the placenta extracts.

Results

Article 1: As a result of a comprehensive literature search, promising novel biomarkers of effect related to bisphenols and also with altered neurodevelopmental (BDNF) and reproductive outcomes (kisspeptin, Kiss) were selected. This is the first effort to comprehensively identify effect biomarkers for HBM purposes.

Article 2 and 3: Urinary BPA concentrations were longitudinally and positively associated with thought problems and somatic complaints at adolescence. BPA concentrations were also longitudinally associated with increased BDNF DNA methylation at CpG6 in a dose-dependent manner. However, urinary childhood BPA concentrations were not associated with cognitive abilities, except for poorer working memory

Article 4: Urinary arsenic (As) and cadmium (Cd) were associated with more internalizing and externalizing problems, respectively. However, inverse associations were found for mercury (Hg) and lead (Pb). Higher As levels were associated with higher BDNF DNA methylation at CpGs 3 to 6, while Cd was associated with lower serum BDNF protein levels. Urinary Hg and Pb did not show any association with effect biomarkers.

Article 5: Higher urinary levels of IMPy, TCPy, and ETU were associated with more behavioral problems. IMPy, MDA, DETP, and 1-N were also significantly associated with decreased serum BDNF levels, while MDA, 3-PBA, and ETU with higher DNA methylation percentages at several CpGs. WQS models suggested a mixture effect on higher behavioral problems and BDNF DNA methylation, with MDA showing the highest weight within the WQS mixture approach. Interestingly, when MDA was assessed individually, only showed an association with lower serum BDNF levels.

Article 6: Most placentas showed estrogenic activities, since induced ER-mediated transactivation and ER-dependent cell proliferation, with a strong inhibition of thyroid hormone (TH) signaling and the AR transactivity; however, the induction of the AhR was found in few placental extracts. All placental extracts agonized or antagonized at least three of the selected endpoints. Effects found in the two estrogenic bioassays were positively and significantly correlated and the AR-antagonism activity showed a positive correlation with both estrogenic bioassay activities. However, the *in vivo* anti-thyroid activities of placental extracts were not correlated with any of the tested in vitro assays.

Conclusion

In this PhD thesis, it has been shown that boys of the INMA-Granada birth cohort are exposed to all the selected environmental chemical compounds, BPA, non-persistent

pesticides, and toxic metals from birth to adolescence. Our results highlight the role of BDNF as a promising, toxicologically-supported effect biomarker for brain function that may help to improve the inference of causal relationships in observational studies addressing environmental exposures and neurodevelopment in children. The implementation and assessment of selected mechanistically-based effect biomarkers of combined activity will also help to better characterize the signaling pathways through which mixtures of lipophilic chemical compounds could elicit adverse health outcomes in humans.

Resumen

Introducción

Los seres humanos están expuestos a miles de sustancias químicas tanto naturales como artificiales. Se sabe que algunos de estos productos químicos podrían ejercer efectos adversos sobre la salud humana, sin embargo, el impacto en la salud asociado con esta exposición a lo largo de la vida no se comprende completamente. El uso de biomarcadores de efecto en estudios de biomonitorización humana (HBM) podría ayudar a comprender los mecanismos de acción por los que los contaminantes ambientales causan efectos adversos para la salud.

Objetivo

El objetivo principal de la presente tesis doctoral fue seleccionar, describir, probar e implementar biomarcadores de efecto para la evaluación de asociaciones exposición-salud durante ventanas críticas del desarrollo humano, relacionadas con alteraciones cognitivas y conductuales. Para este fin, se propusieron los siguientes objetivos específicos:

Objetivo 1: Identificar y priorizar los biomarcadores de efecto existentes para BPA, metales, pesticidas y mezclas complejas de productos químicos.

Objetivo 2: Proporcionar información relevante sobre mecanismos y vías del efecto adverso (AOP) para cubrir las lagunas de conocimiento y mejorar la interpretación de los biomarcadores de efecto.

Objetivo 3: Investigar los efectos de la exposición a contaminantes ambientales sobre la función cognitiva, el cociente intelectual (CI) y la función conductual en adolescentes varones españoles de la cohorte INMA-Granada.

Materiales y métodos

Los niños de la cohorte INMA-Granada fueron evaluados en tres períodos diferentes del desarrollo: prenatal, niñez (9-11 años) y adolescencia (15-17 años). Se midieron compuestos químicos relevantes como BPA, metales, pesticidas y mezclas de compuestos lipofílicos en muestras humanas (orina y placenta). También se evaluaron varios biomarcadores de efecto (niveles proteicos del factor neurotrófico derivado cerebral (BDNF) y metilación del ADN del gen BDNF) en muestras de suero, orina y sangre total. Además, se utilizaron cinco biomarcadores *in vitro* / *in vivo* de actividad combinada (estrogenicidad, anti-androgenicidad, AhR y funcionamiento anti-tiroideo)

para cuantificar las actividades biológicas de la misma mezcla de compuestos lipofílicos presente en extractos de placenta.

Resultados

Artículo 1: Como resultado de una búsqueda exhaustiva de la literatura, se seleccionaron biomarcadores de efecto novedosos relacionados con la exposición a bisfenoles y con alteraciones en el desarrollo neurológico (BDNF) y reproductivo (kisspeptina, Kiss). Este estudio constituyó el primer esfuerzo para identificar de manera integral los biomarcadores de efecto dirigidos a programas de biomonitorización.

Artículo 2 y 3: Las concentraciones urinarias de BPA se asociaron longitudinal y positivamente con problemas de pensamiento y quejas somáticas en la adolescencia. Las concentraciones de BPA también se asociaron longitudinalmente con un aumento de la metilación del ADN del gen BDNF, a nivel de la CpG6, de forma dosis-dependiente. Sin embargo, las concentraciones de BPA en la orina durante la infancia no se asociaron con la función cognitiva, excepto por una peor memoria de trabajo.

Artículo 4: El arsénico (As) y el cadmio (Cd) urinarios se asociaron con más problemas internalizantes y externalizantes, respectivamente. Sin embargo, se encontraron asociaciones inversas para el mercurio (Hg) y el plomo (Pb). Los niveles más altos de As se asociaron con una mayor metilación del ADN del gen BDNF en las CpGs 3, 4, 5 y 6, mientras que el Cd se asoció con niveles más bajos de proteína BDNF en suero. Hg y Pb urinarios no mostraron asociación con los biomarcadores de efecto.

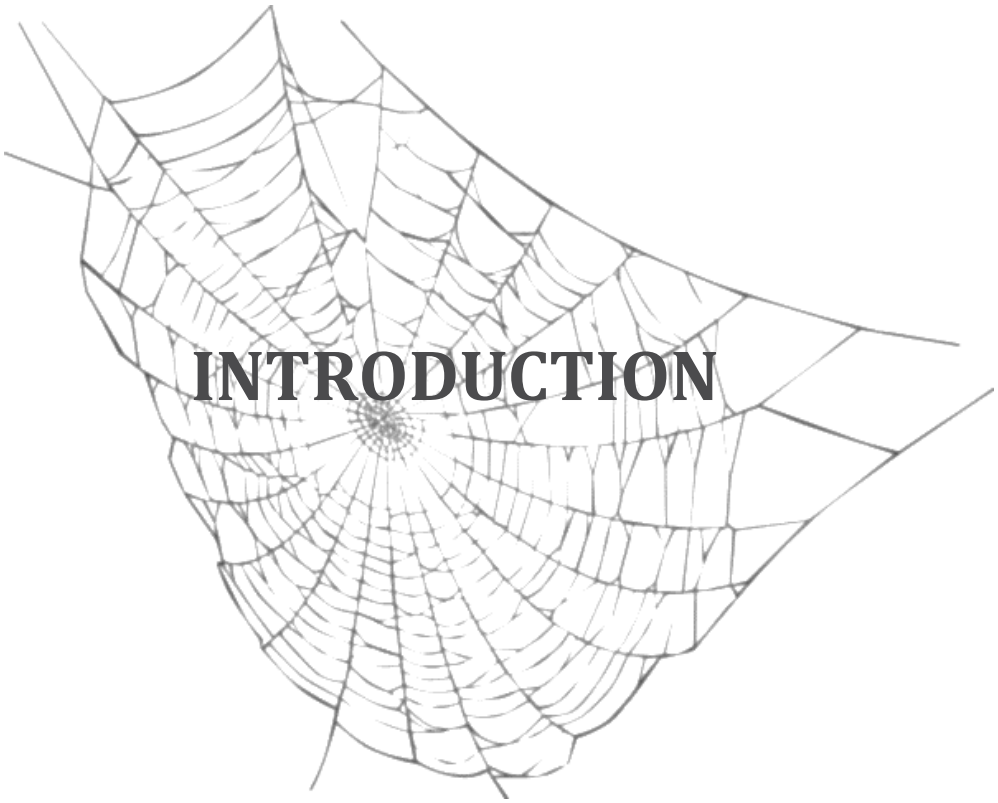
Artículo 5: Los niveles urinarios más altos de IMPy, TCPy y ETU se asociaron con más problemas de comportamiento. IMPy, MDA, DETP y 1-N también se asociaron significativamente con niveles disminuidos de BDNF en suero, mientras que MDA, 3-PBA y ETU con mayores porcentajes de metilación de ADN en varios CpGs. Los modelos WQS sugirieron un efecto de mezcla con más problemas de comportamiento y mayor porcentage de metilación del ADN del gen BDNF. MDA mostró la mayor influencia dentro del enfoque de mezcla WQS. Curiosamente, cuando MDA se evaluó individualmente, solo mostró asociaciones con niveles más bajos de BDNF en suero.

Artículo 6: La mayoría de las placentas mostraron actividad estrogénica, ya que indujeron transactivación mediada por ER y proliferación celular dependiente del ER, con una fuerte inhibición de la señalización de la hormona tiroidea (TH) y la transactividad de AR; sin embargo, la inducción de AhR se encontró en pocos extractos placentarios. Todos los extractos placentarios agonizaron o antagonizaron al menos tres de los criterios de valoración seleccionados. Los efectos encontrados en los dos

bioensayos estrogénicos se correlacionaron positiva y significativamente, además también se correlacionaron positivamente con la actividad antiandrogénica. Sin embargo, las actividades antitiroideas *in vivo* de los extractos de placenta no se correlacionaron con ninguno de los ensayos *in vitro* testados.

Conclusión

En la presente tesis doctoral se ha demostrado que los niños de la cohorte de nacimiento INMA-Granada están expuestos a todos los contaminantes ambientales seleccionados, BPA, pesticidas no persistentes y metales tóxicos desde el nacimiento hasta la adolescencia. Nuestros resultados destacan el papel del BDNF como un biomarcador de efecto sobre la función cerebral que puede ayudar a mejorar la inferencia de las relaciones causales en los estudios observacionales que abordan la exposición ambiental y el desarrollo neurológico en los niños. La implementación y evaluación de biomarcadores de efecto de actividad combinada basados en mecanismos seleccionados también ayudarán a caracterizar mejor las vías de señalización a través de las cuales las mezclas de compuestos químicos lipofílicos podrían provocar resultados adversos para la salud en los seres humanos.



INTRODUCTION

1 Introduction

1.1 Human Biomonitoring

Humans are exposed to myriads of natural and man-made chemical substances, present in the air, ingested food, water, workplace, and numerous consumer products. It is known that some of these chemicals could exert adverse effects on human health, however, the health impact over a lifetime associated with this exposure is not completely understood.

Different strategies have been performed to characterize the exposure to environmental chemicals, mainly based on their measurement from its source (e.g., factories smoke, contaminated lakes, etc.); or the individual's environment (chemicals substances present indoor, or at the workplace). Although these approaches were useful to describe the extent of exposure in ecological systems or occupational settings, they were not enough since the amount of exposure needed to exert an effect (biologically relevant dose) is not necessarily given by the degree of contact with a certain chemical substance (Sexton et al., 2004). Consequently, Human Biomonitoring (HBM), which is defined as *"the method for assessing human exposure to chemicals or their effects by measuring these chemicals, their metabolites, or reaction products in human specimens"* (CDC, 2005), was considered the 'gold standard' strategy. The main target in HBM studies is measuring the total body burden of environmental chemicals exposure using biomonitoring to assess its impact on human health. The accumulation of chemicals into the human body at detectable levels is known as the **body burden**, determined by two types of factors: **general factors**, including the concentration of the pollutant in a given environment, its physicochemical properties and exposure timing; and the **individual factors**, which involves the uptake, the metabolism and the excretion rates of a given environmental chemical (Sexton et al., 2004). Additionally, biomonitoring makes reference to the measurement of chemical pollutants in bodily fluids, such as blood, urine, saliva, or breast milk, and other species such as hair, nails, teeth, and feces.

As a result, HBM data are the most valuable for public health, environmental health research, public health surveillance, awareness-raising, and ultimately for policy-making decisions (Schwedler et al., 2017; Sexton et al., 2004). Because HBM studies are performed longitudinally in time, they can address spatial and time trends, and even lifestyle factors contributing to the exposure, such as diet. HBM is also used for the **prioritization** of chemical compounds and their regulation, for the **verification** of whether policy regulation, such as substance ban or usage restrictions, lead to a decrease

of the internal dose; and as **an alarm system** when elevated body burden and internal exposure is highlighted by exposure biomarkers. The drastic reduction of blood lead after the banned of this compound in petrol is a good example of the valuable role of HBM. Additionally, **individual absorption, metabolism, and excretion rates** are key targets included in HBM data, which allows a precise assessment of population risks and the highlighting of susceptibility groups. For example, BPA was banned for its use in baby bottles by the European Commission in 2011 after the increasing evidence of its deleterious effects on children's development (Regulation (EU) No 10/2011).

To assess the impact of chemicals exposure on human health, biomarkers of exposure, effect, and susceptibility, can be used in HBM studies combined with the health status of individuals, leading to the generation of exposure-effects associations, filling of gaps in knowledge, and new research hypothesis. For a better understanding of the cascade of events that occurred from the exposure to the disease, biomarkers are located into conceptual frameworks that organize and synthesize the scientific knowledge, called adverse outcomes pathways (AOP). The structure in which AOPs are formed begins with the first reaction between the stressor and the organism, known as the molecular initiating event (MIE), and the events that occurred from the MIE to the adverse outcome are called key events (KE).

However, many biomarkers are poorly understood or not fully described within the HBM context. Thus, attention should be paid to defining their properties in detail. For example, evidence regarding the temporal variation of exposure biomarkers should be obtained (e.g., using the metabolite instead of the parent compound to assess the biologically relevant dose); and the biological meaning of some/novel effect biomarker should be validated previous to its use in HBM studies. These previous steps will help for a correct interpretation of exposure-effect relationships and increased confidence of obtained associations. Therefore, and citing the NRC "*the ultimate objective of biomonitoring is to link information on exposures, susceptibility, and effects to understand the public health implications of exposure to environmental chemicals*" (NRC, 2006).

1.2 Environmental Pollutants

Global **environmental pollution**, including greenhouse emissions, acid deposition, or water pollution, is due to the industrialization, urbanization, and energy consumption activities performed by humans (WHO, 2015). **Environmental pollutants** are chemical substances present in the environment, some of them with hazardous

potential for human health. They have been linked with a wide variety of adverse health outcomes, such as respiratory diseases, allergy, increased oxidative stress, cardiovascular-, reproductive- and mental disorders, malignancies, and other clinical alterations (La Merrill et al., 2020). However, less information is available regarding the threads of these compounds, or their possible implication, on chronic non-communicable diseases, such as stroke, cancer, diabetes and, heart and chronic lung diseases (Kelishadi, 2012; Woodruff, 2015). Therefore they are considered public health concerns (Kelishadi, 2012).

1.2.1 Endocrine Disruptors

Some environmental pollutants can interact with the hormonal system leading to detrimental effects on human development. These compounds are known as **endocrine disruptors** (EDs). EDs are a heterogeneous group of man-made products used in the industry for several purposes, such as cosmetics (parabens, benzophenones), plastics (BPA and analogs, phthalates), lubricant and solvents (PCBs or PBDEs), and pesticides (malathion, diazinon, chlorpyrifos, mancozeb, etc.).

Mechanisms through which EDs alter the endocrine system were summarized recently in ‘‘ten key characteristics of ED chemicals’’, as reported by La Merrill et al., (2020), and briefly summarized as follows: **1) Interacting or activating hormone receptors:** EDs can bind to hormonal receptors, mainly nuclear receptors, eliciting signals that should not be produced. For example, BPA is a known activator of the estrogen receptor (ER) (De Falco et al., 2015; H.-R. Lee et al., 2013); **2) Antagonizing hormone receptors:** nuclear receptors acting as ligand-dependent transcription factors can be inhibited by EDs, thus altering the genomic response. Phthalates, BPA, or alkylphenols are known inhibitors of the androgen receptor (AR) (De Falco et al., 2015); **3) altering hormone receptor expression:** EDs chemicals can modulate the expression, internalization, and degradation of hormone receptors, thereby changing the hormonal availability. For example, bisphenol A (BPA) inhibits the degradation of ER and also alters the expression of oxytocin and vasopressin receptors (Cao et al., 2012; Patisaul, 2017); **4) altering signal transduction in hormone-responsive cells:** signal transduction is mediated through both membrane and nuclear hormone receptors, altered by EDs. As an example, the fungicide tolylflutanid reduces insulin receptor substrate 1, thus impairing insulin functioning (Sargis et al., 2012); **5) Inducing epigenetic modifications in hormone-responsive cells:** Epigenetic processes such as DNA and histone methylation are mechanisms used by hormones to exert permanent

modifications. However, EDs can alter the hormones' epigenetic action or directly alter these epigenetic processes, leading to hormonal disruption. It appears that in fact, PCBs can alter hypothalamic RNA expression (Topper et al., 2015); **6) altering hormone synthesis** by blocking or inhibiting intracellular and distant endocrine mechanisms, such as the update of iodine into thyroid cells performed by perchlorate, which decreases thyroid hormone synthesis (Lisco et al., 2020); **7) altering hormone transport across cell membranes:** hormones can cross the cellular membrane passively (the case of steroid hormones) or actively and both mechanisms can be altered by EDs. For example, BPA can reduce the secretion of insulin by decreasing calcium uptake in mouse pancreatic islet β-cells (Villar-Pazos et al., 2017); **8) altering hormone distribution or circulating hormone levels:** EDs can displace hormones from serum binding proteins, thus altering the hormonal bioavailability. In some *in vivo* models, the pesticide malathion reduced testosterone levels in testis, ovaries, and serum (Y. Zhang et al., 2016); **9) altering hormone metabolism or clearance:** once a hormone had fulfilled its activity, it is broken down by blood proteases (i.e. gonadotrophins) or is metabolized by enzymes (e.g. thyroid hormones), both mechanisms can be altered by EDs. PCBs for instance can inhibit the oestrone sulfotransferase, thus inhibiting the sulfation process (Kester et al., 2000); and finally, **10) altering the fate of hormone-producing or hormone-responsive cells** by promoting or disrupting cellular migration, proliferation, differentiation, and death. PCBs alter thyroid hormone signaling, which regulated cerebellum development, thus leading to abnormal morphology later in life (Gauger et al., 2007).

Depending on their physicochemical characteristics, EDs could be divided into persistent or non-persistent substances; **Persistent chemicals** refer to large molecular structures with numerous bonds, which makes them long-lasting, non-polar substances. Consequently, they are hardly metabolized by the detoxification systems of the human body, so they can be bioaccumulated into adipose tissue or different organs, such as the liver or kidneys, with adverse consequences (Krüger et al., 2012). Clear examples from this group of environmental chemicals would be polychlorinated biphenyls (PCBs) or polybrominated diphenyl ethers (PBDEs), but also **metal(oid)s elements** such as mercury (Hg), lead (Pb), cadmium (Cd), and arsenic (As) (Lenters et al., 2019; Sioen et al., 2013). Moreover, metals have a long history of toxicity to human health. Their main sources of exposure are contaminated food or water; for example, Cd and Pb are found in tobacco, but also crops and vegetables, especially Cd; Hg is found in big fishes such as tuna and shellfish, and As is found in industrial processes and contaminated water (Engwa et al., 2019). All of them are transported, distributed, and bioaccumulated into

the human body at tissue and cellular levels, where they bind to proteins and nucleic acids, thus disrupting important cellular functions. Impairments generated from metals exposure go from kidney, lung, and liver damage to mental disorders since they can cross the hematoencephalic barrier (Karri et al., 2016). In this regard, methyl-mercury and lead neurotoxic effects are the most studied, while limited or controversial evidence is available regarding Cd and As neurotoxicity when referring to childhood and adolescence exposure (Engwa et al., 2019; Rodríguez-Barranco et al., 2013).

On the other hand, **non-persistent chemicals** are short molecular substances with a low number of bonds, making them polar substances with a short duration in the human body (short half-life substances). They are metabolized by glucuronidation or sulfonation processes and, afterward, excreted in the urine, where they are used to be measured (Gore et al., 2015). Although these compounds have short half-lives in the human body, they can interact with different physiological systems, thus being hazardous for human health. Examples from this category would be **bisphenol A (BPA)**, phthalates, benzophenones, or some ''modern'' **pesticides** such as chlorpyrifos, diazinon, malathion, deltamethrin, cyfluthrin, carbaryl, or mancozeb. In this regard, **BPA** has been the attention focus during the last years due to its 'promiscuity', since it can bind to a wide range of nuclear receptors, thus up- or downregulating important signals that impair the hormonal homeostasis. BPA is used in the manufacture of polycarbonate plastics and epoxy resin liners of canned food, among others. Thus, its main source of exposure is through the ingestion of contaminated food or water. Among the effects of BPA, reproduction, neurodevelopment, and obesity outcomes have been the most studied in neonatal, early childhood, and adult population. From these outcomes, neurodevelopment is of special concern, since evidence of BPA effects on the developing brain is increasing. Thus, regulatory agencies and governments are beginning to regulate, and even ban, its use in some products, such as in bottled water, can liners, or thermal paper (Molina-Molina et al., 2019). Nevertheless, there are some gaps in knowledge regarding the mechanisms by which BPA can alter the developing brain in crucial windows of development as childhood or adolescence.

Modern **non-persistent pesticides** are increasing worldwide. Organophosphates (OPs) and pyrethroids are the two most common classes of insecticides, although the use of carbamates and dithiocarbamates is increasing as well (Furlong et al., 2017; Hernández et al., 2013). Due to their acute toxicity, the use of indoor OPs was banned, but they are still used in agriculture. Since their use is decreasing, the use of pyrethroids is increasing as biocides and in agriculture fields. As consequence, concentrations of

these compounds are commonly found in the urine of pregnant women and children, but also neonates' meconium and umbilical cord blood (Andersen et al., 2021; Dalsager et al., 2019). Moreover, neurodevelopmental effects have been found for children exposed prenatally or during early childhood to these compounds. The central nervous system (CNS) is extremely susceptible to exogenous stressors during the first stages of development, and changes produced in these stages could have an impact in future stages of life (Grandjean and Landrigan, 2006). Therefore, the study of pesticides' effects on key windows of development is crucial for their correct risk assessment and regulation.

1.2.2 Mixtures

Traditionally, toxicology and epidemiology have used a one-compound-at-a-time approach to assess the effect of environmental chemicals on human health. The advantages of this strategy rely on the quantification of the internal dose, the identification of exposure-effect relationships, and the identification of hazard ratios, which are very convenient for risk assessment. However, this approach is far away from real exposure, since humans are simultaneously exposed to hundreds of chemical substances at low doses (Bopp et al., 2018; Kortenkamp, 2014; Mustieles and Arrebola, 2020). Mixture assessment to xenobiotics involves considerable theoretical and practical challenges. Some decades ago, the cocktail/combined effect was considered to be negligible if single chemicals were below their non-observed adverse effect levels (NOAEL). However, it was proven that a mixture of estrogenic chemicals below their NOAELs tested *in vitro* exerted estrogenic effects in an additive manner (Kortenkamp and Faust, 2018). Consequently, international regulatory authorities and the scientific community realized that the combined effect risks have been underestimated for too long. Moreover, they also realized that previous approaches to assess the combined effect tended to ignore the synergistic, antagonistic, and/or additive interactions among chemicals (Kortenkamp and Faust, 2018).

As a result, there is a need for cumulative risk assessment, which means the development of new methodologies to assess the real exposure scenario. Evaluating multiple chemicals is complex since it could lead to a net of underlying mechanisms resulting in diverse health outcomes or effects. Moreover, these effects could follow linear or non-linear patterns depending on their concentration within the mixture or the physical and chemical properties of the single chemical compound (Hernández and Tsatsakis, 2017). Experimental models assessing chemicals effects pointed towards an

additive joint effect. Using this starting point, it is possible to use mathematical models to predict the potential effect. However, is not feasible to test every compound of a mixture, moreover if we take into account the presence of emerging contaminants, for which there is no information available (Kortenkamp et al., 2009; Pourchet et al., 2020; Vinggaard et al., 2021).

Thus, assessment of the chemical mixture effect is a multistage challenge that requires the expertise of different scientific fields, such as analytical chemists, toxicologists, and epidemiologists (Vinggaard et al., 2021).

1.3 Biomarkers

To describe environmental chemicals exposure and to assess adverse health effects, epidemiological studies have used biological markers, so-called **biomarkers**. This approach was born from the realization that *"traditional"* toxicology and epidemiology alone were not able to answer whether a disease was related to environmental chemicals exposure. Classical toxicological methods were not sensitive enough to identify intermediate events between the exposure and the clinical disease, consequently, exposure-effect epidemiological associations were not reliable enough to confirm the trueness of their associations. As a result, the consecutive steps that drive the exposure to a clinical disease were referred to as the epidemiological "black box" (Figure 1) (DeCaprio, 1997).

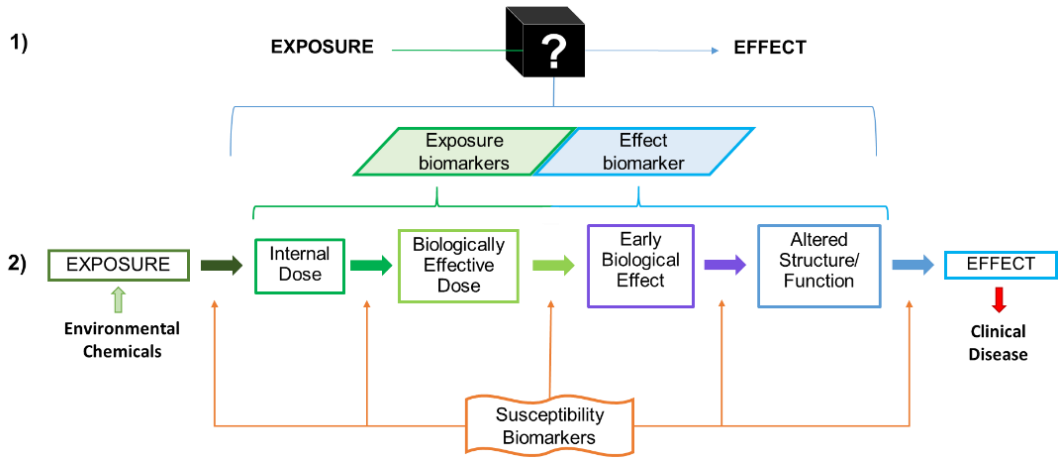


Figure 1. Epidemiological 'Black Box' according to the classic model linking exposure with disease (1) and NRC biomarker paradigm (2). Adapted from Decaprio, 1997.

Afterward, the National Research Council (NRC) divided the so-called *black box* into the following sequence of events: (i) internal dose, (ii) biologically effective dose,

(iii) early biological effect, and (iv) altered structure/function. These events are divided into two groups; events (i) and (ii) would refer to **exposure biomarkers**, whereas (iii) and (iv) to **effect biomarkers**. The intersections between events would correspond to **susceptibility biomarkers**. Nevertheless, these assignments are not exclusive, since the distinction between events is often vague and they should be taken as a continuous rather than a series of different events (Grandjean, 1995).

Thus, biomarkers reflect alterations at molecular and/or cellular levels that occur during temporal and mechanistic pathways, thus connecting environmental chemical exposure with a potentially adverse effect on human health and eventually, a disease (Studies, 2006). For a correct interpretation of the information given by biomarkers, several aspects have to be taken into account, such as the type of biomarker, the matrix in which is determined, and the moment in which is measured.

1.3.1 Biomarkers of exposure

Biomarkers of exposure are also known as biomarkers of **internal dose**, in other words, the absorbed fraction of a xenobiotic that enters the organism by crossing the physiological barriers. Exposure biomarkers, defined by the World Health Organization (WHO) as "t*he chemical or its metabolite or the product of an interaction between a chemical and some target molecule or cell that is measured in a compartment in an organism*" (WHO, 2001) are influenced by numerous factors, such as the characteristics of the xenobiotic, the route of exposure and the individual physiological characteristics.

Consequently, exposure biomarkers are indicators of **xenobiotic bioavailability**. Traditionally, measuring these biomarkers in biological samples such as blood or urine provide data of the internal dose. The measurement of the xenobiotic metabolite in a target biological matrix is considered a more relevant exposure biomarker, since it reflects the amount of xenobiotic that has been metabolized by the organism. In fact, for the assessment of pesticides in epidemiological studies, metabolites rather than parent compounds are often measured, such as urinary 3-phenoxybenzoid acid (3-PBA) to assess pyrethroids rather than measuring deltamethrin or cyfluthrin, or in the case of organophosphates, measuring 3,5,6-Trichloro-2-pyridinol (TCPy) rather than its parent compound, chlorpyrifos. Those cases in which the xenobiotic is measured together with the site of action, (e.g., adducts in total lymphocyte DNA), are considered biomarkers of **biologically effective dose**.

1.3.2 Biomarkers of effect

Effect biomarkers are fundamental indicators of the organism status as well as predictors of clinical disease. According to the WHO, biomarkers of effect are defined as ''*a measurable biochemical, physiologic, behavioral, or other alteration in an organism that, depending on the magnitude, can be recognized as associated with an established or possible health impairment or disease*'' (WHO, 2001). This definition is of outer importance for the present doctoral thesis since it constitutes the basis from which the search, identification, testing, and implementation of novel effect biomarkers have been performed and shown in the successive chapters.

Conversely to exposure biomarkers, effect biomarkers are associated with a **physiological system** rather than with the toxicity of a given xenobiotic. In other words, an effect biomarker can be a predictor only for neurotoxicity, but it can be associated with different environmental chemicals with neurotoxic potentials, such as methyl-mercury, lead, chlorpyrifos, diazinon, or phthalates. Moreover, an ideal effect biomarker should be peripherally measured and it should reflect alterations associated with a disease in the target tissue, especially for those with complicated accessibility, such as the brain.

Regarding the temporal trend followed in the construct of the NRC, effect biomarkers for **early biological effect** would be those which reflect the first results of xenobiotic-organism interactions. This group refers mainly to epigenetic markers, such as chromosomal aberrations, sister chromatid exchange, or enzyme induction/inhibition. Meanwhile, effect biomarkers from an **altered structure or function** would reflect changes that can be appreciated at micro and macromolecular levels. Thus, a wide range of biomarkers could be included, from those measured in biological tissues such as 8-hydroxy-2-deoxy-guanosine (8-OHdG) in urine, or dopamine and hormones in serum, to the anogenital distance, magnetic resonance images (MRI), pain assessment scales, or behavioral tests.

The Human Biomonitoring Initiative in Europe (HBM4EU) is looking at health effects elicited by chemical exposure, using and combining data on biomarkers of exposure to contaminants of particular interest (e.g., phthalates, bisphenols, heavy metals) and on biomarkers of effect, together with information on mechanisms of action from experimental studies. Effect biomarkers should allow for the reliable and simple identification and measurement of specific biological changes produced by the chemical compound of interest, and the measurements must be accurate, precise, reproducible, and interpretable. Additional information is available on the HBM4EU project website:

https://www.hbm4eu.eu/ and can be consulted in the following deliverables and additional deliverables: D14.4, AD14.4, AD14.6, D14.7 (Mustieles et al., 2018; Fernandez et al., 2019; Rodriguez-Carrillo et al., 2020; Fernandez et al., 2021).

1.3.3 Biomarkers of combined effect

The aim of **biomarkers of combined effect**, is to map the biological effects that own a given mixture of environmental chemicals previously isolated from human samples, such as serum, blood, or placenta. Consequently, these kinds of biomarkers are also known as ''biomarkers of internal exposure'', ''biomarkers of combined activity'', or ''biomarkers of *ex vivo* hormonal activity'' (Vinggaard et al., 2021).

The assessment of mixtures using *in vitro* and *in vivo* models to detect and quantify biological effects without the need for screening for hundreds of chemicals have been performed in the ecotoxicological field (*i.e.* monitoring changes on river waters) for years (Abbas et al., 2019; Bopp et al., 2018). This approach can be used with human matrices such as blood, urine, or placenta in the same receptor-based models. However, the presence of endogenous hormones would mask the combined effect of the mixture, thus making this approach useless for risk assessment purposes (Arrebola et al., 2012). Chromatographic methods are extremely useful in this case since they can isolate fractions of chemicals based on their physicochemical characteristics. Thus, allowing the separation of a ‘‘real-world’’ mixture of lipophilic environmental chemicals from endogenous hormones and its subsequent testing in receptor-based assays, taking into account the interaction among chemicals. With this approach, different biological effects can be described for a given mixture, since it can be tested in a wide range of assays: from *in vitro* models testing the activation of the estrogen receptor to *in vivo* models addressing the effect on thyroid functioning (Fernandez et al., 2007c; Leusch et al., 2018). Thus, contributing to the understanding of mixtures effects.

Biomarkers of combined activity can be used within an epidemiological context, thus giving another tool for mixtures risk assessment. However, they are not useful to quantify the chemical components of a given mixture. For that purpose, effect-directed analyses should be performed (Vinggaard et al., 2021). Previous epidemiological studies used these biomarkers to assess the effects of persistent organic pollutants in diverse human health outcomes, such as breast cancer in the adult population or, autism spectrum disorders (ASD) and risk of cryptorchidism and hypospadias in newborns. Results from these studies showed that these mixtures were linked to a higher risk of breast cancer, and urogenital male malformation, respectively (Arrebola et al., 2015;

Bonefeld-Jorgensen et al., 2011; Ghisari and Bonefeld-Jorgensen, 2009; Ibarluzea et al., 2004).

The use of effect biomarkers in the context of HBM are not fully studied and could fill those gaps in knowledge that classical toxicology and epidemiology could not answer. For that reason, in this doctoral thesis, we asked whether biomarkers of combined effect would be of utility to depict different biological effects from the same mixture and whether biomarkers of effect could contribute to the understanding of causal inferences on exposure-effect associations.

1.3.4 Biomarkers of susceptibility

According to the WHO, biomarkers of susceptibility are defined as ''*an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific chemical substance*'' (WHO, 2001). Susceptibility biomarkers reflect the conditions that increase, or decrease, the odds for the transition from one event to another, or even more than one event. This sensitivity could be acquired, (e.g., a disorder that makes individuals more susceptible to environmental chemicals) or inherited, when it is indicated by genetic markers. This is very important, since biomarkers of susceptibility are, in other words, indicators of **interindividual toxicity**. Consequently, they do not follow the timeline stages proposed by the NRC construct. One example of susceptibility biomarkers would be the genetic polymorphisms of enzyme activity, such as the glutathione-S-transferase (GST) or N-acetyltransferase (NAT), key catalyzers of xenobiotics.

1.4 Brain-derived neurotrophic factor as an example of effect biomarker

Brain-derived neurotrophic factor (BDNF), firstly discover in 1982 and isolated from pig brain in 1987 (Leibrock et al., 1989), is a protein from the family of neurotrophins, which also includes nerve growth factor (NGF), neurotrophin 3 (NT3) and neurotrophin 4 (NT4) (Bilgiç et al., 2017). It is expressed throughout the brain, although is highly expressed in the cerebral cortex, cerebellum, hippocampus, and amygdala (Notaras and Buuse, 2018; Zheleznyakova et al., 2016). BDNF plays a key role in a wide range of neurological processes, concretely synaptic plasticity, regulation of glio-, neuro-, and synaptogenesis, neuroprotection, and control of long and short-lasting

synaptic interactions, thus influencing memory and cognition (Binder and Scharfman, 2004; Kundakovic et al., 2015). These roles are given by its characteristic pattern of synthesis, in which different biological active isoforms interact with different receptors, thus up- or down-regulating numerous signaling pathways (Kowiański et al., 2018). Briefly, BDNF is secreted from the endoplasmic reticulum as its precursor form, pre-pro-BDNF, which is biologically inert. Afterward, it is translocated to the Golgi apparatus, where the pre-region is cleaved by intracellular proteases, leading to the formation of the immature isoform, **pro-BDNF**, characterized for showing an N-terminal pro-domain and for being biologically active. This isoform is further cleaved, forming the mature isoform, **m-BDNF**, biologically active with a C-terminal mature domain (Foltran and Diaz, 2016). Interestingly, the stage from pro- to m-BDNF can be performed in different cell compartments: in the trans-Golgi network, performed by furin, in the intracellular secretory vesicles, by convertases, and in the extracellular space, by plasmin or matrix metalloproteases 2 and 9 (MMP2 and MMP9) (Cunha et al., 2010; Kowiański et al., 2018). To exert their physiological processes, both BDNF isoforms are secreted into the extracellular space after membrane depolarization, often showing opposite biological activities due to their receptor-binding preferences. Description of BDNF-receptors signaling pathways are fully explained in the reviews performed by Kowiański et al., (2018) and Reichardt, (2006), briefly summarized as follows.

m-BDNF binds to the tyrosine kinase B receptor (TrKB), which belongs to the tropomyosin-related kinase (Trk) family, triggering the formation of the phosphorylated-TrkB receptor, which activates several enzymes: phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), guanosine triphosphate hydrolases (GTPases) of the Ras homolog (Rho) gene family, and phospholipase C-γ (PLC-γ) (Kowiański et al., 2018; Reichardt, 2006). All these enzymes initiate different signaling cascades with specific cellular functions. For instance, PI3K/Akt modulates the synaptic plasticity dependent on N-methyl-D-aspartate receptors (NMDAR) and PI3K/Akt/mTOR enhances dendritic growth and branching through the regulation of protein synthesis. MAPK is required for the activation of cAMP response element-binding protein (CREB) and extracellular-signal-regulated kinase 1/2 (ERK 1/2), both important for cytoskeleton protein synthesis, early response gene expression, as well as branching and dendritic growth in hippocampal neurons. GTPases cascade also contributes to dendritic growth-inducing the synthesis of microtubule and actin. Finally, PLC-γ protein activates the kinase C (PKC) dependent pathway, which increases synaptic plasticity. In summary, the binding of m-BDNF with TrKB initiates cascades which

mechanisms of action lead to antiapoptotic and prosurvival activities as well as enhancement of dendritic growth and branching.

On the other hand, **pro-BDNF** binds to p75 neurotrophin receptor (p75NTR), which forms the pro-BDNF/p75NTR/sortilin complex, leading to a c-Jun amino-terminal kinase (JNK), nuclear receptor kappa B (NR-κB), and Ras homolog gene family member A (RhoA) activation (Kowiański et al., 2018; Reichardt, 2006). Through the JNK signaling cascade, neuronal apoptosis is activated, thus leading to neuronal cell death. Conversely, the activation of NR- κB promotes survival and maintenance processes for an optimal neuronal cell number. Finally, the RhoA cascade regulates neuronal growth cone. In summary, pro-BDNF binding to its receptor triggers signaling pathways involved in the fate of neurons from different brain areas, thus promoting cell death or survival. Nevertheless, it has been reported that high levels of pro-BDNF led to the elimination of neuronal cells rather than neuronal cell survival (Teng et al., 2005).

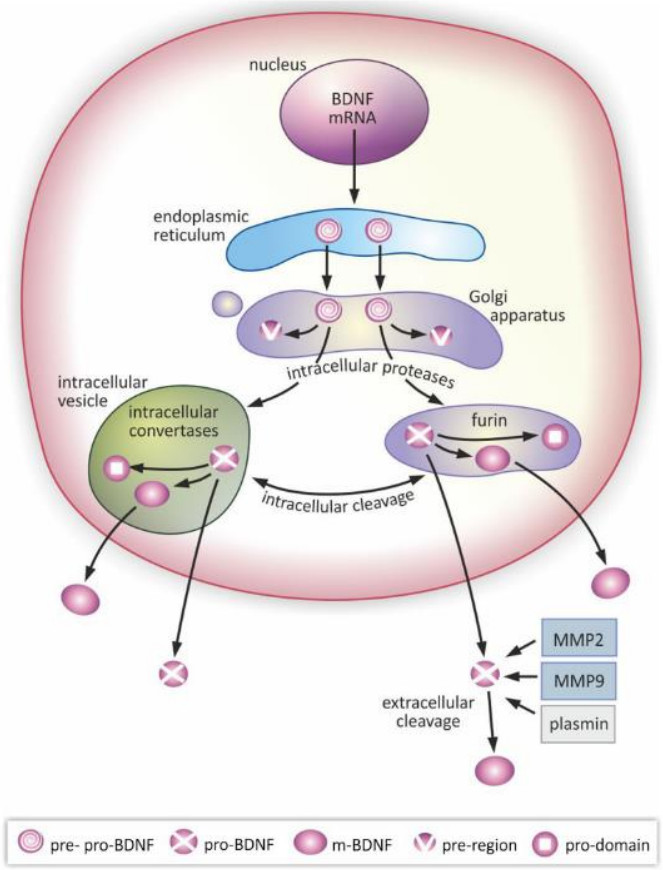


Figure 2. BDNF synthesis patterns, picture taken from Kowiański et al., (2018).

The most outstanding aspect of BDNF is based on the mature and pro-BDNF relationship. Both isoforms should be maintained in a dynamic balance not only during

the entire brain development but during the entire life of the individual. The ratio of m-BDNF/pro-BDNF changes according to the stage of brain development. For example, levels of pro-BDNF are higher during the early post-natal period, when synaptic pruning takes place. Conversely, levels of m-BDNF will be higher during adulthood, since the maintenance of efficient neural circuits and functional connections will be prioritized. Consequently, there would be a higher risk of developing mental health disorders if this balance is broken. Alterations of BDNF protein levels and its DNA methylation patterns are associated with mood and cognitive alterations in previous epidemiological studies.

Briefly, levels of **serum and plasma BDNF** increased after treatment for major depressive and bipolar disorder among treatment responders' patients (Polyakova et al., 2015). Additionally, serum BDNF changed according to the presence of depression in patients with Parkinson's disorder (PD) and was also a good indicator of motor severity (Huang et al., 2020). In a case-control study, BDNF was found as a good indicator for stroke recovery (Pedard et al., 2018); and in a longitudinal study, preterm children with higher levels of neurotrophins (BDNF among them) at 2 weeks of age were associated with lower risk of cognitive impairments at ten years old (Kuban et al., 2018). Moreover, ***bdnf* gene DNA methylation** at promoter I and IV are being proposed as a biomarker for specific diseases, especially for bipolar disorder and major depression (Fuchikami et al., 2011; Zheleznyakova et al., 2016). Conversely, according to some clinical trials, levels of plasma BDNF did not change according to treatment, either schizophrenia relapse (Pillai et al., 2018). Additionally, there were no associations between changes in serum and plasma BDNF levels and depressive symptoms of twenty-four patients with depression undergoing electroconvulsive therapy (Vanicek et al., 2019).

It also has been shown that levels of BDNF are susceptible to change due to exposure to environmental chemicals. Therefore, it could be a valuable effect biomarker reflecting brain impairments due to environmental chemical exposure. However, mechanisms of action by which these compounds could alter BDNF protein or *bdnf* DNA methylation are not fully described (Zheleznyakova et al., 2016). In some epidemiological studies, alterations in children's BDNF protein levels have been associated with prenatal exposure to methyl-mercury and Cd; further, infants exposed prenatally to Cd showed social problems at 12 months of age (Spulber et al., 2010; Wang et al., 2016). Additionally, lower plasma and serum BDNF levels were associated with occupational exposure to manganese (Mn) and with chronic As exposure in the adult population (Karim et al., 2019; Zou et al., 2014). Metals have not been the only environmental pollutants associated with BDNF alterations. Three epidemiological studies from North American and Chinese longitudinal cohorts addressing prenatal

exposure to PAHs also found alterations of peripheral BDNF protein levels (Kalia et al., 2017; Perera et al., 2015; Tang et al., 2014). Moreover, prenatal exposure to BPA has been associated with higher *bdnf* gene DNA methylation percentages at several CpGs (Kundakovic et al., 2015). Results from this study suggested that DNA methylation of *the bdnf* gene measured in whole blood correlated with the same DNA methylation patterns in the hippocampus. This suggests a good biomarker candidate since DNA methylation is more stable during time than serum/plasma BDNF protein levels (Kundakovic et al., 2015).

Although is still controversial, scientific evidence points toward BDNF as a suitable indicator of neurodevelopment. Additionally, there are associations between alterations of its secretion patterns and exposure to environmental contaminants. Therefore, we asked whether this neurotrophin could be useful in HBM studies as a biomarker of effect to neurodevelopmental impairments.

1.5 Relevant human health outcomes: neurodevelopment

Among the health outcomes impaired by environmental chemicals exposure, neurodevelopment is one of the highest concerns for environmental health, since its consequences range from individuals‘ lower quality of life to the welfare of entire societies (Grandjean and Landrigan, 2006). The increasing incidence of autism spectrum disorders (ASD), attention deficit hyperactivity disorders (ADHD), and subclinical decrements in brain functioning among children and adolescent population are known to be partly caused by the exposure to environmental pollutants, constituting the so-called ,'' silent pandemic of neurodevelopmental toxicity‘‘, firstly denounced by Philippe Grandjean and Philip Landrigan (Grandjean and Landrigan, 2014). This statement is based on the characteristics of brain development, since it is a long process in which complex cascades of events, highly vulnerable to exogenous stressors, can be altered depending on whether a xenobiotic (or its metabolite) enters the developing brain, and the period of development in which the exposure takes place (Supke et al., 2021).

The **developing brain** is a susceptible process in which any alteration could lead to irreversible adverse effects for individuals. It starts soon on day 25 after conception when the neural tube begins to form, and it last until early adulthood. During the third week of gestation, neural progenitor cells will be dividing and differentiating into both, neurons and glia, thus forming the basis of the premature nervous system (Stiles and Jernigan, 2010). Along with pregnancy, the early brain structure suffers progressive changes, leading to the characteristic folds that designate the basis of brain regions. At

the cellular level, neurons begin to produce chemical signaling molecules, enabling communication between nerve cells from different areas, thus forming the fiber pathways. At gestational week 10 (end of embryonic period), the basics of the neural system are considered to be established and billions of fibers connect different brain regions, forming complex networks known as the *connectome* (Ardesch et al., 2019; Stiles and Jernigan, 2010).

Although the assemble of the functional brain take place in the prenatal period, it still develops after birth, when the number of connections grows exponentially, determined by sensory inputs. After 2 years of age, the brain reorganizes the connectome through synaptic pruning, in which inefficient connections are removed to maximize its performance (Konkel, 2018). By the age of 6, the brain reaches 90% of its adult volume and by the age of 25, it is considered fully developed (Ardesch et al., 2019; Konkel, 2018; Stiles and Jernigan, 2010). Within this temporal range, adolescence dramatically reshapes teenagers' perception of themselves, as well as their social context through physical, neural, and interpersonal changes to start the transition into adulthood (Gore et al., 2018). Adolescents must exhibit social-communicative skills, reproductive behaviors, adequate anxiety responses, and affective states according to their age and sex (Spear, 2000). Neural mechanisms by which these changes take place are complex since it requires a network of diverse brain areas interacting among them and mediating specific aspects of behavioral functioning (Gore et al., 2018).

In this regard, dendritic spine turnover and inhibitory neurotransmission are the two main neural processes involved during adolescence (Pfeifer and Allen, 2021). For instance, a higher turnover of the dendritic spines during adolescence enhances synaptic plasticity, and thus the experience-dependent learning, while higher inhibition of neurotransmission could be particularly beneficial to follow an adequate social behavior (Pfeifer and Allen, 2021). The increasing incidence of mental health disorders among adolescents, such as social anxiety, depression, or eating disorders may be enhanced by exposure to environmental pollutants (Bjørling-Poulsen et al., 2008; Bouchard et al., 2010; Pfeifer and Allen, 2021; Shoaff et al., 2020; Supke et al., 2021). However, as adolescence has been an understudied period of development, little is known regarding the neurological impact of exposure to environmental chemicals. Thus, we asked to what extent the exposure to persistent and non-persistent chemicals compounds could alter the behavioral functioning of adolescents. Given the transcendence of metals, BPA, and non-persistent pesticides on neurodevelopment and the current state of knowledge regarding their neurotoxicological effects on this population, we decided to evaluate

these compounds. These questions will be addressed in articles #4, 5, and 6 from the results section.

One of the main limitations found in the assessment of neurological impairments due to exposure to environmental chemicals in neuropsychological evaluation. The most common assessment for brain development, in the context of environmental epidemiology, is through **psychological tests**, such as the *Child Behavior Check List 6/18* (CBCL 6/18) for behavioral functioning, or the *Wechsler Intelligence Scale for Children* (WISC) for cognitive functioning, among others. These tools, although scientifically proven to be useful, might be biased by the subjectivity of the evaluator (e.g. when parents make the psychological evaluation) (Howieson, 2019). Additionally, they are not used harmoniously in epidemiological studies, since for the evaluation of a given brain function, there are different scales with different degrees of sensitivity and specificity, thus increasing the uncertainty of exposure-effect associations. Moreover, mechanisms of action through which chemical compounds disrupt brain functioning are not completely understood and cannot be retrieved using this method. Therefore, we asked if the use of a novel effect biomarker could be useful as an indicator of the neurological impact after exposure to environmental chemicals and if it could be of added value for epidemiological studies.

RATIONALE & OBJECTIVE

2 Rationale

Global exposure to environmental pollutants is affecting the human health. To date, there is not enough knowledge about human internal dose of chemicals of high concern, such as BPA, metals, or non-persistent pesticides, among others. Even less evidence is available regarding the human adverse effects exerted by mixtures of environmental chemicals. This prevents definitive conclusions on the adverse effect of some exposures to environmental pollutants on human health. Consequently, new strategies are needed to enhance human exposure-effect relationships. Biomarkers of effect could be valuable indicators of potential adverse effects exerted by exposure to environmental chemicals. In addition, the use of such biomarkers would be of great value in filling existing gaps in knowledge.

As a result, Human Biomonitoring studies are making a great effort to describe chemical exposure at individual and populational levels as well as to assess their effect on human health.

The present doctoral thesis has been developed under the framework of the Human Biomonitoring for Europe initiative (HBM4EU), a joint effort of 30 countries, the European Environment Agency (EEA) and the European Commission (EC), taking part in this project through the European Union (EU) Policy Board. HBM4EU started in 2017 and was co-funded by the EC's Horizon 2020 research program. The main aim of this project is to generate knowledge regarding the environmental exposure to target chemicals and chemicals groups of the European population, the impact of such exposure on human health, and ultimately, to answer policy-relevant questions for chemical prioritization (Ganzleben et al., 2017).

3 Objective

The main aim of the present doctoral thesis was to select, describe, test, and implement effect biomarkers for the assessment of exposure-health associations during critical windows of human development, related to cognitive and behavioral alterations. For this purpose, the following specific objectives were proposed:

a) To identify and prioritize existing biomarkers of effect for BPA, metals, pesticides, and complex mixtures.
b) To provide relevant mechanistic and adverse outcome pathway (AOP) information to cover knowledge gaps and enhance the interpretation of effect biomarkers data.
c) To investigate the effects of chemical exposure with cognitive domains, intelligence quotient (IQ), and behavioral function among Spanish adolescent males from the INMA-Granada cohort.

The work carried out in accordance with the proposed objectives has resulted in six scientific articles, with the following objectives:

1. To identify and prioritize existing biomarkers of effect for BPA, as well as to provide relevant mechanistic and adverse outcome pathway (AOP) information in order to cover knowledge gaps and better interpret effect biomarker data.
2. To investigate the association of urinary BPA concentrations with cognitive domains and intelligence quotient (IQ) in boys.
3. Assess whether childhood BPA exposure (9–11 years) is longitudinally associated with behavioral function at adolescence (15–17 years), and BDNF; the cross-sectional relationship between BDNF biomarkers and behavior in adolescents; and whether BDNF biomarkers may mediate BPA-behavior associations.
4. To explore the relationships among exposure to environmental metals, BDNF biomarkers at two levels of biological complexity, and behavioral function in adolescent males.
5. To investigate the relationship between exposure to various non-persistent pesticides, BDNF, and behavioral functioning in adolescents.
6. To assess, characterize, and compare the combined biological effect of chemical mixtures extracted from the same human placentas by quantifying the signal elicited in five bioassays, covering relevant endocrine activities (*in vitro* proliferative and reporter gene assays for estrogen, androgen, and aryl hydrocarbon, and *in vivo* thyroid function).

MATERIALS & METHODS

4 Material and Methods

4.1 Study population

Most of the specific objectives set out in this doctoral thesis have been tested in the Spanish birth cohort "Environment and Childhood" (INMA-*Infancia y Medio Ambiente* –) is a mother-child birth cohort developed in seven regions of Spain: Asturias, Gipuzkoa, Sabadell, Ribera d'Ebre, Menorca, Valencia, and **Granada**. The main aim of this population-based study is to evaluate the impact of environmental chemicals exposure during key windows of susceptibility, such as pregnancy, early childhood, puberty, and adolescence, on growth and development (Guxens et al., 2012).

The INMA-Granada subcohort started between 2000 to 2002, in which 668 mother-son pairs were recruited at delivery in San Cecilio University Hospital from Granada province (Spain) (Fernandez et al., 2007c). Since then, several follow-ups of the indexed families have been carried out. The second follow-up enrolled a random subsample of 220 boys at 4-5 years in 2004-2006. During the third follow-up, which was developed in 2010-2012, a total of 300 peripubertal boys aged 9-11 years were accepted to participate. Finally, during the last follow-up, carried out in 2017-2019, boys from second and third follow-ups were re-contacted and asked to participate. Thus, a total of 151 adolescent males aged 15-17 years agreed and underwent a medical examination and sampling procedures (Castiello et al., 2020).

At each follow-up, socio-demographical, clinical, and dietary information was obtained through administered questionnaires; in addition, biological samples (placenta, urine, blood, and serum) were collected. All follow-ups followed the principles of the declaration of Helsinki and were approved by the Ethics Committee of San Cecilio University Hospital.

4.2 Biomarkers of exposure assessment

4.2.1 Bisphenol A

Bisphenol A (BPA) was measured in single spot urine samples from children of the INMA-Granada at the second follow-up, aged 9-10 years. Urine samples were collected under non-fasting conditions between 5 p.m. to 8 p.m., using 10 mL polypropylene tubes. Samples were immediately stored at -20 ^{0}C. Total BPA (free plus conjugated) was

quantified using liquid chromatography-mass spectrometry (LC/MS) in the laboratory of the Department of Analytical Chemistry, University of Cordoba (Spain). Before quantification in the spectrometer, samples underwent a pre-treatment. Briefly, 1.25 mL of urine sample was fortified with 2.5 μg/L $^{13}C_{12}$-bisphenol A as internal standard and 625 μL of β-glucuronidase H1 and sulfatase H1 solution. Conditions of each enzyme consisted of 926 U/mL, in 1 M of ammonium acetate buffer at pH 5. Afterward, 2 mL of the treated sample was added into glass tubes, closed with aluminum caps, and incubated overnight at 37^{0}C. Successively, the sample was quantified using an AB Sciex 4000 Qtrap® mass spectrometer coupled to an Agilent 1200 Series LC system with a negative-ion TurboSpray interface. Limit of detection (LOD) and quantification (LOQ) were 0.1 mg/L and 0.2 mg/L, respectively. To avoid BPA contamination due to urine collection containers, labware, or LC equipment, LiChrosol water was filtered using 47 mm Styrene DVB (SDB-XC) disk provided by Empore (3 M, St Paul, Minnesota, USA), and glassware and eppendorfs tubes were rinsed with methanol before their use.

4.2.2 Metal(oid)s elements

Total urinary concentrations (organic and inorganic) of the metals As, Cd, Pb, and Hg were measured in the INMA-Granada adolescent males aged 15-17 years (third follow-up). For that purpose, spot urine samples under non-fasting conditions were collected and stored at -80 ^{0}C until analysis at the Department of Legal Medicine, Toxicology and Physical Anthropology, of the University of Granada (Spain). All-metal(oid)s elements were measured following the same procedure by inductively coupled plasma mass spectrometry using an Agilent 8900 triple quadrupole ICP-MS (Agilent Technologies, Santa Clara, CA, USA) as extensively reported in a previous study (Castiello et al., 2020). Briefly, for quality control and assessment of the analytical procedure, samples were spiked with multielement internal standard solution (400 μg/L) containing Sc, Ge, Ir, and Rh; blanks, calibration standards; and specific certified materials according to the US National Institute of Standards and Technology: Trace Elements in Natural Water Standard Reference Material SRM 1640a and Seronorm (Sero, Billingstad, Norway), and Trace Elements Urine L1 and L2 (references 210605 and 210705, respectively). LOD for total As, Cd, Pb, and Hg were 0.60 μg/L, 0.01 μg/L, 0.16 μg/L, and 0.05 μg/L, respectively.

4.2.3 Non-persistent pesticides

Urinary concentrations of several non-persistent pesticide metabolites were measured in the INMA-Granada adolescent males aged 15-17 years (third follow-up). Five OP metabolites: 3,5,6-trichloro-2-pyrimidol (TCPy), metabolite for chlorpyrifos, 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMPy), metabolite of diazinon, malathion dicarboxylic acid (MDA), metabolite of malathion, diethyl thiophosphate (DETP), metabolite of chlorpyrifos-ethyl, and diethyl dithiophosphate (DEDTP), metabolite for chlorpyrifos-ethyl. Two **pyrethroids**: 2,2,dichlorovinyl-2,2-dimethylcyclopropane-1-carboxilic acid (cis-trans-DCCA), metabolite for alpha-cypermethrin, beta-cyfluthrin, and 3-phenoxybenzoic acid (3-PBA), unspecific metabolite for pyrethroids. One **carbamates** metabolite: 1-naphthol (1-N), a metabolite of carbaryl; and one **dithiocarbamates** metabolite: ethylene thiourea (ETU), metabolite for mancozeb. For that purpose, spot urine samples collected under non-fasting conditions were stored at -80^{0}C at the facilities of the Biomedical Research Center (CIBM), of the University of Granada (Spain). Metabolites were measured following different methodologies according to their chemical characteristics. Assessment of TCPy, IMPy, DETP, DEDTP, 3-PBA, 1-N, and ETU were done in ultra-high-performance liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS), using UHPLC Ultimate 3000 and Q Exactive Focus mass spectrometer, both provided by Thermo Fischer. These metabolites were quantified at the facilities of the ''UNETE research unit'' of the Biomedical Research Center (CIBM), from the University of Granada (Spain). The whole procedure and reagents used are detailed explained in previously reported studies from our research group (Freire et al., 2021; Suárez et al., 2021). Quantification of MDA and DCCA metabolites was performed using liquid chromatography coupled to mass spectrometry (LC-MS/MS), in an Agilent 1290 liquid chromatography and API 4000 mass spectrometry (provided by Agilent and AB Sciex Instrument, respectively). Both metabolites were measured at the facilities of MEDINA Foundation, Granada (Spain). Limits of detection were 0.039 µg/L for TCPy, 0.117 µg/L for IMPy, 0.052 µg/L for MDA, 0.116 µg/L for DETP, 0.142 µg/L for DEDTP, 0.117 µg/L for 3-PBA, 0.055 µg/L for DCCA, 0.156 µg/L for 1-N, and 0.072 µg/L for ETU.

4.2.4 Chemical Mixtures assessment

We examined exposure to environmental chemical mixtures by assessing the combined effects of chemicals extracted from human placentas. Thus, twenty-five placenta samples from healthy women of the INMA-Granada cohort were randomly selected among those kept at the biobank of the San Cecilio University Hospital (Granada,

Spain). Placental homogenates were extracted following a previously validated semipreparative HPLC protocol (Fernandez et al., 2004) to efficiently separate organohalogenated lipophilic chemicals from endogenous hormones and more polar compounds, using a normal-phase column and a gradient with two mobile phases. Briefly, 3 g of placenta homogenate was split into two 5 mL Falcon Tubes with 1.5 g each. Successively, polar and non-polar compounds were extracted from placental homogenates by splicing 3 g of homogenate into 1.5 g per Falcon tube. Afterward, 1.5 mL of distilled water was added and shacked for 1 min; immediately after, 3 mL of ethyl acetate was added, vortexed for 10 min and centrifuged for 10 min, 4050 x g at 4 ^{0}C. The two obtained supernatants were then pooled in a clean glass vial, and the extract was evaporated under a nitrogen stream. The dried extract was dissolved with 700 µL of hexane and dried again under a nitrogen stream. Then, it was resuspended in 400 µL of hexane and injected twice (200 µL) to undergo preparative HPLC. The placental extract was eluted by a specific gradient of two mobile phases: n-hexane (phase A) and n-hexane:methanol:2-isopropanol (40:45:15)(v/v) (phase B) at a flow rate of 1.0 mL/min. The α-fraction, containing non-polar compounds and isolated from endogenous hormones was eluted during the first 11 minutes of the chromatographic run (Fernandez et al., 2004).

4.3 Biomarkers of effect assessment

For a correct implementation of effect biomarkers in human biomonitoring programs, it is necessary to carry out a careful selection and validation process. In HBM4EU, selected biomarkers of effect are been investigated in human observational studies, focusing on specific health problems concerning reproduction, neurodevelopment, and the immune system. In this doctoral thesis work, BDNF was selected as a biomarker of effect to investigate possible alterations of the human brain and behavior due to some environmental chemicals.

4.3.1 Brain-derived neurotrophic factor

Adolescents collected the first-morning urine sample as previously detailed. Peripheral venous blood was collected under non-fasting conditions between 5 p.m to 7 p.m. Blood samples were processed to obtain whole blood and serum aliquots. Urine and serum samples were stored at -80 ^{0}C until analysis, whereas whole blood was sent on dry ice to the Human Genotyping Laboratory at the Spanish National Cancer Research Centre. Genomic DNA was extracted using Maxwell® RSC equipment, quantified with

PicoGreen assay, and normalized to 50 ng/µL. Afterwards, extracted DNA was stored at −80 °C and sent to IRSET (Institut de Recherche en Santé, Environnement et Travail - INSERM UMR1085), Rennes, France, to perform DNA methylation analyses.

4.3.1.1 Serum and urine BDNF: methodology and validation

Total (pro and mature BDNF isoforms) serum and urine BDNF concentration analyses were performed using commercials enzyme-linked immunosorbent assays (ELISA) according to manufacturers' instructions (serum BDNF: Quantikine ® R&D Systems, Minneapolis, MN, US; urine BDNF: RayBio® Raybiotech, Norcross, GA, USA) at the facilities of the Biomedical Research Center (CIBM), University of Granada, (Spain).

Briefly, **serum samples** were defrosted, vortexed, aliquoted in 10 µL, and diluted 100-fold. Afterward, they underwent ELISA analysis. The full description for serum BDNF determination is explained in the results section. Serum BDNF concentrations were expressed in ng/ml. **Urine samples** were defrosted and pre-treated according to the protocol developed by Collins and Koven, (2014) with minor modifications. Afterwards, extracted urine samples underwent enzyme-linked immunosorbent assay following manufacturer's instructions to measure total BDNF concentrations.

To assure the comparability of BDNF values assessed with ELISA kits, a quality control procedure was developed, showed in Fig. 3 (Fernandez et al., 2021).

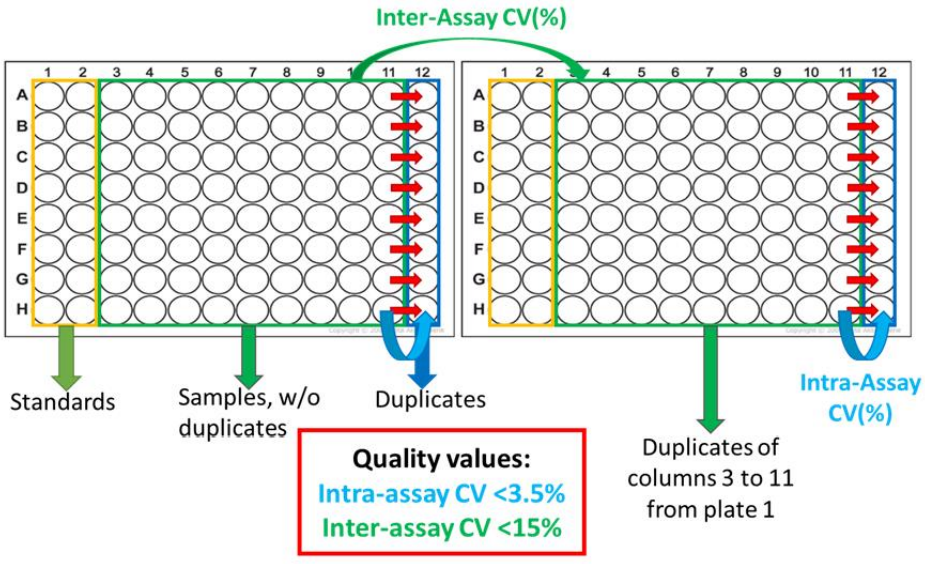


Figure 3. Esqueme of quality control procedure to measure serum and urine BDNF concentrations.

Briefly, two 96-well plates were used per batch of 72 samples. Rows 1 and 2 are charged with standards, and rows 3 to 12 are charged with samples. Concretely, individual samples were added in rows 3 to 11 on plate 1 and their respective duplicates are charged in plate 2. Therefore, an inter-assay coefficient of variability (CV) can be calculated for each sample. To calculate the intra-assay CV, duplicates of row 11 are also charged in row 12. To guarantee the lowest possible variability of measurements, BDNF analyses were performed by a single operator. Samples showing intra and inter-assay CV above 5% and 15%, respectively were re-tested and the mean of the three values was considered as the final BDNF concentration.

4.3.1.2 DNA methylation of the BDNF gene

Briefly, DNA methylation analyses were performed using the bisulfite pyrosequencing technique at IRSET (Institut de Recherche en Santé, Environnement et Travail- INSERM UMR1085), Rennes, France. Genomic DNA concentration and purity were measured using NanoDrop (Thermo Scientific NanoDrop 8000; DNA50 mode). Samples had approximately 1.8–1.9 ratio at 260/280 absorbance, thus the extracted DNA was deemed as pure. The concentration of DNA was further assessed with the QuantiFluor dsDNA system (provided by Promega E2670). Successively, bisulfite conversion of 500 ng of genomic DNA was performed using the Epitec Fast Bisulfite Conversion Kit (Qiagen, 59826). Concentration and purification were remeasured with NanoDrop (Thermo Scientific NanoDrop 8000; RNA40 mode). Afterward, 20 ng of BS-converted DNA was used for downstream PCR amplification of BDNF (Biometra TProfessional Thermoycler, France) with Takara EpiTaq hot-start DNA polymerase (Takara, R110A; 0.6 U/25 µl final concentration). The conditions under which this procedure was performed together with the used primers for BDNF ampliation are detailed in the results section. After PCR amplification, products were purified and loaded in 2 % agarose gel (Qiagen, 28006). Samples were sent for pyrosequencing analyses to the Genomic Platform LIGAN (Lille Integrated Genomics Advanced Network for personalized medicine), France (Pyromark Q24 Advanced Pyrosequencing technology). The degree of DNA methylation was expressed as a percentage and underwent quality control due to variations in PCR efficiency using the Pyromakr Q24 software. Thus, CpGs not passing the quality control were pyrosequenced again to discard technical handling errors. The proportion of CpGs not passing the quality control was less than 4%, which was relatively small.

4.3.2 Biomarkers of combined activity

4.3.2.1 E-Screen

The E-Screen assay was used to assess **cell proliferative effect** induced by placental α-fractions. According to Soto et al., (1995), MCF7-cells were seeded at a density of 4 x 10^3 cells/well in 96-well plates (Falcon®, VWR International Eurolab, Barcelona, Spain) using Dulbecco's Modified Eagle's Medium (DMEM) with phenol red supplemented with 10 % fetal bovine serum (FBS) (Gibco, Invitrogen, Spain) as culturing medium; and incubating at 37 ^{0}C with 5 % CO2 (Soto et al., 1995). After 24 h, culturing medium was replaced with an experimental medium, composed of phenol red-free DMEM supplemented with 10 % dextran-coated charcoal-stripped FBC (DCCS-FBS) (Gibco, Invitrogen, Spain). Dried α-fractions were reconstituted with 1 mL experimental medium, shaken, left to rest for 30 min, and filtered with 0.22 μm filter. They were then diluted x1, x5, and x10 times and tested in triplicates. Wells included 150 μL of experimental medium and 50 μL of α-fraction. Thus, dilutions corresponded to 750 $mg_{placenta}$/mL, 150 $mg_{placenta}$/mL and 75 $mg_{placenta}$/mL, respectively. Plates were incubated for 6 days, then cells were fixed, stained (sulforhodamine B, provided by Sigma-Aldrich, MO, USA) and the solubilized bound dye was read at 492 nm using a Titerek Multiscan plate reader (Flow, Irvine, CA, USA). Quality control was simultaneously performed using 17β-estradiol as a positive control at concentrations 0.1 pM-1000 pM, while the experimental medium was used as a negative control.

4.3.2.2 Estrogen receptor (ER) reporter gene assay

The estrogenic activity of placental α-fractions was assessed with the stably transfected human breast adenocarcinoma MVLN cells, which carry the estrogen response element luciferase reporter vector (M. Pond, France), according to Bjerregaard-Olesen et al., (2015). Cells, seeded at a density of 8.5 x 10^4 cells/well in 96-well plates in culture medium consisting of phenol red-free DMEM (LONZA, Belgium) supplemented with 1% DCCS-FBS (HyClone, Belgium), 6 μg/L insulin (Sigma, USA), 64 mg/L hexamycin (Sandoz, Denmark), 4 mM glutamine (Sigma, USA), and 20 mM HEPES (Gibco, UK); and incubated overnight 37 ^{0}C with 5 % CO2. Experimental medium, composed of phenol red-free DMEM (LONZA, Belgium) containing 0.5 % DCCS-FBS. Dried placental α-fractions were reconstituted with 44 μg/L of EtOH:H2O:DMSO (50:40:10, V/V/V). Successively, 22 μg/L were diluted in the experimental medium, making x55, x275 and x550 dilutions. Afterward, 100 μg/L of diluted fractions were added into plates and tested in triplicate. Thus, tested dilutions corresponded to 1240 $mg_{placenta}$/mL, 248

$mg_{placenta}$/mL and 124 $mg_{placenta}$/mL, respectively. Luciferase activity was measured after 24 h of exposure using an automatic injection of luciferase substrate and a LUMistar luminometer (D-luciferin, free acid, Molecular Probes, L2911, Invitrogen; BMG Labtech, RAMCON). To correct for differences in cell number/well, cell protein levels were measured using fluorometric measures on a WALLAC Victor 2 (Perkin Elmer). Cell viability was done visually at the microscope. For cells showing visual toxicity, low protein levels were also found. Quality control was performed using a dose-dependent 17β-estradiol curve at 1.5 pM-300 pM.

4.3.2.3 Androgen receptor (AR) reporter gene assay

To evaluate the anti-androgenic potential of placental α-fractions, the AR reporter gene assay was performed according to Rosenmai et al., (2020). AR-EcoScreen cell line (JCRB Cell Bank, cat. No. JCRB1328) was seeded at a density of 0.9 x 10^4 in 96-well plates (Costar, Corning, USA) using culturing medium, composed of DMEM-F12 without phenol red (Life Technologies, CA, USA) supplemented with 5% DCCS-FBS (Life Technologies, CA, USA), 50 units/mL penicillin, 50 μg/mL streptomycin (Life Technologies, CA, USA), 200 μg/mL zeocin (Invivogen, CA, USA), and 100 μg/mL hygromycin (Invitrogen, CA, USA). Successively, plates were incubated at 37 ^{0}C with 5 % CO2. After 24 h, the culture medium was replaced by an experimental medium, composed of DMEM-F12 without phenol red supplemented with penicillin/streptomycin and 5 % DCCS-FBS (provided by Sigma Aldrich, MO, USA). Dried α-fractions were reconstituted with 400 μL hexane, reconstituted samples were split into four glass tubes and left to evaporate until dry. Afterward, they were reconstituted again with 250 μL of the experimental medium. Then, they were left to rest for 30 min, filtered using a 0.22 μm filter, and diluted x60, x180, and x600. Successively, cells were exposed to α-fractions by adding 50 μL of diluted fraction and experimental medium into wells. Thus, dilutions tested corresponded to 12.5 $mg_{placenta}$/mL, 4.2 $mg_{placenta}$/mL and 1.25 $mg_{placenta}$/mL, respectively. All treatments (also controls) received a 0.1 nM metribolone (R1881) co-treatment (PerkinElmer, MA, USA), a known AR inductor. After ~20 h of exposure, AR activity was determined by measuring its absorbance. Therefore, 100 μL of Dual-Glo® Firefly Luciferase Reagent, was added into wells, according to manufacturer's instructions (Dual-Glo® Luciferase Assay System, Promega, USA). After 10 min on a shaker table, luminescence was measured using a BioOrbit, Galaxy luminometer. Immediately after, cell viability was measured by quantifying the activity of Renilla luciferase, stably transfected into the used cell line. Thus, 50 μL/well of Dual-Glo® Stop & Glo® reagent was added, plates were left on

shaking plates for 15 min and the luminescence was measured (BioOrbit, Galaxy). The whole assay was performed in three independent experiments, with technical triplicates in each experiment. Quality control consisted of dose-dependent hydroxyflutamide (OHF) (known AR inhibitor) curve, using concentrations from 1 x 10^3- 5000 x 10^3 pM in all experiments.

4.3.2.4 Aryl hydrocarbon receptor (AhR) transactivity assay

To explore whether α-fractions activate AhR, which is known to initiate the transcription of metabolizing enzymes (Rosenmai et al., 2020), the procedure of Long et al., (2003) was followed. Stably transfected rat hepatoma cell line (H4IIE-CALLUX) was seeded at a density of 2.2 x10^4 in 96 well-plates and incubated at 37 ^{0}C with 5% CO2 during ~22 h. Culturing medium was composed of minimum essential medium α (MEM α) supplemented with 5% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL Gibco Amphotericin B (provided by Life Technologies). FBS concentration was reduced to 1% to make the experimental medium. In parallel, dried α-fractions were reconstituted with 400 µL hexane, split into four glass vials, and left to evaporate until dry. Once evaporated, fractions were reconstituted again with experimental medium, vigorously shaken, and left to rest for 30 min. Afterward, they were filtered using 0.22 µm filters and diluted x100, x300, and x1000 times; 50 µL of diluted α-fraction and 150 µL of experimental medium were charged into wells. Dilutions x100, x300, and x1000 corresponded to 7.5 $mg_{placenta}$/mL, 2.5 $mg_{placenta}$/mL and 0.75 $mg_{placenta}$/mL, respectively. Samples were tested in two independent experiments. Luminescence was used to measure the degree of AhR activation after cells were exposed ~22 h by adding 40 µL/well luciferin solution, composed of 0.5 mM luciferin with 0.5 mM AMP in lysis buffer. In parallel, cell viability was assessed using the same protocol, but cells were seeded at a density of 1.1 x 10^4 cells/well. After the end of the assay, the medium was removed and replaced with 50 µL of fresh medium with 3-(4, 5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) at a concentration of 0.45 mg/mL, successively, plates were incubated for 1.5 h. Then, the medium was removed and 50 µL/well of isopropanol was charged into plates. Plates were left on a shaker table for 5 min and afterward, absorbance was measured. For quality control, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was used as positive control, tested at concentrations from 0.5 pM to 3000 pM.

4.3.2.5 Xenopus eleutheroembryonic thyroid assay (XETA)

The *in vivo* assay, XETA, is a short-term, relatively high-performance assay to assess thyroid hormone (TH) disruption by quantifying the fluorescence variations emitted by transgenic tadpoles (OECD, 2019). Thus, to assess the TH disruptive effect of placental α-fractions during embryonic stages, Nieuwkood and Faber (NF) stage 45 Xenopus laevis tadpoles of 1 week old from the Tg(*thibz:eGFP*) line were used, according to Fini et al., (2007). To capture the disruption of the TH, the construct contains a promoter of the TH-sensitive TH/bZIP coupled to a Green Fluorescence Protein (GFP) reporter gene, which may be involved in multiple thyroid axis regulatory stages. Dried α-fractions were reconstituted in 16 µL and 400 µL of DMSO and Devian Water, respectively, split into two parts of 208 µL each and added to wells with the experimental medium until the final volume reached 8 mL. Thus, the final tested concentration corresponded to 187.5 $mg_{placenta}$/well. Four independent experiments were performed. Briefly, fifteen tadpoles/well were placed in 6-well plates (TPP Switzerland) with 8 mL of solvent control (DMSO at 0.01 %) in Evian water, thyroid hormone triiodothyronine T3 (5 nM), or α-fraction spiked with T3 (5 nM). Plates were left at 23 ^{0}C and after 72 h of exposure, tadpoles were anesthetized to proceed with fluorescence measurements. Each tadpole was manually positioned into 96-well plates to obtain individual color images using an Olympus AX-70 binocular equipped with long pass GFP filters and a Q-Imaging Exi Aqa camera (25x objective, and 3 s exposure). The software QC Capture pro (QImaging) was used for images acquisition and quantification using ImageJ. Quantifications were targeted in the region of interest (ROI), which included the whole head area, thus excluding non-specific autofluorescence emitted from the gut area.

4.4 Neuropsychological assessment

4.4.1 Cognitive Function

Cognitive function was assessed in boys aged 9-11 years from the INMA-Granada cohort by a trained psychologist blinded to their exposure levels, at the facilities of San Cecilio University Hospital (HUSC), Granada (Spain). A battery of eleven tests was selected, summarized as follows:

1. **General cognitive intelligence:** The Kaufman Brief Intelligence Test (K-BIT) was applied, which is calculated based on verbal and non-verbal scales scoring (Kaufman and Kaufman, 1997).

2. **Language:** the verbal scale of the K-BIT (Kaufman and Kaufman, 1997), was used, which contains two subtests: verbal knowledge (child must name a series of graphical displayed objects) and general knowledge and riddles (child must name words from their definitions and their visualization with missing letters).
3. **Attention:** Continuous performance test (CPT) was applied, which assessed selective attention and impulsitivity (Conners, 1995). From this task, three dependent variables are obtained: hits, commission errors, and attention index.
4. **Verbal memory:** Complutense-Spain verbal learning test (TAVECI) was used to assess memory and learning processes, including immediate recall, short-term and long-term recall, and recognition (Benedet, 2001).
5. **Visual-motor function:** Trail Making Tests part A (TMTA) was used (Reitan, 1958). Time in seconds (s) is expressed as the dependent variable.
6. **Processing speed:** Weschler Intelligence Scales for Children IV edition (WISC-IV) (Wechsler, 2007) was used and calculated based on two tasks. The first task involves the selection of certain figures. The second involves filling spaces under numbers with their corresponding symbols based on an established model.
7. **Executive function:** Assessed by four components; updating, inhibition, shifting, and abstract reasoning (Diamond, 2013).
8. **Updating measurements:** Two components were measured. First, working memory, in which the child listens to a letter-number sequence and repeats it (Wechsler, 2007). Second, verbal fluency, using the verbal fluency test (FAS). The number of animals pronounced is the dependent value (Benton and Hamsher, 1989).
9. **Inhibition:** Two subtests were performed, the Stroop Color and Word Test (Spanish version) which evaluates cognition and inhibition based on the total score of three conditions; and the Go/No-Go task, which evaluates motor inhibition. Dependent values are the hit and false alarms rates (Donders, 1969).
10. **Shifting:** Part B of the Trail Making Test was used (Reitan, 1958). Time is measured in seconds.
11. **Abstract reasoning:** the non-verbal scale of K-BIT was used (Kaufman and Kaufman, 1997). The number of correct responses is considered as the dependent variable.

4.4.2 Behavioral Function

The behavioral function was evaluated using the Spanish version of the Child Behavior Checklist 6/18 (CBCL 6/18), which was administered by a trained psychologist

at the facilities of the HUSC. This test parental perception of their son's behavior, in this case, adolescents, during the last six months.

The CBCL contains 118 items rated on a three-point scale (0= ‘‘not true’’, 1= ‘‘somewhat true’’, 2= ‘‘very/often true’’) and grouped in eight syndrome scales: anxious/depressed, withdrawn/depressed, somatic complaints, social problems, thought problems, attention problems, rule-breaking behavior, and aggressive behavior; empirically separated into internal domains (addressing emotional problems) and external domains (addressing behavioral problems). These scales are summarized in three composite scales: internalizing problems (sum of anxious/depressed, withdrawn/depressed, and somatic complaints scale scores); externalizing problems (sum of rule-breaking behavior and aggressive behavior scale scores); and total problems, reported as sex and age-normalized T-scores. A higher scale score always indicates more behavioral problems (Achenbach and Rescorla, 2013).

4.5 Statistical Analyses

Statistical analyses and main models are extensively described in each of the articles included in the results section. A summary of the most important details of the statistical analysis of each of the published articles is provided in Table 1. For each article, the statistical method was selected based on (i) the objective of the study, (ii) the main variable and the availability of covariates; (iii) the exposure variable; (iv) the biomarker of effect measured; and (v) the health outcome explored.

To briefly show the statistical characteristics of each study, the following information was reported: study type, independent variable, dependent variable, the window of susceptibility, sample size, statistical method, normalization of the exposure, and the adjustment model.

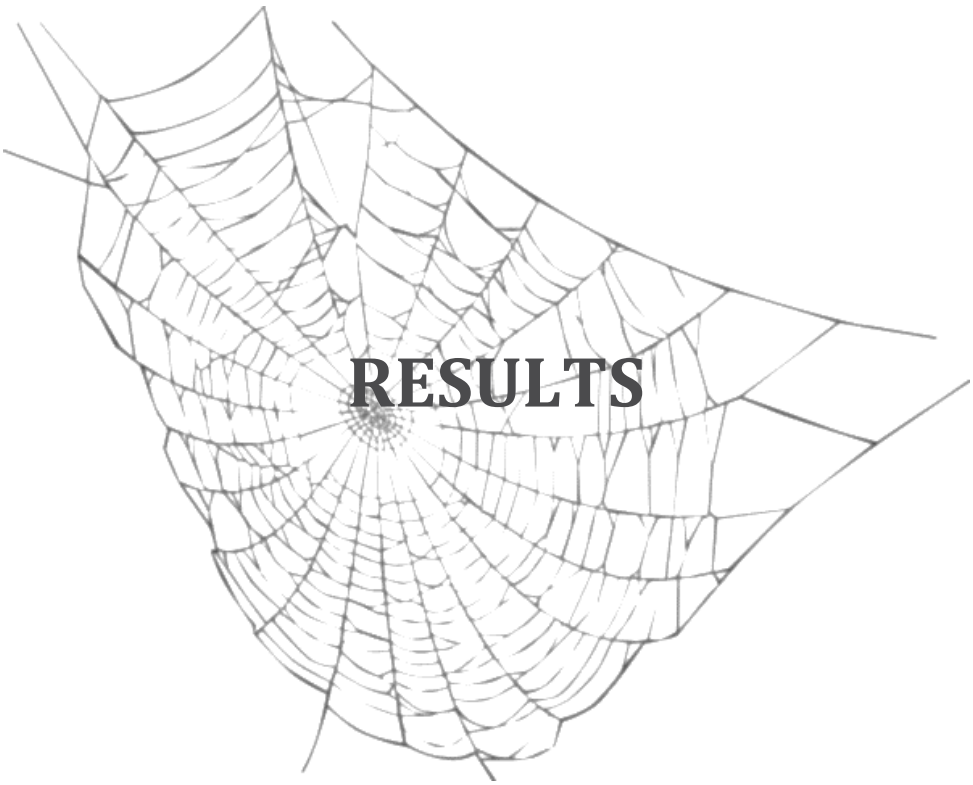
Table 1. Statistical Analyses were performed to address the main objectives enclosed in the present doctoral thesis.

Article	Studies characteristics and statistical methodologies performed		Page
Article 1	**Study type**	Comprehensive review and AOP identification. No statistical analyses were performed.	73
Article 2	**Study type**	Cross-sectional study	98
	Independent variable	Urinary BPA levels	

	Dependent variable	Cognitive function	
	Window of susceptibility	Childhood	
	Sample size	269 boys aged 9-11 years	
	Statistical methods	Mann-Whitney and Kruskal Wallis Spearman's correlation test Multivariate linear regression (ANOVA)	
	Normalization of exposure	Urine BPA concentrations (μg/L) were $\log_e$-transformed, normalized by urinary creatinine levels, and expressed as microgram of BPA per gram of creatinine (μg/g).	
	Adjustment model	Child's age (years), BMI (kg/m^2), smoking exposure at home (yes/no), mother's age at the time of assessment (years), mother's intelligence score, maternal marital status (married/unmarried), maternal education (university/secondary school/up to primary), smoking exposure during pregnancy (yes/no) and breastfeeding (yes/no).	
Article 3	**Study type**	Longitudinal study	112
	Independent variable	Urinary BPA levels	
	Dependent/s variable/s	Behavioral function (CBCL) Serum and urine BDNF protein levels DNA methylation of the BDNF gene	
	Window of susceptibility	Childhood (9-11 years)	
	Sample size	103 adolescents aged 15-17 years	
	Statistical methods	Multiple imputations by regression method Multivariate lineal regression (ANOVA) Mediation	
	Normalization of exposure	Urine BPA concentrations (μg/L) were normalized by urinary creatinine levels and expressed as microgram of BPA per gram of creatinine (μg/g). Afterward, it was log2-transformed	
	Adjustment model	Adolescent's age and BMI z-scores (continuous) at behavioral assessment (15–17 years), maternal education (primary, secondary, or university), urinary cotinine at 9–11 years (continuous), and alcohol consumption at adolescence (yes/no).	
Article 4	**Study type**	Cross-sectional study	125

	Independent variable	Urinary metal and metalloid elements levels: total As, Cd, Hg, and Pb concentrations	
	Dependent variable	Behavioral function (CBCL) Serum and urine BDNF protein levels DNA methylation of the BDNF gene	
	Window of susceptibility	Adolescence (15-17 years)	
	Sample size	125 adolescents with available data on exposure, serum BDNF protein levels, and covariates 113 adolescents with available data on exposure, DNA methylation, and covariates	
	Statistical methods	Multivariate linear regression (ANOVA) Generalized additive model (GAM)	
	Normalization of exposure	Urinary As, Cd, Hg, and Pb concentrations were natural log-transformed	
	Adjustment model	**Model 1:** Adolescent's age at behavioral assessment, BMI (kg/m^2), and creatinine (mg/dL), maternal schooling (primary, secondary, or university), and intelligence, and for all metals simultaneously. **Model 2:** additionally adjusted for passive tobacco smoking and total fish intake of adolescents.	
Article 5	**Study type**	Cross-sectional study	140
	Independent variables	Urinary level of several non-persistent pesticide metabolites: TCPy, IMPy, MDA, DETP, DCCA, 3-PBA, 1-N, and ETU	
	Dependent variable	Behavioral function (CBCL) Serum and urine BDNF protein levels DNA methylation of the BDNF gene	
	Window of susceptibility	Adolescence (15-17 years)	
	Sample size	130 adolescents with available data on exposure, serum BDNF protein, and covariates 118 adolescents with available data on exposure, BDNF gene DNA methylation, and covariates	
	Statistical methods	Multiple imputations using the regression method Multivariate linear regression (ANOVA) Weighted quintile sum (WQS) Mediation	
	Normalization of exposure	Pesticide metabolites were naturally log-transformed to reduce skewness.	

	Adjustment model	Adolescent's age at the time of assessment (15-17 years), BMI (kg/m^2), the season of urine collection (autumn, winter, spring, or summer), creatinine levels (mg/dL), alcohol consumption (yes/no), and maternal schooling (primary/secondary/university)	
Article 6	**Study type**	Experimental study	157
	Main variable	Biological activity of each biomarker of combined effect: E-Screen, ER transactivity assay, AR transactivity assay, AhR transactivity assay, and XETA	
	Window of susceptibility	Prenatal exposure	
	Sample size	24 placental α-fractions	
	Statistical methods	D'Agostino & Pearsons omnibus test Kruskal- Wallis test (Dunn's post hoc test) for XETA analyses Classification of samples as positive in the bio-assays was based on cut-off values calculated from the limit of detection (LOD) for remaining biomarkers of the combined effect. Pearson's correlation test Linear regression analyses (ANOVA)	
	Normalization of tested variables	Biomarkers of combined activity were normalized to the mean on negative control to express data in fold-change.	



RESULTS

5 Results

Article 1: Mustieles, V., D'Cruz, S.C., Couderq, S., Rodríguez-Carrillo, A., Fini, J.-B., Hofer, T., Steffensen, I.-L., Dirven, H., Barouki, R., Olea, N., Fernández, M.F., David, A., 2020. **Bisphenol A and its analogues: A comprehensive review to identify and prioritize effect biomarkers for human biomonitoring.** Environment International. 144, 105811. https://doi.org/10.1016/j.envint.2020.105811

Article 2: Rodríguez-Carrillo, A., Mustieles, V., Pérez-Lobato, R., Molina-Molina, J.M., Reina-Pérez, I., Vela-Soria, F., Rubio, S., Olea, N., Fernández, M.F., 2019. **Bisphenol A and cognitive function in school-age boys: Is BPA predominantly related to behavior?** Neurotoxicology 74, 162–171. https://doi.org/10.1016/J.NEURO.2019.06.006

Article 3: Mustieles, V., Rodríguez-Carrillo, A., Vela-Soria, F., D'Cruz, S.C., David, A., Smagulova, F., Mundo-López, A., Olivas-Martínez, A., Reina-Pérez, I., Olea, N., Freire, C., Arrebola, J.P., Fernández, M.F., 2022. **BDNF as a potential mediator between childhood BPA exposure and behavioral function in adolescent boys from the INMA-Granada cohort.** Science of the Total Environment. 803, 150014. https://doi.org/10.1016/J.SCITOTENV.2021.150014

Article 4: Rodríguez-Carrillo, A., Mustieles, V., D'Cruz, S.C., Legoff, L., Gil, F., Olmedo, P., Reina-Pérez, I., Mundo, A., Molina, M., Smagulova, F., David, A., Freire, C., Fernández, M.F., 2022. **Exploring the relationship between metal exposure, BDNF, and behavior in adolescent males.** International Journal of Hygiene and Environmental Health 239, 113877. https://doi.org/10.1016/j.ijheh.2021.113877

Article 5: Rodríguez-Carrillo, A., D'Cruz, S.C., Mustieles, V., Suárez, B., Mundo-López, A., Molina, M., Peinado, F., Molina-Molina, J.M., Artacho-Cordón, F., Smagulova, F., Arrebola, J.P., Fernández, M.F., David, A., Freire, C. **Non-persistent pesticide exposure, BDNF, and behavioral function in adolescents: Exploring a novel effect biomarker approach.**

Article 6: Rodríguez-Carrillo, A., Rosenmai, A.K.A.K., Mustieles, V., Couderq, S., Fini, J.-B.J.B., Vela-Soria, F., Molina-Molina, J.M.J.M., Ferrando-Marco, P., Wielsøe, M., Long, M., Bonefeld-Jorgensen, E.C.E.C., Olea, N., Vinggaard, A.M.A.M., Fernández, M.F.M.F., 2021. **Assessment of chemical mixtures using biomarkers of combined biological activity: A screening study in human placentas.** Reproductive Toxicology. 100, 143–154. https://doi.org/10.1016/j.reprotox.2021.01.002

ARTICLE 1

Mustieles, V., D'Cruz, S.C., Couderq, S., Rodríguez-Carrillo, A., Fini, J.-B., Hofer, T., Steffensen, I.-L., Dirven, H., Barouki, R., Olea, N., Fernández, M.F., David, A., 2020. **Bisphenol A and its analogues: A comprehensive review to identify and prioritize effect biomarkers for human biomonitoring.** Environment International. 144, 105811. https://doi.org/10.1016/j.envint.2020.105811

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Review article

Bisphenol A and its analogues: A comprehensive review to identify andprioritize effect biomarkers for human biomonitoring

Vicente Mustieles[a,b,c,1,*], Shereen Cynthia D'Cruz[d,1], Stephan Couderq[e,1], Andrea Rodríguez-Carrillo[a], Jean-Baptiste Fini[e], Tim Hofer[f], Inger-Lise Steffensen[f], Hubert Dirven[f], Robert Barouki[g], Nicolás Olea[a,b,c], Mariana F. Fernández[a,b,c,2,*], Arthur David[d,2,*]

[a] *University of Granada, Center for Biomedical Research (CIBM), Spain*
[b] *Instituto de Investigación Biosanitaria (ibs. GRANADA), Spain*
[c] *Consortium for Biomedical Research in Epidemiology & Public Health (CIBERESP), Spain*
[d] *Univ Rennes, EHESP, Inserm, Irset (Institut de recherche en santé, environnement et travail) – UMR_S 1085, F-35000 Rennes, France*
[e] *Evolution des Régulations Endocriniennes, Département "Adaptation du Vivant", UMR 7221 MNHN/CNRS, Sorbonne Université, Paris 75006, France*
[f] *Section of Toxicology and Risk Assessment, Norwegian Institute of Public Health, P.O. Box 222 Skøyen, NO-0213 Oslo, Norway*
[g] *University Paris Descartes, ComUE Sorbonne Paris Cité, Paris, France. Institut national de la santé et de la recherche médicale (INSERM, National Institute of Health & Medical Research) UMR S-1124, Paris, France*

Abbreviations: **AO**, adverse outcome; **AOP**, adverse outcome pathway; **ACR**, albumin-to-creatinine ratio; **AD**, androstenedione; **AhR**, aryl hydrocarbon receptor;**ALT**, alanine aminotransferase; **AMH**, anti-Müllerian hormone; **AP**, alkaline phosphatase; **AR**, androgen receptor; **AST**, aspartate aminotransferase; **BEX2**, brain expressed X-linked 2; **BDNF**, brain-derived neurotrophic factor; **BPA**, bisphenol A; **BRCA1**, breast cancer 1; **CaMKII**, calcium/calmodulin-dependent kinase II; **CMHS**, Canadian measures health survey; **COMT**, catechol O-methyltransferase; **CpG**, 5′—cytosine—phosphate—guanine—3′; **CREB**, cAMP response element-binding protein; **CRP**, c-reactive protein; **DHEA-S**, dehydroepiandrosterone sulfate; **DHT**, dihydrotestosterone; **DNA**, deoxyribonucleic acid; **E1**, estrone; **E2**, 17β-estradiol; **E3**, estriol; **EDCs**, endocrine-disrupting chemicals; **ERR**, estrogen related receptor; **ER**, estrogen receptor; **FAI**, free androgen index; **FSH**, follicle stimulating hormone; **FT**, free testosterone; **FT3**, free triiodothyronine; **FT4**, free thyroxine; **GDNF**, glial cell-derived neurotrophic factor; **GnRH**, gonadotrophin releasing hormone; **GGT**, gamma glutamyl transpeptidase; **GSH**, glutathione, reduced form; **HbA1c**, glycated hemoglobin; **HBM**, human biomonitoring; **HBM4EU**, European Human Biomonitoring Initiative; **HDL-C**, high-density lipoprotein cholesterol; **HNE-MA**, 4-hydroxy-2-nonenal-mercapturic acid; **HOXA10**, homeobox A10; **HOMA-B**, homeostasis model assessment of beta-cell function; **HOMA-IR**, homeostasis model assessment for insulin resistance; **HP**, hypothalamus-pituitary; **HPA**, hypothalamus–pituitary-adrenal; **HPLC**, high performance liquid chromatography; **HPT**, hypothalamus-pituitary-thyroid; **hs-CRP**, high-sensitivity c-reactive protein; **IgE**, immunoglobulin E; **IL**, interleukin; **INHB**, inhibin B; **INSL3**, insulin-like peptide 3; **KE**, key event; **KiSS**, kisspeptin; **LD**, lactate dehydrogenase; **LDL-C**, low-density lipoprotein cholesterol; **LH**, luteinizing hormone; **LINE-1**, long interspersed element-1; **LTP**, long-term potentiation; **LTRs**, long terminal repeats; **MDA**, malondialdehyde; **MIE**, molecular initiating event; **MoA**, mode of action; **NHANES**, National Health and Nutrition Examination Survey; **NIS**, sodium/iodide symporter; **NMDARs**, glutamate N-methyl-D-aspartate receptors; **NRs**, nuclear receptors; **NRC**, National Research Council; **PCOS**, polycystic ovary syndrome; **PlGF**, placental growth factor; **PBMCs**, peripheral blood mononuclear cells; **PREG**, pregnenolone; **PRL**, prolactin; **PXR**, pregnane X receptor; **P4**, progesterone; **RHs**, reproductive hormones; **RNA**, ribonucleic acid; **ROS**, reactive oxygen species; **sFlt1**, soluble fms-like tyrosine kinase-1; **SHBG**, sex hormone-binding globulin; **SP4**, specific protein 4; **STAT3**, signal transducer and activator of transcription 3; **SULT2A1**, sulfotransferase family 2A member 1; **TBA2-MDA**, thiobarbituric acid-malondialdehyde; **TC**, total cholesterol; **TG**, triglycerides; **THs**, thyroid hormones; **TNF-α**, tumor necrosis factor alpha; **TPOab**, thyroid peroxidase autoantibodies; **TSH**, thyroid-stimulating hormone; **TSLP**, thymic stromal lymphopoietin; **TSP50**, testis-specific protease-like protein 50; **TT**, total testosterone; **TT3**, total triiodothyronine; **TT4**, total thyroxine; **T2DM**, type 2 diabetes mellitus; **U.S.**, the United States of America; **WHO**, World Health Organization; **8OHdG**, 8-hydroxy-2′-deoxyguanosine; **8-isoprostane**, 8-*iso*-prostaglandin F2α.

* Corresponding authors at: University of Granada, Center for Biomedical Research (CIBM), Spain (V.Mustieles); Instituto de Investigación Biosanitaria (ibs.GRANADA), Spain (M.F. Fernández); and Univ Rennes, EHESP, Inserm, Irset (Institut de recherche en santé, environnement et travail) - UMR_S 1085, F-35000, Rennes, France (A. David).
E-mail addresses: vmustieles@ugr.es (V. Mustieles), marieta@ugr.es (M.F. Fernández), arthur.david@ehesp.fr (A. David).
[1] Authors contributed equally.
[2] Co-last authors.

A B S T R A C T

Human biomonitoring (HBM) studies have demonstrated widespread and daily exposure to bisphenol A (BPA). Moreover, BPA structural analogues (e.g. BPS, BPF, BPAF), used as BPA replacements, are being increasingly detected in human biological matrices. BPA and some of its analogues are classified as endocrine disruptors suspected of contributing to adverse health outcomes such as altered reproduction and neurodevelopment, obesity, and metabolic disorders among other developmental and chronic impairments. One of the aims of the H2020 European Human Biomonitoring Initiative (HBM4EU) is the implementation of effect biomarkers at large scales in future HBM studies in a systematic and standardized way, in order to complement exposure data with mechanistically-based biomarkers of early adverse effects. This review aimed to identify and prioritize existing biomarkers of effect for BPA, as well as to provide relevant mechanistic and adverse outcome pathway (AOP) information in order to cover knowledge gaps and better interpret effect biomarker data. A comprehensive literature search was performed in PubMed to identify all the epidemiologic studies published in the last 10 years addressing the potential relationship between bisphenols exposure and alterations in biological parameters. A total of 5716 references were screened, out of which, 119 full-text articles were analyzed and tabulated in detail. This work provides first an overview of all epigenetics, gene transcription, oXidative stress, reproductive, glucocorticoid and thyroid hormones, metabolic and allergy/immune biomarkers previously studied. Then, promising effect biomarkers related to altered neurodevelopmental and reproductive outcomes including brain- derived neurotrophic factor (BDNF), kisspeptin (KiSS), and gene expression of nuclear receptors are prioritized, providing mechanistic insights based on *in vitro*, animal studies and AOP information. Finally, the potential of omics technologies for biomarker discovery and its implications for risk assessment are discussed. To the best of our knowledge, this is the first effort to comprehensively identify bisphenol-related biomarkers of effect for HBM purposes.

1. Introduction

Bisphenol A (BPA) is a synthetic high production monomer used inpolycarbonate plastics and epoXy resins in a wide range of consumer products. These include for instance canned food and beverages, plastic bottles, food containers, toys, thermal receipts, and medical equipmentamong many other applications (Calafat et al., 2009; Cao et al., 2009;Carwile et al., 2009; Ehrlich et al., 2014; Fleisch et al., 2010; Iribarne-Durán et al., 2019; Molina-Molina et al., 2019; Vandenberg et al.,2007). More recently, BPA has even been detected in infants' socks, highlighting the novel role of textiles as potential source of bisphenol exposure (Freire et al., 2019). Although diet is one of the predominant sources of BPA exposure in the general population due to the leachingof BPA from packaging materials and can liners into food and beverages (Buckley et al., 2019; Vandenberg et al., 2010), other sources androutes also contribute to human exposure (Freire et al., 2019; Molina- Molina et al., 2019; Morgan et al., 2018). Indeed, non-dietary sources(e.g. dermal and inhalation) have been proposed to be of equal or even higher toxicological relevance than dietary sources based on markedly different toxicokinetics (Liu and Martin, 2017; von Goetz et al., 2017). Assessing human exposure to BPA is challenging, mainly due to its short biological half-life and rapid excretion. Furthermore, problems related to external contamination during sampling and/or analytical processes have been reported in some studies (Teeguarden et al., 2016;Ye et al., 2013). Biomonitoring studies have assessed BPA in blood plasma, serum and tissues; however, the analysis of total urinary BPA (i.e. free BPA and phase II conjugates) after enzymatic deconjugation iswidely recognized as the standard approach (Calafat et al., 2015). Thus, HBM studies have demonstrated widespread and daily exposure to BPA,detecting urinary BPA in more than 90% of the general European and US populations at low concentrations (Becker et al., 2009; Calafat et al., 2008). The advantages of urine as exposure matriX include its non-invasiveness and the possibility to collect repeated samples over time,which is important for a reliable assessment of non-persistent chemicals with short biological half-lives and episodic exposure patterns (Vernet et al., 2019). However, urinary BPA concentrations do not directly inform about bioactive concentrations in specific tissues or internal levels, nor differentiate among exposure routes. Hence, some studies have suggested that measurements in other biological matrices would also beconvenient under specific circumstances (Stahlhut et al., 2016). Additionally, recent data requiring further confirmation suggests that BPA exposure assessment using indirect analytical techniques involving enzymatic deconjugation could underestimate human urinary BPA concentrations depending on the protocol used (Gerona et al., 2019). These inherent limitations related to BPA exposure assessment in HBM studies must be considered when interpreting exposure-health associations.

BPA is a known endocrine disrupting chemical (EDC) that can interfere with hormonal balance even at low doses (Rubin, 2011). Its mechanisms of action are particularly complex since BPA can bind not only to nuclear and membrane estrogen receptors but also to thyroid, glucocorticoid, and peroXisome proliferator-activated receptors. It can also interact with steroidogenic enzymes, among other molecular targets (Acconcia et al., 2015; Mustieles et al., 2018a; Rubin, 2011; Wetherill et al., 2007). Additionally, BPA has been shown to exert epigenetic modifications (e.g. altered DNA methylation) that could partially explain BPA effects on various health endpoints (Ferreira et al., 2015; Kundakovic and Champagne, 2011). Taken together, this biological promiscuity might explain the pleiotropic effects exerted by BPA on behavior, reproduction, and metabolism in both experimental animals (Nesan et al., 2018; Peretz et al., 2014; Wassenaar et al., 2017) and human populations (Chevalier and Fénichel, 2015; Mustieles et al.,2015; Peretz et al., 2014). Other health outcomes suspected of being affected by BPA exposure include hormone-dependent cancers, the immune system and developmental diseases (Bansal et al., 2018; Murata and Kang, 2018; Rochester, 2013). Although associations found in human populations cannot demonstrate causality, BPA effects on different systems and organs are supported by an extensive body of experimental evidence. For some endpoints such as reproduction and behavior, the epidemiological findings are also increasingly consistent (Mustieles et al., 2018a, 2015; Peretz et al., 2014).

BPA has recently been classified as a reproductive toXicant and a substance of very high concern by the European Chemicals Agency (ECHA, 2016, 2017). However, most BPA replacements are structural analogs such as bisphenol S (BPS) and bisphenol F (BPF), which also show hormonal activity (Molina-Molina et al., 2013; Rochester and Bolden, 2015) and are increasingly detected in human urine (Yang et al., 2014b; Ye et al., 2015). Many other bisphenol analogues, including bisphenol AF (BPAF), bisphenol AP (BPAP), bisphenol Z (BPZ),and bisphenol B (BPB) have also been shown to exert estrogenic activities (Chen et al., 2016; Mesnage et al., 2017; Moreman et al., 2017), with the exception of tetramethyl bisphenol F (Soto et al., 2017). Since recent animal studies have shown that

exposure to bisphenol analoguessuch as BPS, BPF and BPAF induce a similar pattern of neurobehavioraldisruption as BPA (Rosenfeld, 2017), there is an urgent need for new monitoring approaches that can timely address the potential risks posedby bisphenol analogues in order to avoid regrettable substitutions of BPA and other environmental chemicals.

The European Human Biomonitoring Initiative (HBM4EU) represents a joint effort of 28 countries and the European Commission, co-funded by Horizon 2020. Its main aim is to coordinate and advance HBM in Europe in a standardized way to provide evidence for policy-making. The HBM4EU consortium identified several critical questions concerning bisphenols, which include the identification of bisphenol-related effect biomarkers and their mechanistic pathways following the adverse outcome pathway (AOP) framework. Effect biomarkers aremeasurable biological changes helpful for establishing dose–response and mechanistic relationships. By providing a link between exposure, internal dose and early health impairment, they could be extremely useful in order to improve HBM and risk assessment of chemicals with avery short half-life (Decaprio, 1997) (Fig. 1). Based on the WHO and the Committee on Human Biomonitoring for Environmental Toxicants(IPCS-WHO, 1993; NRC, 2006), effect biomarkers are defined as "a measurable biochemical, physiologic, behavioral, or other alteration in an organism that, depending on the magnitude, can be recognized as associated with an established or possible health impairment."

Fig. 1 presents a simplified unidirectional conceptualization of the exposure-disease continuum for practical purposes. However, it is worth noting that real-life scenarios are much more complex and dynamic. The development of an adverse effect in humans will depend onthe route of exposure to BPA and the internal dose at the target organ, the critical window of exposure, individual susceptibility, as well as potential non-monotonic dose-responses together with adaptive mechanisms and feedback regulations (Mustieles and Arrebola, 2020). An important characteristic that complicates the evaluation of BPA effects in both experimental toxicology and human settings is the probable existence of non-monotonic dose–response relationships, also typical of endogenous hormones (Hill et al., 2018; Vandenberg et al., 2019, 2012). In relation to biomarkers of effect, different biomarker profiles could be expected for those exposed to the highest levels compared to other exposure ranges, as well as for occupationally-exposed subjects compared to the general population. Thus, the shape of exposure-health associations should be evaluated *a priori*, without assuming the existence of linearity across all exposure ranges.

Based on the needs identified, this work aimed to: i) conduct a comprehensive review of the literature to create an inventory of existing effect biomarkers used in epidemiologic studies related to bisphenols exposure and specific health endpoints of concern; ii) prioritize the most relevant biomarkers of effect for HBM purposes; iii) provide relevant mechanistic and/or AOP information to improve the interpretation of biomarker data; and iv) identify gaps in knowledge and potential novel effect biomarkers to be investigated in future bio- monitoring programs. To the best of our knowledge, this is the first effort to identify the best epidemiological effect biomarkers for BPA and its analogues for HBM purposes.

2. Methods

2.1. Literature search methodology

This comprehensive review covered all scientific publications available in the PubMed/MEDLINE database from January 2008 up to January 2018 with the aim of identifying effect biomarkers linked to bisphenol exposure and human adverse health effects. To decomplexify the very large amount of references found and prioritize the most relevant effect biomarkers, three general steps were followed:

First, relevant search terms including both MeSH and non-MeSH terms (Suppl. Table 1) for both the exposure (bisphenols) and selected health endpoints were selected. To cover as much information as possible, the term "bisphenol" was chosen as the key search term for exposure. Six health endpoints were a priori chosen according to their relevance for BPA-related human health and HBM4EU objectives: i) neurodevelopment, ii) reproductive diseases, iii) endocrine diseases, iv) obesity, cardiovascular and metabolic disorders, v) allergies and immunological diseases and vi) cancer. Afterwards, boolean operators 'AND' and 'OR' were used to combine search terms (Suppl. Table 1). Several PubMed filters were employed to gain precision: a) "Full-text" articles and b) published in the last "10 years". As a result of this exploratory search, 5716 potential references were found (Suppl. Fig. 1). A screening procedure of the abovementioned 5716 abstracts was performed to identify relevant articles reporting effect biomarker information for each outcome of interest. At least two researchers participated in the selection of abstracts for each health endpoint. The following exclusion criteria were applied: a) articles with only exposure data, b) non-original research articles, and c) articles with only

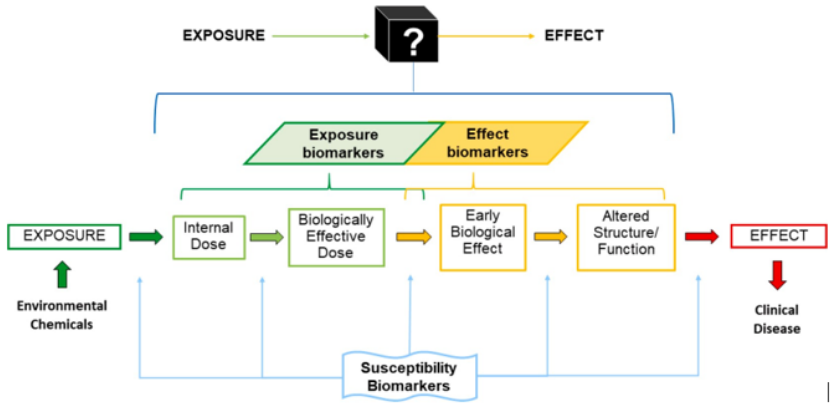

Fig. 1. Conceptual pathway representing the continuum between environmental chemical exposure and clinical disease (adaptation of the National Research Council biomarker paradigm identifying concrete stages in the exposure-disease continuum). Exposure biomarkers measure the actual absorbed dose ("internal dose") and active dose at the putative target organ/tissue ("biologically effective dose"). Effect biomarkers measure early molecular or biochemical/cellular responses in target or non-target tissues ("early biological effect"), functional or structural changes in affected cells or tissues ("altered structure/function"), or actual clinical disease. Susceptibility biomarkers help to identify individuals with genetically mediated predisposition to xenobiotic-induced toxicity (Adapted from De Caprio, 1997).

experimental or *in vivo* studies. Then, duplicate references were removed.Suppl. Fig. 2 shows the flow-chart of the selection process.

Finally, all the information provided by each of the 119 selected studies was collected, analyzed, synthetized and tabulated (Suppl. Tables 2-9). We reported study quality parameters such as study design; characteristics of the population including sample size; exposure assessment (biological matrix, number of samples analyzed, methods followed and quality measures); the effect biomarkers measured in each biological matrix, and the analytical method used; as well as the main results and conclusions. Throughout this in-depth screening, we additionally considered criteria such as the feasibility of the biomarker together with its specificity, sensitivity, and reliability, and if a potential mode of action (MoA) and/or adverse outcome pathway (AOP) were previously described.

After conducting the review and analyzing its results, some gaps in knowledge were identified. Therefore, a fourth post-hoc step was performed by hand-search to gather AOP information (https://aopwiki. org/) and specific BPA mechanistic studies (PubMed database) that may experimentally support the implementation of selected promising noveleffect biomarkers found in this work [brain-derived neurotrophic factor(BDNF), kisspeptin (KiSS) and gene expression of nuclear receptors].

2.2. *Classification of effect biomarkers in the AOP context*

After analyzing all references, effect biomarkers were classified into two main categories based on their level of biological organization: molecular effect biomarkers and cellular/biochemical effect biomarkers. This classification was based mainly on the AOP framework.

AOPs have been developed recently and are conceptual constructs that integrate existing knowledge on the linkage between a direct molecularinitiating event (MIE), through its associated key events (KEs) until thedevelopment of an adverse outcome (AO) at a biological level of organization relevant to risk assessment (Ankley et al., 2010) (see Fig. 2).Within this classification, molecular effect biomarkers include biological markers such as epigenetic modifications (e.g., DNA methylation)and changes in gene expression. These molecular effect biomarkers aremore likely to coincide with early KEs or even molecular initiating events (MIEs) in the AOP framework, meaning that these biomarkers are potentially closer to the exposure. The main limitation is that their predictive potential for adverse effects is most of the time unknown, in particular for epigenetic biomarkers. In contrast, biochemical or cellular biomarkers (e.g., hormones, insulin and glucose levels, etc.) are generally closer to the phenotype and are therefore more likely to be representative of late KEs in the AOP framework. The main strength of biochemical/cellular biomarkers is their predictive potential for disease; however, they may be less specific. Overall, the combination of both molecular and biochemical effect biomarkers, when substantiated by an AOP-like conceptualization of the *in vitro* and *in vivo* evidence, appears as an optimal approach for improving the causal understandingof exposure-disease relationships in HBM studies.

3. Results and discussion

3.1. *Inventory and description of existing effect biomarkers identified inhuman studies*

3.1.1. *Molecular effect biomarkers*

Molecular effect biomarkers identified in this literature search include epigenetic and gene expression biomarkers (Suppl. Table 2) as well as oxidative stress markers (Suppl. Table 3), which are summarizedin Table 1.

3.1.2. *Epigenetic biomarkers.*

Epigenetics refers to heritable alterations in gene expression that do not involve alterations in the DNA sequence. The main epigenetic regulators include DNA methylation, histone modifications, and microRNAs. Overall, epigenetic markers are stable biomarkers that can be transmitted to subsequent generations and studies have already shown that modifications of DNA methylation patterns can potentially serve as relevant effect biomarkers for a wide range of environmental contaminants such as dioxins, plasticizers, pesticides, and hydrocarbons (Manikkam et al., 2012).

A variety of epigenetic markers associated with exposure to bisphenols have been inventoried through this literature search. These epigenetic markers were related to different health outcomes such as reproduction, neurodevelopment, obesity and metabolic disorders. Among the retrieved papers, eight studies analyzed hypo- or hyper- methylation of specific candidate genes, one study focused on DNA hydroxymethylation, and two studies investigated microRNA alterations (Suppl. Table 2).

Regarding hypo- or hyper DNA methylation markers, associations between urinary BPA concentrations and alterations in the methylationpatterns of DNA isolated from saliva of genes involved in immune function, transport activity, metabolism, and caspase activity were observed in pre-pubescent girls from Egyptian rural (n = 30) and

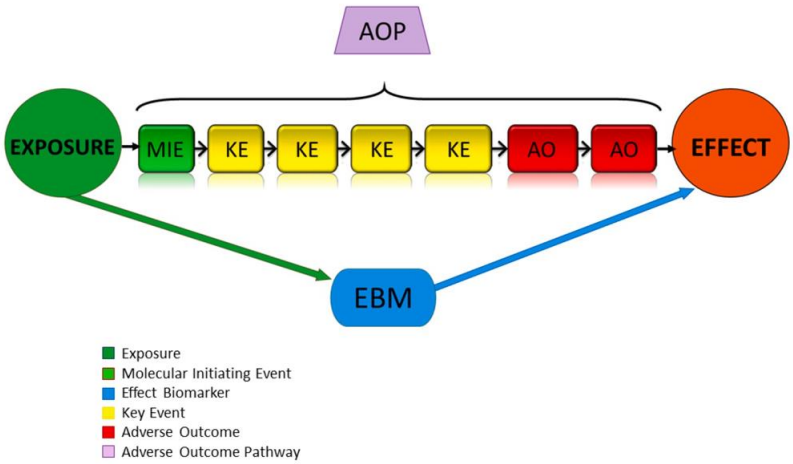

Fig. 2. Visualization of the AOP network and the analogy between intermediate steps and effect biomarkers (EBM) of interest for HBM. Both early KEs (i.e., early biological changes such as epigenetic modifications and altered gene expression) and late KEs (i.e., altered structure or function markers such as sexual hormones and glucose/insulin) in a given AOP have the potential to be assessed or implemented as EBM in epidemiologic studies.

Table 1
Inventory of bisphenol-related epigenetic and oxidative stress effect biomarkers identified in HBM studies.

Biomarkers	Matrix	Health endpoint	Number of studies	Strengths	Limitations	Conclusions
				Epigenetic/Gene expression Biomarkers [a]		
DNA methylation of BDNF Region IV	Blood	Neurodevelopmental disorders	1 (Table S2)	DNA methylation is stable over time compared to gene expression or circulating protein levels, which are subjected to short-term variations. BDNF pathway alteration can affect long-term memory, learning, and depression and anxiety disorders. In mice, DNA methylation of BDNF in hippocampus is correlated with blood methylation.	DNA methylation regions should be carefully selected, mainly the promoter regions, so the status of DNA methylation is related with its gene expression.Although the DNA methylation status of BDNF in blood is a promising biomarker for brain function, its predictive potential and role is not fully understood.	Neurotrophins like BDNF constitute potential effect biomarkers of brain function for bisphenols. Molecular/biochemical biomarkers of brain function constitute an important knowledge gap. The potential of this novel biomarker warrants further research at different biological levels (DNA, RNA, protein…) in HBM studies.
Gene expression of nuclear receptors (ERs, ERRs, AR, TRs, AhR, PPARs)	Blood and Semen	Reproduction Metabolic disorders	2 (Table S2)	Gene expression of nuclear receptors and other targets in PBMCs could be a surrogate of their gene expression in target organs, providing relevant data on potential mechanisms of action.	In most cases, the predictive potential for a given disease is unknown. Notwithstanding, emerging data is supporting their suitability for specific health endpoints.	Although their predictive potential is uncertain, when combined with other related molecular or biochemical effect biomarkers, gene expression markers in PBMCs can help to identify potential mechanisms and increase the biological plausibility of epidemiologic associations.
KiSS gene expression	Placenta	Pregnancy adverse outcomes/ Reproduction Disorders	1(Table S2)	*KISS1* is a major regulator of puberty onset and other reproductive functions. Kisspeptin neuron stimulation is an essential event upstream of GnRH pulse release from the HPG axis, and BPA has been shown to adversely affect kisspeptin neuronal system. Therefore, *KISS1* expression could serve as an early indicator of reproductive dysfunctions associated with BPA exposure.	Kisspeptin carries out a variety of physiological functions from reproduction to metabolism. So precisely identifying the health issue associated with *KiSS* deregulation may be difficult.	Kisspeptin gene dysregulation could be a very early indicator of HPG axis dysfunction and its downstream hormonal events associated with reproduction. Since BPA is a recognized reprotoxicant, assessment of *KISS1* in combination with other biomarkers could help to map the key events underlying BPA's adverse reproductive effects.
Sperm epigenetic marks (LINE-1 methylation and 5-hydroxy-methylcytosine)	Sperm	Reproduction Disorders	1(Table S2) 1(Table S2)	LINES are a group of long terminal repeats and their methylation status could serve as a surrogate measure of global DNA methylation. 5-hydroxymethylcytosine (5hmC), also called as DNA hydroxymethylation, is an intermediate step in DNA demethylation process, and is a relatively stable epigenetic mark. 5hmc can associate with chromatin regulatory proteins and thus could regulate gene transcription.	A limitation of assessing LINEs is their lack of specificity. LINEs are repeat elements, and mapping their genome location would be difficult. Although a global loss of 5hmC has been observed in some cancers, its physiological role still remains elusive.	Semen constitutes a non-invasive sample that can provide effect data at different levels of organization: from cell counts and functional aspects, to seminal hormones, and sperm epigenetic and gene expression markers. Future studies should explore the implementation of molecular epigenetic markers together with more classical semen parameters.
				Oxidative stress biomarkers [a]		
8OHdG and 8-isoprostane	Urine	Oxidative stress	11(8OHdG) and 2 (8-isopr.)(Table S3)	Both markers are easy to measure in urine, which is preferred over serum. 8OHdG is a marker of DNA damage, and 8-isoprostane of lipid peroxidation. They are predictive of diverse chronic diseases including metabolic syndrome, cardiovascular disease and cancers.	The biggest limitation is the lack of specificity for a given exposure or a specific tissue/ organ. Since these markers can be affected by dietary patterns, future studies should consider diet intake. Considerable inter- and intra-day variations, which can be minimized by collecting repeated urine samples. Additionally, there is a small risk of artefactual formation/degradation during sample processing.	In general, positive associations have been reported with 8OHdG in relation to BPA, and also other structural analogues. Although there are fewer studies evaluating 8-isoprostane, positive associations are also dominant. These oxidative stress markers, although not specific to a unique health endpoint, are able to capture disruptions at different levels of biological organization. Thus, they have been demonstrated useful in mediation analyses between exposure biomarkers and health endpoints. These markers should be corrected for urinary dilution. Overall, their use in combination with other more specific markers should be encouraged.

(*continued on next page*)

Table 1 (*continued*)

Biomarkers	Matrix	Health endpoint	Number of studies	Strengths	Limitations	Conclusions
MDA	Urine	Oxidative stress	6 (Table S3)	Several positive associations in relation to BPA exposure have been reported. MDA is specific for lipid peroxidation.	Risk of artefactual generation during preparatory steps involving excessive heating prior to analysis. Not BPA or organ specific.	Although assessment of MDA is preferred in urine than serum, the possibility of artefactual generation still remains. The first methodological option is a HPLC separation prior to UV or fluorescence analysis of the TBA_2-MDA chromophore. Urinary HNE-MA constitutes another promising marker of lipid peroxidation that was recently associated with bisphenol analogues.
3-NO_2Tyr	Plasma	Nitrosative stress	1(Table S3)	The study that investigated this marker in relation to human prenatal BPA exposure was an interspecies comparison. Thus, it is experimentally supported.	Lack of specificity for a given exposure or a specific tissue/organ.	A positive association between BPA and 3-NO_2Tyr (but not 3-ClTyr and diTyr) was found in plasma from pregnant mothers and umbilical cords. This was supported by similar effects in sheep and rodents. Although limited data is available, future studies may evaluate markers of nitrosative stress, especially in cord blood in relation to maternal prenatal BPA exposure.

Abbreviations: AhR (aryl hydrocarbon receptor); AR (androgen receptor); BDNF (brain-derived neurotrophic factor); ERs (estrogen receptors), ERRs (estrogen-related receptors), KiSS (kisspeptin); LINE (long interspersed nuclear elements); MDA (malondialdehyde acid); PPARs (peroxisome proliferator-activated receptors), TRs (thyroid receptors); 8OHdG (8-hydroxy-2′-deoxyguanosine); 3-NO_2Tyr (3-nitrotyrosine).

[a] Effect biomarkers of lower interest or measured in invasive matrices are not reported in the table but in the text.

urban areas (n = 30) using untargeted genome-wide profiling (Kim et al., 2013). More specifically, homeobox A10 (HOXA10) involved in embryo morphogenesis; breast cancer 1 (BRCA1), involved in DNA transcription and repair; and brain expressed X-linked 2 (BEX2) associated with estrogens and cell cycle, were found to be hypo-methylated. Another study reported alterations in DNA methylation in relation to BPA exposure assessed in fetal liver tissue and signal transducer and activator of transcription 3 (STAT3) also evaluated in fetal liver samples (n = 50) (Weinhouse et al., 2015). STAT3 signaling is associated with inflammatory liver cancer (Svinka et al., 2014) and STAT3 methylation has been proposed as a possible biomarker for liver tumor risk (Weinhouse et al., 2015). Increased site-specific methylation of COMT, a gene that encodes for catechol-O-methyltransferase involved in the metabolism of catecholamines and SULT2A1, a phase II metabolism enzyme, was also observed in human fetal liver in response to higher BPA concentrations (Nahar et al., 2014). Hanna et al. (2012) reported an inverse correlation between serum BPA concentrations and altered testis-specific protein 50 (TSP50) gene methylation levels in the blood of women (n = 35) who were undergoing *in vitro* fertilization. Althoughthe exact function of TSP50 is not fully elucidated, an increased expression of TSP50 due to loss of its normal methylation was observed inbreast cancer epithelial cells, but not in normal breast tissues (Shanet al., 2002).

A recent study using data from the Columbia Center for Children's Environmental Health (CCCEH) cohort showed that higher prenatal urinary BPA concentrations during the third trimester of gestation were prospectively associated with altered cord blood DNA methylation of BDNF Exon IV at two CpG sites in a sex-specific manner (n = 41 females, n = 40 males), and these effects were more pronounced in boys compared to girls (Kundakovic et al., 2015). Importantly, prenatal BPA was previously associated with behavior problems in 198 children from this same cohort (Perera et al., 2012). This study was considered of high quality based on its prospective design, robust exposure and effect measurements, and the validation of its results using a rodent model investigating BDNF DNA methylation in both brain tissue and whole blood in response to BPA dosing (Kundakovic et al., 2015). BDNF is a member of the neurotrophin family and is a key regulator of neuronal synaptic plasticity. BDNF is considered a biomarker specific for neurobehavioral disorders as it is involved in the pathogenesis of various psychiatric ailments such as depression, anxiety, schizophrenia and bipolar disorder (Autry and Monteggia, 2012). For instance, altered BDNF levels have been associated with attention deficit, cognitive skill decline and behavioural defects in children (Cubero-Millán et al., 2017; Yeom et al., 2016). From a neurodevelopmental point of view, DNA methylation of BDNF Exon IV constitutes one of the most promising biomarkers identified for BPA.

In another study following a targeted analysis, long interspersed nuclear elements (LINE) were reported to be hypo-methylated in the spermatozoa of Chinese factory workers exposed to BPA (n = 77) compared to non-exposed workers (n = 72) (Miao et al., 2014). LINEs are a group of non-long terminal repeat retrotransposons that comprises approximately 17% of the human genome and are normally heavily methylated in order to prevent genome instability. It is also considered to be a surrogate marker of global DNA methylation. Experimental studies conducted on human fetal liver samples (n = 18) found an association between BPA concentrations in liver and hypomethylation of LINEs, long terminal repeat (LTRs), satellite repeats and DNA elements (Faulk et al., 2016). Moreover, an association between BPA concentrations and global DNA methylation was observed in placenta samples by using targeted LINE-1 assay (Nahar et al., 2015).

DNA hydroxylmethylation is another epigenetic biomarker related to high BPA exposure. In this epigenetic modification, the C5 cytosine of the DNA is replaced with a hydroxymethyl group to form 5-hydroxymethylcytosine (5-hmc), which is considered an intermediate step in the DNA demethylation process (Richa and Sinha, 2014). Zheng et al. (2017) performed

a genome-wide DNA hydroxymethylation study using sperm samples of men who were occupationally exposed to BPA. Compared to controls without occupational BPA exposure, the total levels of 5hmc increased significantly (19.37%) in BPA occupationally-exposed men (Zheng et al., 2017). A global increase in DNA hydroxymethylation profile (72.6% of the genome) in LINE-1 repeats, imprinted genes and other important genes involved in DNA damage response was observed in BPA-exposed workers compared to non-exposed workers (60% of the genome). However, it is worth noting that this study included a relatively small sample size (30 BPA-exposed men and
26 controls) with very high urinary BPA concentrations in exposed workers (Zheng et al., 2017).

MicroRNAs (miRNAs) constitute another important epigenetic biomarker associated with BPA exposure. miRNAs are approximately 22 nucleotide long non-coding RNAs capable of regulating gene expression. Changes in the miRNA expression patterns have been observed under several disease conditions (Tüfekci et al., 2014), and also following exposure to environmental contaminants (Sollome et al., 2016).An epidemiological study performed in Italy (n = 40) observed associations between BPA concentrations in the placentas of patients undergoing therapeutic abortion and upregulation of 34 miRNAs and theirtarget genes (De Felice et al., 2015). More specifically, a strong correlation was found between mir-146a upregulation and BPA concentrations both measured in placental tissue. In contrast, the National Children's Study (NCS) Vanguard Cohort, which involved a larger sample size (n = 110), found no association between altered miRNA levels and BPA concentrations in the placentas (Li et al., 2015). These conflicting results highlight the difficulty to interpret and identify relevant effect biomarkers specific to bisphenols exposure in human populations where exposure is complex (i.e., multiplicity of exposures, exposure misclassification issues) and biological markers not totally specific to chemical families.

The literature search has identified a wide range of epigenetic biomarkers associated with BPA exposure for different health outcomes and different biological matrices (including invasive ones such as liver samples from aborted fetuses). Notwithstanding, a prioritization is needed to ensure that some of these biomarkers can be progressively implemented in HBM studies.

3.1.2.1. Gene expression biomarkers.
Gene expression profiling of mRNA transcripts in any cell type at a given time is a relevant approach to study cellular function at a global level, as well as genotype-phenotype interactions. Thus, variations in gene expression could potentially be used as relevant biomarkers of what is occurring in a given tissue, with implications for the prediction of health outcomes and elucidation of potential mechanisms (Suppl. Table 2). As a limitation, it is often unclear to which extent the assessment of gene expression in non-invasive matrices such as blood represents gene expression in a given target tissue.

Nuclear hormone receptors (NRs) constitute transcription factors that are critical for sensing the hormonal signals that regulate a wide range of physiological processes from metabolism to development (Sever and Glass, 2013). A study from Italy involving 100 male subjectsreported a positive association between urinary BPA concentrations andincreased expression of two estrogen-responsive genes in peripheral blood leukocytes, i.e., the estrogen receptor beta (ERβ) and the estrogen-related receptor alpha (ERRα) (Melzer et al., 2011). This study did not involve women subjects in order to avoid the bias that could possibly occur due to cyclic hormonal variations in premenopausalwomen. Another Italian study analyzed changes in the expression of hormone receptors in peripheral blood mononuclear cells of both fertile and infertile men from three different areas (metropolitan, urban and rural areas) (La Rocca et al., 2015). Higher concentrations of serum total BPA concentrations in infertile men were positively correlated with increased expression of nuclear receptors: ERα, ERβ, AR, AhR andPXR.

Another candidate gene associated with BPA exposure is kisspeptin (KiSS). Kisspeptins are neuroactive (hypothalamic) peptides, encoded by the KISS1 gene, that stimulate gonadotropin releasing hormone(GnRH) and play an essential role in the onset of puberty and maintenance of normal reproductive functions (Clarke et al., 2015). A study conducted on 262 mother–child pairs from China (192 pairs from an e-waste recycling town and 70 from a control area) showed that KISS1 and leptin mRNA expression levels in placental tissue were higher in thee-waste than in the control group (Xu et al., 2015). Moreover, cord blood free (unconjugated) BPA concentrations were positively associated with an increased expression of both markers (Xu et al., 2015). Due to the important role of kisspeptins in regulating puberty, they could be important effect biomarkers for assessing BPA-associated in- duction of precocious puberty in adolescents.

3.1.2.2. Oxidative stress and inflammatory biomarkers. Damage from endogenously produced oxygen radicals occurs to lipids in cellular membranes, proteins and nucleic acids (Halliwell and Gutteridge, 2015). Several oxidative stress biomarkers, e.g. 8-oxo-7,8-dihydro-2′- deoxyguanosine (8-OHdG or 8-oxodGuo), malondialdehyde (MDA) and 8-*iso*-prostaglandin $F_{2\alpha}$ (8-isoprostane), have been studied for decades and are included in several AOPs (e.g. 8-OHdG in AOP 17 and MDA in AOP 260) (Hofer, 2001). However, their use in environmental epidemiological studies investigating health effects related to non- persistent chemical's exposure is recent.

Several oxidative stress biomarkers were identified in 14 epidemiological studies assessing bisphenols (Suppl. Table 3). Overall, the studies indicated that exposure to BPA mainly assessed in urine was positively associated with increased urinary levels of 8-OHdG, MDA and/or 8-isoprostane to some extent (Asimakopoulos et al., 2016; Kim and Hong, 2017; Lv et al., 2016; Watkins et al., 2015; Yang et al., 2014a; Yang et al., 2009; Yi et al., 2011; Zhang et al., 2016). On the contrary, a few studies found no associations (Erden et al., 2014b; Honget al., 2009), while results were inconsistent in others (for instance, Huang et al. (2017) found a positive association with 8-isoprostane, but not with 8-OHdG). No studies reported negative associations for any ofthe oxidative stress biomarkers evaluated and BPA exposure. Two studies reported positive associations between exposure to the structural analogue BPS and higher urinary 8-OHdG levels (Asimakopoulos et al.,2016; Zhang et al., 2016). There are methodological issues of concern related to artefactual MDA formation and lack of HPLC separation in some studies (Grotto et al., 2009), and therefore 8-OHdG and 8-isoprostane excreted and measured in urine appear as the most suitable markers of oxidative stress related to bisphenols exposure.

The mechanisms by which BPA induces oxidative stress are not completely elucidated (Gassman, 2017). The main metabolic pathway for bisphenols is by conjugation leading to glucuronide or sulfate conjugates that are mainly excreted in urine. A minor metabolic pathway described is cytochrome *P*-450-mediated hydroxylation to a catechol, followed by further transformation to an *o*-quinone (Kovacic, 2010), being the latter capable of redox cycling with generation of ROS (Kovacic, 2010; Sakuma et al., 2010).

Other oxidative stress markers measured included o,o'-di-tyrosine (formed from oxidation of two nearby tyrosines within proteins that underwent hydrolysis); 3-chloro-tyrosine (from neutrophil myeloperoxidase release of hypochlorous acid (HOCl) reacting with tyrosine); markers of nitrosative stress (e.g. 8-nitro-guanine and 3-nitro-tyrosine)formed from reaction with the oxidants peroxynitrite anion ($ONOO^-$) or nitrogen dioxide (NO_2), but also total thiols and GSH levels (sulfhydryl-SH groups are susceptible to oxidative damage, which can lower the SH concentration). Additionally, assessment of protein carbonyls (ad- ducts to protein formed from metal-catalyzed oxidation of amino acids or from aldehyde including lipid peroxidation reactions) and activities or genetic polymorphisms (e.g. antioxidant enzymes such as catalase and glutathione peroxidase) were also reported. Although less studied in relation to bisphenols, urinary HNE-MA (4-hydroxy-2-nonenal-mercapturic acid) has potential for becoming a

Table 2
Inventory of bisphenol-related hormonal, metabolic and allergy/immune effect biomarkers in HBM studies.

Biomarkers	Matrix	Health Endpoint	Number of studies	Strengths	Limitations	Conclusions
				Reproductive Biomarkers		
TT, E2, LH, FSH, SHBG Additional estimations: fT, FAI, TT/E_2, FAI/LH, FSH/LH	Serum	HPG axis / Reproduction /Behavior /Metabolism disorders	29 (Table S4)	Strong support from experimental studies. Hormonal concentrations can be compared to population reference values. Relationships and ratio among related hormones can provide information on enzymatic activity and also provides information on feedback loops.	Diurnal and seasonal variations. Requires the consideration of sex and developmental periods (e.g., males vs. females, children vs. adolescents/adults, phase of the menstrual period). A limited number of studies assessed the five components of this set of biomarkers. Not specific to bisphenols or to specific chemical families.	Requires a more comprehensive characterization of the hormonal axis, standardized collection of sample and measurements with quality controls. Careful consideration of timing of developmental period and sex regarding timing of sampling and set of effect biomarkers to assess. This initial set of reproductive hormones must be coupled to other more specific biomarkers of a given tissue, to gain precision.
INSL3/ INHB/ AMH	Serum	Testicular function and descent	1 (INSL3) 8 (INHB) (Table S4)	INSL3 regulates testicular descent and is constitutively expressed in Leydig cells.AMH is a marker of Sertoli cells maturation. INHB reflects Sertoli function and the status of the testis germinative epithelium. Additionally, it regulates FSH secretion from the pituitary.	Although not specific for particular chemical families, it can be more specific for those chemicals targeting male reproductive organs. AMH has not been previously studied in relation to BPA exposure.	When combined with other HPG hormones and semen parameters, these biomarkers can help to gain precision regarding the male reproductive system. Although less explored, some of these markers can also provide information on female reproductive tissues.
DHT/ PREG/17-OHPREG/ DHEA-S/ PRL/ P4/ E_1/ E_3	Serum	Steroidogenesis	11(Table S4)	The assessment of other steroids can help to detect potential disruptions of the steroidogenesis process beyond the more classic reproductive hormones normally studied.	Because not all steroids can be evaluated, the prioritization of additional steroidogenic markers should be substantiated on mechanistic and/or AOP knowledge.	Some of these steroids can provide extra information on specific pathways based on experimental hypotheses.
E1, E_2, and hydroxilated metabolites: 2-OH-E_1, 2-OH-E_2, 4-OH-E_1, 4-OH-E_2	Urine	Estrogenic metabolism /Breast cancer	2 (Table S4)	Assessing urinary concentrations of hydroxy-estrogens provides information on estrogen metabolism and excretion pathways, which may be a predictor of oxidative stress, endocrine alterations and even of the risk of developing breast cancer risk in women.	Effect biomarkers measured in urine should always be corrected for urinary dilution. Any alteration in renal function could bias both exposure and effect biomarker measurements.	As urine constitutes the most accessible matrix in HBM studies, research and validation of effect markers measured in urine is important. An altered estrogen metabolism can be an important factor resulting in negative effects of prolonged exposure to BPA.
KiSS	Serum	Ovulation Pregnancy Puberty Onset	1 (Table S4)	BPA actions on Kisspeptinergic neural systems are experimentally supported. Kisspeptinergic neurons integrate reproductive and metabolic inputs at a central level, to then coordinate downstream signaling involving GnRH release at hypothalamus, and consequently LH and FSH levels at the pituitary.	Limited data on variability of human serum KiSS levels and reference values are not available. The role of KiSS on human physiology is not fully understood. Not specific to chemical families, but more specific to those chemicals such as bisphenols, known to exert effects in the hypothalamus.	KiSS plays an important role on ovulatory control in adult females, pregnancy, and it is crucial for puberty onset. KiSS levels should be combined with other biomarkers of the HPG axis. A cross-talk exists between kisspeptinergic neurons and leptin, that may explain KiSS role on metabolic health and vice versa. This novel effect biomarker should be further explored at different biological levels (DNA, RNA, protein…) in HBM studies.
sFlt1:PlGF	Serum	Placental function / Pregnancy outcomes	1(Table S4)	The sFlt1:PlGF ratio may predict pregnancy complications such as preeclampsia and fetal growth restriction.	Not specific for a given chemical. Absence of population reference values.	The sFlt1:PlGF ratio is a promising and understudied marker of placental function that could help to understand mechanisms of placenta-related adverse effects in relation to BP exposure and other environmental chemicals during pregnancy.
Semen quality parameters	Semen samples	Spermatogenesis / Fertility	7 (Table S5)	Human sperm parameters such as sperm count, concentration, morphology and vitality, present the advantage of examining a localized tissue in a non-invasive way. Additionally, seminal plasma offers the possibility of measuring exposure biomarkers and hormonal parameters in the same matrix.	While sperm collection in adult men is feasible, obtention of samples during puberty and adolescence (one of the most critical periods for spermatogenesis) is more complex. Not specific for a chemical family, but more specific to those chemicals known to impact spermatogenesis.	Semen constitutes a non-invasive sample that can provide both *in situ* exposure and effect data, specific to the male reproductive system, and at different levels of organization: from cell counts and functional aspects, to seminal hormones, and sperm epigenetic and gene expression markers. Future studies should take advantage of integrating these possibilities.

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Table 2 (*continued*)

Biomarkers	Matrix	Health Endpoint	Number of studies	Strengths	Limitations	Conclusions
				Glucocorticoid biomarkers		
Cortisol	Serum and saliva	HPA axis / Stress Response	3 (Table S6)	Emerging evidence suggest that BPA may disrupt the hypothalamic–pituitaryadrenal (HPA) axis altering the stress response in experimental animals. Cortisol plays a crucial role in the stress response, as well as metabolic health. There exist population reference levels.	Not specific for a given chemical.Subjected to daily variations. While repeated blood sampling is more complicated, especially in children, repeated cortisol measurements in saliva is feasible.	Although only 3 epidemiologic studies have studied cortisol in response to BPA exposure, these preliminary results call for further research. A more complete characterization of the HPA axis would be desirable, including the measurement of serum CRH and ACTH levels apart from cortisol.
				Thyroid biomarkers		
TSH, FT_3, FT_4, TT_3, TT_4	Serum	HPT axis /Neurodevelop-ment and Metabolism	11(Table S7)	Thyroid hormones are considered upstream biomarkers of neurodevelopment and metabolic health. Even subclinical dysfunction of thyroid homeostasis during pregnancy may affect offspring neurodevelopment. TSH levels are readily available from neonatal blood tests in most industrialized countries.	Not specific for a given chemical. TSH secretions exhibit circadian rhythms which can mask subtle variations. Circulating TH levels are less affected, due to the existence of an extrathyroidal pool. Not all studies have measured this same set of thyroid biomarkers, hindering interpretations especially in the case of discordant results.	Additional research using more complete datasets pertaining to the HPT axis are needed. Repeated measures throughout time are advisable. Iodine intake should be considered. No previous studies have considered the gene expression or DNA methylation of thyroid receptors in PBMCsin relation to bisphenols exposure.
TPOab	Serum	Antithyroid autoantibodies (Immune System-Thyroid disruption)	6 (Table S7)	Autoimmune thyroid disease causes cellular damage and alters thyroid gland function when sensitized T-lymphocytes and/or autoantibodies bind to thyroid cell membranes, causing cell lysis and inflammatory reactions. The presence of thyroperoxidase (TPO) autoantibodies is an early predictor of thyroid dysfunction.	Not specific for a given chemical. There exist other thyroid related autoantibodies, including the antithyroglobulin antibody (TgAb) and the TSH receptor antibody (TRAb).	The assessment of thyroid autoimmunity provides a more exhaustive characterization of thyroid disruption. TPOab was positively associated with higher serum BPA concentrations in adults, although not in all studies. Future studies should incorporate TPOab in conjunction with classic thyroid hormones to better identify susceptible euthyroid subjects before development of thyroid disease.
				Metabolic biomarkers		
Glucose and Insulin / HOMA HbA1c	Serum	Glucose homeostasis	18 (Glucose and/ or insulin) 10 (HOMA) 4 (HbA1c)(Table S8)	The relationship between fasting glucose and insulin levels calculated through the HOMA index, constitutes a validated biomarker of β-cell function and insulin resistance. There is strong experimental support for low-dose BPA actions on glucose homeostasis. HbA1c provides information on dysregulated glucose levels during the previous months.	Not specific. Differences between children and adult populations should be considered.	The HOMA index is a validated biomarker of interest for glucose homeostasis to detect both subclinical and clinical effects (e.g., classifying prediabetic and type 2 diabetes subjects). HbA1c complements HOMA data with the cumulative glycemic history. Future studies should test whether exposure to bisphenol analogues are associated with an altered glucose homeostasis.
TG, TC, HDL-C, LDL-C	Serum	Lipid metabolism	6 (Table S8)	Serum lipids have been traditionally used in relation to cardiometabolic health. There are reference and cut-off values for these biomarkers.	Not specific. Although these markers could be useful in adult and elder populations, they may not be enough sensitive for children, given the lack of associations reported, with the exception of obese children.	Although serum lipids are important diagnostic components of the metabolic syndrome, its utility in relation to bisphenols exposure is not clear, particularly in children populations.Other metabolic markers different from serum lipids may be more sensitive to bisphenols exposure.
Leptin/ Adiponectin among other adipokines	Serum	Adipose tissue function	6 (Table S8)	Leptin secretion by adipose tissue is proportional to fat mass and regulates energy balance. While leptin upregulates proinflammatory cytokines related to insulin resistance, adiponectin exerts opposing anti-inflammatory and insulin-sensitizing actions.	Not specific. There exists less studied adipokines such as ghrelin, visfatin, omentin, resistin, etc.	The leptin/adiponectin ratio is regarded as a marker of adipose tissue (dys)function, related to insulin sensitivity. Adipokine levels seem to be sensitive markers of BPA-related metabolic disruption in both children and adults. The combined measurement of adipokines levels with glucose homeostasis is especially encouraged in future studies assessing exposure to both BPA and its analogues.

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Table 2 (*continued*)

Biomarkers	Matrix	Health Endpoint	Number of studies	Strengths	Limitations	Conclusions
ALT, AST, GGT, ALP, LD	Serum	Liver function	4 (Table S8)	Reference levels for these classic liver markers exist. ALT is primarily localized in the liver, while AST is present in several tissues. Their ratio helps to identify the etiology of liver damage (e.g., viral, alcohol, steatosis…). LD and ALP are less specific to the liver since they are also present in other tissues. For example, elevated ALP levels can indicate both liver or bone damage. However, if both ALP and GGT levels are increased, will be indicative of a cholestatic disorder.	Not specific. These markers are not always specific for the liver, and other tissues can contribute to altered levels. Liver markers may not be sensitive enough to map subclinical effects of exposure to environmental chemicals in healthy populations. In contrast, they may be interesting for susceptible populations including obese children and adults, elders, subjects with medication affecting liver dynamics or alcohol consumption.	Liver biomarkers can be combined to inform about different forms of liver damage: steatosis, obstruction, fibrosis and/or necrosis. Future studies should confirm or rule out the relationship between liver function biomarkers and bisphenols exposure, especially in susceptible populations.
ACR	Urine	Endothelial function	3 (Table S8)	The ACR is a biomarker of early endothelial dysfunction in both children and adults. Its measurement in urine means that it can be implemented in pediatric populations without the need of sampling blood.	Not specific. ACR is not specific to a given organ, but provides information on the renal and endothelial system function.	Apart from BPA, preliminary data has shown that BPS exposure is also associated with higher ACR levels in children. Therefore, the ACR should be further studied as a potential marker endothelial dysfunction in relation to bisphenol exposure.
CRP/ hs-CRP	Serum	Systemic Inflammation	3 (Table S8) 4 (Table S3) 1 (Table S9)	C-reactive protein (CRP) is an acute phase protein produced by the liver in response to inflammation. It is an important predictor of cardiometabolic and other chronic diseases.	Not specific of chemicals or tissue localization. CRP is neither specific to a given organ or disease. Although hs-CRP is preferred over standard CRP measurements, only one of the studies assessing CRP measured this form.	CRP should be combined with markers more specific of metabolic health (e.g., glycemic biomarkers, adipokines, hepatic markers…). Although CRP is a non-specific marker of systemic inflammation, it may capture disruptions at different levels of biological organization. Thus, CRP may be an interesting biomarker for assessing mediation effects in epidemiologic studies assessing exposure-metabolic disease associations.
IL-6, IL-10,TNF-α	Serum	Interleukins / Inflammation	2 (Table S8) 2 (Table S3) 1 (Table S9)	Inflammatory cytokines are signaling molecules predominantly produced by T immune cells and macrophages, as well as cells regulating inflammatory processes. Thus, they may provide additional information on the tissues or pathways affected.	Not specific of chemicals or tissue localization.	Investigating the serum profile of pro- and anti-inflammatory cytokines may help elucidate pathways activated by bisphenols and other environmental chemicals. Ideally, they should be coupled to other more specific metabolic markers in order to gain precision and facilitate data interpretation in human studies.
Vitamin D	Serum	Metabolic function	3 (Table S8)	Vitamin D receptors have been found in many tissues, including the cardiovascular, immune and reproductive systems.	Not specific. Not specific for a specific organ. More mechanistic studies are needed.	Further studies are warranted to understand the possible interaction between bisphenols and altered levels of the so-called "hormone D" in relation to both reproductive and metabolic adverse effects.
				Allergy/Immune markers		
IgE	Serum	Antibodies / Food and contact allergies	3 (Table S9)	IgE and specific IgE antibodies play an important role in allergic (atopic) processes including asthma.	Not specific. The predictive potential of total IgE differs according to asthma and allergy subtypes.	IgE has been shown to partially mediate the effect of prenatal BPA exposure on the risk of asthma development in children. This potential mediating role of total IgE and specific IgE antibodies in BPA-related allergic sensitization should be further explored.

Abbreviations: ACR (albumin-creatinine ratio); ALP (alkaline phosphatase); AMH (anti-müllerian hormone); ALT (alanine aminotransferase), AST (aspartate aminotransferase); CRP (c-reactive protein); DHEA-S (dehydroepiandrosterone sulfate); DHT (dihydrotestosterone); E1 (estrone); E2 (17-β-estradiol); E3 (estriol); FAI (free androgen index); FSH (follicular stimulating hormone); fT (free Testosterone); FT3 (free triiodothyronine); FT4 (free thyroxine); GGT (gamma-glutamyl transaminase); HbA1c (glycated hemoglobin); HDL-C (high-density lipoprotein cholesterol); HOMA (homeostasis model assessment); HPG (hypothalamus-pituitary–gonadal); hs-CRP (high-sensitivity c-reactive protein); IgE (immunoglobulin E); IL (interleukin); INHB (Inhibin); INSL3 (insulin-like peptide 3); KiSS (kisspeptin); LD (lactate dehydrogenase); LDL-C (low-density lipoprotein cholesterol); LH (luteinizing hormone); PREG (pregnenolone); PRL (prolactin); P4 (progesterone); sFlt1:PlGF (soluble fms-like tyrosine kinase 1 to placental growth factor ratio); SHBG (sex hormone-binding globulin); TC (total cholesterol); TG (triglycerides); TNF-α (tumor necrosis factor alpha); TPOab (thyroperoxidase antibodies); TSH (thyroid stimulating hormone); TT (total testosterone); TT3 (total triiodothyronine); TT4 (total thyroxine); 2-OH-E1 (2-hydroxy-estrone); 2-OH-E2 (2-hydroxy-17β-estradiol); 4-OH-E1 (4-hydroxy-estrone); 4-OH-E2 (4-hydroxy-17β-estradiol); 17OHPREG (17α-hydroxypregnenolone).

becoming a reliable biomarker of lipid peroxidation, which was recently associated with both higher urinary BPA and BPF concentrations (Wang et al., 2019).

Oxidative stress biomarkers comprehensively capture disruptions atvarious levels of biological organization. As a limitation, these bio markers are not specific for a given exposure such as BPA, or for a unique organ or system, and can also originate from external sources such as food.

3.1.3. Biochemical effect biomarkers

Biochemical effect biomarkers identified in the literature search aresummarized in Table 2.

3.1.3.1. Reproductive hormones (RHs). RHs coordinate a myriad of physiological functions. The most important ones include their role in sexual differentiation of both gonads and brain during development, but also on metabolic organs (Bao and Swaab, 2011). Additionally, RHsmaintain these functions during adulthood. In total, 32 studies reporting biomarker data on reproductive hormones were retrieved from the literature search and organized based on similar windows of development and/or study designs (Suppl. Table 4). The most relevant articles are discussed below.

Few studies explored associations between prenatal BPA exposure and maternal or offspring sex hormone levels. Prenatal urinary BPA concentrations were associated with decreased total testosterone (TT) cord blood levels and testosterone:17β-estradiol (T/E2) ratio among male neonates (Liu et al., 2016); whereas cord blood BPA concentrations were associated with lower cord blood insulin-like peptide 3 (INSL3) levels but not with TT in a case-control study of cryptorchidism (Chevalier et al., 2015). INSL3 is a major regulator of testicular descent,and a marker of Leydig cells maturation. Since INSL3 is not acutely regulated by the hypothalamus-pituitary (HP) axis, but is constitutively secreted by Leydig cells, it is considered a valid marker for their numberand status (Sansone et al., 2019) that could be further implemented in environmental epidemiologic studies, together with anti-Müllerian hormone (AMH), an analogous marker of Sertoli cells maturation (Sansone et al., 2019).

Peripuberty and adolescence represent understudied critical periodswhich can also be affected by environmental chemical exposures. Urinary BPA concentrations during the second trimester of gestation were associated with higher serum inhibin B (INHB) levels in peripubertal boys, and with higher TT levels in peripubertal girls (Watkins et al., 2017a, 2017b). INHB is produced by Sertoli cells, and its levels directly reflect the status of the testis germinative epithelium. LowINHB levels have been associated with low testicular function and/or with alterations of testicular parameters at histological examination (Esposito et al., 2018). Urinary BPA concentrations were cross-sectionally associated with increased serum TT levels and reduced cortisolin prepubertal 9–11 year-old boys (Mustieles et al., 2018b). Additionally, urinary BPA concentrations were negatively associated with serum TT levels among male adolescents and positively associated withTT levels in female adolescents in the 2011–2012 National Health and Nutrition Examination Survey (NHANES) (Scinicariello and Buser, 2016). As previously observed in animal studies, there exists a complex relationship between BPA and reproductive hormones, which greatly depends on the dose, sex and timing at which exposure occurs (discussed in detail in Mustieles et al. 2018b).

A case-control study on precocious puberty in girls found that higher urinary BPA concentrations correlated with higher urinary concentrations of TT, E2 and pregnenolone (PREG) (Lee et al., 2014b), while a very small study comparing 28 cases of precocious puberty in girls with 28 controls did not find associations between urinary BPA and serum E2 or KiSS levels (Özgen et al., 2016). However, KiSS levels significantly differed among cases and controls (Özgen et al., 2016). KiSS controls the hypothalamic secretion of GnRH and is consequently implicated in puberty onset, fertility and pregnancy outcomes (Skorupskaite et al., 2014). Given that KiSS appears to be a target of BPA exposure in both rodents and non-human primates (Kurian et al.,2015; Patisaul, 2013), it should be further investigated in HBM studies.BPA has been proposed as a risk factor for PCOS, and three case- control studies were retrieved from the search. Serum BPA concentrations were associated with increased serum TT and androstenedione(AD) levels in adult women (Kandaraki et al., 2011) and with higher TT, free testosterone (FT) and dehydroepiandrosterone sulfate (DHEA-S) levels among adolescents (Akın et al., 2015). Additionally, another case-control study in women with PCOS found a positive association between serum BPA concentrations and the free androgen index (FAI)(Tarantino et al., 2013). These initial results suggest that BPA-related ovarian toxicity may be stronger in PCOS patients. Given the increasing
prevalence of this condition, further research is warranted.

BPA may impact female fertility through actions in both the ovary and the HP axis, converging in abnormal estrous cyclicity (Viguié et al.,2018). One of the main molecular targets inside the ovary would be aromatase inhibition in antral follicles, through which the production of E2 from T would decrease (Viguié et al., 2018). Mok-Lin et al. (2010)studied 84 women undergoing fertility treatment finding that higher BPA concentrations measured in two urine samples were associated with reduced E2 peak levels and reduced oocyte count. These results were confirmed in a follow-up study including 174 women by Ehrlichet al. (2012). However, the latest follow-up of this study with 256 women did not replicate previous findings (Mínguez-Alarcón et al., 2015), which may be influenced by the progressive decline in BPA concentrations observed over the years in the U.S. (LaKind et al., 2019). Bloom et al. (2011) studied 44 women attending another fertility clinic and also found that free serum BPA concentrations were inversely associated with E2 levels, but this time no association was observed between BPA concentrations and oocyte count. Finally, a study including 106 occupationally exposed and 250 non-occupationally exposed women reported increased serum prolactin (PRL) and progesterone (P4) levels among all females in response to higher urinary BPA concentrations. However, when only occupationally exposed participants were considered, higher BPA exposure was additionally associated with increased serum E2, while reduced follicle stimulating hormone (FSH) levels were only observed in non-occupationally exposed women (Miaoet al., 2015). The results from Miao et al. (2015) suggest that different biomarker profiles may be observed in response to different BPA concentrations and/or exposure routes (Miao et al., 2015). In other occupational studies, in which inhalation and dermal absorption are thoughtto be the main exposure routes leading to high internal levels of free BPA (Hines et al., 2018), different effects would also be expected compared to the general population.

Although the study of reproductive biomarkers in women is particularly complex due to hormonal variations during different phases of the menstrual cycle, the still limited but suggestive evidence calls attention for further HBM studies to correctly address this hypothesis strongly substantiated by toxicological data (Peretz et al., 2014; Viguié et al., 2018).

Out of the 10 studies retrieved on BPA and reproductive hormones in adult men, all found significant associations with at least one reproductive hormone; however, not all of them were conducted under the same setting and neither reported consistent relationships (Suppl. Table 4). In men recruited from the general population, Galloway et al. (2010) observed that BPA concentrations measured in one 24-hour urine sample were associated with higher serum TT levels, in line with Lassen et al. (2014) who reported associations between urinary BPA concentrations and increased serum TT, FT, E2, and luteinizing hormone (LH) levels. In contrast, Mendiola et al. (2010) reported that urinary BPA was negatively associated with FAI and the FAI/LH ratio, and positively associated with serum sex hormone-binding globulin (SHBG) levels. In men attending a fertility clinic, urinary BPA concentrations were negatively associated with serum INHB levels and theE2:TT ratio, and positively associated with FSH and the FSH:INHB ratio (Meeker et al., 2010a). Den Hond et al. (2015) observed reduced serum TT levels in

response to higher urinary BPA concentrations from males seeking fertility care. Vitku et al. (2016) assessed BPA concentrations and 11 steroid hormones in the plasma and seminal plasma of male attending a fertility center. Plasma BPA was positively correlated with estrone (E1), E2, PREG, 17-OH-PREG and DHEA, while negatively associated with dihydrotestosterone (DHT) levels. In contrast, seminal BPA was negatively associated with P4, 17-OH-P4 and DHEA, but similarly correlated with E2 and estriol (E3) (Vitku et al., 2016). Among the strengths of Vitku et al. (2016) are the measurement of both BPA exposure and hormone biomarkers using a previously validated hyphenated mass spectrometry methodology in circulating blood and semen. In studies with occupationally and non-occupationally exposed men, higher serum BPA concentrations were associated with reduced serum AD, FT and FAI, and increased SHBG levels (Zhou et al., 2013), as well as with reduced serum AD and increased SHBG levels (Zhuang et al., 2015). Additionally, urinary BPA was associated with increased levels of PRL, E2 and SHBG, as well as with reduced serum levels of FSH, AD and FAI (Liu et al., 2015).

BPA exposure has also been related to lower sperm counts and/or poorer semen quality (Suppl. Table 5). In several cross-sectional studies, urinary BPA concentrations were associated with poorer semen parameters in adults (Knez et al., 2014; Lassen et al., 2014; Li et al., 2011; Meeker et al., 2010b), while in others were not (Goldstone et al., 2015; Mendiola et al., 2010). Interestingly, Vitku et al. (2016) found that BPA concentrations assessed in seminal plasma, but not in blood plasma, were negatively correlated with sperm count, concentration and morphology, suggesting that circulating BPA concentrations may not have the same biological meaning than exposure measured at this localized fluid. Semen is produced by different organs and constitutes a non-invasive biological sample that fits very well the exposure-effect biomarker paradigm discussed in this work, since it can provide *in situ* exposure and effect data specific to the male reproductive system, and at different levels of biological organization: from cell counts and sperm morphology/vitality, to seminal hormones, and sperm epigenetic and gene expression markers (Bonache et al., 2012), among many other molecular markers (Sutovsky and Lovercamp, 2010) and omics approaches (Huang et al., 2019).

Among Korean male and female adults participating in a large national biomonitoring survey, urinary concentrations of E1, E2 and theirhydroxylated metabolites were higher in the participants with highest urinary BPA concentrations compared to the low-exposed group. Estrogen metabolism to 4-hydroxy-E1 and 4-hydroxy-E2 was more active than that to 2-hydroxy-E1 and 2-hydroxy-E2 among participants inthe high BPA exposure group, with possible implications for breast cancer and other endocrine disorders (Kim et al., 2014).

BPA has previously been associated with neonatal outcomes including lower birth weight and preterm birth in epidemiologic studies (Mustieles et al., 2020, 2018c; Pergialiotis et al., 2018), although underlying mechanisms are poorly understood. Ferguson et al. (2015) found that maternal urinary BPA concentrations measured at four times during pregnancy were associated with higher plasma levels of soluble fms-like tyrosine kinase-1 (sFlt-1), and a higher sFlt-1/placental growth factor (PlGF) ratio, suggesting a disrupted placental development. Among the strengths of Ferguson et al., (2015) are the repeated collection and measurement of BPA in urine and of sFlt-1/PlGF in serum up to four times throughout pregnancy using validated methodologies. While PlGF is a pro-angiogenic placental protein, which plays an important role in vascularization, sFlt-1 binds to PlGF making it anti-angiogenic. Lower circulating levels of PlGF and higher levels of sFlt-1 during pregnancy predict pregnancy complications such as preeclampsia and fetal growth restriction (Ferguson et al., 2015). The sFlt-1:PlGF ratio is a promising effect biomarker of placental function that should be explored.

Overall, the results show that serum LH, FSH, E2, TT and SHBG levels are the reproductive markers more frequently evaluated in HBM studies in both children and adult populations (Table 2). The measurement of SHBG levels allows the estimation of FT levels and FAI(Table 2). The E2:TT ratio is used as a measure of aromatase activity. TT:LH and FSH:INHB ratios are employed as biomarkers of Leydig and Sertoli function, respectively (Meeker et al., 2010a). Other useful and less studied effect biomarkers are INHB and AMH levels as markers of Sertoli function, and INSL3 as a marker of Leydig function (Table 2). Additionally, the adrenal androgen DHEA-S and cortisol have been studied as biomarkers of adrenarche in prepubertal children, and KiSS as biomarker of puberty onset. The sFlt1:PlGF ratio should also be further explored as a marker of placental function. Other less studied markers include AD, DHT, PRL, PREG and P4 (Table 2). One of the main shortcomings observed across studies is the absence of a harmonized panel of hormonal markers to assess a specific function in a particular age group. Importantly, several hormonal biomarkers should be assessed in combination to adequately characterize a complete biological pathway. This approach may counteract the variability associated with steroid hormone levels and account for feedback regulations, facilitating data interpretation.

3.1.3.2. Glucocorticoid hormones.

Increasing evidence shows that BPA, among other environmental chemicals, may disrupt the hypothalamic–pituitary adrenal (HPA) axis altering the stress response (Michael Caudle, 2016). However, scarce human data is available (Suppl. Table 6). In a longitudinal birth cohort, higher maternal urinary BPA concentrations at second trimester of pregnancy wereassociated with a dysregulation of the maternal daytime cortisol pattern in saliva, including reduced cortisol at waking and a flatter daytime pattern (Giesbrecht et al., 2016). In a consecutive study, the offspring of these women was followed-up and cortisol levels were measured in infant's saliva before and after an infant stressor (blood draw) at
3 months of age (Giesbrecht et al., 2017). The authors found that maternal prenatal urinary BPA concentrations were associated with increases in baseline cortisol levels among female infants but decreasesamong males (Giesbrecht et al., 2017). In contrast, after the blood draw (i.e. the stressor), maternal BPA concentrations were associated with increased cortisol compared to baseline levels among males, but decreased levels among female infants, suggesting a sex-specific effect of BPA on HPA-axis function (Giesbrecht et al., 2017). In peripubertal boys from the Spanish INMA-Granada cohort, higher urinary BPA concentrations were cross-sectionally associated with reduced serum cortisol levels and a higher TT:cortisol ratio, suggesting a potential effect at the adrenal gland (Mustieles et al., 2018b). More studies are needed to further elucidate the role of BPA exposure on the stress response, preferably with a more complete characterization of the HPA axis (Table 2).

3.1.3.3. Thyroid hormones (THs). THs play critical roles in differentiation, growth, and metabolism and are required for the normal function of all tissues (Yen et al., 2006), most notably for normal brain development (Bernal, 2005). While various MoAsunderlying BPA's effect on the hypothalamic-pituitary-thyroid (HPT) axis are described in experimental studies (Dang et al., 2009; Moriyamaet al., 2002; Sheng et al., 2012), epidemiologic studies are scarce and the majority differ in scope, focus and consequently in the effect biomarkers tested (Table 2 and Suppl. Table 7). Overall, associations between BPA exposure and circulating THs are difficult to interpret in the cases when significant associations are described, calling for additional research using more complete datasets pertaining to the HPT axis. Nonetheless, while not consistently significant, an inverse association between BPA and thyroid-stimulating hormone (TSH) is repeatedly observed in adults (Meeker et al., 2010a) in conjunction with increased free triiodothyronine (FT_3) (Wang et al., 2013) or decreased total thyroxine (TT_4) (Meeker and Ferguson, 2011) in pregnant women (Aung et al., 2017) and in newborns (Brucker-Daviset al., 2011; Chevrier et al., 2013; Romano et al., 2015). Additionally, free thyroxine (FT_4) and serum BPA levels were negatively correlated in men and positively

correlated in pregnant women (Aker et al., 2016; Aung et al., 2018; Sriphrapradang et al., 2013). Assessment of thyroid autoimmunity provides a more exhaustive characterization of thyroid disruption. In the only study focused on thyroid autoimmunity, thyroid peroxidase autoantibodies (TPOab) positivity was significantly higher across increasing serum BPA quartiles in adults (Chailurkit et al.,2016).

Birth cohorts have also reported an inverse relationship between maternal BPA exposure and maternal TSH (Aung et al., 2017) and sex- specific TSH levels in newborns (Chevrier et al., 2013; Romano et al., 2015). Since mild or transient variations in THs during development affect offspring cognitive outcomes (Bernal, 2005), further investigation is warranted.

3.1.3.4. Metabolic biomarkers.

BPA is a suspected obesogenic compound that promotes fat accumulation in experimental animals (Wassenaar et al., 2017), but also a metabolic disruptor able to alter glucose homeostasis at very low doses (Alonso-Magdalena et al., 2015),and to alter satiety signals (Heindel et al., 2017). Below we summarize and discuss the most important biomarkers of whole-body metabolism retrieved (Table 2 and Suppl. Table 8).

Few studies have explored the association between BPA exposure during pregnancy and maternal glucose homeostasis. Urinary BPA concentrations at second trimester, but not first trimester of pregnancy,were positively associated with blood glucose levels 1-hour after a 50-g glucose challenge test at 24–28 weeks of gestation (Chiu et al., 2017). However, a case-control pilot study and a prospective mother–child cohort study that evaluated urinary BPA during the first trimester did not find a higher risk of gestational diabetes mellitus (Robledo et al., 2013; Shapiro et al., 2015).

The HOMA-IR is a validated biomarker based on fasting glucose andinsulin levels used to quantify peripheral insulin resistance and pancreatic beta-cell function (HOMA-B) (Borai et al., 2011). In children, some studies have found higher HOMA-IR values in response to higher BPA concentrations, especially among obese subjects (Khalil et al., 2014; Lee et al., 2013; Menale et al., 2017), while others have not (Eng et al., 2013; Watkins et al., 2016). Adipokine levels seem to be early metabolic indicators of BPA exposure. Leptin secretion by adipose tissue is proportional to fat mass and regulates energy balance. While leptin upregulates proinflammatory cytokines related to insulin resistance, adiponectin exerts opposing anti-inflammatory and insulin-sensitizing actions (López-Jaramillo et al., 2014). Several studies in neonates (Ashley-Martin et al., 2014; Chou et al., 2011), children (Menale et al., 2017; Volberg et al., 2013; Watkins et al., 2016) and adults (Rönn et al., 2014; Zhao et al., 2012) suggest that both leptin and adiponectin (and probably other adipokines) could be sensitive early markers of BPA metabolic disruption, especially important in the case of infants and children. Although the previous studies varied in study design, critical window of development and matrix for BPA exposure assessment, they point to a similar direction. Importantly, Menale et al.(2017) confirmed their observational findings in adipocytes isolated from eight prepubertal children, showing a decrease in adiponectin after *in vitro* BPA dosing. This is in line with described BPA actions in the adipose tissue of experimental animals (Wassenaar et al., 2017), as well as previously reported associations between BPA exposure and obesity in children (Kim et al., 2019; Mustieles et al., 2019) and possibly in adults (Oppeneer and Robien, 2015; Rancière et al., 2015). Therefore, the use of these markers of adipose tissue function should be encouraged. On the contrary, serum lipids [triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C)] do not seem to be sensitive biomarkers of BPA-related metabolic effects in children (Eng et al., 2013; Khalil et al., 2014; Perng et al., 2017).

The relationship between adult BPA exposure and biomarkers of glucose homeostasis has been relatively well-studied, mostly under a cross-sectional design. Higher BPA concentrations have been associated with increased glycated hemoglobin (HbA1c) levels in adults from the NHANES (Silver et al., 2011) and adult men from the Canadian Measures Health Survey (CMHS) (Tai and Chen, 2016). In the NHANES, urinary BPA was positively associated with greater serum insulin levelsand insulin resistance (HOMA-IR) (Beydoun et al., 2014), higher risk of prediabetes (Sabanayagam et al., 2013), and higher chances of type 2 diabetes mellitus (T2DM) independently of other risk factors (Shankar and Teppala, 2011). Other Asiatic cross-sectional surveys have alsoreported similar results for the risk of T2DM (Aekplakorn et al., 2015; Ning et al., 2011), and insulin resistance (Wang et al., 2012). Moreover, a prospective study identified a susceptible group of adults for BPA effects on glucose homeostasis based on a genetic risk score (Bi et al., 2016). Fasting circulating glucose and insulin levels, together with HOMA (-IR and -B) estimations constitute valid biomarkers of insulin resistance, prediabetes and T2DM, which have been consistently associated with BPA exposure (Hwang et al., 2018). Future studies should evaluate whether BPA substitutes can also interfere with glucose homeostasis.

Similar to studies in children, serum lipid levels in adults do not seem to be good metabolic biomarkers for BPA effects (Lang et al., 2008; Milošević et al., 2017; Savastano et al., 2015). In contrast, several associations have been reported for liver enzymes, including clinically abnormal serum levels of gamma glutamyl transpeptidase (GGT), lactate dehydrogenase (LD) and alkaline phosphatase (AP) in adults from the NHANES (Lang et al., 2008; Melzer et al., 2010), and increased serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and GGT levels in elderly (Lee et al., 2014a). More studies are needed to elucidate whether BPA exposure could impact liver function in adults.

Low-grade chronic inflammation is closely related to obesity and metabolic syndrome (Saltiel and Olefsky, 2017). BPA exposure has been associated with higher levels of serum inflammatory markers such as interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α) (Savastano et al., 2015), high-sensitivity c-reactive protein (hs-CRP) (Choi et al., 2017), CRP (Lang et al., 2008) and IL-10 (Song et al., 2017). However, more experimental and epidemiologic data are needed to better understand the possible mediating role of inflammation in BPA-related metabolic effects.

The albumin:creatinine ratio (ACR) is considered a biomarker of early endothelial dysfunction in both children and adults (Bartz et al., 2015). In NHANES, childhood urinary BPA concentrations were cross-sectionally associated with higher urinary ACR levels (Trasande et al., 2013). Another cross-sectional analysis among Chinese adults found similar associations between urinary BPA and a higher risk of low-grade albuminuria (Li et al., 2012). Interestingly, a recent pilot study detecting more BPS than BPA concentrations in children's urine, found that BPS but not BPA concentrations were associated with a higher urinary ACR (Kataria et al., 2017). The ACR should be further studied as a potential biomarker of BPA-related endothelial dysfunction.

Beyond its skeletal effects, vitamin D receptors have been found in many tissues, including the cardiovascular, immune, and reproductive systems (Norman, 2008). In NHANES, higher urinary BPA concentrations were cross-sectionally associated with reduced vitamin D levels in women but not in men (Johns et al., 2016). Maternal prenatal urinary BPA was also associated with reduced maternal serum vitamin D levels and a higher risk of deficiency (Johns et al., 2017). Additionally, amongpatients with chronic obstructive apnea, serum BPA concentrations were negatively associated with serum vitamin D levels (Erden et al., 2014a). Further studies are warranted to understand the possible interaction between bisphenols and altered levels of the so-called "hormone D" (Norman, 2008).

3.1.3.5. Allergy/Immune biomarkers.

Immunoglobulin (Ig)E and specific IgE antibodies play an important role in allergic (atopic) processes including asthma (Froidure et al., 2016). A prospective study among Taiwanese children assessed urinary BPA and serum IgE at 3 and 6 years of age (Wang et al., 2016b). Higher BPA concentrations at age 3 were cross-sectionally associated with increased IgE levels, particularlyin girls. Similar results were found at age 6. BPA concentrations at age 3 were prospectively associated with increased IgE levels at age 6. Additionally, IgE levels mediated 70% of the total effect of BPA on asthma risk (Wang et al., 2016b). In NHANES, urinary BPA was positively associated with allergic asthma in adult females, and with sensitization to various specific allergens in a dose–response manner (Vaidya and Kulkarni, 2012). On the contrary, maternal first-trimester urinary BPA concentrations were non-linearly associated with cord blood IL-33 and thymic stromal lymphopoietin (TSLP), but not IgE levels, in a Canadian mother–child cohort (Ashley-Martin et al., 2015). The possible mediating role of both total and specific IgE antibodies in BPA-related allergic sensitization should be further explored.

3.2. *Prioritization of effect biomarkers in HBM studies*

A wide range of existing bisphenol-related effect biomarkers were identified for most of the human health outcomes screened. Those biomarkers with potential application in HBM studies and supported by mechanistic knowledge organized under the AOP framework were prioritized. First, we discussed the advantages of assessing KiSS and gene expression of nuclear receptors in relation to reproductive effects based on a previously published BPA-related AOP (Viguié et al., 2018). Second, given that biomarkers of brain function were identified as the most important knowledge gap, but no previous BPA-related AOP was available in this area, we built a network integrating mechanistic information on BDNF from three fully-developed AOPs, additionally identifying the pathways through which BPA could lead to altered BDNF function.

3.2.1. *Reproductive effect biomarkers*

Previous studies have observed significant associations between bisphenols exposure and altered reproductive hormone levels (Suppl. Table 4). However, in many cases the interpretation is hindered by diurnal variations in steroid levels, sets of hormones analyzed, analytical techniques used, periods of exposure and feedback loop effects. The implementation of other complementary effect biomarkers at different levels of biological organization could therefore help to achieve a broader and more accurate picture. A recent evidence-based AOP has exhaustively underpinned the endocrine pathways through which female developmental and/or adult BPA exposure may alter estrous cyclicity, thus increasing the risk of adverse fertility and birth outcomes (Viguié et al., 2018).

Fig. 3 synthesizes and depicts the endocrine pathways and tissues potentially disrupted by BPA exposure in females, adapted from Viguié et al. (2018). Briefly, BPA has been consistently shown to reduce aromatase activity in Granulosa cells, thus preventing the preovulatory rise of estrogens at the ovary (Peretz et al., 2014; Viguié et al., 2018). Additionally, BPA has been shown to act at a central level, interfering with the function of Kisspeptinergic neurons, delaying or suppressing the gonadotrophin-dependent peak of LH needed to achieve ovulation as shown in both rodents and primates (Kurian et al., 2015; Ruiz-Pino et al., 2019; Viguié et al., 2018). Thus, BPA might alter estrous cyclicity and ovulation acting locally in the ovary, centrally in the hypothalamus, or both.

In HBM studies, measuring KiSS protein levels in serum, together with traditional hormone markers (LH, FSH, E2 and TT) may allow a better characterization of potential BPA actions on the HP axis (Fig. 3). Serum KiSS levels could also help to identify the menstrual period in which the sample was taken (Zhai et al., 2017). Similar to other effect biomarkers, particular attention must be given to the storage of samples under the best conditions in order to avoid a potential degradation of this protein over time (Gejl et al., 2019). In addition, evaluating KiSS at other biological levels with higher stability over time, such as for example DNA methylation of *KISS1* in peripheral blood mononuclear cells (PBMCs), could help to counteract the variability related to circulating levels. KiSS plays a crucial role during pregnancy and puberty onset, but also during adulthood regulating the ovulatory mechanism (Cortés et al., 2015). Emerging data is also showing a cross-talk between adipose tissue and kisspeptinergic neurons mediated through the adipokine leptin (Cortés et al., 2015). Overall, KiSS represents an understudied but promising effect biomarker of both reproductive and metabolic health.

Apart from fertility clinic settings in which human oocyte retrieval and follicular fluid sampling is possible (Machtinger et al., 2013), assessing whether BPA may decrease aromatase activity in the ovary is not possible in HBM studies using non-invasive matrices. Notwithstanding, the gene expression and/or DNA methylation of estrogenic receptors [estrogen receptor (ER)α, ERβ, estrogen-related receptor (ERR)α, etc.] and perhaps other molecular targets in blood cell populations could help to evaluate the biological plausibility of BPA associations as previously shown (Melzer et al., 2011), especially when complemented with the abovementioned set of reproductive biochemical biomarkers. Indeed, aromatase gene expression in peripheral blood leukocytes from adult women was significantly higher during the follicular phase compared to the luteal phase of the menstrual cycle, and its expression was correlated with circulating E1 and E2 serum levels (Vottero et al., 2006). Thus, aromatase expression in women's blood could represent an interesting surrogate of local aromatase expression in the ovary. Future studies should investigate whether the expression and epigenetic status of molecular markers in PBMCs is predictive of adverse health effects in human populations.

3.2.2. *Neurodevelopmental/neurological effect biomarkers*

The neurotrophin BDNF is a promising effect biomarker that could fill an important knowledge gap regarding neurodevelopmental outcomes associated with bisphenols exposure. However, organized data on potential mechanistic pathways that could lead to altered BDNF levels (and other targets) are needed to support its implementation in HBM studies, as well as identify potential novel molecular markers of brain function. Fig. 4 shows the result of integrating three fully-developed AOPs sharing the key event "reduced BDNF release" leading to thesame AO: impaired learning and memory. Numbers inside boxes correspond to scientific articles referenced below.

In AOP 54, BPA may decrease TH levels through mechanisms involving the sodium/iodide symporter (NIS) and thyroperoxidase (TPO) as illustrated by reduced iodide uptake through non-competitive inhibition of NIS (Wu et al., 2016) [number 11 in Fig. 4] and decreased mRNA levels of *Nis* and *tpo* (Silva et al., 2018) [12] in rat thyroid follicular cell lines, and decreased TPO activity and NIS-mediated thyroidradioiodide uptake in BPA-exposed rats (Silva et al., 2018). Nevertheless, Silva et al. (2018) reported increased serum T4 levels, which contradicts expected effects in this AOP and other studies showing decreased T4 levels in pregnant ewes and their offspring (Viguié et al., 2013) [13] and aged mice with learning and memory deficits following pubertal exposure (Jiang et al., 2016) [14].

AOPs 12 and 13 describe causal events initiated by inhibition of glutamate N-methyl-D-aspartate receptors (NMDARs). AOP 13 focuseson KEs occurring during brain development and AOP 12 adds downstream events during aging. Reduced hippocampal mRNA and/or protein expressions of NMDAR subunits along with negative effects in different forms of learning and memory were reported in male offspring mice (Xu et al., 2010) [1] and rats (Wang et al., 2014) [2] prenatally exposed to BPA; in mice of both sexes following prenatal and postnatal exposure (Tian et al., 2010) [5]; and in mice exposed postnatally (Jardim et al., 2017) [4].

A single neonatal dose of BPA in mice may reduce hippocampal levels of Calcium/calmodulin-dependent kinase II (CaMKII), a protein activated by calcium influx and crucial for learning and memory (Viberg and Lee, 2012) [15]. In addition to impaired learning and memory, prenatal exposure to BPA decreased hippocampal levels of phosphorylated cAMP response-binding element (CREB), known to be triggered by Ca2 + influx (Tao et al., 1998), and its target BDNF in male rats (Wang et al., 2016a) [17], and decreased mRNA levels of NMDAR2B and BDNF in male mice (Kundakovic et al., 2015) [16]. Importantly for HBM purposes, BDNF CpG methylation profiles in mice blood reflected methylation profiles and transcription levels in the hippocampus, suggesting that blood BDNF DNA methylation may be a valid surrogate marker of human brain BDNF expression levels (Kundakovic et al., 2015) [16].

As expected from BDNF's critical role in dendritic arborization and synaptic plasticity (Kowiański et al., 2018), decreased dendritic spine density in region CA1 of the hippocampus (critical for memory formation) was reported in rats following postnatal exposure to BPA (Bowman et al., 2014) [8] and in neonates of non-human primates exposed *in utero* (Elsworth et al., 2013) [6]. Prenatal exposure also reduced the length and number of dendritic branches of postnatal mice,with long-term effects consisting of reduced spine densities in hippocampal CA1 of aged mice (Kimura et al., 2016) [9]. Inhibition of NMDAR1, decreased synaptic proteins (e.g., PSD-95, synapsin I, synaptophysin or spinophilin) and altered synaptic structure were observed in the hippocampus of male mice exposed perinatally (Xu et al., 2013) [7], and rats exposed prenatally (Wang et al., 2014) [2]. Furthermore, perinatal exposure reduced expression of synaptophysin and the excitatory to inhibitory synaptic protein ratio in the hippocampus and cortex of male mice (Kumar and Thakur, 2017) [3].

In turn, neuronal network function as evaluated by electrophysiological techniques may be compromised. BPA decreased the induction of hippocampal long-term potentiation (LTP) in juvenile rats together with reduced NMDAR-mediated postsynaptic current in hippocampal slices. Moreover, consistent with inhibited NMDARs, decreased spine density and pre-synaptic glutamate release, spatial memory was impaired (Hu et al., 2017) [10].

In addition to fewer neurons in various regions of the hippocampus, decreased hippocampal LTP induction and impaired learning and memory, Zhou et al. (2017) [18] demonstrated a dose-dependent DNA damage in adolescent male mice brains after chronic exposure. BPA treatment also reduced the viability of hippocampus-derived neural stem cells and induced apoptosis and neurodegeneration in the hippocampus of rats exposed perinatally (Tiwari et al., 2015) [19] and postnatally with deficits in memory and learning (Agarwal et al., 2015) [20]. The contribution of neuroinflammation to the neurodegenerative effects of BPA is substantiated by increased numbers of microglia in thedentate gyrus of both postnatal rats and adult female voles exposed during early development (Rebuli et al., 2016) [21]. Furthermore, maternal BPA increased microglial and astrocyte activation and elevated TNF-α and IL-6 levels in the prefrontal cortex of female offspring mice (Luo et al., 2014) [22].

Several potential biomarkers of effects related to the aforementioned KEs may be relevant for HBM studies. For instance, in seven yearold girls, buccal DNA methylation levels of the gene encoding NMDAR2B were positively associated with prenatal urinary BPA concentrations (Alavian-Ghavanini et al., 2018). Specific protein 4 (SP4), akey regulator of NMDAR signaling (Priya et al., 2013), has been proposed as a biomarker of early stage psychosis (Fusté et al., 2013; Pinacho et al., 2015), being a sensitive target of BPA exposure *in vivo* (Lam et al., 2011). Additionally, glial cell-derived neurotrophic factor (GDNF) plays an important role in various neuropsychiatric disorders (Ibáñez and Andressoo, 2017) and may be modulated by BPA along with alterations of dopamine and serotonin systems in rats (Castroet al., 2015). Finally, synapsin I, an important protein for neurotransmitter release and synaptic function exhibited similar expression patterns in both PBMCs and the hippocampus of rats and may serve as an early biomarker of cognitive function (Cifre et al., 2018).

Fig. 4 integrates the pathways presented in the three selected AOPs;however, other mechanisms not covered in those AOPs may also be important. For example, NMDAR inhibition plays a fundamental role in this network as a MIE, but also as a KE when regulated *via* nuclear ERsin the hippocampus (El-Bakri et al., 2004). Indeed, ERs colocalize to cells that express BDNF (Sohrabji and Lewis, 2006), and previous experimental evidence suggests that BPA actions in the hippocampus may be mediated by altered estrogenic signaling (Chen et al., 2017; Leranth et al., 2008). Thus, BPA could lead to altered BDNF function through at least two MoAs: disruption of thyroid and estrogenic pathways. At an HBM level, evaluating a combined set of effect biomarkers implicated in this AOP network (mainly THs and BDNF) could better characterize theneurological effects of bisphenols. Moreover, these targets should be

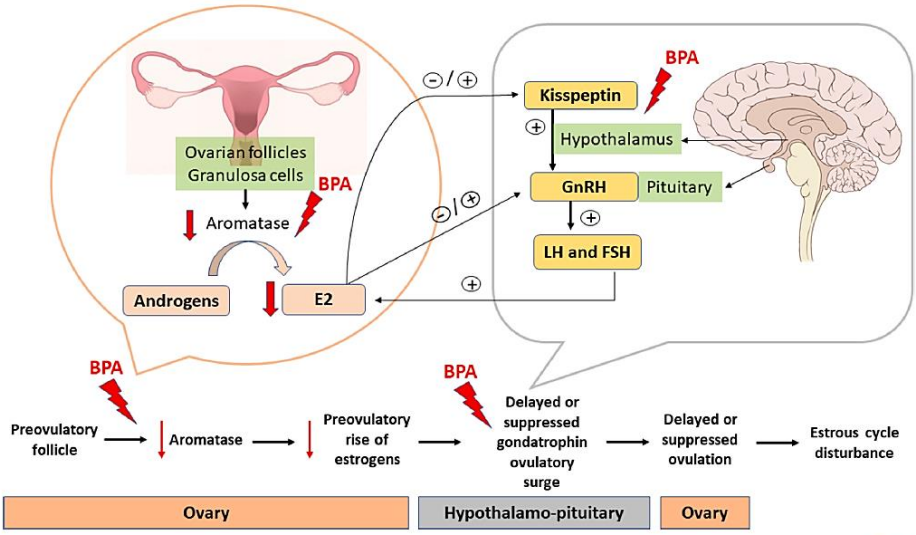

Fig. 3. Hypothesized sequential cascade of BPA events leading to development of altered estrous cycle, adapted from AOP data constructed by Viguié et al. (2018). BPA (bisphenol A); E2 (17β-estradiol); GnRH (gonadotropin-releasing hormone); LH (luteinizing hormone); FSH (follicle stimulating hormone).

assessed at different levels of biological organization (protein levels, gene expression, DNA methylation, etc.) to achieve the most complete picture possible.

A limitation of this AOP network is that the three available AOPs focused on learning and memory, while no AOPs discussing the role of BDNF on behavioral and psychiatric diseases were retrieved from the AOP-Wiki. It's known that BDNF also plays a role in the development of anxiety, depression and mood disorders (Martinowich et al., 2007), andprevious reviews of the epidemiologic evidence found that prenatal BPA exposure is associated with behavioral problems in children (Ejaredar et al., 2017; Mustieles et al., 2015; Rodríguez-Carrillo et al., 2019). Therefore, the role of neurotrophins as potential mediators of bisphenol-related actions on behavioral and emotional problems shouldbe also considered in future epidemiologic studies.

4. Conclusions and future perspectives

This comprehensive literature search has allowed us to create the first inventory of existing effect biomarkers for bisphenols, but also to propose potential novel effect biomarkers that may be implemented in HBM studies. The assessment of mechanistically-based effect biomarkers will help to improve the inference of causal relationships between bisphenols exposure and adverse health outcomes in future HBM and epidemiologic studies. Moreover, parallel efforts for other chemical families are ongoing under the HBM4EU initiative (Baken et al., 2019),which will result in a structured body of work that will enable a more systematic approach for the selection of effect biomarkers in the context of exposure to low-dose complex chemical mixtures. This work will also help to prioritize the selection of effect biomarkers for BPA substitutes, facilitating the evaluation of potential adverse effects in a timely manner, without the need to wait for decades until the onset of an overtdevelopmental or chronic disease state.

As a technical limitation, harmonization of measurement methodologies for effect biomarkers in HBM studies is needed, including the performance of interlaboratory comparisons and quality controls, to avoid variability and misclassification errors, thus improving comparison and replicability among studies. The development of analytical methodologies relying on hyphenated mass spectrometry should be encouraged to progressively substitute ELISA and other immunoassayscommonly employed. In relation to BPA exposure assessment, many
-although not all- of the studies reviewed in this work measured total BPA (free and phase II conjugates) in urine samples using previously validated hyphenated mass spectrometry methodologies following quality assurance and control (QA/QC) measures. In this regard, the HBM4EU initiative has fostered an extensive network of European laboratories that share QA/QC procedures and undergo interlaboratory comparisons to warrant the highest standards for the biomonitoring of exposure to priority compounds. At an epidemiological level, prospective designs with repeated measurements for both exposure and effect biomarkers need to progressively replace cross-sectional studies with only punctual assessments. In addition, future studies should include the implementation of effect biomarkers as potential mediators in the exposure-disease continuum, as a complementary tool to better evaluate exposure-health associations.

Because environmental contaminants such as bisphenols have complex MoA, implementation of effect biomarkers at different levels of biological organization (e.g., DNA, RNA, proteins or metabolites) seems necessary. This point is reinforced considering that, depending on the dose, the target tissue or the window of exposure, the MIE and downstream events may be different. In this review, we proposed to follow the AOP framework for prioritizing the right set of effect biomarkers to be studied. However, there is a limited number of fully developed AOPs. Consequently, efforts are needed to develop more AOPs for manyendpoints and xenobiotics, which is another objective of the HBM4EU initiative. The biomarker paradigm and AOP framework (Figs. 1 and 2) have been followed in this review to provide a practical and visual conceptualization to advance the field of effect biomarkers. Although this structured framework presents many strengths, we acknowledge inherent limitations such as the potential existence of non-monotonic dose–response relationships, the inability to assess dose at target organs, and the difficulty of assessing combined effects in the context of complex chemical mixtures.

In future HBM studies, the combined use of multi-omics technologies to perform global characterization of genes (genomics), genome wide epigenetics, mRNA

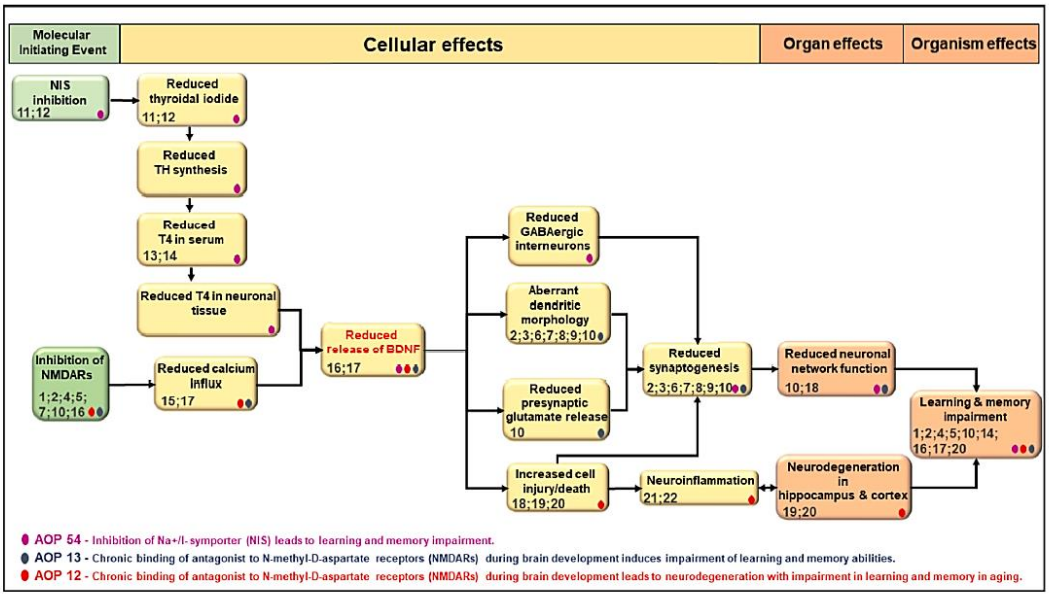

Fig. 4. Network integrating MIEs and KEs from AOPs 12, 13 and 54 leading to a reduced release of BDNF (Boxes), and identification of the steps that can be triggered or promoted by BPA based on available mechanistic data (numbers inside boxes indicate scientific articles referenced in the manuscript).

(transcriptomics), proteins (proteomics) and metabolites (metabolomics) in a specific biological sample seems a very good option to overcome the limitation of looking at the wrong biological level of organization, or the wrong biological targets. Although these technologies are still expensive and require expertise to analyze and interpret the tremendous amount of data generated, systems biology is expanding at a fast rate and the corresponding computational tools are expected to be developed. Moreover, multi-omics should be able to explain complex biological phenomena not only for a class of Xenobiotics but for multiple stressors. Hence, untargeted analyses should be encouraged, preferably coupled to targeted analyses, in orderto uncover the right biological pathways disrupted by xenobiotics, and their corresponding sets of effect markers.

There is an increasing need to rapidly evaluate the safety of exposure to emerging chemicals in human populations. However, many diseases are only triggered after years of chronic exposure to multiple xenobiotics. Therefore, 21st century environmental policymakers may have to consider whether regulation of chemical contaminants should be proactive and informed by changes in molecular profiles predictive of adverse effects, rather than a *sine qua non* reliance on firm health endpoints that may take decades to investigate.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https:// doi.org/10.1016/j.envint.2020.105811.

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ARTICLE 2

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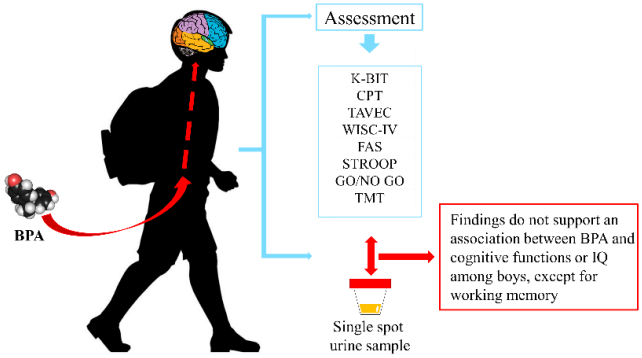
Bisphenol A and cognitive function in school-age boys: Is BPA predominantly related to behavior?

Andrea Rodríguez-Carrillo[a,b], Vicente Mustieles[a,b,c,*], Rocío Pérez-Lobato[a], José M. Molina-Molina[a,b,c], Iris Reina-Pérez[a,b], Fernando Vela-Soria[a,b], Soledad Rubio[d], Nicolás Olea[a,b,c], Mariana F. Fernández[a,b,c,*]

[a] University of Granada, Center for Biomedical Research (CIBM), Spain
[b] Instituto de Investigación Biosanitaria Ibs GRANADA, Spain
[c] Consortium for Biomedical Research in Epidemiology & Public Health (CIBERESP), 18100, Spain
[d] University of Córdoba, Institute of Fine Chemistry and Nanochemistry, 14017 Córdoba, Spain



A B S T R A C T

Background: Bisphenol A (BPA) has been associated with impairments in children's behavior, but few studieshave investigated its relationship with cognitive function.
Objective: To investigate the association of urinary BPA concentrations with cognitive domains and intelligence quotient (IQ) in Spanish boys.
Methods: BPA levels were quantified by liquid chromatography-tandem mass spectrometry (LC–MS-MS) in one spot urine sample from 269 boys of the INMA-Granada cohort, in their follow-up at 9–11 years of age. Cognitive function was evaluated by a trained psychologist using a comprehensive neuropsychological test battery (including general intelligence, language skills, working memory, attention, impulsivity, visual-motor coordination, processing speed and executive function, among others). Cross-sectional associations between BPA levels and neuropsychological standardized scores were analyzed by adjusted linear and logistic regression models. *Results:* Median (P25, P75) BPA concentrations were 4.76 (2.77, 9.03) μg/L and 4.75 (2.75, 10.2) μg/g ofcreatinine (Cr). Boys in the third and fourth quartile of volume-based BPA concentrations showed better pro cessing speed scores than boys in the first quartile (β = 5.47; 95%CI: 1.4, 9.4 and β = 3.57; 95%CI: -0.4, 7.5,respectively); and boys in the third quartile showed better inhibitory control (β = 1.6; 95%CI: -0.3, 3.5) and impulsivity (β= -4.2; 95%CI: -9.0, 0.0). In contrast, boys in the fourth quartile showed poorer working memory scores than those in the first quartile (β= -1.0; 95%CI: -2.1, -0.1). All these associations were attenuated whenCr-standardized BPA concentrations were considered. Cr-based BPA concentrations were also associated with ahigher risk of being below the 20th percentile of working memory scores [OR_a = 1.51; 95%CI: 1.01, 2.25].
Discussion: Our findings do not support an association between urinary BPA concentrations and cognitive function or IQ among boys, except for working memory. BPA was previously found to be associated with behavior problems in the same study population, suggesting that BPA may predominantly affect the behavior of children rather than their cognitive function, in line with previous epidemiologic studies.

1. Introduction

Bisphenol A (BPA) is a man-made chemical extensively used in the manufacture of a wide range of materials, including polycarbonateplastics, epoxy resin liners of canned food, medical devices, some dental sealants, and thermal receipts, among others (Vandenberg et al., 2007; Molina-Molina et al., 2019). Even more, BPA has recently been detectedin socks for infants, highlighting the novel role of textiles as potential sources of chemical exposure (Freire et al., 2019). Diet is considered the predominant source of exposure to BPA in the general population, due its leaching from food packaging and can liners (Vandenberg et al., 2010), followed by dermal absorption and inhalation (Ehrlich et al., 2014; Michałowicz, 2014).

Biomonitoring studies have confirmed the continuous and chronic exposure of humans to BPA, explaining its detection in the urine of more than 90% of the general population in Europe and the USA (Becker et al., 2009; Calafat et al., 2008; Casaset al., 2011; Vandenberg et al., 2010). BPA has been detected in other biological matrices besides urine, such as maternal blood, placenta, amniotic fluid, cord and fetal serum blood, and maternal breast milk, demonstrating the internal exposure to BPA of mothers, fetuses, and newborns (Vandenberg et al., 2010). BPA is a known endocrine-disrupting compound (EDC) with the potential to alter hormonal homeostasis (Mustieles et al., 2018a), even at environmentally relevant doses, through its effects on

on multiple steroid hormone receptors that mediate a myriad of cellular effects (Casals-Casas and Desvergne, 2011; Vandenberg et al., 2010). Severalin vitro and in vivo studies have shown that BPA can interfere with estrogenic pathways by binding to nuclear estrogen receptors (ERs) (Wetherill et al., 2007) as well as other membrane estrogen receptor families (Alonso-Magdalena et al., 2012). It has also been found to exert antagonistic activities after binding to the androgen receptor (Molina- Molina et al., 2013) and to alter the expression of steroidogenic enzymes, as well as interacting with glucocorticoid, PPAR-γ, and thyroid signaling pathways (Mustieles et al., 2015, 2018b).

The developing brain is a key target for BPA, and experimental studies have associated pre-, peri, and post-natal BPA exposure with various alterations in brain structure, behavior, and certain cognitive domains (Nesan et al., 2018). Rodent models have shown that BPA canalter the expression of nuclear estrogen receptors in the amygdala and hypothalamus (Cao et al., 2013; Rebuli et al., 2014), in neuronal cell populations in the substantia nigra (Tando et al., 2007), in the periventricular preoptic area of the hypothalamus (Rubin et al., 2006), andin the locus coeruleus (Tando et al., 2014). Behavioral changes linked to BPA exposure include increased anxiety (Luo et al., 2014; Xu et al., 2012), hyperactivity (Komada et al., 2014), greater aggressiveness (Patisaul and Bateman, 2008), and modifications in socio-sexual (Porrini et al., 2005) and play (Dessì-Fulgheri et al., 2002) behaviors. Rodent studies have also found that BPA exposure induces spatial and non-spatial memory impairments associated with neural modifications, especially in processes involved in synaptic plasticity (Kuwahara et al., 2013; Mhaouty-Kodja et al., 2018; Poimenova et al., 2010; Weinberger et al., 2014; Xu et al., 2010).

Although most published studies have analyzed the relationship of developmental BPA exposure with the behavior of children, which has been reported to be sex-specific (Casas et al., 2015; Findlay and Kohen, 2015; Mustieles et al., 2015, 2018b; Perera et al., 2012; Roen et al., 2015), much less is known about its relationship with their cognitive functions. Therefore, the objective of this study was to explore the relationship of urinary BPA concentrations with cognitive functioning in agroup of Spanish boys aged 9–11 years.

2. Methods

2.1. Study population

The INMA (Infancia y Medio Ambiente – Environment and Childhood) cohort is a population-based study developed in seven Spanish regions with the aim of exploring the possible effects of environmental pollutants during pregnancy and early childhood (Guxens et al., 2012) on child growth and development. From 2000 to 2002, theINMA-Granada sub-cohort enrolled 668 mother-son pairs (Fernandez et al., 2007). For the current study, all families from the INMA-Granadacohort were contacted and invited to participate in the 2010–2012 follow-up, when the children reached the age of 9–11 years. Written consent was obtained from 300 families (44.9%), and BPA concentrations were available for 298 boys. Eighteen of these children were excluded from the present study for the presence of chronic disease that could interfere with cognitive development, i.e., hyperthyroidism(n = 1), diabetes (n = 1), language disorder (n = 2), attention deficit hyperactivity disorder (ADHD) (n = 7), Noonan syndrome (n = 1), Asperger syndrome (n = 2), cerebral palsy (n = 1), Tourette syndrome with ADHD (n = 1), and Charcot–Marie–Toth syndrome with ADHD (n = 1), and for a history of brain tumor surgery (n = 1). Additionally, 11 children were excluded due to an incomplete neuropsychological assessment or the absence of important covariate data. Therefore, the final study sample size comprised 269 mother-son pairs with data available on urinary BPA concentrations, cognitive assessment data, and covariates. The study followed the principles of the declaration of Helsinki and was approved by the Ethics Committee of San Cecilio University Hospital (Granada, Spain).

2.2. BPA exposure assessment

At the follow-up session at the hospital, always held between 5 p.m. and 8 p.m., a single non-fasting spot urine sample was collected in a 10- mL polypropylene tube and immediately stored at -20 °C. Total BPA (free plus conjugated) was quantified at the laboratory of the Analytical Chemistry Department of Cordoba University (Spain) as previously described (Perez-Lobato et al., 2016), using liquid chromatography- mass spectrometry with a limit of detection (LOD) of 0.1 μg/L and a limit of quantification (LOQ) of 0.2 μg/L. Urinary creatinine (Cr) concentrations (mg/dl) were determined at the Public Health Laboratory of the Basque Country (Spain) to account for urine dilution (Fernándezet al., 2015). The researchers who conducted the urine analyses were blinded to the characteristics of the study participants. Collection, storage, and processing of urine samples were conducted under controlled conditions, and account was taken of background BPA contamination from the presence of polymers in components of the urine collection containers and/or equipment or labware. An inter-laboratory comparison was also performed with the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (IPA)—Institute of the Ruhr-University Bochum (Germany), with considerable experience in BPA determination.

2.3. Neuropsychological assessment

The cognitive function of each boy was assessed at the University Hospital facilities by a trained neuropsychologist (RPL) blinded to theirexposure levels, using a comprehensive neuropsychological battery of tests (Perez-Lobato et al., 2015). This battery was chosen because it assesses omnibus features of child neurodevelopment (e.g., IQ, language, attention, processing speed, etc.) and because previous epidemiologic studies have used similar tests to study cognitive domains in relation to BPA exposure (Mustieles et al., 2015):

1. **General cognitive intelligence:** applying the Kaufman Brief Intelligence Test (K-BIT) (Kaufman and Kaufman, 1997) and basing the IQ calculation on verbal and nonverbal scale scores.
2. **Language:** using the verbal scale of the K-BIT (Kaufman and Kaufman, 1997), which has two subtests: i) verbal knowledge, in which the child must name graphically displayed objects to assess receptive vocabulary; and ii) general knowledge and riddles, in which the child must name words from their definition and their visualization with letters missing to assess expressive reasoning.
3. **Attention:** applying the continuous performance test (CPT) (Conners, 1995) to assess sustained and selective attention togetherwith impulsivity. In this task, the child must press any key on the keyboard as quickly as possible whenever the letter 'A' appears in yellow ('go' condition). Dependent variables for analyses are hits (press any key in 'go' condition), commission errors (press any key in 'no go' condition), omission errors (no key pressed in 'go' condition), and attention index.
4. **Verbal memory:** applying the Complutense-Spain Madrid verbal learning test (TAVECI) (Benedet et al., 2001) to assess different memory and learning processes, including immediate recall, short- and long-term recall, and recognition. The examiner reads aloud a list of 15 words five times. The child must state words recalled immediately after each reading and then after intervals of 10 and 20 min.

5 **Visual-motor coordination:** applying the Trail Making Test part A (TMTA) (Reitan, 1958), in which the child must connect consecutive numbers in an alternating sequence as quickly as possible, with the time in seconds (s) as dependent variable.

6 **Processing speed:** calculating the sum of two subtests (symbol search and coding) from Edition IV of the Wechsler Intelligence Scales for Children (WISC-IV) (Wechsler, 2007). The two tasks mustbe completed in a maximum of 2 min. In the first task, the child selects certain figures from a series of figures. In the second task, the child fills in spaces under numbers with corresponding symbols following an established model.

7 **Executive function:** assessing four components: updating, inhibition, shifting, and abstract reasoning (Diamond, 2013).

8 **Updating measurements:** assessing two components: a) Working memory: the child listens to a letter–number sequencing subtest from the WISC-IV (Spanish version) and repeats the numbers fromthe lowest to the highest, followed by repetition of the letters in alphabetic order (Wechsler, 2007); b) Verbal fluency: the children must say as many names of animals as possible during a 1-min period in the categorical verbal fluency test (FAS), with the number of animals correctly pronounced as dependent variable (Benton andHamsher, 1989).

9 **Inhibition:** applying two subtests: a) the Spanish version of the Stroop Color and Word Test (Golden, 2005) to evaluate cognition and inhibition, in which the child must name colored words (condition 1), read color-words printed in black ink (condition 2), and name the color of words printed in colors that conflict with their meaning, e.g. the word 'red' appears in yellow ink (condition 3, inhibition); the dependent variable is the interference score, calculated from the results of the three conditions using a specific formula; b) the Go/No-Go task (Donders, 1969) to evaluate motor inhibition, in which the child must respond to certain stimuli on the computer screen while inhibiting any response to a distracter stimulus, with the hit and false alarm rates as dependent variables.

10 **Shifting:** using part B of the TMT (Reitan, 1958), in which the child must connect consecutive numbers and letters in an alternating sequence as quickly as possible, with the time measured in seconds (s)as dependent variable.

11 **Abstract reasoning (matrix analogies test):** measured with the non-verbal scale of the K-BIT (Kaufman and Kaufman, 1997). The child selects a picture that best completes a visual pattern followinga visual analogy. The dependent variable is the number of correct responses.

A higher score always indicates better cognitive function except for the following tests, in which a higher score indicates worse cognitive function: i) subtests "*commission errors"* and *"omissions"* from the *CPT* test on sustained attention function; ii) *the TMT-A* test of visual-motor coordination function; iii) the *TMT-B* test of shifting; and iv) the "*false- alarm rate"* subtest of the Go/No-Go test on impulsivity and/or inhibition function.

2.4. *Covariates*

Models were adjusted for the same set of covariates as in a previous study of BPA and behavior in the same population (Perez-Lobato et al., 2016), including: breastfeeding and smoking during pregnancy; child's age and body mass index [BMI (kg/m^2)] (Wirt et al., 2015), and mother's age, marital status (married/not married), education level (university/secondary school/up to primary) and mother's intelligence score, measured with the Similarities subtest of the Wechsler Adult Intelligence-Third Edition (WAIS-III) at the time of assessment. The final models also included as potential confounders exposure to environmental tobacco smoke (any/none) in the home, based on questionnaire.

2.5. *Statistical analysis*

The analysis was based on our previous experience of behavior and cognitive functioning in these same children from the INMA-Granadacohort (Pérez-Lobato et al., 2015 and 2016). In the descriptive analysis of parental and children characteristics, absolute frequencies were calculated for categorical variables, and measures of central tendency and dispersion for continuous variables. The relationship of urinary BPA concentrations with the characteristics of children and parents wasanalyzed with the Mann–Whitney or Kruskal–Wallis test for categorical covariates and the Spearman correlation test for continuous covariates.The median value and interquartile range (IQR) of BPA concentrations (μg/L) were calculated. BPA exposure was considered using bothvolume-based urinary BPA (μg/L) and creatinine (Cr)-standardized ur inary BPA (μg/g) concentrations, as independent variables, as recommended (LaKind and Naiman, 2015). These concentrations were $\log_e$-transformed to standardize the data distribution and reduce the influence of outliers. BPA levels were also divided into quartiles toevaluate potential non-linear associations. Mean (standard deviation), median and range values were calculated for neuropsychological test scores, which were analyzed as continuous variables because some scores could not be standardized for Spanish child populations.

Linear regression models were used to analyze the association between BPA concentrations and neuropsychological test scores, withBeta coefficients representing the mean change in neuropsychological scores associated with each unit increase in $\log_e$-BPA concentrations or the mean difference in neuropsychological scores between the second, third, or fourth quartiles and the first quartile of BPA exposure. Their respective 95% Confidence Intervals (CIs) were also calculated. Logistic regression models were also performed using $\log_e$-transformed Cr- standardized BPA concentrations to analyze the risk of being below or above the 20th or 80th percentile of cognitive function scores, calculating multivariable-adjusted odds ratios (ORs) with their corresponding 95% CIs. All models were adjusted for children's age and BMI,tobacco exposure at home, mothers' age, intelligence, marital status, education level, breastfeeding, and smoking during pregnancy.

SPSS v20.0 (IBM, Chicago, IL) was used for data analyses, and the significance level was set at $p \leq 0.05$.

3. Results

3.1. *Characteristics of the study population*

The mean (± standard deviation) age of the children was 9.9 (± 0.3) years, the mean BMI 19.2 (± 3.5) kg/m^2, and around one third of families reported tobacco exposure at home (Table 1). The median age was 39.7 (± 4.9) years for mothers and 42.1 (± 5.4) years for fathers; 23.4% of mothers and 21.6% of fathers had completed university education, around 90% of mothers and fathers were married, 21.6% of mothers reported smoking during pregnancy, and 87% reported breastfeeding the child. The place of residence was urban in 24.5% of families, semirural in 58%, and rural in 17.5% (Table 1).

BPA was found in all urine samples, at a wide range of concentrations. Fig. 1 shows a boxplot of BPA concentrations. Median (P25, P75) urinary BPA concentrations (μg/L) and Cr-standardized BPA concentrations (μg/g) were 4.76 (2.77, 9.03) and 4.75 (2.75, 10.2), respectively. Table 2 displays the distribution of the raw cognitive function scores of the children. Median IQ was 108 and all remaining cognitive scores were within normal ranges.

3.2. *Cross-sectional associations between BPA and cognitive function scores*

When $\log_e$-transformed volume-based urinary BPA concentrations (μg/L) were used as a continuous variable, higher BPA levels tended to be associated with greater cognitive function scores, although in general, confidence intervals included the null value. In adjusted models, inhibitory control [third quartile β = 1.6; 95%CI: -0.3, 3.5] and impulsivity [third quartile β= -4.2; 95%CI: -9.0, 0.0] (Table 5). In contrast, boys in the fourth quartile showed worse working memory than those in the first quartile [fourth quartile β= -1.0; 95%CI: -2.1, -0.1]. All these associations were attenuated when quartiles of Cr-standardized BPA concentrations were considered (Table 5).

Table 1
Geometric mean and 'geometric mean standard deviation' of urinary BPA concentrations (μg/L) by demographic characteristics of the study population (n = 269).

	n	Mean (SD)/%	BPA (μg/L) Median	GM ± GSD	*p* Value[1]
Child Variables					
Age (years)	269	9.9 (0.3)	–	–	0.08
BMI (kg/m^2)	269	19.2 (3.5)	–	–	0.93
Urinary creatinine (mg/dL)	269	90.2 (0.5)	–	–	0.07
IQ	269	108.2 (11.8)	–	–	0.15
Area of residence (%)					
Urban	269	24.5	5.2	4.7 ± 2.7	0.66
Semiurban	269	58.0	4.7	4.7 ± 2.5	
Rural	269	17.5	4.4	4.2 ± 2.8	
Tobacco Exposure (%)					
Yes	269	29.4	4.7	4.7 ± 2.5	0.85
No	269	70.6	4.8	4.4 ± 2.8	
Maternal Variables					
Age (years)	269	39.7 (4.9)	–	–	0.55
Verbal reasoning[2]	269	14.9 (4.6)	–	–	0.76
Marital status (%)					
Married	269	90.7	4.7	4.6 ± 2.5	0.57
Unmarried	269	9.3	5.2	4.6 ± 3.2	
Education Level (%)					
University	269	23.4	5.0	5.2 ± 2.7	0.26
Secondary school	269	31.6	5.0	4.8 ± 2.3	
Up to primary	269	45.0	4.13	4.2 ± 2.7	
Breastfeeding (%)					
Yes	269	87.0	4.7	4.6 ± 2.5	0.88
No	269	13.0	5.1	4.6 ± 3.5	
Smoking during pregnancy[3] (%)					
Yes	269	21.6	4.9	4.7 ± 2.6	0.85
No	269	78.4	4.7	4.6 ± 2.6	
Paternal Variables					
Age (years)	262	42.1 (5.4)	–	–	0.42
Marital status (%)					
Married	263	90.3	4.7	4.6 ± 2.5	0.62
Unmarried	263	9.7	5.1	4.6 ± 2.7	
Education level (%)					
University	266	21.6	4.3	4.2 ± 2.7	0.30
Secondary school	266	32.0	5.0	5.5 ± 2.5	
Up to primary	266	45.4	4.8	4.3 ± 2.6	

GM: geometric mean; GSD: geometric standard deviation; SD = standard deviation; IQ: intelligence quotient.
[1] *p*-Value: Statistical significance value reached for hypothesis testing (Mann-Whitney test for breastfeeding, smoking during pregnancy, Kruskal–Wallis test for area of residence, maternal and paternal education. Finally, for continuous variables, bivariate analysis with Spearman correlation test).
[2] Verbal reasoning, measured by Similarities subtest of WAIS-III.
[3] Mother's habit.

higher BPA concentrations were associated with lower impulsivity [β = -1.70; 95%CI: -3.36, -0.05], faster processing speed [β = 1.57; 95%CI: 0.09, 3.04], and better inhibitory control [β = 0.75; 95%CI: 0.06, 1.43] (Table 3). However, when $\log_e$-transformed Cr-standardized urinary BPA concentrations (μg/g) were used as continuous variable, adjusted models showed an attenuation of previous associations towards impulsivity [β = -1.67; 95%CI: -3.32, -0.00], processing speed [β = 1.29; 95%CI: -0.20, -2.77], and inhibitory control [β = 0.61; 95%CI: -0.08, 1.30] (Table 4).

When volume-based urinary BPA concentrations were categorized in quartiles, the processing speed was faster for boys in the third and fourth quartile of BPA concentrations than for those in the first quartile [third quartile β = 5.47; 95%CI: 1.4, 9.4; fourth quartile β = 3.57; 95%CI: -0.4, 7.5], while boys in the third quartile showed better

Logistic regression analyses also showed that higher loge-transformed Cr-standardized BPA concentrations were associated with ahigher risk of being below the 20th percentile of working memory, in both unadjusted and adjusted models [OR = 1.44; 95%CI: 1.00, 2.08; OR a = 1.51; 95%CI: 1.01, 2.25]. No associations were observed with the remaining cognitive functions evaluated (Table 6).

4. Discussion

The results of this study do not support a relationship between urinary BPA concentrations and cognitive abilities in boys at 9–11 years of age. Initially, some associations were observed with specific cognitive functions, but these were attenuated or disappeared when the models were adjusted for covariates or when Cr-standardized BPA concentrations were considered, with the exception of working memory. Given the comprehensive assessment of neuropsychological functions conducted, the absence of associations is surprising, especially in the context of previous associations found with behavior throughout the literature (Mustieles et al., 2015).

A previous study of the same study population, including the same 269 boys as in the current analysis, found that higher volume-based andCr-standardized BPA concentrations were both associated with worse behavioral scores in all scales, mainly with more somatic complaints and more social and thought problems, and that there were no substantial differences between unadjusted and adjusted models (Perez- Lobato et al., 2016). In contrast, no consistent associations were observed between urinary BPA concentrations and cognitive functions in the present study, even when models were adjusted for the same set of covariates, except for a potential association between higher BPA concentrations and poorer working memory.

Our findings are in line with previous epidemiological studies on child behavior and cognitive functions, suggesting that BPA exposure may be predominantly associated with behavior domains rather than cognitive functions. Although epidemiologic evidence on the impact of BPA exposure in children has been strengthened over the past few years, most researchers have assessed behavior rather than cognitive function. Moreover, the few studies on cognitive function have published less consistent results than those obtained for children's behavior (Ejaredar et al., 2017; Mustieles et al., 2015, 2018b). Thus, in the EDENbirth cohort, Philippat et al. (2017) reported the association of prenatalurinary BPA concentrations with relationship problems and hyperactivity–inattention in children at 3 and 5 years of age (Philippat et al., 2017). In the same cohort, Nakiwala et al. (2018) found no association in the boys between prenatal BPA exposure and the verbal performance or IQ (Nakiwala et al., 2018) (see Supplemental Table 1). A similar pattern was observed in the Spanish INMA-Sabadell cohort, in which prenatal BPA exposure was associated with an increased risk of ADHD-hyperactivity symptoms and psychomotor development but not with cognitive development (Casas et al., 2015). Likewise, in a study of the MIREC mother-child cohort (Braun et al., 2017a), higher prenatal urinary BPA concentrations were related to more behavior problems in the children at 3 years of age, but no significant associations were found with most of cognitive functions (Braun et al., 2017a). Finally, exposureto dental composite restorations based on bisphenol A-glycidyl methacrylate was associated with behavior problems but not cognitive functions in the New England Children's Amalgam Trial (NECAT) of children aged 6–10 years (Maserejian et al., 2012a, b; Maserejian et al., 2014). Further details of these epidemiological studies are summarizedin Supplemental Table 1.

Prenatal urinary BPA concentrations have been consistently associated with more behavior problems

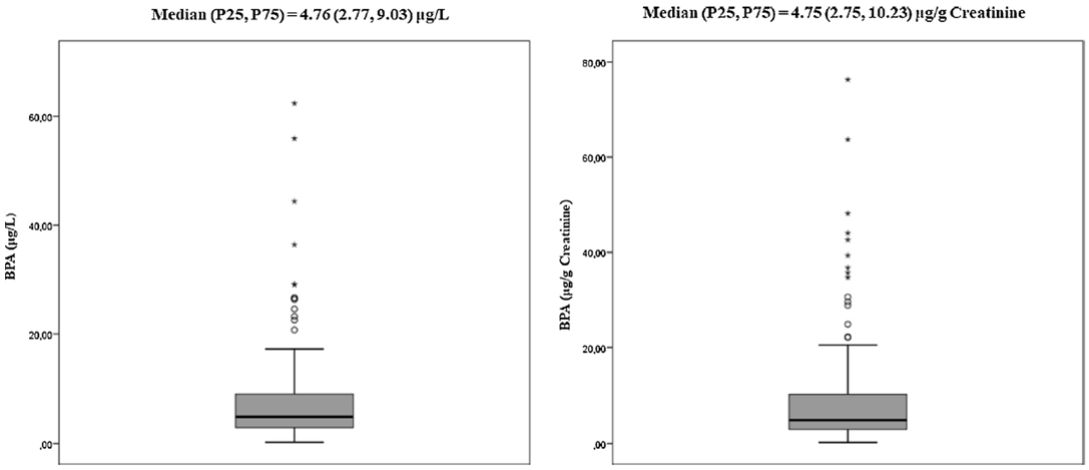


Fig. 1. Boxplots of urinary BPA concentrations (µg/L) and urinary creatinine-adjusted BPA (µg/g) in 269 boys from the INMA-Granada cohort.

and worse executive function during infancy, persisting into early and late childhood among childrenfrom the HOME cohort Study (Braun et al., 2011, 2017b; Stacy et al., 2017). However, prenatal BPA exposure has not been associated with any specific cognitive domain or with the IQ over time (Braun et al., 2017a; Stacy et al., 2017). Only BPA exposure was cross-sectionally associated with worse overall cognition, verbal abilities and speed of mental processing among boys in the HOME study at 8 years of age (Stacy et al., 2017), and only working memory, a component of executive function, remained negatively and significant associated with BPA exposure among boys in the MIREC study at 3 years of age (Braunet al., 2017a). Another exception is the association of higher prenatal urinary BPA concentrations with a greater risk of poorer language skillsamong male but not female toddlers in the Odense Child Cohort, suggesting a possible sex-specific effect of BPA on early language development, although no association was found with ADHD symptoms (Jensen et al., 2019).

Table 2
Children's scores in neuropsychological tests.

Cognitive Function	Test	n	Median	Mean	SD	Range
General Cognitive	IQ[b]	269	108.0	108.2	11.8	77 to 141
Verbal expression and comprehension	K-BIT					
	Full verbal[b]	269	52.0	51.2	5.5	32 to 73
Sustained attention	CPT					
	Correct detection[b]	258	65.0	63.7	5.7	35 to 70
	Commission errors[c]	258	7.0	9.6	11.6	0 to 110
	Omissions[c]	258	5.0	6.4	5.6	0 to 35
	Attention Index[b]	258	1.0	0.7	0.4	0 to 1
Verbal Memory	TAVEC					
	Immediate Recall Trial 1[b]	269	7.0	7.0	1.7	3 to 12
	Immediate Recall Trial 5[b]	269	12.0	11.9	2.1	6 to 15
	Short-term recall[b]	269	11.0	11.0	2.4	4 to 15
	Long-term recall[b]	269	12.0	11.5	2.5	4 to 15
Visual-motor coordination	TMT-A time (s)[c]	268	27.0	29.0	10.8	9 to 86
Processing speed	WISC-IV					
	Verbal Performance[b]	268	99.0	97.5	11.4	64 to 130
Executive Functions						
Working Memory	WISC-IV					
	Letter-number sequencing[b]	269	17.0	17.4	2.7	10 to 25
Verbal Fluency	FAS[b]	267	17.0	17.0	4.2	6 to 28
Impulsivity/Inhibition	STROOP					
	Words[b]	267	114.0	113.9	10.3	86 to 141
	Colors[b]	267	79.0	79.2	9.0	55 to 109
	Words and Colors[b]	267	49.0	48.6	7.0	30 to 71
	Inhibitory Control (Interference)[b]	267	2.0	2.2	5.3	−16.7 to 20.8
	GO NO GO					
	Hit rate[b]	255	1.0	1.0	0.04	0.5 to 1.0
	False-alarm rate[c]	255	0.05	0.06	0.59	0.00 to 0.39
Shifting	TMT-B time (s)[c]	268	53.0	60.1	31.8	23 to 360
Abstract reasoning	Matrix analogies[b]	269	31.0	31.1	4.7	18 to 44

CPT = Continuous performance test; FAS = Verbal fluency test; IQ = Intelligence quotient; K-BIT = Kaufman brief intelligence test; SD = Standard deviation; s = seconds; STROOP = Stroop color and word test; TAVECI = Complutense-Spain Madrid verbal learning test; TMT = Trail making test; WISC-IV = Wechsler Intelligence Scale for Children;
[a]For all tests, direct scores were used in the analysis.
[b] Higher scores determines better cognitive function.
[c] Higher score determines worse cognitive function.

Table 3
Distribution of cognitive function scores by log_e BPA (μg/L) concentrations among 269 boys from the INMA-Granada cohort.

Cognitive Functions		BPA (log_e transformed, unadjusted for covariates)		BPA (log_e transformed, adjusted for covariates[e])	
	n	β	95%CI	β	95% CI
Intelligence quotient (IQ)[b]	269	1.13	−0.36;2.62	0.21	−1.26; 1.69
Verbal comprehension[b]	269	0.31	−0.39;1.01	0.16	−0.57; 0.89
Impulsivity[c]	258	−1.39	−2.87;0.09†	−1.70	−3.36; -0.05*
Attention Index[b]	258	−0.03	−0.09;0.02	−0.04	−0.10;0.02
Long-term recall[b]	269	−0.01	−0.32;0.31	−0.14	−0.47;0.20
Immediate Recall Trial 5[b]	269	0.01	−0.26;0.27	−0.13	−0.41; 0.15
Visual-motor coordination (s)[c]	268	0.17	−1.20:1.54	0.33	−1.10; 1.77
Processing speed[b]	268	1.57	0.13;3.01*	1.57	0.09;3.04*
Working Memory[b]	269	−0.05	−0.40;0.29	−0.25	−0.61;0.11
Verbal Fluency[b]	267	0.18	−0.34;0.71	0.21	−0.35;0.76
Inhibitory Control (Interference)[b]	267	0.65	−0.18;1.32†	0.75	0.06;1.43*
Hit rate[b]	255	0.00	−0.00;0.01	0.00	−0.00;0.01
False-alarm rate[c]	255	−0.00	−0.01;0.01	−0.00	−0.01;0.00
Shifting (s)[c]	268	−0.99	−5.03;3.05	−2.12	−5.76;1.52
Abstract reasoning[b]	269	0.28	−0.36;0.87	0.01	−0.58;0.60

CI: confidence interval; s = seconds. * $p \leq 0.05$; † $p \leq 0.1$.
[a]We show the most relevant abilities measured. For all tests, direct scores were used in the analysis.
[b]A higher score indicates better cognitive function.
[c]A higher score indicates worse cognitive function.
[d]Immediate Recall Trial 5 (TAVECI).
[e]Models were adjusted for child's age (years), BMI (kg/m^2), smoking exposure at home (yes/no), mother's age at time of assessment (years), mother's intelligence score, maternal marital status (married/unmarried), maternal education (university/secondary school/up to primary), smoking exposure during pregnancy (yes/no) and breastfeeding (yes/no).

Table 4
Distribution of cognitive function scores by log_e BPA Cr-standardized (μg/g) concentrations among 269 boys from the INMA-Granada cohort.

Cognitive functions[a]		BPA/Creatinine (μg/g) (log_e transformed, unadjusted for covariates)		BPA/Creatinine (μg/g) (log_e transformed, adjusted for covariates[e])	
	n	β	95% CI	β (SE)	95% CI
Intelligence quotient (IQ)[b]	269	0.55	−0.94;2.03	−0.07	−1.54;1.41
Verbal comprehension[b]	269	0.09	−0.60;0.80	0.16	−0.58;0.89
Impulsivity[c]	258	−1.30	−2.77;0.18†	−1.67	−3.32; -0.00*
Attention Index[b]	258	−0.03	−0.09;0.03	−0.04	−0.10;0.03
Long-term recall[b]	269	−0.02	−0.33; 0.30	−0.18	−0.51;0.15
Immediate Recall Trial 5[b]	269	0.06	−0.21;0.32	−0.10	−0.38;0.18
Visual-motor coordination (s)[c]	268	0.08	−1.28;1.44	−0.00	−1.44;1.44
Processing speed[b]	268	1.31	−0.12;2.75†	1.29	−0.20;2.77†
Working Memory[b]	269	−0.07	−0.41;0.27	−0.20	−0.56;0.16
Verbal Fluency[b]	267	0.17	−0.36;0.70	0.28	−0.28;0.84
Inhibitory Control (Interference)[b]	267	0.48	−0.19;1.14	0.61	−0.08;1.30†
Hit rate[b]	255	0.00	−0.00;0.01	0.00	−0.00;0.01
False-alarm rate[c]	255	−0.00	−0.01;0.00	−0.01	−0.01;0.00
Shifting (s)[c]	268	−0.07	−4.08;3.94	−1.44	−5.10;2.21
Abstract reasoning[b]	269	−0.09	−0.68;0.50	−0.18	−0.77;0.41

SE = standard error; CI: confidence interval; s = seconds. * $p \leq 0.05$; † $p \leq 0.1$.
[a]We show the most relevant abilities measured. For all tests, direct scores were used in the analysis.
[b]A higher score indicates better cognitive function.
[c]A higher score indicates worse cognitive function.
[d]Immediate Recall Trial 5 (TAVECI).
[e]Models were adjusted for child's age (years), BMI (kg/m^2), smoking exposure at home (yes/no), mother's age at time of assessment (years), mother's intelligence score, marital status (married/unmarried), maternal education (university/secondary school/up to primary), smoking exposure during pregnancy (yes/no) and breastfeeding (yes/no).

Differences in findings on the effects of BPA exposure between children's behavior and their cognitive functions may in part be attributable to methodological factors. Thus, trained psychologists usually conduct the assessment of cognitive function at a specific time point, whereas the parents report on the behavior of their children in daily life. It is also possible that sex-specific behaviors may be more sensitiveto BPA exposure in comparison to cognitive domains and the IQ, whichmay also explain the different patterns described in the literature (Mustieles et al., 2015, 2018b). In general, the relationship of BPA exposure to behavior problems rather than cognition appears to be a consistent pattern in the epidemiologic literature, although further studies on cognition are required.

Experimental studies, mostly in rodents, have shown that BPA exposure can interfere with diverse brain areas and functions, leading notonly to behavioral changes but also to impairments in memory and spatial learning, among other functions (Braun et al., 2017c; Gore et al., 2018). These results could be at least partially explained by hormonal mechanisms related to an altered estrogen-androgen balance (Mustieles et al., 2015). These include subtle epigenetic disruptions of nuclear estrogenic receptors and developmental alterations in sexually-dimorphic brain areas and functions in response to low BPA doses, as shown in the CLARITY-BPA studies, among others (Arambula et al., 2018, 2017,2016; Kundakovic et al., 2013). A recent review of the CLARITY-BPA studies confirms this altered neuroendocrine development, and the abrogation of brain and behavioral sexual dimorphisms, even at BPA doses below those considered "safe" for humans by regulatory agencies (Patisaul, 2019).

The median urinary BPA concentration in this study was 4.75 μg/L (4.76 μg/g Cr), similar to some previous findings in children of the same age (Calafat et al., 2008) and younger populations (Braun et al., 2011; Casas et al., 2011; Perera et al., 2012); however, it was higher than observations in other studies of children of the same age, including: the German Environmental Survey on Children (Becker et al., 2009), 2.13 ng/mL; the 2005–2006 NHANES study (Lakind and Naiman, 2011), 2.7 ng/mL, the 2007–2011 Canadian Health measures survey(Findlay and Kohen, 2015), 1.3 ng/mL; and the DEMOCOPHES study on Spanish children at similar age (6–11 y) (Covaci et al., 2015), 1.83 ng/ mL; among others (Hong et al., 2013). Urinary BPA concentrations canbe influenced by the type and timing of sample collection (non-fasting between 17:00 and 20:00 h in the present

Table 5
Distribution of cognitive function scores by quartiles of urinary bisphenol A and Cr-standardized bisphenol A concentrations among 269 boys from the INMA-Granada cohort.

		BPA Quartiles (µg/L)				BPA/creatinine quartiles (µg/g)			
Cognitive functions[a]	n	Reference Scores	Coefficients (CIs)			Reference Scores	Coefficients (CIs)		
		Mean (SD) (0.3-2.8 µg/L)	2nd (2.8-4.5 µg/L)	3rd (4.8-8.9 µg/L)	4th (9.1-62.4 µg/L)	Median (SD) (0.3-2.8 µg/g)	2nd (2.8–4.8 µg/g)	3rd (4.8-10.2 µg/g)	4th (10.3-76.3 µg/g)
Intelligence quotient (IQ)[b]	269	107.0 (11.5)	−0.3 (-4.3;3.8)	−1.0 (-4.7;3.3)	1.2 (-2.7;5.2)	106.9 (10.81)	−0.3 (-4.2;4.0)	−0.6 (-4.4;3.6)	0.7 (-3.1;4.9)
Verbal comprehension[b]	269	51.0 (6.3)	−0.3 (-2.3;1.7)	−0.1 (-1.9;2.0)	0.6 (-1.4;2.6)	50.9 (5.9)	−0.0 (-2.0;2.1)	−0.2 (-2.1;1.9)	0.7 (-1.2;2.8)
Attention									
Impulsivity[c]	258	11.7 (18.5)	−1.6 (-6.3;2.9)	−4.2 (-9.0;0.0)†	−3.2 (-7.7;1.2)	12.0 (16.0)	−2.2 (-7.0;2.2)	−4.1 (-8.9;0.1)†	−4.0 (-8.6;0.4)†
Attention Index[b]	258	0.8 (0.4)	−0.1 (-0.2;0.1)	−0.1 (-0.3;0.1)	−0.1 (-0.3;0.0)	0.8 (0.4)	−0.1 (-0.2;0.1)	−0.1 (-0.3;0.1)	−0.1 (-0.3;0.1)
Verbal Memory									
Long-term recall[b]	269	11.3 (2.6)	0.1 (-0.8;1.0)	0.0 (-0.9;0.9)	−0.5 (-1.4;0.4)	11.4 (2.4)	−0.3 (-1.3;0.5)	−0.4 (-1.3;0.5)	−0.4 (-1.4;0.4)
Immediate Recall Trial 5[b]	269	11.8 (2.1)	−0.4 (-1.3;0.5)	−0.4 (-1.2;0.5)	−0.6 (-1.4;0.3)	11.8 (2.1)	−0.2 (-1.3;0.4)	−0.2 (-1.2;0.5)	−0.2 (-1.3;0.4)
Visual-motor coordination[c]	268	28.7 (10.7)	0.0 (-3.9;4.0)	1.0 (-2.6;5.1)	0.8 (-3.0;4.7)	28.7 (10.9)	0.3 (-3.5;4.5)	1.3 (-2.3;5.5)	−0.1 (-3.7;4.1)
Processing speed[b]	268	94.9 (11.6)	2.3 (-1.8;6.3)	5.4 (1.4;9.4)*	3.5 (-0.4;7.5)†	96.7 (11.0)	−0.3 (-4.5;3.7)	1.1 (-3.1;5.0)	2.5 (-1.7;6.4)
Executive Function									
Working Memory[b]	269	17.6 (2.2)	−0.4 (-1.4;0.6)	−0.5 (-1.5;0.5)	−1.0 (-2.1;-0.1)*	17.4 (2.2)	−0.2 (-1.3;0.7)	0.0 (-1.0;1.0)	−0.6 (-1.6;0.3)
Verbal Fluency[b]	267	16.6 (3.9)	0.3 (-1.2;1.9)	0.0 (-1.5;1.6)	0.7 (-0.9;2.2)	16.5 (3.7)	0.3 (-1.2;1.9)	0.5 (-1.0;2.0)	0.6 (-0.9;2.1)
Impulsivity/Inhibition									
Inhibitory Control (Interference)[b]	267	1.4 (4.7)	0.1 (-1.8;2.0)	1.6 (-0.3;3.5)†	1.6 (-0.3;3.4)	1.7 (4.3)	0.4 (-1.5;2.4)	1.0 (-0.8;3.0)	0.7 (-1.2;2.6)
Hit rate[b]	255	1.0 (0.7)	0.0 (-0.0;0.0)	0.0 (-0.0;0.0)	0.0 (-0.0;0.0)	1.0 (0.1)	0.0 (-0.0;0.0)	0.0 (-0.0;0.0)	0.0 (-0.0;0.0)
False-alarm rate[c]	255	0.1 (0.1)	−0.0 (-0.0;0.0)	−0.0 (-0.0;0.0)	−0.0 (-0.0;0.0)	0.1 (0.1)	−0.0 (-0.0;0.0)	−0.0 (-0.0;0.0)	−0.0 (-0.0;0.0)
Shifting[c]	268	63.4 (27.9)	−8.3 (-18.2;1.7)	−5.5 (-15.4;4.4)	−1.7 (-11.5;8.2)	62.5 (25.3)	−5.6 (-15.8;4.5)	−2.2 (-12.0;7.8)	−3.0 (-12.9;7.0)
Abstract reasoning[b]	269	30.8 (4.2)	0.3 (-1.3;1.9)	−0.5 (-2.1;1.2)	0.3 (-1.3;1.9)	31.0 (3.9)	−0.3 (-1.9;1.4)	−0.2 (-1.8;1.4)	−0.2 (-1.7;1.5)

CI: confidence interval; SD = standard deviation.

Linear regression models were adjusted for child's age, BMI (kg/m2), and exposure to environmental tobacco smoke (yes/no) in the home based on questionnaire, mother's age at the time of assessment, maternal intelligence score, marital status (married/not married), education level (university/secondary school/up to primary), maternal smoking during pregnancy (yes/no) and breastfeeding (yes/no).

[a]We show the most relevant abilities measured. For all tests, direct scores were used in the analysis.

[b]A higher score indicates better cognitive function.

[c]A higher score indicates worse cognitive function.

[d]Immediate Recall Trial 5 (TAVECI).

* $p \leq 0.05$; † $p \leq 0.1$.

Table 6
Risk of being below or above the 20th or 80th percentile of cognitive function scores by log_e BPA Cr-standardized (μg/g) concentrations among 269 boys from the INMA-Granada cohort.

Cognitive functions[a]		BPA/Creatinine (μg/g) (log_e transformed, adjusted for covariatesd)		BPA/Creatinine (μg/g) (log_e transformed, adjusted for covariatesd[d])	
	n	OR	95% CI	OR	95% CI
Intelligence quotient (IQ)[b]	269	0.92	0.67;1.26	0.97	0.69;1.38
Verbal comprehension[b]	269	0.88	0.64;1.21	0.91	0.63;1.31
Attention					
Correct detections[b]	258	1.20	0.86;1.68	1.16	0.80;1.69
Impulsivity[c]	258	1.03	0.76;1.39	1.06	0.76;1.47
Omission errors[c]	258	1.13	0.83;1.54	1.10	0.78;1.56
Attention Index[b]	258	1.17	0.87;1.56	1.20	0.88;1.65
Verbal memory					
Immediate recall[b]	269	1.11	0.80;1.55	1.32	0.91;1.91
Short-term recall[b]	269	0.76	0.53;1.07	0.88	0.61;1.28
Long-term recall[b]	269	1.09	0.79;1.50	1.20	0.85;1.68
Visual-motor coordination[c]	268	–	–	–	–
Processing speed[b]	268	0.87	0.63;1.21	0.83	0.56;1.21
Executive Function					
Working Memory[b]	269	1.44	1.00;2.08*	1.51	1.01;2.25*
Verbal Fluency[b]	267	1.26	0.90;1.75	1.17	0.81;1.70
Impulsivity/Inhibition					
STROOP words[b]	267	0.99	0.71;1.40	1.05	0.72;1.53
STROOP colors[b]	267	0.76	0.55;1.06	0.85	0.60;1.22
STROOP words and colors[b]	267	0.96	0.69;1.35	1.05	0.73;1.52
Inhibitory Control (Interference)[b]	267	0.99	0.72;1.36	0.96	0.67;1.37
Hit rate[b]	255	0.83	0.57;1.24	0.82	0.53;1.28
False-alarm rate[c]	255	0.77	0.56;1.07	0.71	0.49;1.02
Shifting (s)[c]	268	0.87	0.64;1.18	0.83	0.60;1.16
Abstract reasoning[b]	269	1.18	0.84;1.65	1.11	0.76;1.62

OR: Odds Ratio; CI: confidence interval; s = seconds. * $p \leq 0.05$; † $p \leq 0.1$.
[a] We show the most relevant abilities measured. For all tests, scores were dichotomized into percentile 20 when a lower score meant worse cognitive function or percentile 80 when higher scoring meant worse cognitive function, except for attention index which was dichotomized into 0–1.
[b] A higher score indicates better cognitive function. Therefore, a higher OR means a higher risk of being below the 20th percentile.
[c] A higher score indicates worse cognitive function. Therefore, a higher OR means a higher risk of being above the 80th percentile.
[d] Models were adjusted for child's age (years), BMI (kg/m^2), smoking exposure at home (yes/no), mother's age at time of assessment (years), mother's intelligence score, marital status (married/unmarried), maternal education (university/secondary school/up to primary), smoking exposure during pregnancy (yes/no) and breastfeeding (yes/no).

study), country-specific BPA regulations, and differences in food intake and lifestyle. Although no consistent associations with cognitive functions were observed in this study, with the exception of working memory, the relative effect sizes observed (changes of 1–2 points in the neuropsychological scales) are in the subclinical range. However, given that human BPA exposure is ubiquitous, adverse effects could occur at a population level (Bellinger, 2004). Importantly, a large number of people exposed to a small risk may generate many more cases of disease than a small number exposed to a high risk (Rose, 1985).

Our study has some limitations. First, the use of a single spot urine sample to characterize BPA exposure may increase the risk of exposure misclassification. However, this would probably lead to an underestimation rather than overestimation of BPA effects due to the non-persistent nature and short-term variability of this chemical (Betts, 2013). Second, the cross-sectional design of the study does not allow the inference of causal relationships. Third, no assessment was made of possible effects of co-exposure to other chemical compounds, such as phthalates and organohalogenated compounds, among others. Fourth, since multiple comparisons were performed, we cannot rule out that some of the few observed associations may be due to chance.

However, this possibility is counteracted by our interpretation of results, which was based on the model robustness (comparison between unadjusted and adjusted models and between volume-based and creatinine-adjusted BPA concentrations), and on the comparison of associations observed in the current study with the available epidemiologic literature (Supplemental Table 1). Finally, sex-dependent relationships cannot be ruled out, because the study only included boys. The main study strengths include its contribution to the scant data available on BPA exposure and cognitive functions and the application by a trained psychologist of a comprehensive battery of tests. In addition, the analyses controlled for a large number of relevant covariates based on data from multiple questionnaires, physical examinations, and laboratory analyses of samples collected at the follow-up.

5. Conclusion

Results obtained in boys aged 9–11 years from the Spanish INMA-Granada cohort do not support an association between urinary BPA concentrations and cognitive abilities of the children, except for poorer working memory. Given our previous finding of a positive association between BPA and behavior problems in the same study population, these data suggest that BPA may be predominantly associated with theirbehavior.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neuro.2019.06.006.

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ARTICLE 3

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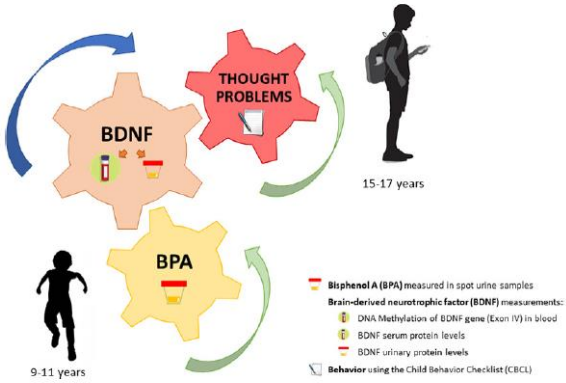
BDNF as a potential mediator between childhood BPA exposure and behavioral function in adolescent boys from the INMA-Granada cohort

Vicente Mustieles [a,b,c], Andrea Rodríguez-Carrillo [a,1], Fernando Vela-Soria [a,*], Shereen Cynthia D'Cruz [d], Arthur David [d], Fatima Smagulova [d], Antonio Mundo-López [b], Alicia Olivas-Martínez [a], Iris Reina-Pérez [a], Nicolás Olea [a,b,c], Carmen Freire [a,b,c], Juan P. Arrebola [b,c,e,2], Mariana F. Fernández [a,b,c]

[a] *University of Granada, Biomedical Research Center (CIBM), Spain*
[b] *Instituto de Investigación Biosanitaria (ibs. GRANADA), Spain*
[c] *CIBER de Epidemiología y Salud Pública (CIBERESP), Spain*
[d] *Univ Rennes, EHESP, Inserm, Irset (Institut de recherche en santé, environnement et travail) - UMR_S 1085, F-35000 Rennes, France*
[e] *Universidad de Granada, Departamento de Medicina Preventiva y Salud Pública, Granada, Spain*

GRAPHICAL ABSTRACT



abstract

Background: Bisphenol A (BPA) exposure has been linked to altered behavior in children. Within the European Human Biomonitoring Initiative (HBM4EU), an adverse outcome pathway (AOP) network was constructed supporting the mechanistic link between BPA exposure and brain-derived neurotrophic factor (BDNF).
Objective: To test this toxicologically-based hypothesis in the prospective INMA-Granada birth cohort (Spain).
Methods: BPA concentrations were quantified by LC-MS/MS in spot urine samples from boys aged 9–11 years, normalized by creatinine and log-2 transformed. At adolescence (15–17 years), blood and urine specimens were collected, and serum and urinary BDNF protein levels were measured using immunoassays. DNA methylation levels at 6 CpGs in Exon IV of the BDNF gene were also assessed in peripheral blood using bisulfite- pyrosequencing. Adolescent's behavior was parent-rated using the Child Behavior Checklist (CBCL/6-18) in148 boys. Adjusted linear regression and mediation models were fit.
Results: Childhood urinary BPA concentrations were longitudinally and positively associated with thought problems (β = 0.76; 95% CI: 0.02, 1.49) and somatic complaints (β = 0.80; 95% CI: −0.16, 1.75) at adolescence. BPA concentrations were positively associated with BDNF DNA methylation at CpG6 (β = 0.21; 95% CI: 0.06, 0.36) and mean CpG methylation (β = 0.10; 95% CI: 0.01, 0.18), but not with total serum or urinary BDNF protein levels.

When independent variables were categorized in tertiles, positive dose-response associations were observed between BPA-thought problems (p-trend = 0.08), BPA-CpG6 (p-trend ≤ 0.01), and CpG6-thought problems (p-trend ≤ 0.01). A significant mediated effect by CpG6 DNA methylation was observed (β = 0.23; 95% CI: 0.01, 0.57), accounting for up to 34% of the BPA-thought problems association.

Conclusions: In line with toxicological studies, BPA exposure was longitudinally associated with increased BDNF DNA methylation, supporting the biological plausibility of BPA-behavior relationships previously described in the epidemiological literature. Given its novelty and preliminary nature, this effect biomarker approach should be replicated in larger birth cohorts.

1. Introduction

Bisphenol A (BPA) is a highly produced synthetic monomer used in polycarbonate plastics and epoxy resins. Among many consumer products, BPA is found in the inner lining of cans and tins (Cao et al., 2009; González et al., 2020; Kim et al., 2020), polycarbonate plastic bottles (Carwile et al., 2009), thermal receipts (Ehrlich et al., 2014; Molina- Molina et al., 2019), medical equipment (Iribarne-Durán et al., 2019), and textiles (Freire et al., 2019). Human BPA exposure is ubiquitous and more than 90% of the European population still has detectable concentrations in their urine (Covaci et al., 2015; Tschersich et al., 2021), despite the fact that BPA analogues have been recently introduced as replacements (Wu et al., 2018). BPA has also been measured in serum, placenta, breastmilk and amniotic fluid, demonstrating internal exposure (Vandenberg et al., 2010).

As a paradigmatic endocrine disrupting chemical (EDC), BPA is known to interfere with diverse aspects of hormone signaling at low doses (Heindel et al., 2020; Ma et al., 2019). Apart from its reprotoxic (Peretzet al., 2014) and metabolism disrupting activities (Akash et al., 2020), BPA is a developmental neurotoxicant in experimental animals (Nesan et al., 2018; Patisaul, 2019). The human literature appears increasingly consistent for altered behavior in children (Ejaredar et al., 2017; Mustieles et al., 2015; Mustieles and Fernández, 2020), although the potential mechanisms underlying observational associations remain poorly investigated.

Research on novel effect biomarkers is among the aims of the European Human Biomonitoring for Europe (HBM4EU) Initiative. Effect biomarkers are measurable biological changes that allow the evaluationof dose–response relationships and may provide a mechanistic link be-tween exposure, early health impairment and health outcomes, consequently improving HBM and risk assessment of environmentalchemicals (Baken et al., 2019; Mustieles et al., 2020). We have recentlyreviewed all the effect biomarkers used in epidemiological studies in relation to BPA exposure, identifying brain-derived neurotrophic factor (BDNF) as a promising biomarker of brain function (Mustieles et al., 2020). An adverse outcome pathway (AOP) network was also constructed, supporting that BPA may interfere with BDNF signaling through different but converging biological mechanisms (thyroid, estrogenic and glutamatergic-related pathways), potentially leading to behavioral and cognitive impairments (Fig. 1).

Discovered in 1982, BDNF is a member of the neurotrophin family of growth factors (Binder and Scharfman, 2004). Although it can be foundthroughout the brain, its expression is particularly high in the hippo-campus, amygdala, cerebellum and cerebral cortex in both rodents and humans (Miranda et al., 2019).

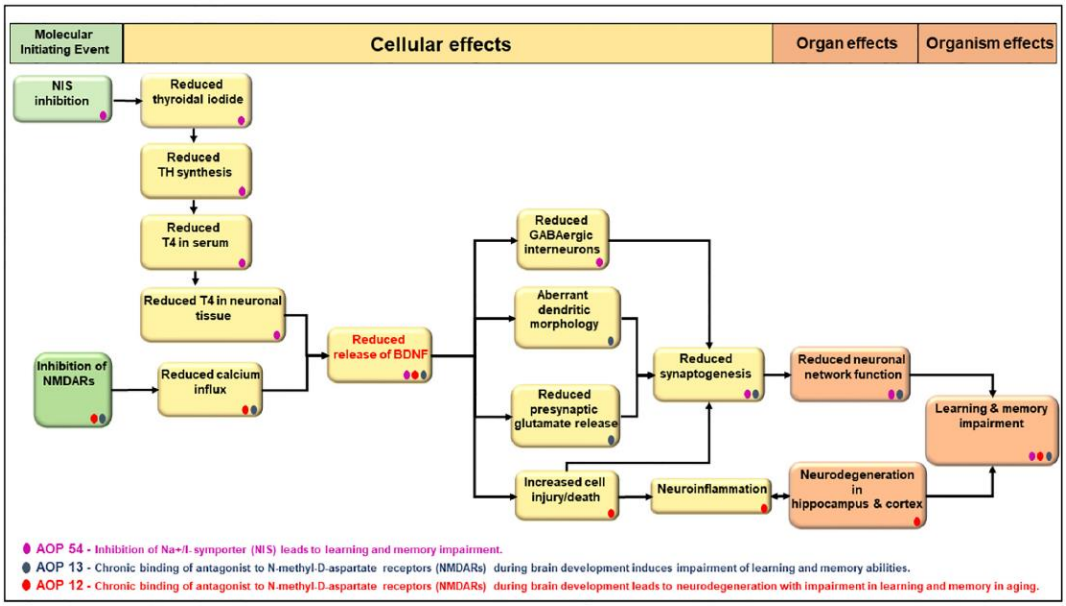


Fig. 1. Adverse outcome pathway network leading to a reduced release of BDNF.
Modified from Mustieles et al. (2020). Integration of 3 fully-developed AOPs (12, 13 and 54) from the AOP wiki (https://aopwiki.org/aops). Boxes represent molecular initiating events (MIEs) and key events (KEs) leading to learning and memory impairment. BPA has been shown to interfere with most of these key events in toxicological studies. In addition to thyroid and *N*-methyl-D-Aspartate (NMDAR) pathways, BPA can also interfere with estrogenic pathways to influence BDNF regulation and behavioral outcomes including anxiety and de-pression (Mustieles et al., 2020).

The precursor pro-BDNF is synthesized and stored in dendrites or axons, and then is used to producethe mature BDNF protein. Of note, the pro- and mature-BDNF forms show opposite actions on neuronal function, providing an additional level of regulation. While pro-BDNF preferentially binds the p75 neurotrophin receptor leading to apoptosis, the mature BDNF form activates tyrosine kinase receptors promoting cell survival and synaptic plasticity (Miranda et al., 2019).

Epigenetic mechanisms, especially DNA methylation, influence BDNF expression and regulation (Ikegame et al., 2013). Patients with psychiatric disorders generally show decreased neural BDNF levels, often associated with increased DNA methylation at specific BDNF promoters (Ikegame et al., 2013). Importantly, DNA methylation changes inthe BDNF gene are consistent across tissues including brain and blood,supporting its use as a peripheral biomarker of psychiatric disorders based on both rodent (Kundakovic et al., 2015) and human post-mortem studies (Stenz et al., 2015). On the other hand, serum total BDNF levels have been previously associated with depression andother psychiatric disorders as shown by different meta-analyses (Polyakova et al., 2015; Rodrigues-Amorim et al., 2018; Toll, 2015). Although less studied, urinary total BDNF levels have also been proposed as a biomarker of executive function in adults (Koven and Collins, 2014).The current work aimed to test our previous toxicologically-based hypothesis (Mustieles et al., 2020) focusing on the BPA exposure –BDNF – behavior triad in the Environment and Childhood (INMA)-Gra-nada birth cohort of boys by investigating: i) whether childhood BPAexposure (9–11 years) is longitudinally associated with behavioralfunction at adolescence (15–17 years); ii) the longitudinal relationship between childhood BPA exposure and BDNF biomarkers measured atadolescence (protein levels in serum and urine, and blood DNA methylation); iii) the cross-sectional relationship between BDNF biomarkersand behavior in adolescents; and iv) whether BDNF biomarkers may mediate BPA-behavior associations.

2. Methods

2.1. Study population

This study forms part of the INMA Project, a multicenter population- based birth cohort study formed by seven cohorts designed to investigate the effect of environmental exposures and diet during pregnancy on fetal, child and adolescent development in different geographical areas of Spain (Guxens et al., 2012). The INMA-Granada cohort initially recruited 668 mother-son pairs with the aim to investigate associations between prenatal exposure to environmental chemicals and male urogenital malformations (Fernandez et al., 2007). A random sample of the initial cohort was re-contacted and asked to participate in follow- up clinical visits at the ages of 4–5 years (N = 220) and 9–11 years (N = 300). In the last follow-up (2017–2019), all boys that participatedin the two previous visits were re-contacted. Of these, 155 boys aged 15–17 years agreed to participate and their parents signed the informedconsent (Castiello et al., 2020). The principles of the declaration of Helsinki were followed, and the initial study and all follow-ups were approved by the Biomedical Research Ethics Committee of Granada. Thephysical and neuropsychological evaluation was performed at the Pedi-atric Unit of San Cecilio University Hospital (HUSC) in Granada.

The current analysis included 130 boys with available urinary BPA concentrations at 9–11 years of age and behavioral data at 15–17 years completed by parents. Between 107 and 121 boys were included in BPA-BDNF biomarker associations. Between 103 and 116 boys were included in BDNF-behavior associations. Finally, 103 children with complete data on exposure, BDNF and outcome were included in the mediation analysis (Fig. 2). Although no significant differences in sociodemographic or clinical characteristics were observed between the children included in this analysis (n = 130) and the remaining who also participated in the previous follow-up at 9–11 years of age (n = 139), childhood BPA concentrations tended to be higher and maternal education lower in the current analysis (Table S1).

2.2. Childhood BPA exposure assessment

Children provided a single non-fasting spot urine sample at the 9–11 year-old visit, between 17:00 and 20:00 h. Urine was collectedin 10-mL polypropylene tubes and immediately stored at −20 °C. Total BPA (free plus conjugated) was determined by liquid chromatography-mass spectrometry at the laboratory of the Department of Analytical Chemistry of the University of Cordoba (Spain) as previously described in detail (Perez-Lobato et al., 2016). The limits of detection (LOD) and quantification (LOQ) were, respectively, 0.1 μg/L and 0.2 μg/L. Extended analytical information and procedures, including quality control and assurance (QA/QC) followed are provided in Perez-Lobato et al. (2016). The collection, storage, and processing of urine biospecimens was performed under controlled conditions, and accountwas taken for potential BPA external contamination from collection containers, equipment or labware. Urinary creatinine concentrations (mg/dL) were assessed at the Public Health Laboratory of the Basque Country (Spain) to account for urine dilution. BPA concentrations were normalized by urinary creatinine and expressed as μg of BPA/g ofcreatinine.

2.3. BDNF biomarkers at adolescence

On the day of their hospital visit at 15–17 years of age, each adolescent collected the first morning urine void and peripheral venous bloodwas collected from participants under non-fasting conditions between17:00–19:00 h. Blood samples were immediately processed to obtain serum and whole blood aliquots. Urine and processed blood samples were subsequently stored at −80 °C. Whole blood was sent on dry ice to the Human Genotyping Laboratory at the Spanish National Cancer Research Centre, where genomic DNA was extracted with the Maxwell® RSC equipment, quantified using the PicoGreen assay and normalized to 50 ng/μL. The extracted DNA was always stored at −80 °C until use.

Total serum BDNF levels (ng/mL) were measured using the commercial Quantikine® enzyme-linked immunosorbent assay (ELISA) kit(R&D Systems, Minneapolis, MN, USA). Serum samples were defrosted, vortexed, separated in two aliquots of 10 μL, diluted 100 times and tested according to manufacturer's instructions at the Biomedical Research Center (CIBM) of the University of Granada (Spain). Each sample was tested in duplicate in different plates and the mean of these two values was calculated in order to reduce measurement variation. Intra-and inter-assay coefficients of variability were <5% and <15%, respectively.

Total urinary BDNF levels were measured using the commercial RayBio® ELISA kit (Raybiotech, Norcross, GA, USA). Urine samples were defrosted, vortexed, and pre-treated following the protocol established by Koven and Collins (2014), with minor modifications. Samples were assessed at the Biomedical Research Center (CIBM) of the University of Granada (Spain) following manufacturer's instructions. Each sample was assessed in duplicate and the mean value was calculated. Intra- and inter-assay coefficients of variability were <5% and <15%, respectively. Creatinine concentrations (mg/dL) in the urine of adolescents were assessed at the Instituto de Investigación Biosanitaria de Granada (ibs.Granada, Spain) to account for urine dilution. Urinary BDNF concentrations were normalized by creatinine andexpressed as μg of BDNF/g of creatinine.

DNA methylation was analyzed using the bisulfite pyrosequencing technique at IRSET (Institut de Recherche en Santé, Environnement et Travail - INSERM UMR1085), Rennes, France. Briefly, genomic DNA concentration and purity was measured using NanoDrop (Thermo Scientific NanoDrop 8000; DNA50 mode). All the samples had approximately 1.8–1.9 ratio at 260/280 absorbance indicating that the extracted

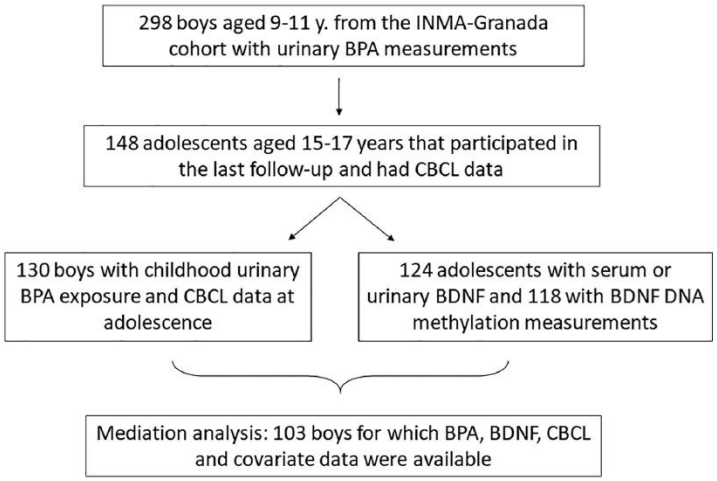


Fig. 2. Participant flow-chart in the Environment and Childhood (INMA)-Granada cohort follow-up visits from the age of 9–11 years to 15–17 years. BPA (Bisphenol A); CBCL (Child Behavior Checklist); BDNF (Brain-derived neurotrophic factor).

DNA was pure. Since accurate quantification of DNA is extremely important forepigenetic studies, DNA concentration was further verified using QuantiFluor dsDNA system (Promega E2670) which is a highly sensitive system for measuring only double-stranded DNA (dsDNA). Subsequently, 500 ng of genomic DNA was bisulfite converted (BS) using Epitect Fast Bisulfite Conversion kit (Qiagen, 59826) following manufacturer's proto-col. The concentration after bisulfite conversion and purification was remeasured using NanoDrop (Thermo Scientific NanoDrop 8000; RNA40 mode) as recommended for BS-DNA. 20 ng of BS-converted DNA was used for downstream PCR amplification (Biometra TProfessional Thermoycler, France) of BDNF by using Takara EpïTaq hotstart DNA polymerase (Takara, R110A; 0.6 U/25 μl final concentration) that could amplify BS-converted DNA, under the following conditions: initial denaturation 98 °C for 30 s followed by denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C at 30 s, for a total of 40 cycles. The primers used for BDNF amplification (0.4 μM final concentration) are provided in Table S2, of which the reverse primer was biotinylated. The targeted region was Exon IV of BDNF (genomic coordinates: chr11:27,723,070–27,723,280 retrieved from UCSC Genome Browser Human February 2009 (GRCh37/hg19), which has been previously validated in rodents and humans (Kundakovic et al., 2015) and contains 6 CpGs including a CREB-binding site (cAMP response element-binding site). Following PCR amplification, the products were purified using MinElute PCR purification kit (Qiagen, 28,006) and were loaded on a 2%agarose gel and a single BDNF product (210 bp size) was observed indicating BDNF primer specificity and absence of primer-dimers. The sampleswere sent to the Genomic Platform LIGAN (Lille Integrated Genomics Advanced Network for personalized medicine), Lille (France), and were pyrosequenced using Pyromark Q24 Advanced Pyrosequencing technology. The degree of methylation at each CpG was expressed as percentageof DNA methylation.

The Pyromark Q24 software measures the percentage of methylation at each CpG and has a built-in quality control system for each run.The software uses non-CpG peaks as reference peaks and determines how well they match with the theoretical pyrogram generated based on the original BDNF sequence to be analyzed. CpG sites that deviate from an expected peak size are highlighted by the software. Since this could happen due to variations in PCR efficiency at certain regions, samples with CpGs that did not pass the quality control were pyrosequenced again to discard an error from technical handling. The number of CpGs that did not finally pass the quality control was small (<4% of all CpG measurements performed). Given that the quality control was CpG-specific, one individual could for example have data quantified for CpGs 1-to-5 but lack data on CpG6 or any other CpG. This smallpercentage of missing CpGs was multiple imputed (see Statistical analysis section). Extended details on the fine-tuning and quality controls performed for the measurement of BDNF biomarkers can be found elsewhere, as part of the HBM4EU project (Fernández et al., 2021).

2.4. *Behavioral assessment*

Adolescent's behavioral function was evaluated using the parent- reported Child Behavior Checklist (CBCL/6-18), a validated questionnaire that evaluates the parental perception about the behavior of their children and/or adolescents during the previous six months (Achenbach and Rescorla, 2001). The CBCL includes 118 items rated on a three-point Likert scale (0 = "Not True",1= "Somewhat or Some- times True", or 2 = "Very/Often True"), that are grouped into eight syn- drome scales (anxious/depressed, withdrawn/depressed, somatic complaints, social problems, thought problems, attention problems, rule-breaking behavior, and aggressive behavior). These scales are grouped into two empirically-derived composite scales: i) the internalizing domain as a measure of emotional problems (sum of scores on theanxious/depressed, withdrawn/depressed, and somatic complaints scales); and ii) the externalizing domain as a measure of behavioral problems (sum of scores on the rule-breaking behavior and aggressive behavior scales). Three other scales are considered mixed-syndrome scales that do not belong to either domain: social, thought, and attention problems (Achenbach and Rescorla, 2001). The total problems composite scale finally quantifies general impairment and correspondsto the sum of scores from all eight syndrome scales, together with a group of 17 "Other problems" items that do not belong to any specific syndrome scale. Raw scores for each scale were converted to sex- and age-normalized t-scores, which were used to evaluate behavior as a continuous outcome in the main analyses. Higher scores mean more behavioral problems in all scales. Children with CBCL/6–18 T-scores ≥60 on internalizing or externalizing problem scales and T-scores ≥65 on diagnostic scales are

considered as borderline/clinical cases (Achenbachand Rescorla, 2001). Parents assessed the behavioral functioning of adolescents under the supervision of a trained psychologist (AM) blindedto the BPA exposure status of the children.

2.5. Covariates

Information on sociodemographic, lifestyle factors and anthropometric data were obtained from validated questionnaires and physicalexaminations by trained stuff during the follow-ups of the children and from their clinical records. Maternal education (categorized as upto primary, secondary school or university) and age of boys (months) were gathered from the questionnaires. Pediatricians measured the weight (kg) and height (cm) of the adolescents without shoes and in light clothing using an electronic scale (TANITA model 354, Seca Corporation, Hamburg, Germany), and age- and sex-specific body mass index (BMI) z-scores were calculated using the 2007 World HealthOrganization (WHO) growth reference standards (de Onis, 2007). Urinary cotinine levels were measured at 9–11 years of age to account for second-hand smoke exposure. Cotinine was determined by competitive enzyme immunoassay (EIA) using commercial EIA microplate kitsat the Public Health Laboratory of the Basque Country (Fernández et al., 2015). Alcohol consumption frequency and type of beverage (fermented vs. distilled drinks) was self-reported by adolescents, and classified as never or less than 1 drink/month vs. more than 1 drink/ month.

2.6. Statistical analysis

Study participant characteristics were described using measures of central tendency and dispersion for numerical variables and frequencies for categorical variables. Creatinine-normalized urinary BPA concentrations were log2-transformed to minimize the skewness of the distribution. For BDNF DNA methylation measures, there was a small percentage of specific CpGs that did not pass the quality control (<4%) among individuals that had the remaining CpGs adequately quantified. Given that CpGs showed varying degrees of correlation among them (Table S3), missing CpG data were multiple imputed (20 imputations) using the regression method to avoid potential selection bias issues (i.e., slight differences in sample size for each CpG investigated).

Covariates were chosen a priori based on previous knowledge and/or those that modified the estimate (regression coefficient) of the exposure variable by >10%. To avoid an overadjustment, and to improve the comparability of exposure-mediator-outcome associations, all modelswere adjusted for the same set of covariates: adolescent's age (months)and BMI (z-scores) at behavioral assessment, since age at assessment predicted CBCL scores in this population and childhood adiposity is known to play a relevant role in neurodevelopment (Steegers et al., 2021); maternal education (primary, secondary or higher) as a well- known measure of socioeconomic status and parenting environment (Koutra et al., 2012; Patra et al., 2016), children's urinary cotinine levels (mg/dL) at 9–11 years of age, as tobacco exposure during childhood is an important predictor of neurobehavior (Chen et al., 2013); and alcohol consumption at adolescence since it has been identified as a relevant predictor of BDNF regulation in this period (Miguez et al., 2020).

Multivariable linear regression models were fit to assess: i) Longitudinal associations between childhood log2-transformed creatinine-normalized BPA concentrations and continuous t-scores foreach behavioral scale at adolescence; ii) Longitudinal associations between childhood log2-transformed BPA concentrations and continuousvalues of BDNF biomarkers at adolescence; and iii) Cross-sectional associations between selected log2-transformed BDNF biomarkers and t- scores of selected CBCL scales, both assessed at adolescence. Beta coefficients and 95% CIs represent the mean change in the dependent variable, for each doubling in the independent variable. In order to explore potential dose-response associations within positive findings, we additionally categorized independent variables (BPA concentrations and BDNF biomarkers) in tertiles, taking the lowest tertile as the reference. Statistical tests for trend across tertiles were calculated by entering the independent variable as an ordinal level indicator (1, 2, 3) of each tertile in the regression model.

To determine whether selected BDNF biomarkers are potential mediators of the longitudinal association between BPA exposure and adolescent's behavior, mediation analysis was performed to calculate the total, direct and indirect effects. To reduce the number of comparisons, mediation analysis was guided by associations previously observed in multivariable regression models. Beta coefficients and 95% CIs were estimated after 10,000 bootstrapped replications. The total effect represents the relationship between the exposure (i.e., BPA) and outcome (i.e., behavior) without accounting for any mediator. The natural direct effect represents the proportion of the statistical relationship between exposure and outcome that is not attributable to the mediator (i.e., BDNF). The mediational or natural indirect effect represents the proportion of the statistical relationship between exposure and outcome that is driven by the mediator. The percentage mediated was calculated as: indirect effect / (direct effect + indirect effect) × 100.

SPSS v25.0 (IBM, Chicago, IL) was used for data analyses. Mediation analysis was performed using the PROCESS macro v3.5 for SPSS (http://processmacro.org/index.html). The significance level was set at P-value <0.05 and all tests were two-tailed. A P-value between 0.05 and 0.10 was considered as being suggestive of statistical significance. Notwithstanding, results were interpreted considering their internal validity and coherence, as well as the existing toxicological and epidemiological support, rather than solely depending on statistical significance (Amrhein et al., 2019). Given the targeted and predefined toxicological hypothesis investigated in this work (Mustieles et al., 2020), and the moderate number of comparisons tested, we did not perform a *post-hoc* correction for multiple comparisons to avoid a disproportionate increase in the frequency of type II errors (Rothman, 2014).

3. Results

3.1. Characteristics of the study population

Mean (standard deviation - SD) age of children at urine collection was 9.90 (0.32) years. Children's mean (SD) urinary concentrations ofcreatinine and cotinine were 100 (39.8) mg/dL and 15.9 (32.8) ng/mL,respectively. Adolescents completed the follow-up with a mean (SD) age and BMI of 16.6 (0.38) years and 23.6 (5.08) kg/m^2, respectively, and 38.5% consumed alcoholic beverages more than once per month. Regarding mothers, more than two-thirds had completed primary (37.7%) and secondary (36.2%) education, while 26.2% had completed university studies (Table 1). The distribution of CBCL behavior t-scores of adolescents is presented in Table S4. Internalizing problems (30.0%) were more prevalent than externalizing behaviors (13.8%) (Table S4).

BPA concentrations were quantified in all urine samples at the 9–11 year-old visit, showing a large range of concentrations (between 0.46 and 76.4 μg/g), and a median and interquartile range (IQR) of 5.41 (3.05, 10.6) μg/g (Table 1). Serum and urinary total protein BDNF levels measured in adolescents showed a median (IQR) of 31.5 (25.4, 38.8) ng/mL and 2.14 (1.56, 3.09) μg/g, respectively (Table 1). The mean percentage of BDNF DNA methylation in the six CpGs investigatedin blood samples from the adolescents showed a median (IQR) of 3.70 (3.45, 4.04), with a minimum value of 2.70% and a maximum of 5.54%.The range and distributions of methylation percentages for each individual CpG are presented in Fig. S1. Pearson correlation coefficients were assessed between CpG's percentage of DNA methylation and serum and urinary BDNF levels (Table S3). Most CpGs tended to positively correlate among them (suggesting the possibility of co-methylation), while CpG1 showed a different pattern of correlation (Table S3). A higher percentage of methylation in most CpGs tended to correlate with lower serum protein BDNF levels, with the exception of CpG1.

Table 2
Longitudinal associations between childhood urinary BPA concentrations (9–11 years) and parent-reported behavior of adolescent boys aged 15–17 (n = 130).

Behavioral functions (CBCL)	BPA (μg/g of creatinine)*	
	β (95% CI)	P-value
Syndrome scores		
Anxious/depressed	0.34 (−0.44, 1.11)	0.392
Withdrawn	0.17 (−0.66, 0.99)	0.690
Somatic complaints	**0.80 (−0.16, 1.75)**	**0.102**
Social problems	−0.11 (−0.87, 0.66)	0.779
Thought problems	**0.76 (0.02, 1.49)**	**0.045**
Attention problems	−0.35 (−1.16, 0.46)	0.394
Rule-breaking problems	0.42 (−0.29, 1.13)	0.245
Aggressive behavior	0.00 (−0.77, 0.77)	0.998
Composite scores		
Internalizing problems	0.39 (−0.50, 1.28)	0.382
Externalizing problems	0.23 (−0.35, 0.81)	0.433
Total problems	**0.80 (−0.13, 1.73)**	**0.092**

Data are presented as Beta estimates and 95% Confidence Intervals [β (95% CIs)]. Models were adjusted for age and BMI z-scores (continuous) at behavioral assessment (15–17 years), maternal education (primary, secondary or university), urinary cotinine at 9–11 years (continuous) and alcohol consumption at adolescence (yes/no). Higher Child Behavior Checklist (CBCL) t-scores mean more behavioral problems for all scales. * Continuous BPA concentrations normalized by urinary creatinine were log2-transformed and treated as the independent variable.

On the contrary, urinary protein BDNF levels were not correlated with most CpGs, with the exception of CpG1, for which a positive borderline-significant correlation was observed (Table S3). Serum and urinary BDNF protein levels were not significantly correlated, although an inverse relationship was observed between both biomarkers.

3.2. Longitudinal associations of childhood BPA exposure with behavior atadolescence

Childhood urinary BPA concentrations tended to be associated with poorer behavior in most CBCL scales at adolescence, except for social and attention problems (Table 2). Each doubling in urinary BPA concentration was associated with a 0.76-point (95% CI: 0.02, 1.49) increase in t-scores for the thought problems scale (Table 2). When BPA concentrations were categorized in tertiles, a dose-response function with thought problems was confirmed (Fig. 3.A, p-trend = 0.08). Children in the upper BPA tertile showed a mean increase of 2 points in thought problems t-scores (range 50–82 points, Table S4) compared to those in the lowest tertile (Fig. 3.A). BPA exposure was additionally associated with increased somatic (β = 0.80; 95% CI: −0.16, 1.75) and total problems (β = 0.80; 95% CI: −0.13, 1.73), although confidence intervals included the null value, and a dose-response shape was not observed for total problems (Fig. S2).

Table 1
Descriptive analysis of BPA concentrations, BDNF biomarkers, and sociodemographic characteristics of boys evaluated at both 9–11 and 15–17 years of age (n = 130) from the Spanish INMA-Granada cohort.

Percentiles	Min	p10	p25	p50	p75	p90	Max
Child BPA (μg/g)	0.46	1.71	3.05	5.41	10.6	18.9	76.3
Adolescent serum BDNF protein levels (ng/mL)	17.2	20.3	25.4	31.5	38.8	47.4	56.0
Adolescent urinary BDNF protein levels (μg/g)[a]	0.16	1.09	1.56	2.14	3.09	4.31	15.2
Adolescent urinary BDNF protein levels (ng/mL)[a]	0.15	2.15	2.68	4.52	5.45	6.15	7.40
Adolescent BDNF mean CpG methylation (%)	2.70	3.20	3.45	3.70	4.04	4.64	5.54

Characteristics	N (%)/mean (SD)
Maternal education	
Primary	49 (37.7%)
Secondary	47 (36.2%)
University	34 (26.2%)
Adolescent alcohol intake (Yes)	50 (38.5%)
Child age at urine collection (years)	9.90 (0.32)
Child urinary creatinine (mg/dL)	100 (39.8)
Child urinary cotinine (ng/mL)	15.9 (32.8)
Adolescent age at follow-up (years)	16.6 (0.38)
Adolescent BMI at follow-up (kg/m^2)	23.6 (5.08)
Adolescent BMI z-scores	0.58 (1.33)

BPA (Bisphenol A); BDNF (Brain-derived neurotrophic factor); BMI (body mass index); p: percentile.
[a] Adolescent raw urinary BDNF protein levels were expressed as ng/mL, while creatinine-corrected urinary BDNF levels were expressed as μg/g.

3.3. Longitudinal associations of childhood BPA exposure with BDNFbiomarkers at adolescence

Childhood urinary BPA concentrations tended to be associated with a higher percentage of BDNF DNA methylation at adolescence in all CpGs investigated (Table 3). BPA concentrations were positively and significantly associated with higher DNA methylation at CpG6 (β = 0.21; 95% CI: 0.06, 0.36) and the mean methylation of the six CpGs assessed (β = 0.10; 95% CI: 0.01, 0.18). Suggestive associations with CpG3 and CpG5 were also observed (β = 0.09; 95% CI: −0.00, 0.17;β = 0.11; 95% CI: −0.00, 0.22). Notably, the magnitude of the association observed for CpG6 doubled those of CpGs 3, 5 and CpG mean. When BPA concentrations were categorized in tertiles, dose-response associations were observed for these three CpGs, with CpG6 showing again the most robust association (Figs. 3.B and S2). Childhood BPA exposure was not associated with total BDNF protein levels measured in either serum or urine at adolescence (Table 3).

3.4. Cross-sectional associations between BDNF biomarkers and behavior atadolescence

Given that BPA exposure was more clearly associated with thought problems compared to the remaining CBCL scores (Table 2 and Fig. S2), we decided to focus on this scale. When BDNF DNA methylationwas considered as the independent variable, most CpGs tended to be positively associated with thought problems, being CpG1 the unique ex ception (Table 4). CpG6 was significantly associated with thought problems (β = 2.59; 95% CI: 0.31, 4.87), and a suggestive positive associationwas also observed for CpG5 (β = 3.42; 95% CI: −0.22, 7.05) [Table 4]. When the CpGs previously associated with BPA (Table 3) were categorized in tertiles, dose-response associations were observed for CpGs 5and 6 and the mean CpG methylation, but not CpG3 (Fig. S2). This dose-response association was stronger and especially evident forCpG6. Thus, boys in the upper tertile of CpG6 BDNF DNA methylation showed a mean increase of 4 points in thought problems t-scores (range 50–82 points, Table S4) compared to those in the lowest tertile(Fig. 3.C). Regarding serum and urinary total BDNF levels, no cross-sectional associations were observed with thought problems (Table 4).

3.5. Mediation analysis

As summarized in Fig. 3, BPA was longitudinally and dose- dependently associated with increased thought problems (Fig. 3.A), and with a higher percentage of BDNF DNA methylation, especially at CpG6 (Fig. 3.B) at adolescence. Additionally, CpG6 methylation was cross-sectionally and dose-dependently associated with increased thought problems (Fig. 3.C). Based on these results, we decided to evaluate whether there was a mediation effect in the subset of 103 boys with available data for the exposure, mediator and outcome. In adjusted models, a significant indirect effect of CpG6 DNA methylation (β = 0.23; 95% CI: 0.01, 0.57) was observed, accounting for up to 34% of the BPA-thought problems association (Fig. 4). No significant indirect effects were observed for CpG5 and the mean CpG methylation in relation to thought problems, or somatic complaints (Table S5).

4. Discussion

In the INMA-Granada birth cohort, higher childhood urinary BPA concentrations were longitudinally associated with increased behavior problems at adolescence, especially thought problems. Childhood BPA exposure was also longitudinally associated with a higher percentage of DNA methylation at the promoter region IV of the BDNF gene measured at adolescence, especially evident at CpG number 6.

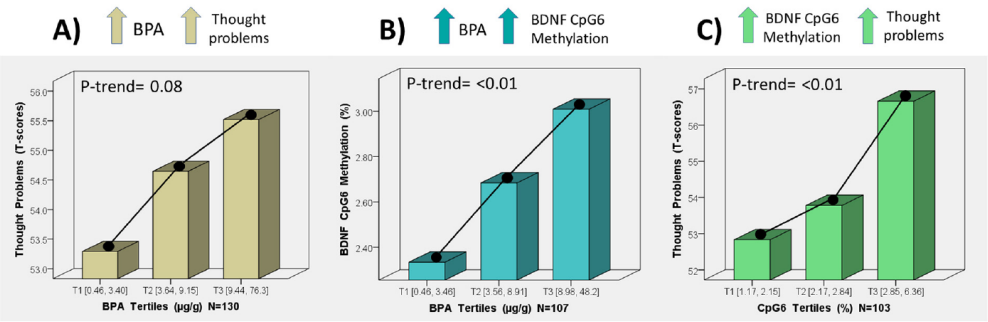


Fig. 3. Relationships among BPA exposure, CpG6 DNA methylation and thought problems categorizing the independent variable in tertiles to assess dose-response trends within the most robust findings.
Childhood urinary BPA concentrations categorized in tertiles were longitudinally, dose-dependently and positively associated with thought problems (A) and with the percentage of BDNF DNA methylation at CpG6 of Exon IV (B). Additionally, CpG6 DNA methylation percentage categorized in tertiles was cross-sectionally, dose-dependently and positively associated with thought problems (C). Models were adjusted for age and BMI z-scores (continuous) at neuropsychological evaluation (15–17 years), maternal education (primary, secondary or university), urinary cotinine at 9–11 years (continuous) and alcohol consumption at adolescence (yes/no). Higher t-scores mean more behavioral problems. Note: The scales of Y-axes represent the range of mean values for tertiles. The full range is 50–82 points for thought problems t-scores, and 1.17%–6.36% in the case of CpG6 BDNF DNA methylation.

Moreover, increased BDNF DNA methylation predicted the occurrence of thought problems, and CpG6 mediated the association between BPA and thought problems. Our findings suggest that BPA exposure may alter BDNF epigenetic regulation, leading to altered neurobehavior during a critical and understudied period of development such as adolescence (Fuhrmann et al., 2015; Pfeifer and Allen, 2020).

In this same cohort, we previously found that higher urinary BPA concentrations were cross-sectionally associated with increased thought, somatic and social problems in 269 boys at the age of 9–11 years (Perez-Lobato et al., 2016). Although in the current work the number of adolescent boys assessed at 15–17 years was lower due to attrition during the follow-up (n = 130), we prospectively confirmed previous associations with thought and somatic problems, which may signal greater vulnerability to the subsequent development of a mental disorder in adulthood (Paus et al., 2008). The thought problems scale includes obsessive thoughts, compulsive behaviors and strange ideas among other items, and has been linked to psychosis during adulthood (Salcedo et al., 2018).

Moreover, the co-occurrence of high scores in both the thought problems and somatic complaints scales has been related to mania (Morgan and Cauce, 1999). Nevertheless, the interpretation of our results must be done at a population instead of a clinical level (Bellinger, 2012, 2004).

Previous studies have reported associations between prenatal BPA exposure and child internalizing problems (Braun et al., 2017, 2011; Grohs et al., 2019; Harley et al., 2013; Perera et al., 2016; Philippatet al., 2017), including somatic complaints (Evans et al., 2014; Li et al., 2020). Regarding thought problems, higher prenatal BPA exposure was associated with increased scores in this scale at 7–9 years in children from the Columbia Center for Children's Environmental Health (CCCEH) cohort, although postnatal BPA exposure in the same children was not cross-sectionally associated with this scale (Roen et al., 2015). Postnatal studies have been more scarce, although our findings may be compatible with those previously described by Hong et al. (2013) and Harley et al. (2013) who found cross-sectional associations between postnatal BPA exposure and CBCL total problems and internalizing problems, respectively. Noteworthy, BPA exposure has been related to both internalizing and externalizing behaviors in toxicological and observational studies (Mustieles and Fernández, 2020), hindering comparisons but suggesting that poorer emotional regulation and executive function may underlie these altered behavioral phenotypes. In this context, the effect biomarker approach followed in this work complemented the information provided by neuropsychological scales, increasing the internal coherence and validity of our findings.

Table 3
Longitudinal associations between child urinary BPA concentrations (9–11 years) and BDNF biomarkers in adolescent boys (15–17 years) from the INMA-Granada cohort.

BDNF measurements	BPA (µg/g of creatinine)*		
	β (95% CI)	P-value	N
BDNF protein levels			
Serum BDNF (ng/ml)	−0.19 (−1.47, 1.10)	0.773	120
Urinary BDNF (µg/g)	0.13 (−0.09, 0.35)	0.254	121
Blood BDNF DNA methylation			
CpG1 (%)	0.03 (−0.09, 0.14)	0.635	107
CpG2 (%)	0.06 (−0.02, 0.13)	0.142	107
CpG3 (%)	**0.09 (−0.00, 0.17)**	**0.056**	107
CpG4 (%)	0.09 (−0.08, 0.26)	0.290	107
CpG5 (%)	**0.11 (−0.00, 0.22)**	**0.055**	107
CpG6 (%)	**0.21 (0.06, 0.36)**	**0.006**	107
CpG mean (%)	**0.10 (0.01, 0.18)**	**0.027**	107

Data are presented as Beta estimates and 95% Confidence Intervals [β (95% CIs)]. Models were adjusted for age and BMI z-scores (continuous) at behavioral assessment (15–17 years), maternal education (primary, secondary or university), urinary cotinine at 9–11 years (continuous) and alcohol consumption at adolescence (yes/no). * Continuous BPA concentrations normalized by urinary creatinine were log2-transformed and treated as the independent variable.

Table 4
Cross-sectional associations between BDNF biomarkers and behavior in adolescent boys (15–17 years) from the INMA-Granada cohort.

BDNF measurements*	Thought problems (CBCL)		
	β (95% CI)	P-value	N
BDNF protein levels			
Serum BDNF (ng/ml)	−0.79 (−3.55, 1.97)	0.571	115
Urinary BDNF (µg/g)	−0.48 (−1.82, 0.86)	0.481	116
Blood BDNF DNA methylation			
CpG1 (%)	−2.22 (−7.03, 2.60)	0.363	103
CpG2 (%)	0.85 (−4.11, 5.81)	0.735	103
CpG3 (%)	0.88 (−3.70, 5.46)	0.704	103
CpG4 (%)	2.64 (−2.02, 7.31)	0.263	103
CpG5 (%)	**3.42 (−0.22, 7.05)**	**0.065**	103
CpG6 (%)	**2.59 (0.31, 4.87)**	**0.026**	103
CpG mean (%)	3.52 (−2.07, 9.11)	0.214	103

Data are presented as Beta estimates and 95% Confidence Intervals [β (95% CIs)]. Models were adjusted for age and BMI z-scores (continuous) at behavioral assessment (15–17 years), maternal education (primary, secondary or university), urinary cotinine at 9–11 years (continuous) and alcohol consumption at adolescence (yes/no). * Continuous BDNF biomarkers were log2-transformed and treated as the independent variable.

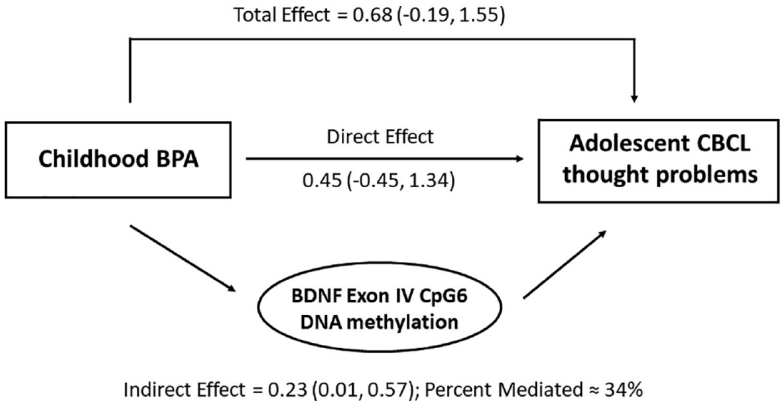


Fig. 4. Mediation model showing associations between childhood urinary BPA concentrations, BDNF DNA methylation and thought problems t-scores at adolescence (n = 103). Beta coefficients and 95% CIs are reported for the total, direct and indirect (i.e., mediated) effects. Continuous log2-transformed BPA concentrations and CpG6 methylation percentage, and continuous thought problems t-scores were used. Models were adjusted for age and BMI z-scores (continuous) at neuropsychological evaluation (15–17 years), maternal education (primary, secondary or university), urinary cotinine at 9–11 years (continuous), and alcohol consumption (yes/no) at adolescence.

Similar to toxicological studies in rodents (Kundakovic et al., 2015;Mustieles et al., 2020), our findings support that childhood BPA exposure promotes a higher degree of DNA methylation at the promoter region IV of the BDNF gene. Kundakovic et al. (2015) orally treated pregnant BALB/c mice with either BPA (200 μg/kg per day) or vehicle throughout gestational days 0–19, demonstrating lasting DNA methylation changes in Exon IV of the BDNF gene in the hippocampus and bloodof the exposed offspring, which is in line with a CLARITY-BPA toxicological report in adult rats (Cheong et al., 2018). Kundakovic et al. (2015) additionally tested their hypothesis in a subset of children from the abovementioned CCCEH cohort. The authors found that boys -but not girls- born to mothers in the highest category of prenatal urinary BPA concentrations showed a significantly higher cord blood DNA methylation of BDNF Exon IV at CpG sites 1 and 2 compared to boys in the lowestexposure group (n = 40 boys with either low or high prenatal BPA exposure). Importantly, prenatal BPA was previously associated with behavioral problems in 198 children from the CCCEH cohort (Perera et al., 2012) including thought problems at 7–9 years of age (Roenet al., 2015). Although in the current study BPA exposure was more robustly associated with CpGs 5 and 6, a possible association towards higher methylation at CpG 2 -but not CpG 1- was also noticed (Table 3). Our findings point to the same direction as Kundakovicet al. (2015), although BDNF DNA methylation was evaluated at different developmental periods (neonates vs. adolescents). Indeed, this may explain the divergence in the involvement of specific CpGs, since BDNF regulation varies throughout development (Kowiański et al., 2018).

The mediational effect observed for CpG6 DNA methylation between BPA exposure and thought problems is supported by: i) the strong andpredefined toxicological hypothesis (Kundakovic et al., 2015; Mustieles et al., 2020); and ii) the consistent dose-response associations betweenthe BPA CpG6 – thought problems triad (Fig. 3). Our results highlightBDNF epigenetic regulation as a plausible key event in BPA- neurobehavior associations that should be further investigated in largerbirth cohorts.

All CpGs investigated showed a methylation status below 10% (Fig. S1), supporting previous reports describing BDNF as a low methylated gene in absolute terms (Cattaneo et al., 2016). Indeed, due to this characteristic, microarray-based DNA methylome measurementsdo not seem a reliable method for BDNF methylation assessment compared to bisulfite-pyrosequencing (Forest et al., 2018; Sugden et al., 2020). Despite low absolute methylation status in the BDNF gene, slight variations in methylation at Exon IV have been linked to functional gene expression changes in experimental animals and human in vitro models(Kundakovic et al., 2015; Martinowich et al., 2007; Pruunsild et al., 2011; Zheleznyakova et al., 2016). Our results support this point, sincein the case of CpG6, even small mean methylation differences of around2% between extreme tertiles (T3: 3.8% vs. T1: 1.8%) predicted a difference of almost 4-points in thought problems t-scores (T3: 56.7 vs. T1: 52.8) [Fig. 3.C].

Serum total protein BDNF levels measured in INMA-Granada adolescents (median: 31.5 ng/mL) were in line with a previous report in 223 male teenagers around 14 years of age showing a mean of 27.0 ng/mL(Pedersen et al., 2017). While no previous study has assessed urinary BDNF concentrations in adolescents, current urinary BDNF levels (median: 4.52 ng/mL) were higher compared to young adults (0.6 ng/mL) (Koven and Collins, 2014). Despite serum total BDNF levels have beenrelated to psychiatric diseases (Polyakova et al., 2015; Rodrigues- Amorim et al., 2018) and that urinary BDNF has been proposed as a biomarker of executive function in adults (Koven and Collins, 2014), we did not find associations with BPA exposure.

The fact that we found longitudinal associations between BPA exposure and blood BDNF DNA methylation but not with serum or urinary total BDNF levels may be due to several reasons. First, we expect DNA methylation to be more stable over time (months or even years) compared to circulating protein levels (Kundakovic et al., 2015), facilitating the detection of prospective associations. However, the temporal variability of BDNF biomarkers is unknown for DNA methylation and scarcein the case of urinary and serum BDNF protein levels (Molendijk et al., 2012). Second, total BDNF protein levels were measured, not differentiating between the pro- and mature forms, which may have reduced the ability to detect associations (Jiang et al., 2017; Lin et al., 2021). Finally, while peripheral BDNF DNA methylation seems to be well correlated with its methylation status in the brain (Stenz et al., 2015), serum BDNF may be influenced by peripheral sources such as platelets (Gejl et al., 2019), and urinary BDNF by local production in the bladder (Antunes-Lopes and Cruz, 2019), which would tend to mask associations. Further research is needed to confirm our findings and identify the most

predictive BDNF biomarkers, as well as to assess their stability over time through repeated measures.

A higher percentage of DNA methylation in most CpGs correlated with lower serum protein BDNF levels, with the exception of CpG1 (Table S3). This is in line with our initial hypothesis that a higher percentage of DNA methylation would reduce BDNF gene expression and protein synthesis. However, we were not able to elucidate whether this negative correlation was mainly accounted by reduced levels of the mature BDNF form, the pro-BDNF form, or both. Interestingly, urinary BDNF levels showed and inverse relationship with CpG1 DNA methylation, and with serum BDNF levels, suggesting that serum and urinary BDNF levels may have a different biological meaning.

BPA exposure in INMA-Granada boys is higher compared to some studies (Covaci et al., 2015; Tschersich et al., 2021), but similar to others(Braun et al., 2011; Calafat et al., 2008; Perera et al., 2012). Timing of urine collection in the present study (i.e., evening), but also food intakeand lifestyle patterns may partially explain the higher levels, since a subset of the same boys at 4–5 years of age showed similar urinary BPA concentrations compared to the 9–11 years-old visit (Casas et al.,2011).

Overall, our findings suggest that BDNF methylation status at Exon IVis a physiologically valid molecular effect biomarker of children's behavior that may mediate some of the well-known toxicological effects of BPA exposure on brain and behavior (Nesan et al., 2018; Patisaul, 2019). Given that many previous epidemiological studies have reported associations between prenatal/postnatal BPA exposure and neurodevelopment(reviewed in Mustieles et al., 2015; Mustieles and Fernández, 2020), BDNF biomarkers, as well as other neurological effect biomarkers (Cediel Ulloa et al., 2021), could be implemented in future biomonitoring studies to improve the inference of causal relationships. Moreover, effect biomarkers of brain function will be useful for the timely assessment of BPA structural analogues such bisphenol S and F which show similar neuroendocrine disruption potential (Rosenfeld, 2017; Tanner et al., 2020).

Among the strengths of this work are the predefined hypothesis based on toxicological data organized following the AOP framework (Mustieles et al., 2020), together with the assessment of BDNF at complementary levels of biological organization. For BDNF DNA methylation, the gold standard (bisulfite-pyrosequencing) was used. The need for a more systematic implementation of effect biomarkers has been recently highlighted (Zare Jeddi et al., 2021), and together with our previous theoretical work (Mustieles et al., 2020), this study exemplifies howto go from toxicological knowledge to the implementation and validation of novel effect biomarkers in HBM studies. Another strength is that BPA exposure was evaluated during late childhood and behavior during adolescence, which are important but understudied periods of brain development (Konrad et al., 2013). The longitudinal design confirmed previous cross-sectional associations between BPA and behavior in the same cohort (Perez-Lobato et al., 2016), reducing the possibility of reverse causality issues. Additionally, we observed dose-response relationships among the exposure-BDNF-behavior triad, and indicationsof potential mediation by BDNF DNA methylation. Overall, this effect biomarker approach grounded on toxicological data helped to establish dose–response and mechanistic relationships, increasing the biological plausibility and internal consistency of the findings.

Regarding limitations, BPA exposure was assessed in one spot urinesample, which may lead to exposure misclassification due to its non-persistent nature and short-term viability. However, this would likely result in attenuation bias, rather than an overestimation of effects (Vernet et al., 2019). The sample size was small, reducing our ability to detect potential associations, and limiting the number of covariates to be controlled for in the models. Notwithstanding, this dataset was sufficient to observe interrelated associations coherent with the hypothesized toxicological pathway. A limitation of our mediation analysis is that BDNF methylation was measured in samples collected at the same time that adolescent's behavior was assessed. Notwithstanding, we do not expect a substantial alteration in the temporal ordering of the exposure-mediator-outcome (Gelfand et al., 2009), since DNA methylation constitutes the most stable “omics” signature over time (Gallego-Paüls et al., 2021), probably providing information on the past months before the measurement. Future studies testing the temporal variability of BDNF biomarkers will help to improve the interpretation of exposure-BDNF associations. Another limitation is that ourstudy design only included boys and sex-dependent associations couldnot be tested (Mustieles and Fernández, 2020). Apart from anxiety, depression and other psychiatric diseases, BDNF has also an important role in long-term memory and learning (Cunha et al., 2010). Although we investigated behavioral outcomes, unfortunately no evaluation of cognitive abilities was performed during the last INMA-Granada follow-up when boys were aged 15–17 years. While adolescence is an importantand understudied period of brain development, BPA exposure during pregnancy was not available in this cohort, being unable to compare how prenatal and postnatal BPA exposures interact to influence adolescent's neurobehavior. In addition to BPA, other environmental chemicals such as phthalates (Ponsonby et al., 2016), lead (Sachanaet al., 2018) and arsenic (Karim et al., 2019), are also known to alter BDNF regulation in experimental animals, and future works should consider the influence of chemical mixtures. Finally, residual confounding arising from unmeasured or uncontrolled covariates including lifestyle patterns (e.g., physical activity, diet, etc.) cannot be ruled out.

5. Conclusions

Childhood BPA exposure was longitudinally associated with a higher percentage of BDNF DNA methylation at adolescence, partially accounting for BPA-behavior associations. Our results highlight the role of BDNF as a promising and toxicologically-supported effect biomarker of brainfunction that may help to improve the inference of causal relationships in observational studies dealing with environmental exposures and children's neurodevelopment. Given the modest sample size analyzed in this pilot study and the novelty of these findings, future studies should replicate them under different settings, windows of development, and in the context of chemical mixtures.

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Credit authorship contributing statement

Vicente Mustieles: Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. Andrea Rodríguez-Carrillo: Validation, Writing – original draft, Visualization, Writing – review & editing. Fernando Vela-Soria: Investigation, Data curation, Writing – review & editing. Shereen Cynthia D'Cruz: Investigation, Writing – review & editing. Arthur David: Writing – review & editing. Fatima Smagulova: Writing – review & editing. Antonio Mundo-López: Writing – review & editing. Alicia Olivas-Martínez: Investigation, Writing – review & editing. Iris Reina-Pérez: Writing – re-view & editing. Nicolás Olea: Funding acquisition, Writing – review & editing. Carmen Freire: Resources, Project administration, Writing – re-view & editing. Juan P. Arrebola: Supervision, Methodology, Writing – review & editing. Mariana F. Fernández: Supervision, Conceptualization, Funding acquisition, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have

appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.150014.

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ARTICLE 4

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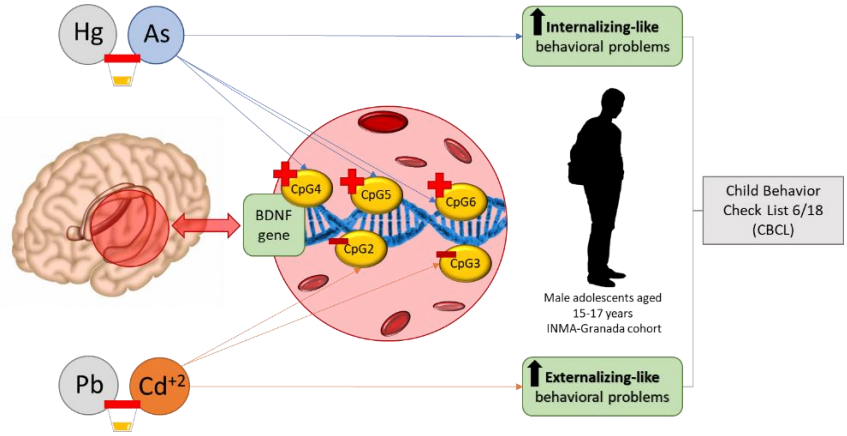
Exploring the relationship between metal exposure, BDNF, and behavior inadolescent males

Andrea Rodríguez-Carrillo [a, b, c], Vicente Mustieles [a, c, d], Shereen Cynthia D'Cruz [e], Louis Legoff [e],Fernando Gil [f], Pablo Olmedo [f], Iris Reina-Pérez [a,b,c], Antonio Mundo [b], Marina Molina [b], Fatima Smagulova [e], Arthur David [e], Carmen Freire [b,c,d,*], Mariana F. Fernández [a,b,c,d]

[a] *Department of Radiology and Physical Medicine, School of Medicine, University of Granada, 18016, Granada, Spain*
[b] *Biomedical Research Center (CIBM), University of Granada, 18016, Granada, Spain*
[c] *Instituto de Investigación Biosanitaria de Granada (ibs.GRANADA), 18012, Granada, Spain*
[d] *Consortium for Biomedical Research in Epidemiology and Public Health (CIBERESP), Spain*
[e] *Univ Rennes, EHESP, INSERM, IRSET (Institut de Recherche en Santé, Environnement et Travail), UMR_S 1085, F-35000, Rennes, France*
[f] *Department of Legal Medicine, Toxicology and Physical Anthropology, School of Medicine, University of Granada, 18016, Granada, Spain*



A B S T R A C T

Background: Brain-derived neurotrophic factor (BDNF) plays an important role in brain development by regulating multiple pathways within the central nervous system. In the Human Biomonitoring for Europe Project (HBM4EU), this neurotrophin is being implemented as a novel effect biomarker to evaluate the potential threatsof environmental chemicals on neurodevelopment.
Objectives: To explore the relationships among exposure to environmental metals, BDNF biomarkers at two levelsof biological complexity, and behavioral function in adolescent males.
Methods: Data were gathered from 125 adolescents on: spot urine sample total concentrations of the neurotoxic metal(oid)s arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb); serum BDNF protein concentrations; and concurrent behavioral functioning according to the Child Behavior Check List (CBCL/6–18). In 113 of the participants, information was also collected on blood BDNF DNA methylation at six CpGs. Associations were evaluated by multivariate linear regression analysis adjusted for confounders.
Results: As, Cd, Hg, and Pb were detected in 100%, 98.5%, 97.0%, and 89.5% of urine samples, respectively. Median serum BDNF concentration was 32.6 ng/mL, and total percentage of BDNF gene methylation was 3.8%. In the adjusted models, urinary As was non-linearly associated with more internalizing problems and Cd withmore externalizing behaviors. The percentage BDNF DNA methylation at CPGs #5 and the mean percentage CpG methylation increased across As tertiles (p-trend = 0.04 and 0.03, respectively), while 2nd tertile and 3rd tertile of Cd concentrations were associated with lower serum BDNF and higher CpG3 methylation percentage. Additionally, when BDNF was categorized in tertiles, serum BDNF at the 3rd tertile was associated with fewer behavioral problems, particularly withdrawn (p-trend = 0.04), social problems (p-trend = 0.12), and thought problems (p-trend = 0.04).
Conclusion: Exposure to As and Cd was associated with BDNF gene DNA methylation BDNF gene and serum BDNF, respectively. Associations with DNA methylation may be attributable to a higher variability over time in circulating BDNF concentrations than in the methylation status of this gene. Caution should be taken wheninterpreting the results relating postnatal Pb and Hg to behavioral functioning. Further studies are needed to verify these findings.

1. Introduction

The human brain develops from week eight of gestation up to late adolescence and even early adulthood (Rice and Barone, 2000; Stiles and Jernigan, 2010), being considered fully developed at around 25 years of age (Stiles and Jernigan, 2010). Hence, children are especially vulnerable to environmental neurotoxic compounds, including certain metals (Zhou et al., 2019). Current evidence suggests that exposure to environmental chemicals plays a major role in the so-called "silent pandemic of neurodevelopmental toxicity", *i.e.,* the rising incidence of behavioral and cognitive problems in children and adolescents,including autism spectrum disorders

(ASDs) and attention-deficit hyperactivity disorder (ADHD) (Bellinger, 2009; Grandjean and Landrigan, 2014). However, although developmental susceptibility to environmental chemicals may extend into adolescence, the potential adverse health effects of environmental exposure in this age group have not been fully elucidated (Mustieles et al., 2020; Pfeifer and Allen, 2021).

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophic family, has been associated with a wide range of neuropsychological processes, including neuro- and glio-synaptogenesis, synaptic plasticity, and neurite growth, among others (Kowiański et al., 2018; Sasi et al., 2017). This is largely explained by the characteristic pattern of BDNF synthesis, in which several biologically active isoforms interact with multiple receptors, thereby triggering, upregulating, or downregulating numerous signaling pathways (Kowiański et al., 2018). BDNF has been proposed as a biomarker of effect for brain functioning, allowing the exploration of potential causal pathways between exposureto particular endocrine disruptors (e.g., metals, bisphenol A, polycyclic aromatic hydrocarbons) and neurobehavioral outcomes in epidemiological studies (Kalia et al., 2017; Kundakovic et al., 2015; Mustieles et al., 2020; Perera et al., 2015; Tang et al., 2014). Exposure of humans to the neurotoxic environmental metals mercury (Hg) (particularly methyl-Hg), cadmium (Cd), lead (Pb), and arsenic (As) has been associated with disturbances in the pattern of BDNF synthesis, mainly detected as alterations in serum concentrations of total BDNF (Karim et al., 2019; Spulber et al., 2010; Y. Wang et al., 2016; Zhou et al., 2019; Zou et al., 2014). However, the biological meaning of BDNF gene DNA methylation patterns remain poorly understood. In a mouse study, Kundakovic et al. reported that blood BDNF gene methylation levels at six CpGs reflected the methylation profile and transcription levels in the hippocampus; they suggested that blood BDNF DNA methylation levels might be a surrogate marker of brain BDNF expression in humans (Kundakovic et al., 2015).

Humans are simultaneously exposed to multiple environmental chemicals. There are particular concerns about metallic/metalloid elements, which are ubiquitous in the environment, given that some of them are known to be neurodevelopmental toxicants, even at very low doses (Grandjean and Landrigan, 2006; Jakubowski, 2011; Rodríguez-Barranco et al., 2016; Schoeman et al., 2009). These elements arefrequently detected in human urine, blood, and hair samples (Gil and Hernández, 2015). Chronic exposure of humans to As, Cd, Hg, and Pb has been implicated in various adverse effects (ATSDR 2020; 2016, 2012, 1999), and epidemiologists have increasingly addressed the effects of this exposure on neurodevelopment in relation to anxiety, depression, Alzheimer's disease, and ASD, among others (Freire et al., 2018; Jaishankar et al., 2014; Long et al., 2019; Mravunac et al., 2019; Sanders et al., 2014; Shah-Kulkarni et al., 2020; Yousef et al., 2011; Zhou et al., 2019). However, the behavioral effects of environmental metal exposure remain controversial, in part because fully standardizedinstruments are not available to assess the behavioral functioning of children and adolescents (Ciesielski et al., 2012; Khan et al., 2011;Lucchini et al., 2012; Roberts et al., 2013; Sanders et al., 2015).

Effect biomarkers based on toxicologic findings have been identified by the Human Biomonitoring for Europe Project (HBM4EU) after comprehensive searches of the literature (Baken et al., 2019; Mustieles et al., 2020; Steffensen et al., 2020). The most promising biomarkers are being tested in several European cohorts to assess their value as indicators of the potential adverse effects of environmental chemicals. BDNF has been highlighted as a brain development marker that might complement neuropsychological tests (Mustieles et al., 2020). The hypothesis of the present study was that BDNF is involved in the causal pathway between metal exposure and adverse effects on behavioral function and therefore serves as an adequate epidemiological biomarker to evaluate exposure-mediator-effect relationships. The study objectives were therefore: to assess the relationship between exposure to As, Cd, Pb, and/or Hg and behavioral functioning in adolescent males; and to investigate the role of the BDNF biomarker measured at two levels of biological organization (BDNF gene DNA methylation and serum protein concentration).

2. Material and methods

2.1. Study population

This study is part of the *INMA*-INfancia y Medio Ambiente (Environment and Childhood) Project, a multicenter population-based birth cohort study designed to investigate the effects of environmental exposures and diet during pregnancy and early life on fetal, child, and adolescent development in different parts of Spain (Guxens et al., 2012). The INMA-Granada cohort recruited 668 mother-son pairs in Granada,Southern Spain, in 2000–2002 (Fernandez et al., 2007). Randomly selected adolescents from the baseline cohort were re-contacted to seektheir participation in clinical follow-ups at the ages of 4–5 (n = 220, 32.9%) and 9–11 years (n = 298, 44.6%). Participants who attended both follow-up sessions (n = 269) were invited to participate in the most recent follow-up at the age of 15–17 years (2017–2019) Agreement was obtained from 151 (56.13%) of these, who underwent physical examinations at the Pediatrics Unit of our third-level university hospital in Granada (Castiello et al., 2020). All 151 participants provided a urine sample, and 135 of them also provided a blood sample. The present study included the adolescents with available data on urinary metal concentrations, behavioral outcomes, serum total BDNF protein concentrations, and relevant covariates (n 125); information on BDNF gene DNA methylation patterns was also available for 113 of these adolescents (see Fig. 1). The parents/guardians of the adolescents signed informed consent to their participation in the study, which was approved by the Biomedical Research Ethics Committee of Granada (Spain).

2.2. Analysis of urinary metal concentrations

A single spot urine sample was collected from the first morning voidof each participant on the day of their hospital visit. Samples were storedat 80 °C until analysis. Urinary concentrations of total (both organic and inorganic) As, Cd, Hg, and Pb were measured at the laboratory of the Department of Legal Medicine, Toxicology and Physical Anthropology, University of Granada, using inductively coupled plasma mass spectrometry with an Agilent 8900 triple quadrupole ICP-MS (Agilent Technologies, Santa Clara, CA, USA) as previously described (Castiello et al., 2020). Quality control and quality assessment procedures included spiked samples with 400 μg/L of a multielement internalstandard solution with Sc, Ge, Ir, and Rh; intermediate calibration standards; blanks; and the following certified reference materials (US National Institute of Standards and Technology): Trace Elements in Natural Water Standard Reference Material SRM 1640a and Seronorm (Sero, Billingstad, Norway), and Trace Elements Urine L1 and L2 (references 210605 and 210705, respectively)]. Limits of detection (LODs) were 0.60 μg/L for As, 0.01 μg/L for Cd, 0.05 μg/L for Hg, and 0.16 μg/Lfor Pb (Supplementary Material, Table S1). Urinary creatinine was measured by the Jaffle method in a Roche Cobas C-311 system using a commercial kit (Creatinine Jaffé Gen 2, CREJ2) and expressed as mg/dL.

2.3. Serum BDNF and whole blood BDNF gene DNA methylation

Peripheral venous blood samples were drawn from participants under non-fasting conditions between 5 p.m. and 7 p.m. on the same dayas the collection of the urine sample. Blood samples were immediately processed to obtain serum and whole blood aliquots, which were subsequently stored at

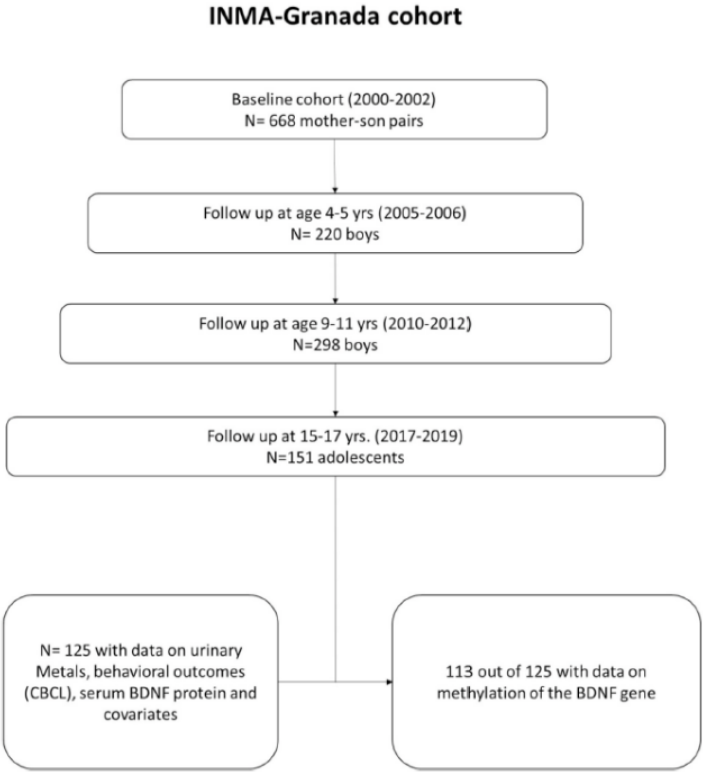


Fig. 1. Flow-chart showing the time-line of follow-ups conducted in the INMA-Granada cohort and the final sample of 15–17-year-old adolescents in the present study.

– 80 C°. Whole blood was sent in dry ice to the Human Genotyping Laboratory at the Spanish National Cancer Research Center, where genomic DNA was extracted using Maxwell® RSC equipment, quantified by PicoGreen assay, and diluted to 50 ng/μL. Extracted DNA was always stored at – 80 C° until use.

Total serum BDNF concentrations (mature and immature isoforms of BDNF) were measured with an enzyme-linked immunosorbent assay using the commercial Quantikine® ELISA kit (R&D Systems, Minneapolis, MN, USA) at the Biomedical Research Center (CIBM), Granada, Spain. Briefly, samples were defrosted, vortexed, aliquoted in 10 μL, and diluted 100-fold. Next, 50 μL of diluted sample was tested in duplicate,placed in a 96-well plate coated with an anti-BDNF monoclonal antibody, and incubated at room temperature for 2 h. The plate was then washed four times with 400 μL wash buffer solution, followed by the addition of 200 μL BDNF-specific monoclonal antibody in each well. The plate was incubated for 1 h at room temperature and then washed as described above. Finally, 200 μL of a mixture containing stabilized hydrogen peroxide and tetramethylbenzidine was added to each well, and the plate was incubated for 30 min at room temperature protected from light. Then, 50 μL of Stop Solution (sulfuric acid) was added to each well, and samples were immediately read by luminometry at 450 nm wavelength. Serum total BDNF protein concentrations had intra- and inter-assay coefficients of variation of <5% and 15%, respectively.

DNA methylation of the BDNF gene was determined by bisulfite pyrosequencing analysis at the IRSET (Institut de Recherche en Santé, Environnement et Travail - INSERM UMR1085) in Rennes (France), as described in detail elsewhere (Mustieles et al., 2022). Genomic DNA levels were quantified using the QuantiFluor dsDNA system (Promega E2670). Successively, 500 ng of genomic DNA was bisulfite converted (BS) with Epitect Fast Bisulfite Conversion kit (Qiagen, 59826), and theconcentration and purification were then remeasured with NanoDrop (Thermo Scientific NanoDrop 8000; RNA40 mode). Downstream PCR amplification (Biometra TProfessional Thermoycler, France) was performed using BDNF primers, 20 ng of BS-converted DNA, and Takara EpiTaq hot-start DNA polymerase at a final concentration of 0.6 U/25 μL (Takara, R110A) under the following conditions: initial denaturation at 98 °C for 30 s denaturation, anneling at 55 °C for 30 s, and extension at 72 °C for 30 s, running at total 40 cycles. Primers used for BDNF amplification (0.4 μM final concentration) are reported in Table S1, and the reverse primer was biotinylated. Exon IV of BDNF was the target region (genomic coordinates: chr11:27,723,070–27,723,280 retrieved from UCSC Genome Browser Human February 2009 (GRCh37/hg19), previously validated in rodents and humans (Kundakovic et al., 2015), which contains 6 CpGs, including a CREB-binding site (cAMP response element-binding site). After PCR amplification, the products were purified using the MinElute PCR purification kit (Qiagen, 28006) and then loaded on a 2% agarose gel to ensure amplification of a single BDNF product. Samples were sent to the LIGAN (Lille Integrated Genomics Advanced Network for personalized medicine) Genomic Platform in Lille (France) for pyrosequencing using Pyromark Q4 Advanced Pyrosequencing technology. The methylation level at each CpG was expressed as percentage DNA methylation.

2.4. *Behavioral functioning assessment*

The validated Spanish version of the Child Behavior Checklist (CBCL/6–18) was used to evaluate the behavioral function of the participants (Sardinero García et al., 1997). This questionnaire was completed by the parents/guardians of

each participant in relation to his behavior during the previous six months (Achenbach and Rescorla, 2013; Sardinero García et al., 1997). The CBCL contains 118 items ratedon a three-point scale (not true, somewhat true, very/often true) and grouped in the following eight syndrome scales: anxious/depressed, withdrawn/depressed, somatic complaints, social problems, thought problems, attention problems, rule-breaking behavior, and aggressive behavior. These scales are summarized in three composite scales: internalizing problems (sum of anxious/depressed, withdrawn/depressed, and somatic complaints scale scores); externalizing problems (sum of rule-breaking behavior and aggressive behavior scale scores); and total problems (reported as sex and age-normalized T-scores). A higher scale score always indicates more behavioral problems (Achenbach and Rescorla, 2013).

2.5. *Covariates*

Data on sociodemographic characteristics and lifestyle factors were collected by administering *ad hoc* questionnaires to the participants andtheir parents/guardians. The weight, height, and body mass index (BMI) of participants were measured following standardized procedures, extensively detailed in Castiello et al. (2020). Covariates used in the present study included data collected at the 15- to 17-year-old follow upvisit on the characteristics of the adolescents: age (in months, continuous), area of residence (categorized as urban or sub-urban/rural), annual family income (<25000, 25000–35000, or >35000 €), and passive smoking (yes or no); on the characteristics of their mothers: age(in years, continuous), intelligence (verbal reasoning measured by the similarities subtest of WAIS-III at the 9- to 10-year-old follow-up), marital status (stable partner: yes or no), schooling (up to primary, secondary, or university), current employment status (employed orunemployed) and alcohol consumption (yes or no). The adolescents alsocompleted a validated food frequency questionnaire to obtain information on their overall fish consumption (monthly intake of <3 portions, 3–5 portions, or >5 portions) (Notario-Barandiaran et al., 2020).

2.6. *Statistical analysis*

Descriptive analyses were performed to summarize the sociodemographic and lifestyle characteristics of the study participants. Urine samples with undetected levels of As, Cd, Hg, and Pb were assigned a value of LOD/√2. Detection frequencies and/or percentiles were calculated for raw urinary metal concentrations (μg/L) and the effect biomarkers, i.e., serum BDNF and percentage DNA methylation at 6 CPIs of Exon-IV from the BDNF gene. Spearman's correlation analysis was conducted to assess relationships between metal levels concentrations, expressed as μg/L.

Multivariate linear regression models were performed for: i) the association of metal exposure with behavioral outcomes; ii) the association of metal exposure with the BDNF biomarkers of effect (serum BDNF and methylation profile of the BDNF gene) and iii) the association of these biomarkers with behavioral outcomes.

Urinary metal concentrations were left-skewed and therefore modeled as (natural) log-transformed variables. Associations with metals and effect biomarkers were considered as continuous variables and also categorized in tertiles to investigate possible non-linear relationships. Next, generalized additive models (GAM) were constructedfor a more precise assessment of non-linear associations between metal exposure and behavioral outcomes. Confounders were carefully selected based on: i) substantive knowledge supporting their relevance for neurodevelopment and/or metals exposure; ii) their use in previous epidemiological studies; and iii) change in regression coefficient (beta) by more than 10%. Thus, two adjusted models were performed for all exposure-effects analyses.

First model (Model 1) was adjusted for the age and BMI of adolescents, given that the age determines the stage of brain development and BMI is known to have an important impact on children's behavior (Hughes et al., 2020; Richards and Xie, 2015). We usedunadjusted urinary metal concentrations and urinary creatinine concentrations as separate independent variables in accordance with previous observations reporting that this is a better approach to control formeasurement error bias due to variability of urine concentrations (Barr et al., 2005; O'Brien et al., 2016). Because multiple metals may simultaneously affect behavioral functioning, regression models were mutually adjusted for all metals. In models with continuous exposure variable, all metals were introduced as continuous variables, whereas in models with categorical exposure variable, all metals were introduced categorized into tertiles. This approach was performed given that our sample size was not large enough to conduct advanced analysis of mixture effects. Model 1 was further adjusted by maternal schooling andintelligence, since these variables have their own influence on neurodevelopment and have been also extensively used in epidemiological studies evaluating neurodevelopmental outcomes (Patra et al., 2016; Wirt et al., 2015). Model 2 (fully-adjusted) was additionally controlled for adolescents' passive smoking and fish intake. Second-hand tobacco is a potential source of exposure to heavy metals, especially for Cd (Campbell et al., 2014; NavasAcien, 2018; Spulber et al., 2010), while tobacco smoke has been negatively associated with the neuro- development of children (Chen et al., 2013; Lee et al., 2011; Spulber et al., 2010). Fish consumption was included because it is a major source of exposure to As, Hg, and Pb, although it has also been positively associated with neurodevelopment due to its content of fatty acids such as omega-3 (Gil and Gil, 2015; Mozaffarian and Rimm, 2006). Finally, multicollinearity was assessed in all regression models by calculating the variance inflation factor (VIF). Additionally, a sensitivity analysis was performed including one single element at a time to test the consistency across models. Associations showing $p < 0.05$ were considered significant. Nevertheless, given the relative small sample size, statistical significance was additionally evaluated based on internal validity, coherence and previous toxicological and epidemiological evidence of the observed associations (Amrhein et al., 2019). SPSS v26.0 (IBM, Chicago, IL) and R statistical software version 3.4.3 were used for data analyses.

3. Results

3.1. *Descriptive analyses*

Table 1 displays the general characteristics of the study participantsand their mothers. The mean (standard deviation - SD) age of the adolescents was 16.9 (0.4) years and their mean BMI was 23.33 (4.99) kg/ m^2. Almost three-quarters of the participants lived in urban areas, just under half were passive smokers, around one-third reported a monthly fish intake of less than 3 portions, and just over one-third had a family income of 25000–35000 €/year. Their mothers had a mean age of 39.5years, almost all had a stable partner, just under one-third had a university education, more than three-quarters were employed, and aroundhalf of them regularly consumed alcohol (Table 1).

All urine samples contained quantifiable concentrations of As (median = 24.20 μg/L), 98.5% contained concentrations of Cd (median = 0.08 μg/L), 97.0% concentrations of Hg (median = 0.76 μg/L), and 89.5% concentrations of Pb (median 0.42 μg/L) (Table 2). Significant positive correlations were found between As and Cd, Hg and Pb concentrations (Spearman's rho = 0.22, 0.52 and 0.19, respectively) and between Cd with Hg and Pb concentrations (Spearman's rho = 0.52 and 0.36, respectively) but not between Hg and 0.36, respectively) but not between Hg and Pb concentrations (Supplementary Material, Table S2).

Table 1
General characteristics of study participants (n = 125).

Variables	Mean ± SD or n (%)
Adolescents	
Age (years)	16.6 ± 0.4
BMI (kg/m^2)	23.6 ± 5.2
Creatinine (mg/dL)	184.6 ± 57.6
Area of residence	
Urban	96 (72.2)
Sub-urban/rural	37 (27.8)
Passive smoking	
Yes	55 (41.4)
No	76 (57.1)
Fish consumption	
<3 portions per month	45 (33.8)
3–5 portions per month	40 (30.1)
>5 portions per month	43 (32.3)
Mothers	
Age (years)	39.6 ± 4.7
Schooling	
Up to primary	50 (40.0)
Secondary	44 (35.2)
University	31 (24.8)
Occupational status	
Employed	78 (62.4)
Unemployed	47 (37.6)
Marital status	
Stable partner	115 (92.0)
No stable partner	10 (8.0)
Alcohol consumption	
Yes	65 (52.0)
No	60 (48.0)
Annual family income (euros)	
<25000	48 (36.1)
25000-35000	59 (44.1)
>35000	29 (21.8)
Verbal reasoning*	15.6 ± 5.1

SD: Standard deviation; BMI: Body mass index.
*Verbal reasoning measured by similarities subtest of WAIS-III at 9-11-year follow-up.

The median concentration of serum BDNFwas 32.6 ng/mL and the median percentage DNA methylation values forCpGs 1 to 6 were: 4.5%, 3.2%, 3.2%, 5.7%, 3.2%, and 2.4%, respectively; the median percentage total CpG methylation was 3.8%(Table 2). The distribution of CBCL T-scores is exhibited in Supplementary Material (Table S3). Globally, there was a lower prevalence of externalizing problems (16%) than of internalizing problems (32%) in this study population.

1.1. *Metal exposure and adolescents' behavior*

Table 3 displays the associations between tertiles of urinary metal concentrations and CBCL T-scores. The overall patterns pointed towardsa non-linear relationship of urinary As and Cd concentrations with behavioral problems, with As exposure being associated with more internalizing problems, such as anxiety, somatic and thought problems;and Cd with more externalizing problems, such as social, attention problems and aggressive behavior. Further, second and third tertiles (intermediate and high levels) of urinary As and Cd levels were associated with greater anxiety and more somatic complaints, aggressive behaviors, and social and internalizing problems; however, some of these associations did not reach the statistical significance. These associationspersisted but some were attenuated after adjustment for passive smoking and fish intake (Table 3). GAM analyses confirmed the presence of non-linear relationships for As and Cd (Supplementary Material, Figs. S1 andS2). Both Hg and Pb exposure showed associations with lower CBCL scores in several subscales, especially withdrawn, somatic complaints, and social and internalizing problems, which remained after adjustment for passive smoking and fish intake (Table 3).

Models considering continuous urinary metal concentrations showed associations of Cd with more social problems and aggressive behavior, although these relationships did not reach the statistically significance. However, Hg concentrations were significantly associated with fewer social problems (Table S4). The VIF was below 1.5 for each independentvariable, ruling out multicollinearity. Finally, sensitivity analyses not showed substantial differences between models, neither in the direction of associations, and neither in overall patterns (Table S7).

1.2. *Metal exposure and BDNF*

The overall pattern showed lower serum BDNF levels across tertiles of urinary As and Cd concentrations, with significant associations between Cd and serum BDNF and between As and CpG5 and total CpGs methylation percentages. However, these associations were not observed for Hg and Pb (Table 4).

In relation to the BDNF gene methylation profile, concentrations of As in the third tertile (higher level) were associated with higher percentage of methylation at CpGs #4, 5, and 6 and for total CpGs, with statistically significant results for CpG 5 and total DNA methylation. Associations were also found between urinary Cd in the second *versus* first tertile and lower BDNF gene methylation at CpGs #2 and 3 (Table 4). In the fully-adjusted model, the aforementioned associations remained and some became stronger (Table 4). Urinary Pb concentrations were positively but non-significantly associated with higher DNA methylation patterns, while urinary Hg showed associations with decreasing BDNF gene DNA methylation (Table 4). Models with continuous data showed non-significant associations of As with higher total CpG methylation and of Pb with higher methylation at CpG 1, whereas Cd was negatively but also non-significantly associated with methylation at CpG3 (Table S5). VIF values for each independent variable in all models were <1.5, ruling out multicollinearity. Sensitivity analyses showed an attenuation of associations between As and BDNF DNA methylation percentages, however, direction and overall tendencies were still observed (Table S8).

1.3. *BDNF and adolescents' behavior*

Continuous serum BDNF concentrations suggested association with a lower score for the withdrawn subscale (β= - 0.12, 95% CI = - 0.27, 0.03) (Fig. 2A). Lower scores were obtained by adolescents in the second and third tertiles of serum BDNF concentrations than by those in the first (lowest)

Table 2
Distribution of urinary metal concentrations (μg/L), serum BDNF concentrations (ng/mL), and percentage of BDNF gene DNA methylation values at six CpGs (%).

		As		Cd		Hg		Pb	
% Detection (n = 125)		100		98.5		97.0		89.5	
Percentiles	25	8.26		0.05		0.29		0.27	
	50	24.20		0.08		0.76		0.42	
	75	44.07		0.12		1.02		0.70	
		Serum BDNF	**CpG1**	**CpG2**	**CpG3**	**CpG4**	**CpG5**	**CpG6**	**Total CpGs**
n		125	111	113	113	105	108	101	112
Percentiles	25	25.41	3.82	2.90	2.84	5.35	2.69	2.02	3.45
	50	32.59	4.46	3.20	3.21	5.70	3.16	2.34	3.77
	75	39.40	4.87	3.51	3.64	6.32	3.67	3.12	4.06

Table 3
Adjusted models for the association between tertiles of urinary metal concentrations and CBCL scores (n = 125).

CBCL scores	Model 1				Model 2			
	As tertiles (μg/g)							
	1st (0.58–6.19)	2nd (6.47–16.18)	3rd (16.35–465.4)	p-trend	1st (0.58–6.19)	2nd (6.47–16.18)	3rd (16.35–465.4)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β (95% CI)	β (95% CI)	
Syndrome scores								
Anxious depressed	53.9 (4.9)	3.38 (0.39;6.37)*	1.71 (−1.34;4.76)	0.32	54.0 (4.9)	4.0 (0.87;7.13)**	1.77 (−1.31;4.84)	0.34
Withdrawn	57.0 (6.1)	0.83 (−2.64;4.29)	1.15 (−2.38;4.69)	0.52	57.0 (6.2)	0.95 (−2.68;4.57)	1.44 (−2.12;4.99)	0.43
Somatic complaints	55.2 (6.1)	5.77 (2.08;9.46)**	3.06 (−0.70;6.82)†	0.15	55.3 (6.2)	5.58 (1.66;9.50)**	2.86 (−0.99;6.70)†	0.20
Social problems	55.0 (5.8)	0.79 (−2.2;3.78)	1.34 (−1.71;4.39)	0.38	54.8 (5.8)	1.1 (−2.05;4.24)	1.49 (−1.59;4.57)	0.34
Thought problems	53.3 (4.3)	2.61 (−0.25;5.46)†	1.43 (−1.49;4.34)	0.37	53.2 (4.3)	2.56 (−0.45;5.57)†	1.36 (−1.60;4.31)	0.42
Attention problems	54.6 (4.6)	2.48 (−0.36;5.31)†	0.65 (−2.25;3.54)	0.72	54.5 (4.6)	2.58 (−0.43;5.60)†	0.60 (−2.36;3.55)	0.79
Rule-breaking behavior	54.6 (5.8)	−0.53 (−3.16;2.11)	−1.29 (−3.98;1.41)	0.34	54.7 (5.9)	−0.31 (−2.95;2.34)	−1.34 (−3.94;1.25)	0.29
Aggressive behavior	55.7 (6.3)	0.22 (−2.81;3.24)	0.18 (−2.90;3.27)	0.91	55.5 (6.3)	1.01 (−2.09;4.12)	0.35 (−2.7;3.40)	0.85
Composite scores								
Internalizing problems	52.4 (10.4)	5.28 (0.25;10.31)*	4.16 (−0.98;9.29)†	0.13	52.4 (10.6)	5.87 (0.52;11.22)*	4.43 (−0.82;9.68)†	0.12
Externalizing problems	52.2 (9.9)	0.59 (−4.14;5.32)	−0.54 (−5.37;4.29)	0.81	52.0 (10.0)	1.96 (−2.82;6.74)	−0.22 (−4.91;4.47)	0.87
Total problems	52.3 (9.7)	3.12 (−1.52;7.76)	1.98 (−2.76;6.71)	0.43	52.1 (9.8)	3.88 (−0.97;8.73)†	2.20 (−2.56;6.96)	0.41
CBCL scores	Cd tertiles (μg/g)							
	1st (0.03–0.05)	2nd (0.04–0.05)	3rd (0.05–0.55)	p-trend	1st (0.01–0.03)	2nd (0.04–0.05)	3rd (0.05–0.55)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β (95% CI)	β (95% CI)	
Syndrome scores								
Anxious depressed	55.2 (5.2)	0.29 (−2.91;3.48)	0.89 (−2.22;4.01)	0.56	55.2 (5.2)	0.67 (−2.66;4.0)	1.37 (−1.88;4.61)	0.40
Withdrawn	56.9 (6.8)	1.71 (−1.89;5.32)	0.94 (−2.58;4.45)	0.63	56.9 (6.8)	1.57 (−2.15;5.28)	1.37 (−2.25;4.99)	0.47
Somatic complaints	59.6 (8.1)	−0.05 (−4.05;3.94)	−3.42 (−7.31;0.47)†	0.07	59.6 (8.1)	0.48 (−3.67;4.63)	−2.78 (−6.83;1.26)	0.160
Social problems	53.4 (4.8)	4.85 (1.85;7.85)*	3.05 (0.13;5.98)*	0.06	53.4 (4.8)	4.50 (1.37;7.63)**	2.85 (−0.20;5.90)†	0.10
Thought problems	53.7 (4.9)	1.83 (−1.19;4.85)	1.98 (−0.97;4.92)	0.06	53.7 (4.9)	2.38 (−0.75;5.50)†	2.47 (−0.57;5.51)†	0.12
Attention problems	54.5 (5.1)	2.84 (−0.11;5.79)†	0.66 (−2.22;3.53)	0.19	54.5 (5.1)	2.94 (−0.15;6.03)†	0.77 (−2.23;3.78)	0.69
Rule-breaking behavior	54.4 (5.6)	−1.10 (−3.83;1.67)	−0.56 (−3.24;2.12)	0.71	54.4 (5.6)	−0.70 (−3.42;2.01)	0.12 (−2.52;2.76)	0.90
Aggressive behavior	53.9 (5.61)	2.28 (−0.88;5.44)	3.97 (0.89;7.05)*	0.01	53.9 (5.6)	2.56 (−0.64;5.76)†	4.26 (1.14;7.38)**	0.01
Composite scores								
Internalizing problems	55.6 (10.3)	1.54 (−3.77;6.84)	−0.82 (−5.99;4.36)	0.72	55.6 (10.3)	1.65 (−3.92;7.22)	−0.19 (−5.62;5.24)	0.91
Externalizing problems	50.6 (9.4)	1.87 (−3.08;6.82)	3.18 (−1.64;8.01)	0.19	50.6 (9.4)	2.37 (−2.57.7.32)	3.87 (−0.95;8.68)†	0.11
Total problems	52.5 (9.0)	3.57 (−1.26;8.40)	1.66 (−3.05;6.37)	0.53	52.5 (9.0)	3.84 (−1.15;8.83)†	2.21 (−2.65;7.07)	0.41
CBCL scores	Hg tertiles (μg/g)							
	1st (0.02–0.23)	2nd (0.23–0.48)	3rd (0.49–3.24)	p-trend	1st (0.02–0.23)	2nd (0.23–0.48)	3rd (0.49–3.24)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β (95% CI)	β (95% CI)	
Syndrome scores								
Anxious depressed	55.9 (5.8)	−1.02 (−4.16;2.13)	−2.38 (−5.83;1.07)	0.17	56.3 (5.8)	−1.29 (−6.29;0.95)	−2.67 (−6.29;0.95)	0.14
Withdrawn	58.8 (8.2)	−3.04 (−6.59;0.51)†	−4.15 (−8.25;-0.26)*	0.04	59.0 (8.4)	−2.94 (−6.58;0.70)†	−4.57 (−8.62;-0.53)*	0.03
Somatic complaints	59.3 (7.8)	−3.36 (−7.22;0.51)†	−0.51 (−4.75;3.73)	0.87	59.5 (8.0)	−3.79 (−7.79;0.21)†	−1.25 (−5.70;3.20)	0.65
Social problems	56.6 (6.7)	−2.56 (−5.63;0.51)†	−5.37 (−8.73;-2.00)**	<0.001	56.3 (6.8)	−1.9 (−5.06;1.25)	−4.68 (−8.20–1.17)**	0.01
Thought problems	54.4 (5.1)	−0.73 (−3.71;2.25)	−1.61 (−4.88;1.64)	0.32	54.5 (5.3)	−1.10 (−4.17;1.97)	−2.35 (−5.76;1.07)	0.17
Attention problems	55.8 (5.3)	0.28 (−2.67;3.23)	−1.65 (−4.89;1.59)	0.29	55.5 (5.4)	0.49 (−2.58;3.56)	−1.51 (−4.92;1.91)	0.35
Rule-breaking behavior	54.9 (5.96)	−1.85 (−4.55;0.84)	−1.31 (−4.27;1.64)	0.40	55.1 (6.1)	−1.99 (−4.63;0.65)†	−1.77 (−4.71;1.17)	0.25
Aggressive behavior	56.0 (6.1)	−1.06 (−4.16;2.05)	−3.26 (−6.67;0.14)†	0.05	56.0 (6.2)	−0.94 (−4.06;2.19)	−3.28 (−6.76;0.20)†	0.06
Composite scores								
Internalizing problems	56.4 (10.9)	−3.18 (−8.39;2.04)	−2.51 (−8.23;3.21)	0.40	56.8 (11.1)	−3.20 (−8.64;2.25)	−2.95 (−9.01;3.11)	0.35
Externalizing problems	52.7 (9.66)	−1.57 (−6.43;3.30)	−2.47 (−7.81;2.87)	0.36	53.0 (9.7)	−1.47 (−6.30;3.37)	−2.74 (−8.12;2.64)	0.31
Total problems	54.6 (9.5)	−2.78 (−7.58;2.01)	−3.79 (−9.05;1.47)	0.15	54.7 (9.7)	−2.58 (−7.51;2.35)	−3.99 (−9.47;1.49)	0.15
CBCL scores	Pb tertiles (μg/g)							
	1st (0.01–0.18)	2nd (0.18–0.31)	3rd (0.31–2.64)	p-trend	1st (0.01–0.18)	2nd (0.18–0.31)	3rd (0.31–2.64)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β (95% CI)	β (95% CI)	
Syndrome scores								
Anxious depressed	55.5 (6.3)	0.31 (−2.7;3.31)	−2.28 (−5.25;0.69)†	0.14	55.7 (6.3)	0.20 (−2.92;3.33)	−2.30 (−5.42;0.82)	0.15
Withdrawn	57.4 (5.1)	−0.86 (−4.28;2.56)	−1.04 (−4.42;2.35)	0.54	57.4 (5.1)	−0.69 (−4.20;2.83)	−1.26 (−4.77;2.25)	0.48
Somatic complaints	60.3 (7.6)	−4.54 (−8.27;-0.81)*	−3.31 (−7.01;0.38)†	0.07	60.5 (7.6)	−4.62 (−8.50;-0.74)*	−3.37 (−7.24;0.51)†	0.09
Social problems	55.0 (5.7)	−0.17 (−3.12;2.78)	−0.76 (−3.68;2.16)	0.60	54.8 (5.7)	0.58 (−2.47;3.62)	−0.43 (−3.48;2.61)	0.78
Thought problems	54.8 (5.0)	−0.17 (−3.03;2.70)	−0.94 (−3.77;2.01)	0.51	54.8 (5.1)	−0.06 (−3.02;2.90)	−0.74 (−3.70;2.22)	0.62
Attention problems	55.9 (5.5)	−1.14 (−3.99;1.71)	−1.55 (−4.37;1.28)	0.27	55.8 (5.5)	−0.75 (−3.73;2.23)	−1.34 (−4.32;1.64)	0.37
Rule-breaking behavior	53.6 (4.4)	−0.27 (−2.88;2.33)	0.91 (−1.67;3.48)	0.49	53.7 (4.5)	0.31 (−2.25.2.88)	1.48 (−1.08;4.04)	0.25
Aggressive behavior	55.6 (5.7)	−0.11 (−3.10;2.88)	−0.84 (−3.8;2.12)	0.57	55.4 (5.7)	0.67 (−2.35;3.68)	0.01 (−3.00;3.03)	0.99
Composite scores								
Internalizing problems	57.5 (8.2)	−4.59 (−9.59;0.41)†	−4.19 (−9.14;0.76)†	0.09	57.7 (8.2)	−4.50 (−9.75;0.74)†	−4.32 (−9.57;0.92)†	0.10
Externalizing problems	52.7 (7.5)	−1.89 (−6.56;2.78)	−1.30 (−5.92;3.33)	0.57	52.5 (7.5)	−0.70 (−5.36;3.97)	0.05 (−4.62;4.71)	0.98
Total problems	55.3 (7.2)	−3.3 (−7.89;1.30)	−3.24 (−7.79;1.31)	0.15	55.2 (7.3)	−2.43 (−7.20;2.32)	−2.55 (−7.30;2.19)	0.28

Model 1: adjusted for adolescent's age and BMI, maternal schooling and intelligence, and for all metals simultaneously.
Model 2: additionally adjusted for passive tobacco smoking and total fish intake of adolescents.
For all subscales, higher score indicates more behavioral problems.
**p < 0.0; *p < 0.05; †p < 0.10.

tertile in the withdrawn [(β_{T2} = —3.77, 95% CI = —7.00;-0.53), (β_{T3} = —3.49, 95% CI = —6.95; —0.02)], social problems (β_{T3} = —2.52, 95% CI= -5.69,0.65), and thought problems (β_{T3} 2.88, 95% CI 5.78; 0.01) subscales, observing a significant linear trend for both withdrawn (p-trend 0.04) and thought problems (p-trend 0.04) (Table S6). A lower score in the total problems scale was observed in participants in the second *versus* first tertile of serum BDNF concentrations (β 3.85, 95% CI 8.28; 0.58).

When total DNA methylation of the BDNF gene was considered as a continuous variable, no significant association was found with the behavior of the adolescents, although the percentage BDNF gene methylation appeared in general to be inversely related to the behavioral scores (Fig. 2). Similar results were obtained when tertiles of total BDNF gene DNA methylation were considered (data not shown).

4. Discussion

The results of this exploratory study among Spanish adolescent males (aged 15–17 years) suggest a relationship between urinary As and Cd exposure and behavioral problems, possibly through their effects on BDNF secretion patterns (serum BDNF protein levels and BDNF gene DNA methylation percentage). In these adolescents, intermediate urinary As and Cd concentrations were associated with more internalizing and externalizing problems, respectively. Furthermore, results suggest that serum BDNF protein concentrations were lower in adolescents exposed to moderate and high As and Cd levels. High As concentrations were also associated with increased percentage BDNF gene DNA methylation and moderate urinary Cd concentrations suggested associations with decreased BDNF gene DNA methylation percentages. Interestingly, increased serum BDNF levels were associated with fewer behavioral alterations (*i.e.,* withdrawn and social, thought, and total problems). Hg and Pb concentrations were found to be inversely related to behavioral functioning. No statistically significant relationships were found between Hg or Pb concentrations and percentage BDNF gene DNA methylation or serum BDNF protein concentrations.

4.1. *Epidemiological evidence on the association of As and Cd exposurewith neurobehavior*

Urinary Cd concentrations were within the range reported for adolescents by the National Health and Nutrition Examination Survey(NHANES, 2009–2014) and the German Human Biomonitoring Commission (Sanders et al., 2019; Schulz et al., 2011). However, urinary As concentrations were higher in the present population. Previous epidemiological studies have assessed the potential harmful effects of postnatal exposure to As and Cd on neurobehavioral function, but the resultshave not been conclusive. On the one hand, two systematic reviews found no association between As exposure and behavioral outcomes in children between 5 and 15 years of age (Rodríguez-Barranco et al., 2013; Tolins et al., 2014). On the other hand, two epidemiological studies in children aged between 6 and 12 years reported that urinary As (total and inorganic) was associated with poorer attention (Rodríguez-Barranco et al., 2016) and with depressive problems (Lin et al., 2017), more in line with the present findings. In other epidemiological studies, postnatal newborn hair concentrations of Cd were associated with withdrawn and social and attention problems in 7- to 16-year-old Chinese children (Bao et al., 2009), and urinary Cd was related to worse prosocial behavior in 10-year-old children (Gustin et al., 2018). However, no significant association was found between blood Cd concentrations and more behavioral problems in children at 2, 5, or 7 yearsof age (Cao et al., 2009). In the present study, urinary Cd concentrations were associated with CBCL subscales for externalizing behaviors (*i.e.*, social problems and aggressive behavior) and for somatic and thought problems. These patterns seem to point towards an association of As and Cd exposure with altered behavioral functioning in adolescents.

The above comparisons with the present findings should be interpreted with caution. First, because most previous studies measured As and Cd prenatally or during early or late childhood, whereas the presentstudy focused on adolescence. Neurological mechanisms and the susceptibility of behavioral functions to these compounds differ among developmental periods (Gore et al., 2018; Spear, 2000; Stiles and Jernigan, 2010); which may explain the absence of evidence on the association between metal exposure and behavioral domains during this period of development (Rodríguez-Barranco et al., 2013; Spear, 2000).Second, data on metal concentrations may differ according to the matrix used (*e.g.*, urine, blood, hair, or drinking water). Urine is a useful matrixfor assessing chronic exposure to Cd in biomonitoring studies because of its long half-life, reflecting long-term exposure, whereas concentrationsof As in urine correspond to acute exposure (Gil and Hernández, 2015). Finally, wide variations in the instruments used to assess behavioral functioning may also explain discrepancies among studies (Rodríguez-Carrillo et al., 2019).

4.2. *Possible effects of As and Cd on neurobehavior through alteration of BDNF expression patterns*

The suggestive association of As and Cd exposure with behavioral functioning might be explained by their binding to N-methyl-D-aspartate (NMDA) receptors in the hippocampus (Karri et al., 2016). This would lead to a reduction in BDNF concentrations and consequent behavioral and cognitive impairments, consistent with the adverse outcome pathways (AOPs) described by Mustieles et al. (2020) (Fig. 3). The hippocampus is responsible for the formation of emotional responses and the acquisition of memory and learning, which are both associated with social behavior (Ciranna, 2006). It is especially susceptible to exogenous and endogenous stressors, and the resulting changes in its structure and function can play a crucial role in the development of mood disorders (Zaletel et al., 2017).

Exposure to As may affect behavioral function through a direct action on the BDNF gene, given that As can alter DNA methylation patterns, possibly by interacting with transcription factor binding sites (TFBS) and inhibiting DNA repair mechanisms (Demanelis et al., 2019;Karim et al., 2019) (mechanisms of action shown in Fig. 3, numbers 1 and 2). This may explain the present findings of increased BDNF gene methylation in adolescents with higher urinary As concentrations (Fig. 3). The present mechanism is also consistent with experimental findings of an association between memory deficits and decreased hippocampal BDNF and CAMP responsive element binding protein 1 (CREB) in mice exposed to As (Sun et al., 2015). As can also exert an indirect effect on BDNF *via* the following pathways: first, by the inhibition of NMDA receptors, which play a key role in Ca^{+2} influx mechanisms, leading to reduced BDNF concentrations (Wang et al., 2016) (Fig. 3, number 3); second, through an imbalance of the oxidative stress homeostasis, thereby increasing reactive oxygen species (ROS) and reducing glutathione (GSH) (Karri et al., 2016; Mimouna et al., 2018), which favors cell injury or death and leads to neuroinflammation and ultimately to the degeneration of hippocampal brain cells, reducing the expression of BDNF (Karri et al., 2016) (Fig. 3, number 4); and, finally, by altering the metabolism of neurotransmitters such as GSH or serotonin, which play an important role in the expression and

Table 4
Adjusted models for the association of tertiles of urinary metal concentration with serum BDNF (n = 125) and BDNF gene methylation (n = 113).

	Model 1				Model 2			
	As tertiles (μg/g)							
	1st (0.58–6.19)	2nd (6.47–16.18)	3rd (16.35–465.4)	p-trend	1st (0.58–6.19)	2nd (6.47–16.18)	3rd (16.35–465.4)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β (95% CI)	β (95% CI)	
sBDNF	35.2 (10.1)	−1.15 (−5.97;3.66)	−2.91 (−7.87;2.05)	0.25	35.1 (10.3)	−0.77 (−5.87;4.34)	−2.69 (−7.64;2.26)	0.27
metBDNF								
CpG 1	4.5 (0.6)	−0.09 (−0.37;0.55)	−0.10 (−0.58;0.37)	0.66	4.5 (0.6)	0.12 (−0.35;0.60)	−0.08 (−0.56;0.40)	0.73
CpG 2	3.1 (0.5)	0.17 (−0.11;0.45)	0.20 (−0.09;0.50)	0.16	3.1 (0.5)	0.12 (−0.18;0.41)	0.18 (−0.11;0.48)	0.22
CpG 3	3.2 (0.5)	0.15 (−0.17;0.47)	0.22 (−0.11;0.54)	0.18	3.2 (0.5)	0.10 (−0.22;0.42)	0.19 (−0.14;0.51)	0.24
CpG 4	5.8 (0.9)	0.25 (−0.39;0.90)	0.62 (−0.05;1.28)†	0.06	5.8 (0.9)	0.20 (−0.47;0.87)	0.60 (−0.10;1.27)†	0.07
CpG 5	3.0 (0.6)	0.33 (−0.09;0.76)†	0.54 (0.11;0.98)*	0.01	3.0 (0.6)	0.23 (−0.19;0.65)	0.49 (0.07;0.91)*	0.02
CpG 6	2.4 (0.5)	0.25 (−0.37;0.87)	0.74 (0.09;1.39)*	0.02	2.4 (0.8)	0.12 (−0.48;0.72)	0.67 (0.05;1.29)*	0.03
CpG t	3.6 (0.5)	0.25 (−0.09;0.58)	0.41 (0.06;0.75)*	0.02	3.7 (0.5)	0.19 (−0.15;0.52)	0.38 (0.04;0.72)*	0.02
	Cd tertiles(μg/g)							
	1st (0.01–0.03)	2nd (0.04–0.05)	3rd (0.05–0.55)	p-trend	1st (0.01–0.03)	2nd (0.04–0.05)	3rd(0.05–0.55)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β (95% CI)	β(95% CI)	
sBDNF	34.6 (9.0)	−0.78 (−5.80;4.20)	−2.14 (−7.14;2.87)	0.38	34.6 (9.0)	−1.20 (−6.42;4.02)	−3.00 (−8.25;2.25)	0.25
metBDNF								
CpG 1	4.6 (1.0)	−0.29 (−0.76;0.19)	−0.13 (−0.63;0.36)	0.62	4.6 (1.0)	−0.36 (−0.84;0.13)	−0.17 (−0.67;0.33)	0.55
CpG 2	3.4 (0.5)	−0.39 (−0.67;-0.10)*	−0.14 (−0.43;0.16)	0.43	3.4 (0.5)	−0.41 (−0.69;-0.20)*	−0.14 (−0.43;0.16)	0.46
CpG 3	3.4 (0.5)	−0.37 (−0.69;-0.10)*	−0.14 (−0.48;0.19)	0.46	3.4 (0.5)	−0.41 (−0.72;-0.10)*	−0.18 (−0.51;0.15)	0.34
CpG 4	6.1 (1.0)	−0.43 (−1.07;0.22)	0.06 (−0.62;0.74)	0.82	6.1 (1.0)	−0.43 (−1.09;0.24)	0.04 (−0.66;0.73)	0.85
CpG 5	3.3 (0.7)	−0.21 (−0.65;0.22)	0.17 (−0.28;0.62)	0.42	3.3 (0.7)	−0.22 (−0.65;0.21)	0.14 (−0.30;0.31)	0.48
CpG 6	2.6 (0.8)	0.00 (−0.67;0.67)	0.46 (−0.24;1.15)	0.16	2.6 (0.8)	0.04 (−0.62;0.69)	0.41 (−0.26;1.07)	0.20
CpG t	3.9 (0.5)	−0.29 (−0.62;0.10)†	−0.00 (−0.36;0.35)	0.95	3.9 (0.5)	−0.32 (−0.70;0.00)†	−0.05 (−0.40;0.29)	0.83
	Hg tertiles(μg/g)							
	1st (0.02–0.23)	2nd (0.23–0.48)	3rd (0.49–3.24)	p-trend	1st (0.02–0.23)	2nd(0.23–0.48)	3rd(0.49–3.24)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β(95% CI)	β(95% CI)	
sBDNF	34.9 (9.5)	−0.14 (−5.06;4.79)	−0.65 (−5.96;4.65)	0.80	34.7 (9.8)	0.34 (−4.79;5.47)	0.28 (−5.33;5.88)	0.92
metBDNF								
CpG 1	4.6 (1.0)	−0.28 (−0.73;0.17)	0.29 (−0.22;0.80)	0.31	4.6 (10.0)	−0.21 (−0.68;0.25)	0.37 (−0.17;0.91)	0.20
CpG 2	3.3 (0.5)	−0.14 (−0.43;0.14)	−0.13 (−0.45;0.20)	0.41	3.3 (0.5)	−0.17 (−0.47;0.12)	−0.15 (−0.49;0.19)	0.35
CpG 3	3.4 (0.6)	−0.16 (−0.48;0.16)	−0.20 (−0.56;0.17)	0.27	3.3 (0.6)	−0.19 (−0.51;0.14)	−0.17 (−0.54;0.20)	0.34
CpG 4	6.1 (1.1)	−0.35 (−0.99;0.29)	−0.42 (−1.16;0.32)	0.24	6.1 (1.1)	−0.43 (−1.08;0.23)	−0.46 (−1.23;0.31)	0.21
CpG 5	3.4 (0.8)	−0.39 (−0.72;0.14)	−0.46 (−0.95;0.0)†	0.06	3.3 (0.8)	−0.34 (−0.76;0.10)†	−0.44 (−0.93;0.0)†	0.06
CpG 6	2.7 (1.0)	−0.32 (−0.96;0.32)	−0.64 (−1.39;1.1)†	0.09	2.7 (1.0)	−0.40 (−1.03;0.20)	−0.56 (−1.30;0.2)†	0.12
CpG t	3.9 (0.6)	−0.19 (−0.53;0.14)	−0.20 (−0.59;0.18)	0.27	3.8 (0.6)	−0.24 (−0.57;0.09)	−0.21 (−0.59;0.17)	0.26
	Pb tertiles(μg/g)							
	1st (0.01–0.18)	2nd (0.18–0.31)	3rd (0.31–2.64)	p-trend	1st (0.01–0.18)	2nd (0.18–0.31)	3rd(0.31–2.64)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β (95% CI)	β(95% CI)	
sBDNF	34.1 (9.8)	−1.01 (−5.78;3.75)	0.36 (−4.34;5.06)	0.87	33.9 (9.9)	−0.72 (−5.70;4.27)	0.81 (−4.16;5.78)	0.73
metBDNF								
CpG 1	4.4 (0.7)	0.35 (−0.15;0.84)	0.18 (−0.28;0.64)	0.48	4.4 (0.7)	0.38 (−0.12;0.90)†	0.18 (−0.29;0.65)	0.51
CpG 2	3.1 (0.5)	0.23 (−0.08;0.5)†	−0.02 (−0.30;0.26)	0.78	3.1 (0.5)	0.23 (−0.07;0.50)†	−0.04 (−0.32;0.25)	0.70
CpG 3	3.2 (0.5)	0.18 (−0.17;0.52)	0.06 (−0.26;0.38)	0.75	3.2 (0.5)	0.12 (−0.22;0.46)	0.00 (−0.31;0.32)	0.96
CpG 4	5.7 (0.8)	0.46 (−0.24;1.16)	0.29 (−0.35;0.93)	0.39	5.7 (0.8)	0.42 (−0.29;1.13)	0.26 (−0.40;0.91)	0.46
CpG 5	3.2 (0.6)	0.16 (−0.31;0.62)	−0.1 (−0.52;0.33)	0.60	3.2 (0.6)	0.10 (−0.36;0.55)	−0.17 (−0.58;0.24)	0.38
CpG 6	2.5 (0.7)	0.31 (−0.40;1.01)	−0.01 (−0.64;0.63)	0.55	2.5 (0.7)	0.18 (−0.51;0.87)	−0.13 (−0.74;0.49)	0.62
CpG t	3.7 (0.4)	0.30 (−0.05;0.70)†	0.07 (−0.26;0.40)	0.78	3.7 (0.4)	0.26 (−0.09;0.60)†	0.02 (−0.30;0.34)	0.99

Model 1: adjusted for adolescent's age and BMI, maternal schooling and intelligence, and for all metals simultaneously.
Model 2: additionally adjusted for passive tobacco smoking and total fish intake of adolescents.
For all subscales, a higher score indicates more behavioral problems.
sBDNF: serum BDNF; metBDNF: BDNF gene methylation.
*p < 0.05; †p < 0.10.

production of BDNF (Htway et al., 2019; Ramos-Chávez et al., 2015). For example, adult male mice prenatally exposed to As exhibited a down-regulation of BDNF expression and social isolation-like behavior, possibly mediated by an As-induced alteration of the serotonergic system (Htway et al., 2019). The present results indicate that greaterexposure to As could be associated with higher DNA BDNF gene DNA methylation percentage at several CpGs. If so, it would reduce BDNF gene expression levels and protein concentrations, potentially generating more behavioral problems. Some of the associations found with BDNF gene methylation and protein levels may be in line with the effects described in the above animal models.

As in the case of As, the neurotoxic activity of Cd has also been implicated in the disruption of various pathways. It has been found to cross the blood-brain barrier, enter the CNS, and disrupt the hippocampal membrane function (Kumar et al., 1996; Wang and Du, 2013) (Fig. 3, number 5). In murine studies, Cd exposure was reported to inhibit acetylcholine esterase (AchE) and Na+/K+-ATP-ase pump, reducing neuronal activity in pups (Gupta et al., 1991), Cd-induced redox homeostasis imbalance increased neuronal death in rats (Wang and Du, 2013) (Figs. 3 and 5), and Cd was found to mimic the ubiquitous intracellular ion Ca+2, thereby inhibiting its influx pathways (Xu et al., 2011) (Figs. 3 and 6). However, inadequate information is available to accurately determine whether these pathways have a direct or indirect effect on hippocampal BDNF expression. Some animal studies also found a downregulation of BDNF expression after Cd exposure (Kadry and Megeed, 2018; Mimouna et al., 2018). In the present investigation, adolescents with urinary Cd concentrations in the second tertile (intermediate level) showed associations with decreased serum BDNF concentrations and a tendency towards reduced BDNF gene DNA. Na^+/K^+-ATP-ase pump, reducing neuronal activity in pups (Gupta et al., 1991),

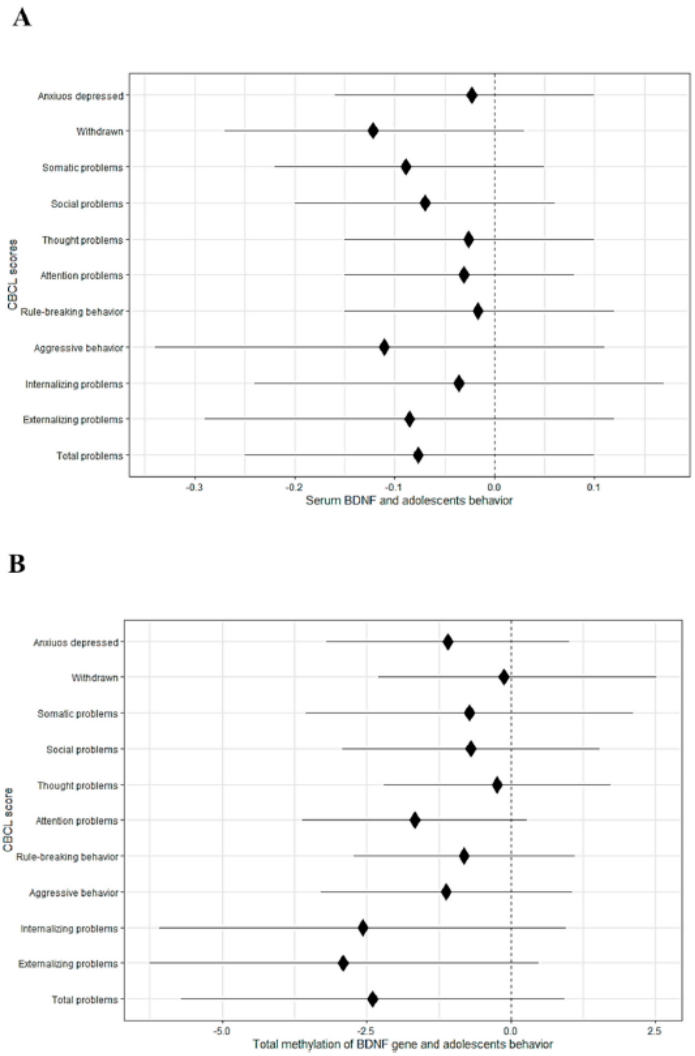


Fig. 2. Forest plot showing associations of serum BDNF concentrations (Fig. 2A) and total BDNF gene DNA methylation at six CpGs (Fig. 2B) with behavioral outcomes.

Cd-induced redox homeostasis imbalance increased neuronal death in rats (Wang and Du, 2013) (Figs. 3 and 5), and Cd was found to mimic the ubiquitous intracellular ion Ca^{+2}, thereby inhibiting its influx pathways (Xu et al., 2011) (Figs. 3 and 6). However, inadequate information is available to accurately determine whether these pathways have a direct or indirect effect on hippocampal BDNF expression. Some animal studies also found a downregulation of BDNF expression after Cd exposure (Kadry and Megeed, 2018; Mimouna et al., 2018). In the present investigation, adolescents with urinary Cd concentrations in the second tertile (intermediate level) showed associations with decreased serum BDNF concentrations and a tendency towards reduced BDNF gene DNA.

4.3. Hg and Pb exposure, adolescents' behavior and BDNF effectbiomarker

Urinary Hg and Pb concentrations were also within the range described for adolescents by the National Health and Nutrition Examination Survey (NHANES, 2009–2014) and German Human Biomonitoring Commission (Sanders et al., 2019; Schulz et al., 2011). Unexpectedly, Hg and Pb concentrations were not associated with neurobehavioral problems in these adolescents. As anticipated, moderate urinary Pb concentrations tended to be associated with higher percentage DNA methylation at CpGs #1, 4 and with total CpG methylation,while urinary Hg concentrations were associated with lesser BDNF gene DNA methylation. No association was observed between serum BDNF protein concentrations and the studied metals.

Adverse effects of prenatal and postnatal exposure to Pb and Hg on cognitive function and intelligence are well documented in humans (Canfield et al., 2003; Cecil et al., 2008; Debes et al., 2006; Freire et al.,2018; Hu et al., 2006; Jusko et al., 2008; Lanphear et al., 2005; Llopet al., 2012; Wright et al., 2008). However, the potential impact of Hg and Pb on behavioral functioning remains unclear, although some studies found associations of postnatal exposure to Pb and Hg with anxiety, social problems, and ADHD (Debes et al., 2006; Liu et al., 2014;Roy et al., 2009). Caution should be taken in interpreting the present results on postnatal Pb and Hg and behavioral functioning, given that urinary concentrations of Hg and Pb may reflect short-term rather than long-term exposure (Gil and Hernández, 2015) and may not serve as appropriate biomarkers to evaluate potential effects on behavior. In addition, some of these apparently protective associations may be explained by dietary and lifestyle confounders. For instance, fish consumption is a potential source of toxic metals as well as beneficial nutrients for brain development (Cano-Sancho and Casas, 2021; Gil and Gil, 2015). Although fish consumption was controlled for in the presentstudy, residual confounding or dietary misclassification cannot be ruled out.

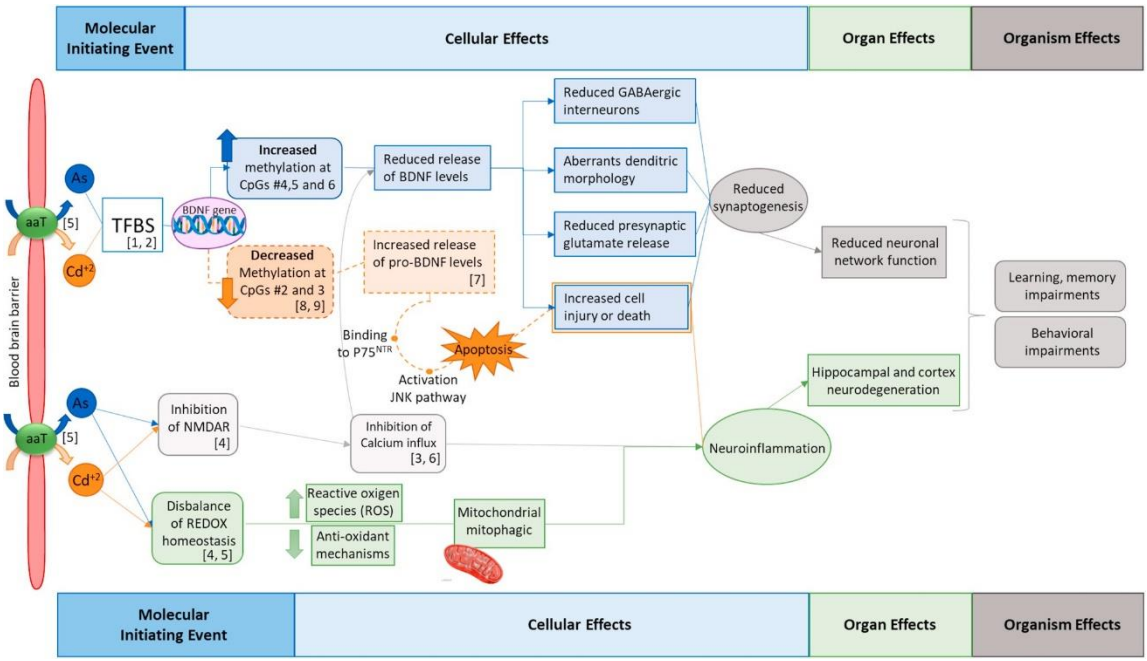


Fig. 3. Hypothesized adverse outcome pathway (AOP) based on the AOPs published by Mustieles et al. (2020) and other specific toxicological references for As (Demanelis et al., 2019; Karim et al., 2019; Karri et al., 2016; Wang et al., 2016) and Cd (Guan et al., 2019; Wang and Du, 2013; Xia et al., 2020; Xu et al., 2011;Zaletel et al., 2017). AOP followed by As and Cd in the hippocampus after crossing the blood brain barrier. As: Arsenic; aaT: amino acid transporter; BDNF: brain-derived neurotrophic factor; Cd: Cadmium; JNK: c-Jun N-terminal kinase; NMDAR: N-Methyl-D-aspartate receptors; pro-BDNF: immature isoform of BDNF; TFBS: Transcription factor binding sites. The observed downregulation of BDNF methylation might lead to higher concentrations of the immature BDNF isoform (pro-BDNF), known to activate cellular apoptosis by binding to P75 neurotrophin receptor (NTR) (Zaletel et al., 2017) [7], possibly explaining the suggested adverse association of Cd with behavior (Fig.3). Similar results were found in a zebrafish model showing increased BDNF expression after Cd exposure alongside locomotor alterations (Xia et al., 2020) [8] and in a genome-wide study finding that Cd exposure reduced global DNA methylation in drosophila melanogaster (Guan et al., 2019) [9]. However, further research is needed to verify this hypothesis, given the absence of published data on the effects of Cd on pro-BDNF secretion and fact that this BDNF form was not measured in the present study.

4.4. *Strengths and limitations*

Study limitations include the small sample size, reducing the statistical power of analyses and preventing the assessment of the mixture effect of the selected metals on BDNF and behavioral function, as well as potential interactions among them. Instead, we simultaneously adjusted the models for all metals in order to assess the effect exerted by a singlemetal while accounting for the influence of the remaining elements. Future studies in larger populations would be needed to address the combined effect of metals mixtures on BDNF and neurodevelopment. The cross-sectional design also means that causal relationships could not be inferred. Furthermore, the study investigated the concentration of total As and Hg, with no speciation procedure. Recent data from the Environment and Childhood study show that the primary source of Hg exposure is fish (Signes-Pastor et al., 2017), where Hg is present as methyl-Hg, the most neurotoxic form. The source of exposure of As, however, remains unknown, although rice (inorganic As) and seafood (organic As) consumption seem to be major sources of As exposure in the Spanish population (Signes-Pastor et al., 2017). Therefore, it is not clearwhether our study population is mostly exposed to inorganic or organic As. Nevertheless, this lack of specificity would tend to underestimate rather than overestimate As effects on neurodevelopment. Additionally, previous studies have also reported associations between urinary total As and behavioral function (Rodríguez-Barranco et al., 2016).

While urinary Cd and As levels appear to be a straightforward choice, it may not be the best biomarker for Pb and Hg exposure, since their short biological half-lives makes them suitable biomarkers for current or recent exposure (Gil and Hernández, 2015). Conversely, urinary Cd levels are a suitable biomarker of long-term and lifetime exposure to this metal (Gil and Hernández, 2015; Järup and Åkesson, 2009). Urinary As is also considered as adequate biomarker of short-term exposure, since it concentrations remains relatively stable among individuals with consistent dietary patterns (Hughes, 2006; Marchiset-Ferlay et al., 2012). Therefore, results for Cd and As could be more reliable comparedto those of Pb and Hg. Finally, spurious associations may have been identified due to the application of multiple analyses, although several significant associations are supported by toxicological and epidemiological studies and are unlikely to be the result of chance. Moreover, theestimated coefficients and confidence intervals should be taken as a global representation of the pattern of relationships between the study variables. Study strengths include the novel exploration approach of BDNF as biomarker of neurodevelopment, assessed at different levels of biological organization (DNA methylation and serum protein). In future epidemiological studies, this approach could contribute to elucidate the neurodevelopmental effects of metals and metalloids, especially As andCd. Another strength is the effort to characterize the effect of As and Cd on behavioral

functioning, given the scant available evidence on the impact of these pollutants. Finally, there has been inadequate research of this type in adolescence, which is characterized by important changesin neurological mechanisms.

5. Conclusion

Within an epidemiological context, serum BDNF protein levels and BDNF gene DNA methylation profile might serve as effect biomarkers to characterize the relationship of postnatal exposure to toxic metals, such as As and Cd, with behavioral problems in adolescents. However, due tostudy limitations, our results need to be verified in future larger epidemiological studies on metal exposures during this and other critical windows of neurodevelopment. Biomarkers of brain function are needed in human biomonitoring studies to better address current gaps in knowledge between environmental exposures and neurodevelopmental disorders.

Declaration of Competing interest

The authors declare no actual or potential conflicts of interest. The funders had no role in the study design, data collection or analysis, decision to publish, or preparation of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113877.

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Non-persistent pesticide exposure, BDNF, and behavioral function in adolescent males: Exploring a novel effect biomarker approach

Andrea Rodríguez-Carrillo [a, b, c], Shereen Cynthia D'Cruz [e], Vicente Mustieles [a, c, d], Beatriz Suárez [b, c, d], Fatima Smagulova [e], Francisco Peinado [c], Juan P. Arrebola [f], Mariana F. Fernández [a, b, c, d], Arthur David [e] and Carmen Freire [b, c, d]

[a] *Department of Radiology and Physical Medicine, School of Medicine, University of Granada, 18016, Granada, Spain*
[b] *Biomedical Research Center (CIBM), University of Granada, 18016, Granada, Spain*
[c] *Instituto de Investigación Biosanitaria de Granada (ibs.GRANADA), 18012, Granada, Spain*
[d] *Consortium for Biomedical Research in Epidemiology and Public Health (CIBERESP), Spain*
[e] *Univ Rennes, EHESP, INSERM, IRSET (Institut de Recherche en Santé, Environnement et Travail), UMR_S 1085, F-35000, Rennes, France*
[f] *University of Granada, Department of Preventive Medicine and Public Health, 18016 Granada, Spain.*

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Background: Numerous contemporary, non-persistent pesticides may elicit neurodevelopmental impairments. Brain-derived neurotrophic factor (BDNF) has been proposed as a novel effect biomarker of neurological function that could help to understand the biological responses of some environmental exposures.
Objectives: To investigate the relationship between exposure to various non-persistent pesticides, BDNF, and behavioral functioning among adolescents.

Methods: The concentrations of organophosphate (OP) insecticide metabolites, 3,5,6-trichloro-2-pyridinol (TCPy), 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMPy), malathion diacid (MDA), and diethyl thiophosphate (DETP); metabolites of pyrethroids, 3-phenoxybenzoic acid (3-PBA), and dimethylcyclopropane carboxylic acid (DCCA) the metabolite of insecticide carbaryl, 1-naphthol (1-N); and the metabolite of ethylene-bis-dithiocarbamate fungicides ethylene thiourea (ETU), were measured in spot urine samples, as well as serum BDNF protein levels and blood DNA methylation of Exon IV of BDNF gene in boys aged 15-17 years belonging to the Spanish INMA-Granada cohort. Adolescents' behavior was reported by parents using the Child Behavior Check List (CBCL/6-18). This study included 140 adolescents of whom 118 had data on BDNF gene DNA methylation. Multivariable linear regression, weighted quantile sum (WQS) for mixture effects, and mediation models were fit.

Results: IMPy, MDA, DCCA, and ETU were detected in more than 70% of urine samples, DETP in 53%, and TCPy, 3-PBA, and 1-N in less than 50% of samples. Higher levels of IMPy, TCPy, and ETU were significantly associated with more behavioral problems, including social, thought problems, and rule-breaking symptoms. IMPy, MDA, DETP, and 1-N were significantly associated with decreased serum BDNF levels, while MDA, 3-PBA, and ETU were associated with higher DNA methylation percentages at several CpGs. WQS models suggest a mixture effect on more behavioral problems and BDNF DNA methylation at several CpGs. A mediated effect of serum BDNF within IMPy-thought and IMPy-rule breaking associations was suggested.

Conclusion: BDNF biomarkers measured at different levels of biological organization provided novel information regarding the potential disruption of behavioral function due to contemporary pesticides, highlighting exposure to diazinon (IMPy) and the combined effect of IMPy, MDA, DCCA, and ETU concentrations. However, further research is warranted.

Keywords: BDNF, non-persistent pesticides, HBM4EU, behavior, effect biomarkers

1. Introduction

The adolescence dramatically reshapes teenagers' perception of themselves, as well as their social context through complex neural mechanisms (Gore et al., 2018). They must exhibit social communicative skills, reproductive behaviors, adequate anxiety responses, and affective states according to their age and sex, among other factors (Spear, 2000). Exposure to environmental neurotoxic chemicals could be involved in the increasing incidence observed for mental health disorders such as social anxiety, depression, or eating disorders, among adolescents (Bjørling-Poulsen et al., 2008; Bouchard et al., 2010; Pfeifer and Allen, 2021; Shoaff et al., 2020; Supke et al., 2021).

A growing body of epidemiological research suggests that prenatal exposures to contemporary non-persistent pesticides, mainly organophosphate (OP) and pyrethroid insecticides, are associated with neurodevelopmental impairments (González-Alzaga et al., 2014; Hernández et al., 2016; Oulhote and Bouchard, 2013; Wagner-Schuman et al., 2015). However, limited evidence is available regarding the potential impact of pesticide exposures during childhood or adolescence (Bouchard et al., 2010; Dalsager et al., 2019a; Damgaard et al., 2006) with inconclusive results (Oulhote and Bouchard, 2013; Quirós-Alcalá et al., 2014; van Wendel de Joode et al., 2016; Wagner-Schuman et al., 2015).

The general population is widely exposed to many non-persistent pesticides. Although some of them are banned in the European Union (EU) (EFSA, 2019) (Supplementary Material, Table S1), and the use of others (e.g. some OPs) is decreasing, the employment of pyrethroids is increasing as agriculture and indoor biocides, being frequently detected in urinary samples of mothers, children, and neonates (Andersen et al., 2021; Dalsager et al., 2019b). Parent compounds of some specific and non-specific pesticide metabolites are known developmental neurotoxicants (Sun et al., 2008; Marinovich et al., 1997).

Under the framework of the Human Biomonitoring for Europe Initiative (HBM4EU), brain-derived neurotrophic factor (BDNF) has been proposed as a promising effect biomarker to improve the causal inference between exposure to environmental chemicals and altered neurodevelopment (Mustieles et al., 2022, 2020; Rodríguez-Carrillo et al., 2022). BDNF plays an important role in synaptic plasticity and is expressed throughout the brain, especially in the hippocampus, cortex, and other basal forebrain areas, which are susceptible to both exogenous and endogenous stressors. Thus, alterations in hippocampal BDNF expression could contribute to the development of mood disorders (Polyakova et al., 2015; Zaletel et al., 2017). In previous epidemiological studies, BDNF disruption has been assessed by measuring its protein levels in serum or plasma, or its degree of DNA methylation in blood concerning behavioral outcomes, including depression, bipolar disorder, attention-deficit hyperactivity disorder (ADHD), and even suicidal behavior at different stages of life (Bilgiç et al., 2020; Heinrich et al., 2017; Kim et al., 2007; Kundakovic et al., 2015; Polyakova et al., 2015). Recent results from our research group highlighted the advantages of including BDNF as an effect biomarker to address the relationship between exposure to bisphenol A and toxic metals, and altered/inadequate neurodevelopment (Mustieles et al., 2022; Rodríguez-Carrillo et al., 2022).

The aim of this study was to explore the potential role of BDNF on the relationship between exposure to non-persistent pesticides and behavior in adolescents, and to assess the mixture effect of pesticides exposure on behavioral symptoms and BDNF levels. To do so, we assessed the cross-sectional relationship of urinary pesticide metabolites, both individually and combined, with: i) the behavioral function of adolescents and ii) the effect biomarker BDNF measured at two levels of biological organization (DNA methylation and serum protein levels); and iii) the association of BDNF with adolescents' behavior. In addition, and if relevant, we aimed to test the potential mediation role of BDNF in exposure-effect relationships.

2. Material and Methods

2.1. Study population

The Environment and Childhood (INMA) Project is a multicenter population-based birth cohort study that aimed to investigate the impact of environmental exposures and diet on health and development during key windows of susceptibility, i.e. pregnancy, childhood, and adolescence, in different geographical areas of Spain (Guxens et al., 2012). During the last follow-up of the INMA-Granada cohort (2017-2019), a total number of 151 adolescents aged 15-17 years agreed to participate in the clinical visit at the San Cecilio University Hospital (HUSC) of Granada, which included physical examination and behavioral assessments (Castiello et al., 2020). All participants provided a first-morning urine sample, and most of them (n=135, 89%) also provided a blood sample. The present study included participants with available data on i) urinary pesticide metabolite concentrations and behavioral outcomes (n=140); ii) urinary pesticide metabolite concentrations, behavioral outcomes, and serum total BDNF protein levels (n=130); and iii) urinary pesticide metabolites, behavioral outcomes, and blood DNA methylation of the Exon IV of BDNF gene at six CpGs (n=118). Further details on the INMA-Granada cohort recruitment and follow-ups have been previously described (Castiello et al., 2020; Fernandez et al., 2007; Freire et al., 2018). The informed consent was signed by the parents' participants and the study protocol was approved by the Biomedical Research Ethics Committee of Granada.

2.2. Analysis of urinary pesticides metabolites concentrations

A first morning spot urine sample was collected from each participant on the day of the clinical visit and kept at -80ºC until analysis. Urine samples were analyzed for 3,5,6-trichloro-2-pyridinol (TCPy), 2-isopropyl-6-methyl-4-pyrimidinol (IMPy), and malathion diacid (MDA), specific metabolites of the OP insecticides chlorpyrifos/chlorpyrifos-methyl, diazinon, and malathion, respectively; dialkyl phosphates diethyl thiophosphate (DETP) and diethyl dithiophosphate (DEDTP), non-specific metabolites of several OP insecticides; 3-phenoxybenzoic acid (3-PBA) and dimethylcyclopropane carboxylic acid (DCCA) (sum of cis and trans isomers); 1-naphthol (1-N), primary metabolite of the carbamate insecticide carbaryl; and ethylene thiourea (ETU), major metabolite of ethylene-bis-dithiocarbamate (EBDC) fungicides such as mancozeb (Supplementary Material, Table S1).

The urinary pesticide metabolites TCPy, IMPy, DETP, DEDTP, 3-PBA, 1-N, and ETU were measured by ultra-high-performance liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS), using an UHPLC Ultimate 3000 (Thermo Fischer) and a Q Exactive Focus mass spectrometer (Thermo Fischer) at the ''UNETE Research Unit" of the Biomedical Research Center (CIBM), University of Granada (Spain). The urinary acid metabolites MDA and DCCA were measured by liquid chromatography coupled to mass spectrometry (LC-MS/MS), using an Agilent 1290 liquid chromatography (Agilent) and API 4000 mass spectrometry (AB Sciex Instruments) at the facilities of the MEDINA Foundation, Granada (Spain). All metabolites, including MDA and DCCA, were first extracted and calibrated according to Suárez et al., (2021). No deconjugation procedure was performed. Standards solutions and Internal Standards (IS) were added to 1 mL of urine samples. Successively, 2 mL of aqueous ammonium hydroxide solution at 1 % was added and kept at room temperature for 20 minutes. Cleanup and preconcentration were performed with solid-phase extraction (SPE). Briefly, activation was done using 3 mL of methanol, 3 of deionized water, and 2 mL of aqueous ammonium hydroxide solution at 1% (v/v). Cartridge was rinsed with 2 mL of formic acid at 1 % with deionized water and dried under vacuum. Further information regarding the analytical method and quality control procedures have fully described previously (Freire et al., 2021; Suárez et al., 2021).

Data regarding retention times, analytical parameters, calibration curves, mean accuracy, selected reaction monitoring (SRM), and relative standard deviation (RSD) values are reported in Supplementary Material (Table S2). Limits of detection (LOD) were 0.039 µg/L for TCPy, 0.117 µg/L for IMPy, 0.052 µg/L for MDA, 0.116 µg/L for DETP, 0.142 µg/L for DEDTP, 0.117 µg/L for 3-PBA, 0.055 µg/L for DCCA, 0.156 µg/L for 1-N, and 0.072 µg/L for ETU (Table S2). To account for urine dilution, concentrations of creatinine in urine were assessed using a commercial kit (CREJ2) through the Jaffe method in a Roche Cobas C-311 system (mg/dL).

2.3. *Serum BDNF and whole blood DNA methylation of BDNF gene assessment*

On the same day of urine sample collection, peripheral venous blood samples were collected from participants under non-fasting conditions between 5 and 7 p.m. and were immediately processed to obtain serum and whole blood aliquots and stored at -80 ºC.Whole blood was sent on dry ice to the Human Genotyping Laboratory at the Spanish National Cancer Research Centre. DNA extraction was performed using Maxwell® RSC equipment and quantified by PicoGreen assay, showing an average concentration of 50 ng/µL.

Total serum BDNF levels, which contains mature and immature isoforms of BDNF, were measured with an enzyme-linked immunosorbent assay using the commercial Quantikine® ELISA kit (R&D Systems, Minneapolis, MN, USA) at the CIBM, University of Granada (Spain), following manufacturer's instructions. Full procedure was described elsewhere (Rodríguez-Carrillo et al., 2022). Serum total BDNF protein showed an intra- and inter-assay coefficient of variation (%) of <3% and 15%, respectively.

DNA methylation of the BDNF gene was performed by bisulfite pyrosequencing analysis at IRSET (Institut de Recherche en Santé, Environnement et Travail - INSERM UMR1085), Rennes, France. Exon IV of BDNF gene was the targeted region (genomic coordinates: chr11:27,723,070–27,723,280 retrieved from UCSC Genome Browser Human February 2009 (GRCh37/hg19), previously validated in rodents and humans (Kundakovic et al., 2015) which contains 6 CpGs including a CREB-binding site (cAMP response element-binding site). Further information regarding measurement of BDNF DNA methylation was published according to Mustieles et al., (2022). Biotinylated primers used for BDNF amplification (0.4 µM final concentration) are provided in Table S2. The degree of methylation at each CpG was expressed as percentage of DNA methylation.

2.4. *Behavioral functioning assessment*

Behavioral function of adolescents was evaluated using the Spanish version of the validated Child Behavior Checklist (CBCL/6-18) (Achenbach and Rescorla, 2013; Sardinero García et al., 1997). In this questionnaire, parents report on their sons' behavior during the previous 6 months. It is based on 118 items rated on a three-point scale (not true, somewhat true, or very/often true) and contains a total of eight syndrome scales: anxious/depressed, withdrawn/depressed, somatic complaints, social problems, thought problems, attention problems, rule-breaking behavior, and aggressive behavior. These scales are then summarized in three composite scales obtained from the sum of the mentioned syndrome scales, as follows: internal problems (sum of scores from anxious/depressed, withdrawn/depressed, and somatic complaints scales), externalizing problems (the sum of scores from the rule-breaking behavior and aggressive behavior scales) and total problems. All scales were normalized by age and sex and finally reported as T-scores. For all above-mentioned scales, higher scoring means more behavioral problems (Achenbach and Rescorla, 2013).

2.5. *Statistical analysis*

Absolute and relative frequencies (categorical variables) and central tendency measures and dispersion profiles (continuous variables) were calculated to describe sociodemographic and lifestyle characteristics of the study participants. For descriptive purposes, detection frequencies were calculated for urinary pesticides concentrations, while percentiles (25th, 50th and 75th) were calculated for both pesticide metabolites and BDNF effect biomarker levels. DEDTP was detected in only 1 urine sample and was thus excluded from further analyses. For pesticide metabolites detected above 70% of samples, concentrations below the LOD were imputed with LOD/√2 (Schisterman et al., 2006). The total concentration of OP (ΣOPs) and pyrethroid (ΣPYR) urinary metabolites was further calculated by summing concentrations of their respective metabolites (i.e., ΣOPs= TCPy + IMPy + MDA + DETP; ΣPYR= DCCA + 3-PBA). A small number of DNA methylation measurements (<4% of all CpG measurements performed) could not be quantified, and thus were multiple imputed (20 times) using the regression method. Spearman's correlation test was used to assess the relationship among concentrations of non-persistent pesticides metabolites.

Multivariable linear regression models were performed to assess the association of i) urinary pesticide metabolite concentrations with adolescents' behavioral function; ii) urinary pesticide metabolites with biomarkers of effect (serum BDNF and methylation profile of the BDNF gene), and iii) BDNF biomarkers with behavioral outcomes. Before regression analysis, pesticide metabolites detected in more than 70% of urine samples (IMPy, MDA, DCCA and ETU) were natural log-transformed to reduce distribution skewness (including ΣOPs and ΣPYR), while those detected in less than 70% of samples were dichotomized into detected vs. non-detected values. BDNF biomarkers (normally distributed) and behavioral outcomes were modeled as continuous variables. Metabolites detected in >70% of samples as well as ΣOPs, ΣPYR, and all BDNF effect biomarkers were further categorized into tertiles to assess possible non-linear associations. DETP (detected in 54% of samples) was categorized as follows: from below the LOD to the 50th percentile of urinary concentration, from the 50th to 75th percentile, and above the 75th percentile.

All models were adjusted for adolescent's age (continuous), body mass index (BMI, continuous kg/m2), alcohol consumption (less than one beverage/month or at

Table 1. General characteristics of adolescents and their mothers (n=140).

Adolescents	Mean ± SD/ n (%)
Age (years)	16.2 ± 0.4
BMI (kg/m^2)	23.2 ± 4.9
Area of residence	
Urban	97 69.5)
Suburban/rural	43 (30.5)
Passive smoking	
Yes	59 (43.2)
No	81 (56.8)
Alcohol consumption	
Never or <1 beverage per month	85 (60.3)
≥1 beverage per month	55 (39.7)
Season of urine collection	
Spring	33 (24.1)
Summer	16 (12.1)
Autumn	62 (42.6)
Winter	29 (21.3)
Annual family income (euros)	
<25,000	54 (38.3)
25,000-35,000	54 (38.3)
>35,000	32 (23.4)
Mothers	
Age (years)	39.6 ± 4.7
Maternal education	
Primary	58 (41.0)
Secondary	46 (33.1)
University	36 (25.9)

SD: standard deviation; BMI: Body mass index.

least 1 beverage/month) and maternal education (primary education, secondary education, or university education), which were selected based on their known influence on neurodevelopment and previous studies exploring pesticide exposure and behavioral function (Patra et al., 2016; Pyman et al., 2021; Wirt et al., 2015). Maternal education, a proxy for socioeconomical status, had a 6% of missing values (n=10). To maintain the initial sample size, this covariable was imputed using multiple imputation through regression method (20 imputations). Season of urinary collection was added to models with pesticide metabolites, since it may have an influence on pesticides exposure (Fortenberry et al., 2014). To control for urine dilution variability and reduce potential error bias, creatinine concentration (ng/mL) was included in models as a separate covariate as recommended in previous studies (Barr et al., 2005; O'Brien et al., 2016). Regression estimates reflect the mean change in behavioral outcome score/serum BDNF level/methylation percentage for each log unit increase in urinary pesticide metabolite concentration in linear models. For models with the independent variable categorized into tertiles, regression estimates reflect the mean change in the dependent variable taking the first tertile (T1) as reference; in models with dichotomized pesticide exposure, regression estimates reflect the mean change in the dependent variable for detected versus undetected concentrations.

Weighted Quantile Sum (WQS) analysis was performed to assess the combined effect of IMPy, MDA, DCCA, and ETU (>70% detection) on behavioral functioning and BDNF biomarkers. The WQS index is regressed from multivariable linear models, which constructs the unidirectional weighted index from quantiled chemical exposure variables, thus reducing potential multicollinearity and dimensionality while providing an overall mixture effect estimate (Tanner et al., 2020). Weights, expressed as percentages, show the relative strength of each mixture component. Pesticide metabolites were binned as quartiles. Since we hypothesized that the mixture effect would be associated with more behavioral problems and BDNF DNA methylation, but with lower serum BDNF protein levels (Mustieles et al., 2020), the index was constructed using weights in positive mode when addressing CBCL subscales and BDNF DNA methylation, and as negative mode when addressing serum BDNF. Mean weights of chemicals exceeding 25% (100%/4 chemicals) were considered as chemicals of concern, meaning that their contribution to the outcome would be larger than expected by chance. Bootstrapping was set to 100. Finally, to evaluate the stability and generalizability of our results, repeated holdout validation was performed. This method combines cross-validation and bootstrap resampling by splitting data into 40-60% training test sets and repeating WQS regression 100 times as previously performed in other epidemiological studies (Galbán-Velázquez et al., 2021; Tanner et al., 2020).

To assess the role of BDNF as potential mediator of significant/suggestive exposure-effect associations, total, direct, and indirect effects were analyzed using mediation analysis in the PROCESS macro v3.5 (http://processmacro.org/index.html). According to Hayes (2009), the magnitude of the effect and confidence intervals (95% CI) were estimating conducting 10000 bootstrapped replicates. The indirect or mediated- effect represents the proportion of the statistical relationship between the exposure (i.e., pesticides) and outcome (i.e., CBCL) that is driven by the mediator (i.e., BDNF). The percentage of mediation was calculated as: indirect effect/ (direct + indirect effect) x 100, where the direct effect represents the proportion of the statistical relationship between the exposure and the outcome not attributable to the mediator. The significance level was set at $p<0.05$, however, results were also interpreted based on patterns of associations, internal validity, and coherence with previous toxicological and epidemiological evidence (Amrhein et al., 2019). SPSS v26.0 (IBM, Chicago, IL) and R statistical software version 3.4.3, package WQS (https://cran.r-project.org/ web/ packages/gWQS/index.html), were used for data analyses.

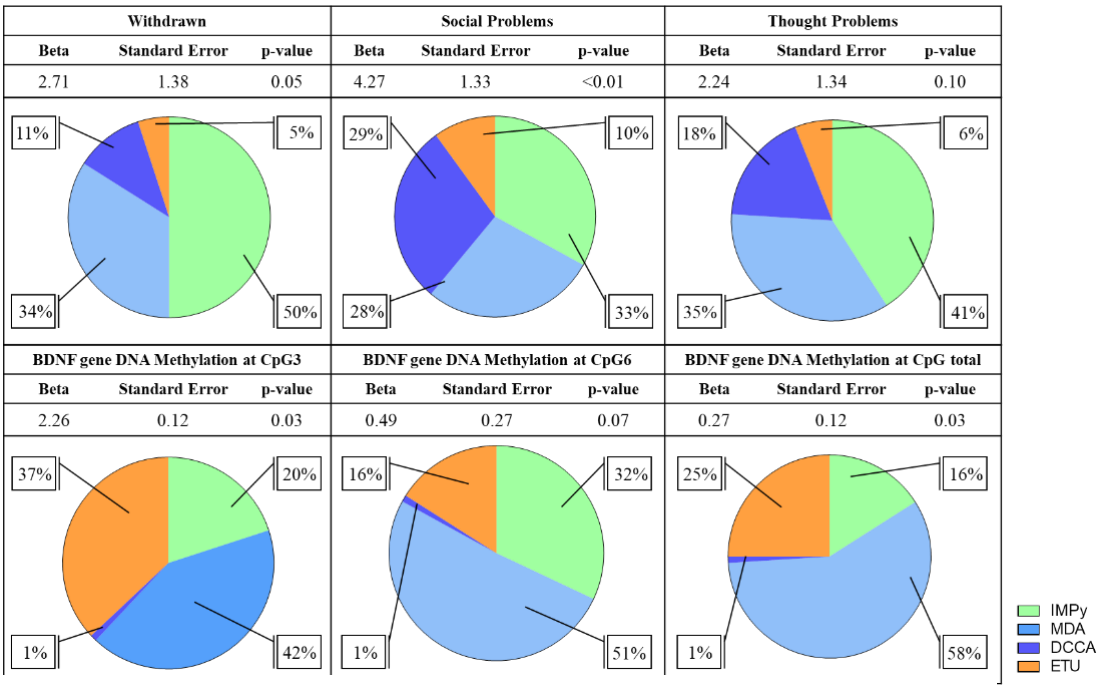

Figure 1. Mixture effect of urinary IMPy, MDA, DCCA, and ETU (IMPy= 2-isopropyl-4-methyl-6-hydroxypyrimidine; MDA= malathion dicarboxylic acid; DCCA= 2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxiclic acid; ETU= ethylene thiourea.) on behavioral functioning (n=140) and BDNF gene DNA methylation at several CpGs (n=118) (BDNF= Brain-derived neurotrophic factor;). Models were adjusted for adolescents' age, BMI, season of urine collection and maternal education.

3. Results

3.1. Characteristics of study participants

Sociodemographic characteristics of study participants are described in Table 1. Adolescents had a mean (standard deviation, SD) age and BMI of 16.2 (0.4) years and 23.2 (4.9) kg/m2, respectively. Less than a half of participants consumed more than one alcoholic beverage per month (39.7 %) and had mothers with university education (25.9 %). Most urine samples were collected in autumn (42.6 %) (Table 1).

Urinary DCCA was detected in all urine samples (median=1.06 ng/mL). MDA, IMPy, and ETU were detected in 83.0%, 74.8% and, 74.2% of samples, respectively (median=0.30, 0.25, and 0.26 ng/mL, respectively). DETP was detected in 54.3% (median=0.25 ng/mL), and TCPy, 3-PBA, and ETU in <40% of samples (Table 2). Urinary concentrations of IMPy and TCPy were positively and significantly correlated with 3-PBA (Spearman's rho, ρ=0.20 and 0.17, respectively), DETP with DCCA and 1-N (ρ = 0.20 and 0.21, respectively); no significant correlation was found for MDA and ETU (Table S3).

Median total serum BDNF level was 32.59 ng/mL, median blood DNA methylation percentages of #1 to 6 CpGs of the BDNF gene were: 4.45%, 3.18%, 3.21%, 5.69%, 3.17%, and 2.42%, respectively; the median total CpGs DNA methylation was 3.70% (Table 2). Finally, adolescents' externalizing problems (16%) were less prevalent than internalizing problems (32%) (Table S2).

3.2. Pesticide's exposure and adolescents' behavioral functioning

Multivariable linear regression models based on tertiles of urinary concentrations of pesticide metabolites showed that adolescents with IMPy concentrations at the third tertile (T3), versus first (T1), were associated with more social problems, rule-breaking, aggressive behavior, externalizing, and total problems [β= 3.34 (95%CI= 0.65,6.02), β= 3.76 (95%CI= 1.06,6.45), β= 3.77 (95%CI= 1.07,4.46), β= 5.50 (95%CI= 1.58,9.42), β= 4.60 (95%CI= 0.68,8.52), respectively] (Table 3). An apparent dose-dependent association with more thought problems was also found [βT2= 2.33 (95%CI= -0.24,4.90) βT3= 2.56 (95%CI= -0.04,5.16)] (Table 3). Detected vs. undetected urinary TCPy was significantly associated with more social and thought problems (Table 3). ΣOPs at T3 was associated with more rule-breaking behavior and externalizing problems [β= 3.40 (95%CI= 0.67,6.14), β= 4.33 (95%CI= 0.33,8.33), respectively]. DETP at T3 was associated with less withdrawn symptoms (β= -3.54, 95%CI= -6.85,0.23).

The pyrethroid metabolite DCCA at T2 and T3, was associated with more attention problems, rule-breaking, and aggressive behaviors, although without reaching statistical significance. Detected versus undetected urinary 3-BPA was associated with less somatic, attention, and internalizing problems. No relevant associations were observed for ΣPYR (Table 3). Finally, urinary ETU at T2 and T3 was associated with more social problems and less anxiety problems [(β= 3.18, (95%CI= 0.64,5.71), β= -2.70, 95%CI= -5.25-0.14), respectively] (Table 3). No associations were observed for MDA and 1-N. Statistical analyses using pesticide metabolites as continuous showed similar results than those reported from categorized exposures (Table S4).

The WQS model revealed some associations regarding the mixture effect of IMPy, MDA, DCCA and ETU on increasing withdrawn problems, with MDA and IMPy presenting the greatest influence on this effect (34% and 50%, respectively). In the case of social problems, IMPy, DCCA, and MDA had the greatest weights (33%, 29%, and 28% of the association, respectively) (Figure 1). The mixture effect model also revealed associations with more thought problems, with IMPy and MDA presenting the greatest influence (41% and 35%, respectively); however, statistical significance was not reached (Fig. 1). Remaining associations are available in Table S6.

3.3. Urinary pesticide metabolites and BDNF levels

Adolescents with urinary IMPy, MDA, DETP, ETU, and 1-N concentrations at T3 were associated with lower serum BDNF levels [β= -4.29 (95%CI= -8.33,-0.25) β= -6.74 (95%CI= -11.38,-2.10), β= -3.82 (95%CI= -8.25,0.61), β= -3.27 (95%CI= -7.36,0.82), respectively] (Table 4). Interestingly, exposure to ΣOPs was associated significantly and dose-dependently with lower serum BDNF levels [βT2= -5.05 (95%CI= -9.24,-0.85), βT3= -7.88 (95%CI= -12.09,-3.67)].

Adolescents with urinary MDA at T2 were associated with increased DNA methylation at CpG 2 (β= 0.26, 95%CI= 0.04,0.46). T3 urinary MDA levels were associated with higher DNA methylation at CpGs 1, 3, and total CpGs, although without reaching statistical significance. ΣOPs levels at T3 also showed a suggestive association with higher DNA methylation at CpG2 (β2= 0.21, 95%CI= -0.02,0.45) (Table 4).

Table 2. Distribution of urinary pesticide metabolites concentrations (ng/mL) (n=140), serum BDNF protein levels (n=130) and methylation levels at CpGs (n=118).

Pesticide metabolites		**IMPy**	**MDA**	**TCPy**	**DETP**	**ΣOPs**	**DCCA**	**3-PBA**	**ΣPYR**	**1-N**	**ETU**
% Detection		74.8	83.0	32.5	54.3	-	100	19.9	-	38.0	74.2
	25	0.08	0.14	<LOD	<LOD	0.67	0.12	<LOD	0.21	<LOD	0.05
Percentiles	50	0.25	0.30	<LOD	0.25	1.29	1.06	<LOD	1.17	<LOD	0.26
	75	0.81	0.50	0.08	0.74	2.27	3.45	0.083	3.53	0.34	0.70
Effect biomarkers		**Serum BDNF (ng/mL)**			**CpG1 (%)**	**CpG2 (%)**	**CpG3 (%)**	**CpG4 (%)**	**CpG5 (%)**	**CpG6 (%)**	**∑CpG (%)**
	25	25.41			3.89	2.90	2.85	5.18	2.70	2.04	3.45
Percentiles	50	32.59			4.45	3.18	3.21	5.69	3.17	2.42	3.70
	75	39.40			4.87	3.50	3.64	6.30	3.68	3.13	4.04

BDNF= Brain-derived neurotrophic factor; IMPy= 2-isopropyl-4-methyl-6-hydroxypyrimidine; MDA= malathion dicarboxylic acid; TCPy=3,5,6-trichloro-2-pyridinol; DETP= diethyl thiophosphate; ΣOPs= sum of organophosphates; DCCA= 2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxiclic acid; 3.PBA= 3-phenoxybenzoic acid; ΣPYR= sum of pyrethroids; 1-N= 1-naphthol; ETU= ethylene thiourea.

Detected versus undetected concentrations of urinary 3-PBA were associated with higher BDNF DNA methylation at CpGs #4, 5, 6 and total CpGs methylation [β= 0.65 (95%CI= 0.03,1.26), β= 0.38 (95%CI= -0.01,0.76), β= 0.57 (95%CI= 0.02,1.12), β= 0.30 (95%CI= 0.00,0.60), respectively] (Table 4). Finally, ETU concentrations at T3 and T2 were associated with more DNA methylation percentages at CpGs: # 2, 3, 4, 5, 6 and total CpGs [CpG2: βT3= 0.27 (95%CI= 0.05,0.49); CpG3: βT3= 0.41 (95%CI= 0.15,0.67); total CpGs βT3= 0.32 (95%CI= 0.04,0.60)] (Table 4). In the linear regression analysis, overall patterns evidenced with the logistic regression were preserved although the associations tended to be attenuated (Table S5).

The WQS model revealed associations between the mixture effect and higher BDNF DNA methylation at CpG3 and total CpGs DNA methylation, with ETU and MDA showing the greatest influence on the mixture effect (37% and 42%, respectively, for methylation at CpG3, and 58% and 25%, respectively, for total CpGs methylation) (Figure 1). Finally, the mixture effect was also associated with higher DNA methylation at CpG6 (MDA and IMPy showing the greatest influence, 51% and 32%, respectively), although without reaching statistical significance (Figure 1). Remaining associations are shown in Table S6.

3.4. BDNF and adolescents' behavior

Briefly, adolescents with BDNF protein levels at T2 were associated with lower thought (β= -2.93, 95%CI= -5.47; -0.38), rule-breaking (β= -2.71, 95%CI= -5.39; -0.02), and total problems (β= -3.57, 95%CI= -7.72; 0.58) (Fig. 2, red and green diamonds), while those with BDNF at T3 were associated with lower thought (β= -3.03, 95%CI= -5.65; -0.40) and rule-breaking behavior problems (β= -2.35, 95%CI= -5.11; 0.42) (Fig. 2, red and green diamonds, respectively).

However, continuous BDNF levels were not associated with any CBCL subscale may suggesting non-dose response associations (Table S7). Adolescents with CpG6 DNA methylation at T3 were associated with higher thought problems (β= 2.86, 95%CI= 0.14; 5.57). Conversely, adolescents showing CpG 2 DNA methylation at T3 were associated with lower anxiety (β= -3.17, 95%CI= -6.00; -0.35), somatic (β= -3.81, 95%CI= -7.30; -0.31), attention (β= -4.60, 95%CI= -8.04; -1.16) and internalizing problems (β= -5.33, 95%CI= -9.88; -0.79) (Fig S1, red diamonds). Additionally, adolescents with CpGs 1 at T2 were associated with lower thought problems (β= -3.40, 95%CI= -6.05; -0.80), and CpG3 at T3 with lower externalizing problems β= -4.75, 95%CI= -9.16; -0.34 (Fig S1, red diamonds).

3.5. Mediation analysis

Because urinary IMPy levels were dose-dependently associated with higher thought and rule-breaking problems, and with lower serum BDNF levels (Tables 3 and 4), and additionally serum BDNF levels non-linearly associated with lower thought and rule-breaking problems (Figure 2); we investigated the potential mediation role of BDNF in exposure-effect relationships. The potential mediation role of categorized serum BDNF protein within IMPy (categorized)-thought problems (continuous) and IMPy (categorized)-rule breaking behavior (continuous) relationships was explored. The covariate-adjusted mediation model revealed a non-significant indirect effect between serum BDNF and IMPy-thought problems (β= 0.17; 95%CI: -0.07, 0.57), which accounted for 21.5% of the association. Direct and total effects were not statistically significant. Additionally, serum BDNF showed a potential small indirect effect within IMPy-rule-breaking problems that accounted for 7.6% (β= 0.11; 95%CI: -0.08, 0.45) of the association, although both total and direct effects were significant (Figure 3).

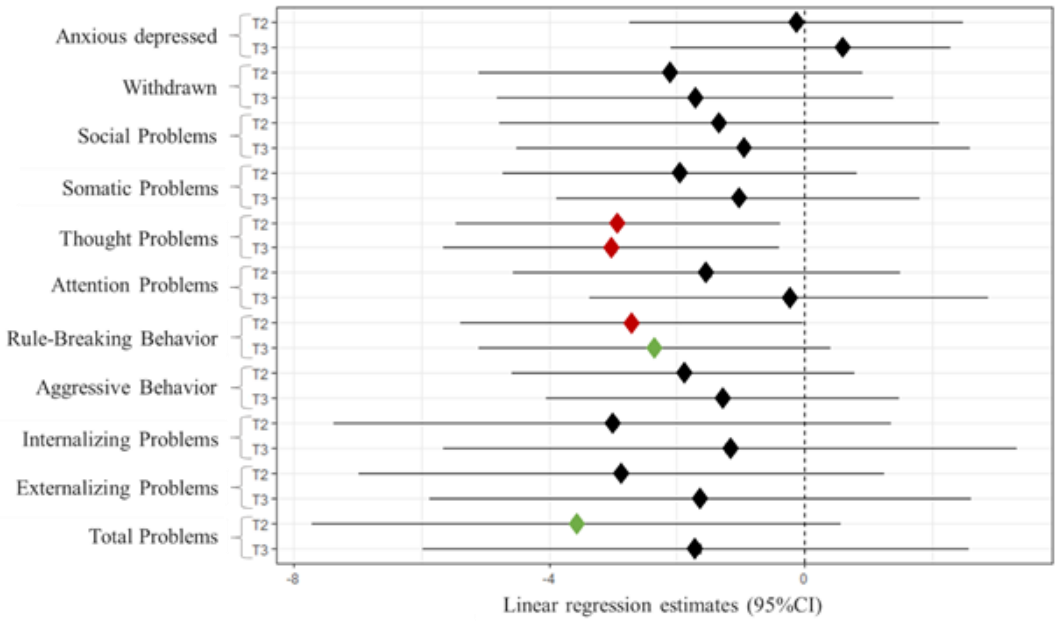

Figure 2. Forest plot showing linear regression estimates (95%CI) for the association between tertiles of serum BDNF levels (T2 and T3) and adolescents' behavior (n=130). Model was adjusted for adolescent's age (months), BMI (continuous), alcohol consumption (>1 beverage/month; <1 beverage/month), and maternal education (primary/secondary/university). Red diamonds represent statistical associations with p-values below 0.05; green diamonds represent p-values below 0.1; black diamonds represent p-values above 0.1.

Table 3. Regression estimates change (β, 95% CI) of the associations between urinary pesticide metabolites concentrations and CBCL behavior scoring (n= 140).

		Syndrome Scores								Composite scores		
		Anxious depressed	**Withdrawn**	**Somatic complaints**	**Social problems**	**Thought problems**	**Attention problems**	**Rule-breaking behavior**	**Aggressive behavior**	**Internalizing problems**	**Externalizing problems**	**Total problems**
IMPy	T2	1.85 (-0.79,4.49)	1.82 (-1.30,4.94)	-0.59 (-3.75,2.56)	1.47 (-1.19,4.13)	2.33 (-0.24,4.90)†	1.10 (-1.87,4.07)	0.76 (-1.90,3.43)	2.47 (-0.20,5.13)†	2.19 (-1.83,6.21)	2.46 (-1.43,6.34)	2.54 (-1.34,6.42)
	T3	1.70 (-0.96,4.37)	2.04 (-1.11,5.19)	-0.99 (-4.17,2.20)	3.34 (0.65,6.02)*	2.56 (-0.04,5.16)†	2.28 (-0.72,5.28)	3.76 (1.06,6.45)**	3.77 (1.07,6.46)**	1.13 (-2.93,5.20)	5.50 (1.58,9.42)**	4.60 (0.68,8.52)*
	p-trend	0.76	0.57	0.24	0.04	0.20	0.18	<0.01	<0.01	0.59	<0.01	0.07
MDA	T2	1.47 (-1.21,4.07)	-0.06 (-3.20,3.07)	0.71 (-2.36,3.78)	-0.28 (-2.94,2.38)	-0.16 (-2.72,2.41)	0.67 (-2.25,3.59)	1.61 (-1.07,4.29)	0.17 (-2.52,2.86)	1.82 (-2.11,5.75)	1.22 (-2.69,5.12)	2.19 (-1.66,6.04)
	T3	0.47 (-2.50,3.44)	0.35 (-3.05,3.75)	-1.42 (-4.94,2.09)	0.37 (-2.67,3.41)	0.08 (-2.86,3.01)	-0.67 (-4.02,2.67)	0.73 (-2.34,3.79)	-0.17 (-3.25,2.91)	-0.36 (-4.86,4.14)	-0.37 (-4.84,4.09)	-0.31 (-4.71,4.09)
	p-trend	0.70	0.82	0.46	0.83	0.97	0.73	0.59	0.92	0.93	0.91	0.96
TCPy	D vs ND	0.88 (-1.39,3.14)	-0.98 (-3.66,1.69)	-1.05 (-3.73,1.64)	2.13 (-0.16,4.42)†	2.48 (0.29,4.67)*	1.05 (-1.49,3.60)	-0.61 (-2.95,1.74)	0.21 (-2.13,2.56)	-0.09 (-3.53,3.36)	-0.74 (-4.14,2.67)	0.58 (-2.80,3.95)
DETP	T2	0.33 (-2.36,3.03)	-1.44 (-4.57,1.68)	-2.04 (-5.21,1.13)	-1.62 (-4.36,1.11)	-1.98 (-4.60,0.65)	-0.54 (-3.56,2.49)	-0.84 (-3.59,1.92)	-1.91 (-4.66,0.85)	-1.79 (-5.86,2.27)	-2.44 (-6.46,1.57)	-2.75 (-6.72,1.23)
	T3	0.08 (-2.77,2.93)	-3.54 (-6.85,-0.23)*	0.00 (-3.36,3.36)	-1.01 (-3.91,1.88)	-0.42 (-3.20,2.36)	0.36 (-2.85,3.56)	1.67 (-1.25,4.59)	-0.15 (-3.07,2.77)	-1.67 (-5.98,2.64)	-0.17 (-4.42,4.09)	-1.01 (-5.22,3.21)
	p-trend	0.88	0.05	0.94	0.49	0.61	0.79	0.32	0.86	0.47	0.92	0.64
DCCA	T2	0.58 (-2.04,3.21)	-1.39 (-4.48,1.70)	0.13 (-2.99,3.24)	1.72 (-0.95,4.38)	1.10 (-1.47,3.68)	2.09 (-0.84,5.03)	0.29 (-2.41,2.98)	0.91 (-1.80,3.61)	-0.06 (-4.05,3.92)	0.73 (-3.19,4.65)	1.28 (-2.61,5.17)
	T3	-0.33 (-3.01,2.35)	-1.00 (-4.16,2.15)	0.74 (-2.44,3.92)	1.54 (-1.18,4.26)	0.82 (-1.81,3.45)	1.17 (-1.82,4.17)	1.88 (-0.87,4.63)	1.27 (-1.49,4.03)	0.47 (-3.59,4.54)	2.43 (-1.58,6.43)	1.93 (-2.05,5.90)
	p-trend	0.81	0.53	0.64	0.26	0.53	0.44	0.18	0.36	0.82	0.23	0.34
3-PBA	D vs ND	-1.52 (-4.20,1.16)	-0.77 (-3.94,2.40)	-5.18 (-8.25,-2.12)**	-2.16 (-4.88,0.57)	-0.70 (-3.34,1.95)	-2.79 (-5.78,0.20)†	-2.01 (-4.78,0.75)	-0.34 (-3.12,2.44)	-3.73 (-7.76,0.30)†	-0.60 (-4.64,3.44)	-2.99 (-6.96,0.98)
1-N	D vs ND	-1.29 (-3.47,0.89)	1.48 (-1.08,4.05)	0.02 (-2.58,2.61)	0.39 (-1.84,2.62)	0.25 (-1.90,2.39)	-0.27 (-2.73,2.18)	-0.65 (-2.91,1.60)	-0.40 (-2.66,1.85)	0.34 (-2.97,3.65)	0.47 (-2.81,3.75)	0.46 (-2.79,3.71)
ETU	T2	0.01 (-2.49,2.50)	1.52 (-1.47,4.52)	-1.73 (-4.34,1.27)	3.18 (0.64,5.71)*	1.59 (-1.25,4.44)	0.41 (-2.09,2.90)	-0.56 (-3.18,2.07)	1.15 (-1.46,3.76)	-0.87 (-4.69,2.96)	0.10 (-3.69,3.89)	0.28 (-3.47,4.02)
	T3	-2.70 (-5.25,-0.14)*	0.80 (-2.26,3.86)	-1.15 (-4.23,1.92)	0.48 (-2.12,3.07)	-0.15 (-3.06,2.77)	-0.89 (-3.44,1.66)	-1.16 (-3.85,1.53)	-0.78 (-3.45,1.89)	-3.00 (-6.91,0.92)	-2.60 (-6.48,1.27)	-2.75 (-6.58,1.09)
	p-trend	0.04	0.60	0.45	0.69	0.94	0.50	0.39	0.58	0.13	0.19	0.16
ΣOPs	T2	2.56 (-0.13,5.25)†	-0.26 (-3.46,2.94)	0.10 (-3.12,3.32)	1.87 (-0.87,4.61)	1.62 (-1.04,4.27)	1.31 (-1.73,4.36)	1.19 (-1.55,3.93)	1.42 (-1.35,4.19)	1.61 (-2.50,5.72)	2.44 (-1.56,6.45)	2.01 (-1.98,6.00)
	T3	1.98 (-0.70,4.66)	0.91 (-2.29,4.10)	1.04 (-2.18,4.25)	2.25 (-0.49,4.99)	2.21 (-0.44,4.86)	1.59 (-1.45,4.63)	3.40 (0.67,6.14)*	2.47 (-0.30,5.23)†	2.53 (-1.58,6.63)	4.33 (0.33,8.33)*	3.61 (-0.38,7.59)†
	p-trend	0.17	0.55	0.51	0.11	0.11	0.31	0.01	0.08	0.23	0.03	0.08
ΣPYR	T2	0.22 (-2.36,2.80)	-1.03 (-4.07,2.01)	-1.86 (-4.91,1.18)	0.89 (-1.73,3.52)	0.36 (-2.18,2.90)	2.26 (-0.62,5.13)	-2.07 (-4.67,0.53)	0.38 (-2.27,3.03)	-0.22 (-4.15,3.71)	-0.74 (-4.57,3.09)	0.50 (-3.33,4.32)
	T3	-0.58 (-3.46,1.76)	-0.74 (-3.81,2.33)	-0.00 (-3.08,3.07)	1.23 (-1.42,3.88)	1.03 (-1.54,3.60)	0.87 (-2.04,3.78)	1.46 (-1.16,4.09)	1.27 (-1.41,3.95)	0.13 (-3.84,4.10)	2.52 (-1.35,6.39)	1.66 (-2.20,5.53)
	p-trend	0.53	0.63	0.97	0.36	0.43	0.53	0.31	0.35	0.95	0.21	0.40

D= Detected; ND= Non detected; IMPy= 2-isopropyl-4-methyl-6-hydroxypyrimidine; MDA= malathion dicarboxylic acid; TCPy=3,5,6-trichloro-2-pyridinol; DETP= diethyl thiophosphate; ΣOPs= sum of organophosphates; DCCA= 2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxiclic acid; 3.PBA= 3-phenoxybenzoic acid; ΣPYR= sum of pyrethroids; 1-N= 1-naphthol; ETU= ethylene thiourea. Models were adjusted for adolescents' age (continuous), BMI (continuous), alcohol consumption (>1 beverage/month; <1 beverage/month), season of urine collection (spring/summer/autumn/winter), creatinine concentrations (mg/dL), and maternal education (primary/secondary/university). * p≤0.05; ** p≤0.01; † p<0.10.

Table 4. Regression estimates change (β, 95%CI) of the associations between tertiles of urinary pesticide metabolites concentrations and serum BDNF protein levels (n=130) and BDNF gene DNA methylation percentage (n=118).

		BDNF protein	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpGt
IMPy	T2	-1.77 (-6.03,2.50)	0.00 (-0.37,0.37)	-0.11 (-0.34,0.13)	0.04 (-0.24,0.32)	0.10 (-0.51,0.71)	0.01 (-0.37,0.39)	0.18 (-0.36,0.73)	0.04 (-0.26,0.33)
	T3	-4.29 (-8.33,-0.25)*	0.12 (-0.24,0.49)	0.02 (-0.21,0.26)	-0.01 (-0.28,0.27)	-0.16 (-0.76,0.44)	0.01 (-0.37,0.39)	0.09 (-0.45,0.63)	0.01 (-0.28,0.31)
	p-trend	0.04	0.39	0.57	0.88	0.46	0.81	0.99	0.94
MDA	T2	-2.71 (-6.88,1.46)	0.21 (-0.15,0.57)	0.26 (0.04,0.46)*	0.12 (-0.16,0.39)	0.04 (-0.57,0.65)	0.18 (-0.21,0.56)	-0.07 (-0.62,0.48)	0.12 (-0.17,0.42)
	T3	-6.74 (-11.38,-2.10)**	0.31 (-0.08,0.71)	0.21 (-0.04,0.46)†	0.24 (-0.06,0.54)	0.25 (-0.41,0.91)	0.23 (-0.18,0.64)	0.05 (-0.54,0.65)	0.22 (-0.10,0.53)
	p-trend	<0.01	0.11	0.08	0.12	0.46	0.26	0.87	0.18
TCPy	Detected vs undetected	0.09 (-3.59,3.76)	-0.10 (-0.41,0.21)	-0.04 (-0.23,0.16)	-0.14 (-0.37,0.10)	-0.04 (-0.56,0.47)	0.07 (-0.25,0.39)	0.08 (-0.38,0.54)	-0.03 (-0.28,0.22)
DETP	T2	-0.68 (-7.87,0.52)†	-0.09 (-0.45,0.26)	-0.00 (-0.23,0.23)	0.04 (-0.23,0.32)	0.09 (-0.51,0.68)	-0.03 (-0.40,0.34)	0.13 (-0.40,0.65)	0.02 (-0.27,0.31)
	T3	-3.82 (-8.25,0.61)†	-0.19 (-0.57,0.19)	0.09 (-0.15,0.33)	0.13 (-0.16,0.42)	0.19 (-0.44,0.82)	-0.02 (-0.42,0.38)	0.31 (-0.25,0.87)	0.08 (-0.22,0.39)
	p-trend	0.09	0.31	0.49	0.34	0.57	0.97	0.25	0.57
DCCA	T2	2.93 (-1.21,7.07)	0.07 (-0.29,0.42)	-0.18 (-0.41,0.05)	-0.01 (-0.28,0.27)	-0.30 (-0.88,0.30)	-0.18 (-0.55,0.19)	-0.07 (-0.60,0.46)	-0.11 (-0.40,0.18)
	T3	0.03 (-4.34,4.40)	0.14 (-0.23,0.51)	-0.07 (-0.31,0.16)	-0.10 (-0.38,0.18)	-0.35 (-0.97,0.27)	-0.17 (-0.56,0.21)	-0.21 (-0.76,0.34)	-0.13 (-0.42,0.17)
	p-trend	0.93	0.44	0.56	0.48	0.25	0.38	0.46	0.40
3-PBA	Detected vs undetected	-2.60 (-6.90,1.64)	0.01 (-0.37,0.39)	-0.00 (-0.24,0.24)	0.21 (-0.08,0.50)	0.65 (0.03,1.26)*	0.38 (-0.01,0.76)†	0.57 (0.02,1.12)*	0.30 (0.00,0.60)*
1-N	Detected vs undetected	-3.91 (-7.35,-0.46)*	-0.20 (-0.51,0.10)	0.13 (-0.07,0.32)	0.01 (-0.22,0.24)	0.37 (-0.14,0.86)	0.25 (-0.06,0.56)	0.30 (-0.15,0.75)	0.14 (-0.10,0.39)
ETU	T2	-1.23(-5.43,2.97)	0.20 (-0.16,0.57)	0.23 (0.01,0.46)*	0.27 (0.01,0.54)*	0.68 (0.09,1.27)*	0.36 (-0.02,0.73)†	0.40 (-0.14,0.93)	0.36 (0.07,0.64)*
	T3	-3.27 (-7.36,0.82)	0.18 (-0.17,0.54)	0.27 (0.05,0.49)*	0.41 (0.15,0.67)**	0.53 (-0.05,1.11)†	0.22 (-0.15,0.58)	0.32 (-0.21,0.84)	0.32 (0.04,0.60)*
	p-trend	0.16	0.46	0.07	0.01	0.08	0.35	0.29	0.05
ΣOPs	T2	-5.05 (-9.24,-0.85)*	0.16 (-0.21,0.53)	-0.51 (-0.28,0.18)	-0.10 (-0.38,0.18)	-0.54 (-1.15,0.06)†	-0.35 (-0.72,0.03)†	-0.45 (-0.99,0.08)†	-0.22 (-0.51,0.07)
	T3	-7.88 (-12.09,-3.67)**	0.23 (-0.13,0.59)	0.21 (-0.02,0.45)†	0.20 (-0.08,0.47)	-0.02 (-0.61,0.58)	0.04 (-0.33,0.41)	0.21 (-0.32,0.73)	0.14 (-0.14,0.43)
	p-trend	0.00	0.22	0.06	0.13	0.95	0.73	0.37	0.27
ΣPYR	T2	-1.96 (-6.14,2.23)	-0.02 (-0.38,0.34)	-0.15 (-0.38,0.08)	0.06 (-0.21,0.34)	0.09 (-0.51,0.69)	0.07 (-0.31,0.44)	1.00 (-0.44,0.64)	0.02 (-0.27,0.32)
	T3	-0.74 (-4.92,3.44)	0.24 (-0.12,0.59)	-0.04 (-0.26,0.19)	-0.05 (-0.32,0.22)	-0.17 (-0.76,0.41)	-0.10 (-0.47,0.26)	-0.06 (-0.58,0.47)	-0.03 (-0.32,0.25)
	p-trend	0.71	0.18	0.77	0.69	0.55	0.57	0.82	0.82

BDNF= Brain-derived neurotrophic factor; IMPy= 2-isopropyl-4-methyl-6-hydroxypyrimidine; MDA= malathion dicarboxylic acid; TCPy=3,5,6-trichloro-2-pyridinol; DETP= diethyl thiophosphate; ΣOPs= sum of organophosphates; DCCA= 2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxiclic acid; 3.PBA= 3-phenoxybenzoic acid; ΣPYR= sum of pyrethroids; 1-N= 1-naphthol; ETU= ethylene thiourea. Models were adjusted for adolescents' age (months), BMI, alcohol consumption (>1 beverage/month; <1 beverage/month), season of urine collection (spring/summer/autumn/winter), creatinine concentrations (mg/dL), and maternal education (primary/secondary/university). * p≤0.05; ** p≤0.01; † p<0.10.

4. Discussion

Our results suggest a possible association between IMPy, ΣOPs, and ETU levels with behavioral problems among Spanish adolescent males, which could be partly explained by BDNF protein levels. A possible combined effect for some pesticides with more withdrawn, social, and thought problems, and higher BDNF DNA methylation percentage at CpG 3, and total CpGs methylation was observed. At the same time, serum BDNF levels were associated with more thought problems and rule-breaking behavior, and BDNF DNA methylation percentage at CpG6 with more thought problems.

4.1. Organophosphate's exposure

Urinary TCPy and MDA concentrations were generally lower than previously reported for children in Spain, the USA, and Costa Rica (Hyland et al., 2019; Roca et al., 2014; van Wendel de Joode et al., 2016), while IMPy and DETP were higher than reported for Spanish and US children (Hernández et al., 2019; Hyland et al., 2019; Roca et al., 2014). In our study, exposure to diazinon (IMPy) and chlorpyrifos (TCPy) metabolites was associated with more social-related problems among male adolescents aged 15-17 years. Results regarding TCPy are partially consistent with the results of three epidemiological studies among Egyptians and Latino American subjects (12-21 years of age) working or living near plantations, which found that urinary TCPy was associated with deficits in cumulative neurobehavioral performance and a higher prevalence of depression, ADHD, irritability, and superficial sensation of abnormality (Ismail et al., 2017; Rohlman et al., 2016; van Wendel de Joode et al., 2016). To our knowledge, no previous study assessed exposure to diazinon concerning child neurodevelopment; however, some in-vivo studies found behavioral functioning alterations after diazinon exposure, supporting our data (Hawkey et al., 2020; Shin et al., 2001; Velki et al., 2017).

Interestingly, IMPy and DETP were associated with lower serum BDNF protein levels, MDA with lower serum BDNF and higher BDNF DNA methylation percentage, while no association was observed for TCPy. ΣOPs was also strongly and dose- dependently associated with lower serum BDNF levels. Although OP pesticides are known to exert adverse effects on the nervous system through acetylcholinesterase (AChE) inhibition (Bjørling-Poulsen et al., 2008; Richendrfer and Creton, 2015), growing evidence also suggest non-cholinergic mechanisms, such as alterations of synaptic formation and neuronal cell development (Rauh et al., 2011). Thus, OP pesticides are known to alter the dopaminergic system, which plays a key role in the regulation of BDNF through the inhibition of tyrosine hydroxilase (TH) activity (Küppers and Beyer, 2001) (Fig. 4, key event #3). This enzyme anabolizes L-tyrosine, precursor of the dihydroxyphenylalanine (DOPA), leading to decreasing dopamine levels (Shin et al., 2001) (Fig. 4, #1). In vivo studies observed TH inhibition after diazinon and malathion exposure, leading to behavioral alterations and increased anxiety behavior in Japanese medaka and rats, respectively (Ahmed et al., 2017; Shin et al., 2001) (Fig. 4, #1 and #2). Additionally, exposure to chlorpyrifos decreased cholinergic system and down-regulated BDNF expression in zebrafish, resulting in increased impulsive rates (Perez-Fernandez et al., 2020) (Fig. 4, #5). Finally, another in vivo study observed that malathion-exposed rats showed increased reactive oxidative stress species (ROS) and reduced hippocampal BDNF expression (Ardebili Dorri et al., 2015) (Fig. 4, #4). This evidence could support, from a mechanistic perspective, our findings regarding OP pesticides exposure and decreased BDNF.

4.2. Pyrethroids

3-PBA urinary concentrations among the Spanish adolescents were lower, and concentrations of DCCA higher, than reported for North American children aged 6-15 years (Oulhote and Bouchard, 2013; Quirós-Alcalá et al., 2014). Urinary 3-PBA levels were associated with fewer somatic problems, whereas DCCA tended to be associated with more behavioral problems. Previous studies in North America found an increased risk of ADHD in 8- to 15-year-old boys with high urinary 3-PBA concentrations (Wagner-Schuman et al., 2015), and more behavioral difficulties in children aged 6-11 years with high urinary DCCA concentrations (Oulhote and Bouchard, 2013). In contrast, in other studies among 6- to 15-year-old children from North and Latin America, 3-PBA and DCCA were not associated with behavioral functioning (Oulhote and Bouchard, 2013; Quirós-Alcalá et al., 2014; van Wendel de Joode et al., 2016). Discrepancies among studies could be due, at least in part, to differences in design, age of the children, and pesticide exposure levels. Moreover, studies used different tests to assess behavioral outcomes, which may be another source of heterogeneity.

The observed association between 3-PBA and increased BDNF gene DNA methylation suggests that exposure to pyrethroids, even at low levels, could alter BDNF gene methylation patterns. There are many mechanisms by which pyrethroids could exert their neurotoxic potential, fully described in Figure 4. First, permethrin interacts with DNA methyltransferases (DNMT), altering DNA methylation patterns in vivo (Bordoni et al., 2015)

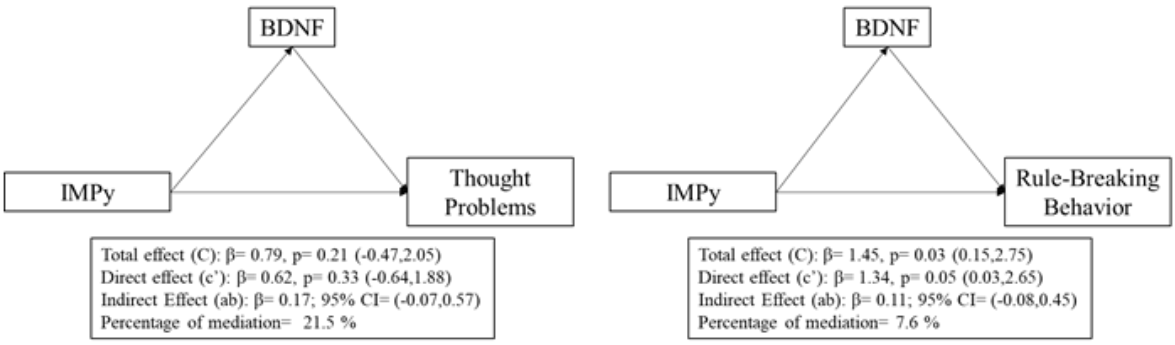

Figure 3. Analysis exploring the role of serum BDNF protein as a potential mediator of the association between urinary IMPy concentrations and thought and rule-breaking problems in adolescents (n=130). Beta coefficients are displayed for total, direct, and indirect effects. BDNF: Brain-derived neurotrophic factor; IMPy= 2-isopropyl-4-methyl-6-hydroxypyrimidine. Both IMPy and BDNF levels were modeled based on tertiles and thought and rule-breaking behavioral problems were normalized using t-scores. Model was adjusted for adolescents' age (months), BMI, alcohol consumption (>1 beverage/month; <1 beverage/month), season of urine collection (spring/summer/autumn/winter), creatinine concentrations (mg/dL), and maternal education (primary/secondary/university). * p≤0.05; ** p≤0.01; † p<0.10.

(Fig. 4; #10). Second, via a compensatory mechanism for retardation of sodium channels voltage-dependent (Nav) activation through down-regulation of Nav expression, linked to decreased BDNF expression, as reported in two studies with deltamethrin-exposed mice (Imamura et al., 2006; Magby and Richardson, 2017) (Fig. 4; #6 and #7). Third, inhibition of NMDAR after permethrin and deltamethrin exposure using in vivo/vitro models led to a down-regulation of the cAMP response element-binding protein (CREB), involved in BDNF expression, resulting in hippocampal BDNF mRNA repression (Imamura et al., 2000; Zhang et al., 2018) (Fig. 4; #8 and #9). In addition, cypermethrin and bifenthrin exposure increased ROS leading to neuroinflammation and long-lasting behavioral impairments in murine models (Gargouri et al., 2018; Nasuti et al., 2007) (Fig. 4, #11 and #12). This AOP like-network build-up a potential mechanism pathway supporting our findings regarding 3-PBA and alteration of BDNF gene DNA methylation.

4.3. Carbamates and dithiocarbamates

In this study, urinary 1-N concentrations were lower than previously reported among German children aged 5-7 years from the general population; urinary ETU concentrations were, however, within the range of those reported for French and Latin American children, aged 5-9 years and living near agricultural fields (Raherison et al., 2019; van Wendel de Joode et al., 2016; Wilhelm et al., 2008). The observed associations between urinary ETU levels with more social and less anxiety problems do not support the results of a Costa Rican study that found no association between ETU and behavioral problems in children assessed at 6-9 years (van Wendel de Joode et al., 2016); although are partially supported by the results of a Mexican study that showed an association between prenatal ETU and more social problems in 1-year-old infants (Mora et al., 2018). Differences in windows of exposure (i.e., prenatal versus postnatal exposure) and age at behavioral assessment (infants versus adolescents), among others, impair the comparison of findings. Experimental studies have nonetheless found that prenatal exposure to mancozeb elicited worse behavioral outcomes, which was mechanistically supported by decreased hippocampal proteins, such as calcium/calmodulin- dependent kinases II (CaMKII), glutamate receptor 1 (GluR1), and synaptophysin (Lee et al., 2015). Mancozeb and others dithiocarbamate fungicides are widely used for the growth of fruits and vegetables, but there is very limited information regarding their effects on neurodevelopment (Ekman et al., 2013). In this regard, a major common metabolite of these fungicides, ETU, is a known thyroid inhibitor, since it prevents the ionization of thyroglobulin, thus inhibiting the synthesis of T3 and T4 (Mutic et al., 2017), which ultimately could lead to decreasing BDNF levels, as proposed by Mustieles et al., (2020). However, in this cohort, ETU was not associated with thyroid hormones (Freire et al., 2021).

Urinary 1-N was also associated with less serum BDNF levels, and urinary ETU with more BDNF DNA methylation percentage at several CpGs. Different pathways supporting these findings have been fully described in Figure 4 (Bjørling-Poulsen et al., 2008; Maranghi et al., 2013). First, inhibition of thyroid receptor (TR) and thyroid peroxidase (TPO) observed in murine models after exposure to 1-N and ETU, resulting in impaired thyroid hormone secretion and reduction of hippocampal BDNF synthesis, ultimately leading to neurodevelopmental alterations (Maranghi et al., 2013; Marinovich et al., 1997; Shafiee et al., 2016; Sun et al., 2008) (Fig. 4; #13, #14, #15, #16). Second, in vivo/vitro models observed CaMKII inhibition after 1-N exposure, impairing the phosphorylation of CREB and the transcription of BDNF, thus decreasing BDNF-mediated neurite growth (Islam et al., 2019; Lee et al., 2015; Saito et al., 2013) (Fig. 4; #18, #17, #19). Finally, 1-N and ETU can uncouple the mitochondrial electron transport chain, leading to mitochondrial dysfunction, generating ROS, and ultimately promoting neuroinflammation and subsequent behavioral impairments (Bjørling-Poulsen et al., 2008; Domico et al., 2007; Gupta et al., 2007; He et al., 2020; Muthaiah et al., 2013) (Fig. 4; #20, #21, #22, #23 and #24).

4.4. Mixture effect

When the combined effect of IMPy, MDA, DCCA, and ETU was considered using WQS analysis a possible association with withdrawal, social, and thought problems was found, with MDA and IMPy accounting with the highest weights. Remarkably, when MDA was assessed individually, no statistical association was found. This may be due to an additive effect of the OPs compounds present in the mixture. However, further results would be needed to confirm these observations. The mixture effect also revealed associations with higher BDNF gene DNA methylation and lower BDNF protein levels. MDA showed to be the highest contributor for increased DNA methylation in the mixture model, but again when assessed individually, no associations were found. Thus, the mixture approach may have unmasked MDA effects on the adolescents' neurodevelopment, highlighting the importance of addressing mixture effects.

In previous epidemiological studies, prenatal exposure to mixtures of environmental chemicals, including pesticides, was adversely associated with different aspects of brain development, such as lower IQ, cognitive functioning, and higher risk of ADHD in children, but pesticides presented low weights in these studies (Guo et al., 2020; Kalloo et al., 2021; Lenters et al., 2019; Vuong et al., 2020). None of the aforementioned studies included IMPy, MDA, DCCA, or ETU in their mixtures assessment exclusively. Regarding mixture effects on BDNF, an in vitro study found an up-regulation of BDNF mRNA after exposure to a mixture of diverse chemical compounds, including chlorpyrifos, cyfluthrin, and deltamethrin (Özdemir et al., 2018). Due to the novelty of this assessment, results should be interpreted with caution and further studies are needed to confirm our results.

4.5. Strengths and limitations

Study limitations include its cross-sectional design, which limits causal inference, and the small sample size. Additionally, some spurious associations may have been identified due to the performance of multiple tests, although our results are supported and contextualized by previous toxicological and epidemiological evidence. Thus, our results are unlikely to be the result of chance, particularly for IMPy and ETU. Due to the short biological half-lives of the analyzed pesticides, their urinary metabolites likely reflect exposure within the previous 24-48 hours, which together with the assessment of spot urine samples increases the risk of exposure misclassification. Additionally, TCPy and 3-PBA were measured as conjugated, thus no deconjugation process was developed. However, this would lead to an underestimation rather than an overestimation of pesticides effect on BDNF and behavior.

Moreover, in cases of continuous low-dose exposure to non-persistent pesticides such as pyrethroids, their metabolites can be considered as biomarker of long-term exposure (Thiphom et al., 2013). Another limitation is that the assay's LOD of 3-PBA was relatively high in the present study, which may hamper the identification of significant effects. To address the potential exposure misclassification, measurement of repetitive urine samples should be performed in future studies to improve the exposure assessment. Among the strengths, we should highlight the novel approach exploring the role of BDNF as a biomarker of brain function at different levels of biological organization on the association between non-persistent pesticides exposure and neurodevelopment. To the best of our knowledge, this is also the first study to explore the combined effect of several pesticides' exposure on BDNF and behavioral functioning among adolescents, an understudied period of development, and to conduct a mediation analysis to further deepen our knowledge into exposure-effect associations.

5. Conclusion

Urinary concentrations of IMPy and ETU, metabolites of the (banned) insecticide diazinon and dithiocarbamate fungicides, respectively, were associated with more behavioral problems among Spanish adolescent males. These associations could possibly be due to alterations on BDNF. In addition, a possible combined effect of the IMPy, MDA, DCCA and ETU mixture on increasing behavioral problems and BDNF gene DNA methylation was found. These associations could possibly be due to alterations in BDNF levels and/or in the DNA methylation of the BDNF gene. The use of biomarkers of effect in epidemiological studies could have an added value, since they provide additional information that could help to elucidate and understand exposure-effect relationships of a given hypothesis. This study is the first to present a complex panel of exposure-effect associations on a very relevant topic. However, longitudinal studies with larger sample sizes are needed to confirm these results.

Declaration of Competing Interest

The authors declare no actual or potential conflicts of interest. The funders had no role in the study design, data collection or analysis, decision to publish, or preparation of the manuscript.

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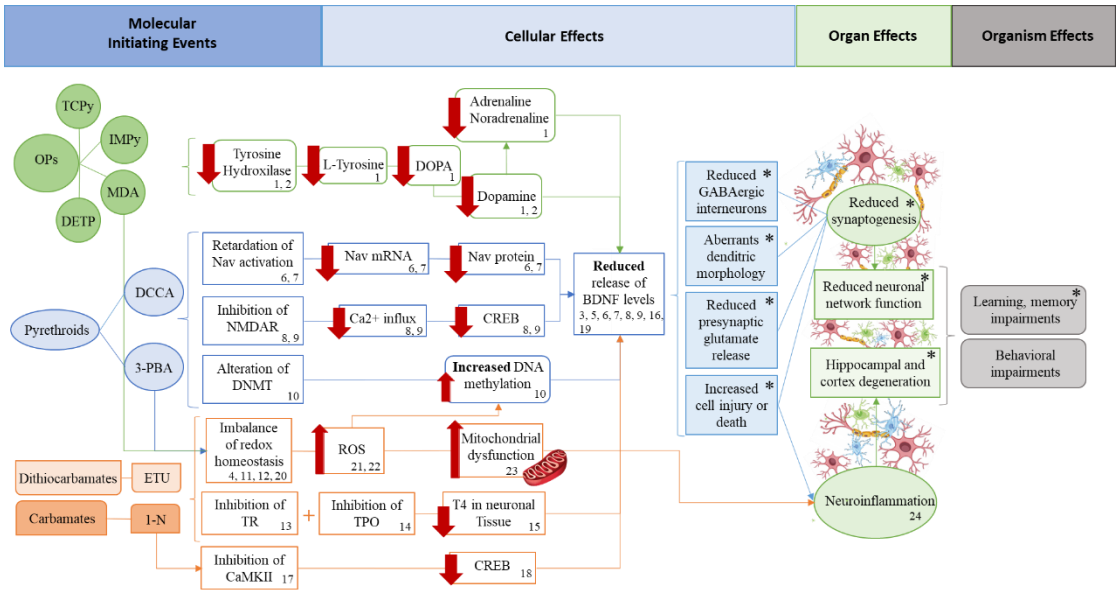

Figure 4. Proposed Adverse Outcome Pathway- like through which pesticide exposure could impair behavioral functioning based on results from this epidemiological study and results from mechanistic studies published previously. 1-N= 1-naphthol; 3-PBA= 3-phenoxybenzoic acid; BDNF= brain-derived neurotrophic factor; CaMKII= calcium/calmodulin-dependent kinases II; CREB= cAMP response element binding protein; DETP= diethyl thiophosphate; DCCA= 2,2,dichlorovinyl-2,2-dimethylcyclopropane-1-carboxilic acid; DNMT= DNA methyl transferases; DOPA= dihydroxyphenylalanine; ETU= ethylenethiourea; IMPy= 2-isopropyl-4-methyl-6-hydroxypyrimidine; MDA= Malathion dicarboxylic acid; Nav= Sodium channels voltage-dependent; NMDAR= N-methyl D-aspartate receptor; OPs= Organophosphates; ROS= reactive oxygen species; T4= Thyroxine; TCPy= 3,5,6-trichloro-2-pyridinol; TH= Tyrosine hydroxilase; TPO= Thyroid peroxidase; TR= Thyroid receptor. OP pesticides inhibit tyrosine hydroxilase, this enzyme anabolizes L-tyrosine, precursor of the dihydroxyphenylalanine (DOPA), leading to decreasing dopamine levels and consequently adrenaline and noradrenaline [1]. Decreased dopaminergic neurotransmitters leads to reduced hippocampal BDNF levels, converging in behavioral impairments [2, 3]. Additionally, MDA increases ROS, leading to reduction of BDNF [4]. Retardation of Nav activation due to pyrethroids exposure induced a compensation mechanism where Nav expression (Nav mRNA and Nav protein) in reduced, decreasing BDNF levels [5, 6, 7]. Pyrethroids can inhibit NMDARs, thus decreasing calcium influx and decreasing activation of CREB, leading to lower BDNF secretion and possibly subsequent behavioral impairments [8, 9]. Additionally, pyrethroids can also induce alterations of DNMT, thus increasing BDNF gene DNA methylation and decreasing serum BDNF protein [10]. In addition, pyrethroids imbalance the redox homeostasis, increasing ROS and leading to neuroinflammation, with deleterious consequences for brain development [11, 12]. 1-N and especially ETU are known inhibitors of thyroid receptor (TR) and thyroid peroxidase (TPO), which lead to impaired thyroid hormone secretion, thus decreasing thyroxine (T4) concentrations in brain tissue, reducing BDNF synthesis and ultimately leading to neurodevelopmental alterations [13, 14, 15, 16 and *]. 1-N inhibits CaMKII, which decreases activation of CREB and therefore BDNF gene transcription [17, 18, 19]. Finally, 1-N and ETU imbalance redox homeostasis by uncoupling the mitochondrial electron transport chain, leading to mitochondrial dysfunction and generation of ROS and neuroinflammation, impairing learning, memory and behavioral functioning [22, 23, 24]. *(Mustieles et al., 2020).

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ARTICLE 6

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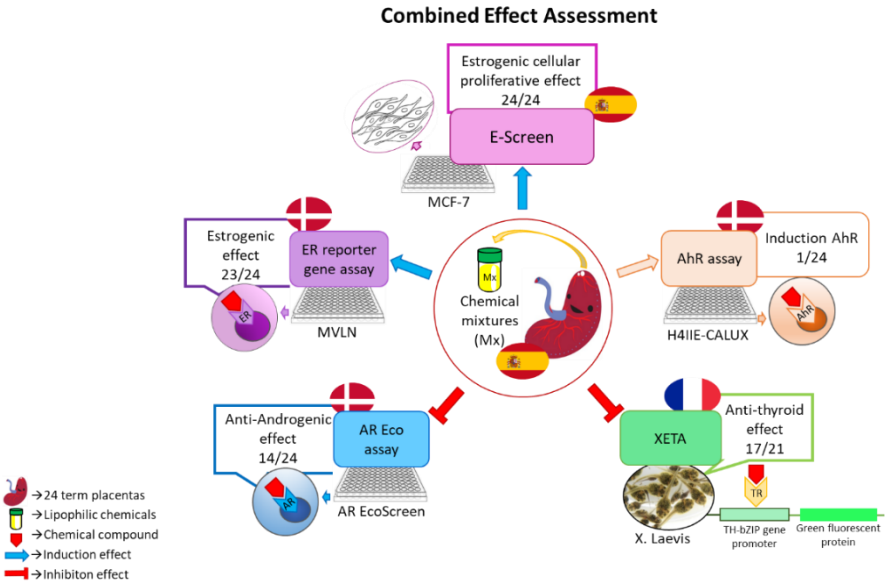
Assessment of chemical mixtures using biomarkers of combined biological activity: A screening study in human placentas

Andrea Rodríguez-Carrillo [a,b,c,d], Anna Kjerstine Rosenmai [e], Vicente Mustieles [a,b,c,d,*], Stephan Couderq [f], Jean-Baptiste Fini [f], Fernando Vela-Soria [a,b,c,d], Jose Manuel Molina-Molina [a,b,c,d], Patricia Ferrando-Marco [c], Maria Wielsøe [g], Manhai Long [g], Eva Cecilie Bonefeld-Jorgensen [g,h], Nicolás Olea [a,b,c,d], Anne Marie Vinggaard [e,1], Mariana F. Fernández [a,b,c,d,*,1]

[a] University of Granada, Center for Biomedical Research (CIBM), Spain
[b] Department of Radiology and Physical Medicine, School of Medicine, University of Granada, 18016, Granada, Spain
[c] Instituto de Investigación Biosanitaria Ibs GRANADA, Spain
[d] Consortium for Biomedical Research in Epidemiology & Public Health (CIBERESP), 18100, Spain
[e] Division of Diet, Disease Prevention and Toxicology, National Food Institute, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark
[f] Physiologie moléculaire et Adaptation, Département "Adaptation du Vivant," UMR 7221 MNHN/CNRS, Muséum National d'Histoire Naturelle, Paris 75005, France
[g] Centre for Arctic Health & Molecular Epidemiology, Department of Public Health Aarhus University, Denmark
[h] Greenland Centre for Health Research, University of Greenland, Nuuk, Greenland

A B S T R A C T

Humans are simultaneously exposed to complex mixtures of chemicals with limited knowledge on potential health effects, therefore improved tools for assessing these mixtures are needed. As part of the Human Biomonitoring for Europe (HBM4EU) Project, we aimed to examine the combined biological activity of chemical mixtures extracted from human placentas using one *in vivo* and four *in vitro* bioassays, also known as biomarkers of combined effect. Relevant endocrine activities (proliferative and/or reporter gene assays) and four endpoints were tested: the estrogen receptor (ER), androgen receptor (AR), and aryl hydrocarbon receptor (AhR) activities,as well as thyroid hormone (TH) signaling. Correlations among bioassays and their functional shapes were evaluated. Results showed that all placental extracts agonized or antagonized at least three of the abovementioned endpoints. Most placentas induced ER-mediated transactivation and ER-dependent cell proliferation, together with a strong inhibition of TH signaling and the AR transactivity; while the induction of the AhR was found in only one placental extract. The effects in the two estrogenic bioassays were positively and significantly correlated and the AR-antagonism activity showed a positive borderline-significant correlation with both estrogenic bioassay activities. However, the *in vivo* anti-thyroid activities of placental extracts were not correlated with any of the tested *in vitro* assays. Findings highlight the importance of comprehensively mapping the biological effects of "real-world" chemical mixtures present in human samples, through a battery of *in vitro* and *in vivo* bioassays. This approach should be a complementary tool for epidemiological studies to further elucidate the combined biological fingerprint triggered by chemical mixtures.



1. Introduction

Humans are exposed to hundreds of environmental chemicals at low doses [1]. Many of these environmental pollutants have been detected indifferent human matrices such as urine, serum and placenta [2–5] and constitute an important part of the human exposome [6]. Appropriate risk assessment of chemical mixtures with human relevance relies on accurately characterizing the mixture composition and the available toxicological information on the constituents of the mixture. However, for many chemicals the information of both, exposure and toxicity is lacking [7]. Thus, predicting the effects of exposure to chemical mixtures in human populations is one of the main challenges of current toxicology and environmental epidemiology [8,9].

Epidemiologic investigations have traditionally followed a one-compound-at-a-time strategy to assess possible associations between environmental chemical exposure and adverse health effects. A disadvantage of this approach is that it ignores additive effects or synergistic and/or antagonistic interactions with other chemicals [10]. To address this challenge, complementary statistical multi-pollutant models to disentangle independent associations among several co-exposures have been applied [11,12]. However, these valuable statistical

approaches also present inherent deficiencies and limitations, for example, that they only evaluate a limited number of chemicals with pre-existing toxicological knowledge, probably underestimating the effects of exposure to real-life mixtures.

To examine the effects of "real-world'' mixtures present in humans,including both known and unknown environmental chemicals, alternative approaches are needed. Chromatographic methods are of great utility since they allow the isolation of fractions containing compoundswith similar physicochemical characteristics, separating them from endogenous hormones [13,14]. The isolated fractions can be tested in a battery of *in vitro* assays covering different molecular initiating events and/or molecular targets, enabling the characterization of various biological effects exerted by the chemical mixture. This approach has beensuccessfully applied in the ecotoxicological field to monitor changes in water biological activity without the need to screen for hundreds of chemicals, and/or to identify emerging pollutants [15–17]. Likewise, human matrices can be used to evaluate the combined effect of chemicalmixtures in epidemiologic studies, and identify emerging exposures. However, this research field is still immature and awaits further exploration [18].

As reported by authors of the EU-project SOLUTIONS, there is a need to balance the way to deal with mixture exposures and their combined effects, since single-chemical exposure approaches tend to ignore data gaps (i.e., missing contaminants), while effect-based approaches may lead to increased uncertainty factors such as the need to further developsuspect and non-targeted screening techniques [19]. Thus, combining both approaches may provide the best results. Previous epidemiologicalstudies have shown that it is possible to obtain chemical fractions fromhuman matrices in which, for example, the most persistent and lipophilic contaminants, without the presence of endogenous hormones, areisolated by various analytical techniques [20–24]. In this context, bioassays testing the combined biological effect of chemical fractions isolated from human samples are usually referred to as "biomarkers of combined activity'', "biomarkers of combined effect", "biomarkers of *exvivo* hormonal activity", and/or "biomarkers of combined internal exposure" [20,25,26]. The advantage of this approach is that the biological effect is measured taking into account all bioactive chemicals as well as plausible interactions among them that may lead to synergistic or antagonistic mixture effects. The total joint effect of human extracts is hypothesized to provide a more holistic strategy to address the true cause of the disease provided that the adverse outcome is related to a specific receptor activity or pathway.

In this regard, among the studies performed on human samples, some effect endpoints have included estrogenic and antiandrogenic activities. The so-called "total effective xenoestrogen burden" (TEXB) has been implemented in several epidemiological studies for endocrine-related diseases such as male urogenital malformations and breast cancer [27, 28], by assessing the combined in vitro estrogenic activity of chemical mixtures extracted from different human matrices including serum, placenta and adipose tissue [13,23,29]. A higher total effective xenobiotic burden of anti-androgens (TEXB-AA), which evaluates the combined anti-androgenic activity exerted by mixtures of pollutants present in human placental extracts, was also associated with an increased risk of urogenital malformations in boys [20]. Other signaling pathways, including the aryl hydrocarbon receptor (AhR) and thyroid function, have also been explored, but to a lesser extent compared with theabovementioned endpoints [18].

There are some gaps in knowledge associated with the use of *in vitro*and *in vivo* biomarkers of combined activity that need to be addressed,such as the standardization across laboratories regarding the procedures performed for the correct execution of bioassays and comparisons among a panel of different bioassays using the same biological matrix. Therefore, the main objective of this study was to assess, characterize and compare the combined biological effect of chemical mixtures extracted from the same human placentas by quantifying the signal elicited in five bioassays, covering relevant endocrine activities (*in vitro* proliferative and reporter gene assays for estrogen, androgen and aryl hydrocarbon, and *in vivo* thyroid function).

2. Materials and methods

2.1. Study population

Twenty-five placenta samples were randomly selected among those kept at the biobank of the San Cecilio University Hospital (Granada, Spain) from healthy women participating in the INMA -INfancia y MedioAmbiente (Environment and Childhood)-, Granada birth cohort study, recruited from October 2000 to July 2002 [30]. Participants for which a limited quantity of placenta was available were excluded before the random selection. Characteristics of the study population were obtained from medical records and validated questionnaires [27]. Placentas were collected at time of delivery, weighed without fetal membranes/maternal decidua, and frozen at -80 °C. The INMA study followed the principles of the declaration of Helsinki and was approvedby the Ethics Committee of San Cecilio University Hospital. All participants signed the informed consent allowing the use of biological samples for environmental research purposes.

2.2. Study design

This work aimed to address the combined effects of "real-world" chemical mixtures of persistent and lipophilic chemicals present in human placenta samples using several bioassays, taking into account allbioactive chemicals as well as plausible interactions among them. Placental homogenates were extracted using a validated semi-preparative chromatographic separation protocol [24,29] at the facilities of the University of Granada (UGR), Spain. Afterwards, driedhigh-performance liquid chromatography (HPLC) fractions were sent on dry ice to the participating institutions in order to conduct specific bioassays. Five bioassays were performed: the E-Screen, conducted at the University of Granada (UGR) Spain; the estrogen receptor (ER) reporter gene assay, performed at

Table 1
Bioassays and institutions in which they were performed.

Laboratory	Biological material	Bioassays (Biomarkers of combined effect)
UGR (Spain)	24 α-Fractions	E-Screen. Estrogenic proliferative effect.
AU (Denmark)	24 α-Fractions	ER reporter gene assay. ER induction effect.
DTU (Denmark)	24 α-Fractions	AR reporter gene assay. Anti-androgenic effect. AhR reporter gene assay. Aryl Hydrocarbon receptor induction.
CNRS (France)	24 α -Fractions	Xenopus eleutheroembryonic thyroid assay. Thyroid hormone disruption.

AU: Aarhus University; CNRS: National Center of Scientific Research; DTU: Technical University of Denmark; UGR: University of Granada.

the Aarhus University (AU), Denmark;the androgen receptor (AR) and aryl hydrocarbon receptor (AhR) reporter gene assays, tested at the Technical University of Denmark (DTU); and the Xenopus eleutheroembryonic thyroid assay (XETA), tested at the National Center of Scientific Research (CNRS) in France (Table 1).

1.1. *Placenta extraction protocol*

To ensure the representativity of the whole placenta tissue, half of the placenta was cut, defrosted, placed in the glass container of a mixer (Büchi Mixer B-400 Büchi Laboratories AG, Flawil, Switzerland) and homogenized (Fig. 1.A). Placental homogenates were extracted following a previously validated semipreparative HPLC protocol [27,29]to efficiently separate organohalogenated lipophilic chemicals from endogenous hormones and more polar compounds, using a normal-phase column and a gradient with two mobile phases. Of the initial 25 placentas, one was excluded due to technical issues during extraction. The placental homogenization and the semi-preparative HPLC extraction protocol are graphically summarized in Fig. 1.

Briefly, 3 g of placenta homogenate was split in two 5 mL Falcontubes with 1.5 g each. Then, 1.5 mL of distilled water was added into thetubes, which were vortexed for 1 min (Fig. 1.B). Successively, the mixture was extracted by adding 3 mL of ethyl acetate and vortex-shaked again for 10 min. Afterwards, the mixture was centrifuged for 10 min at 4050 g. The two supernatants were pooled in a clean glass vial, and the extract was evaporated to dryness at room temperature under a nitrogen stream. The dried extract was dissolved with hexane (700 μL) and dried once more under nitrogen stream. Then it was dissolved in 400 μL of hexane and injected twice (200 μL) into a column to undergo preparative HPLC. The placental extract was eluted by a specific gradient of two mobile phases: n-hexane (phase A) and n-hexane: methanol:2-isopropanol (40:45:15)(v/v) (phase B) at a flow rate of 1.0 mL/min. Specifically, the α-fraction represents the first 11 min of elution of the chromatographic run. The whole process was repeated 5 times and the obtained α-fractions were sent on dry ice to each participant laboratory (Fig. 1.B). The available knowledge to date has shown the presence of common persistent and lipophilic pollutants in the α-fraction, and the absence of steroid endogenous hormones [13,31,32]. Examples of chemical compounds present in different minutes of elution of the α-fraction can be consulted in Table S1 of the Supplementary Material.

1.2. *Bioassays*

Each laboratory reconstituted the dried α-fractions and made specific dilutions to be tested following the requirements of each specific bioassay (e.g. sensitivity, range and cytotoxicity threshold). To facilitatethe comparison among bioassays, the concentrations of placental α-fractions tested were expressed as mg of placenta extracted per well volume in mL ($mg_{placenta}$/mL). The corresponding calculations for each bioassay are explained in Table S2 of the Supplementary Material.

1.2.1. *E-Screen*

MCF-7 cells were used to assess the proliferative effect induced by placental α-fractions as described previously [27]. Cells were seeded at adensity of 4 10^3 cells/well in 96-well plates (obtained by Falcon®, VWR International Eurolab, Barcelona, Spain) in culturing medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) with phenol red supplemented with 10 % fetal bovine serum (FBS) (Gibco, Invitrogen, Spain) and left at 37 °C with 5% CO_2. After 24 h, culturing medium was replaced with experimental medium, consisting of phenolred-free DMEM supplemented with 10 % dextran-coated charcoal-stripped FBS (DCCS-FBS) (Gibco, Invitrogen, Spain). Dried α-fractions were reconstituted in 1 mL of experimental medium, vigorously shaken,left to rest for 30 min, filtered through a 0.22 μm filter, and diluted 1, 5and 10 times, and tested in triplicates. Each well plate included 150 μL of experimental medium together with 50 μL of placental α-fractions. Dilutions x1, x5 and x10 corresponded to 750 $mg_{placenta}$/mL, 150 $mg_{placenta}$/mL and 75 $mg_{placenta}$/mL, respectively (Table S2). After 6 days of exposure, cells were fixed, stained with sulforhodamine B (Sigma-Al- drich, MO, USA) and the solubilized bound dye was read at 492 nm in aTitertek Multiscan plate reader (Flow, Irvine, CA, USA). 17β-estradiol was tested as a positive control at concentrations of 0.1 pM-1000 pM. Experimental medium was used as negative control.

1.2.2. *Estrogen receptor (ER) reporter gene assay*

The activation of ERs by placental α-fractions was evaluated with the stably transfected human breast adenocarcinoma MVLN cells carrying the estrogen response element luciferase reporter vector (provided by M. Pons, France). The procedure followed was previously described by Bjerregaard-Olesen and colleagues [33]. Briefly, cells were seeded at a density of 8.5 10^4 cells/well in 96-well plates with culture medium that included phenol red-free DMEM (LONZA, Belgium) supplemented with 1% DCCS-FBS (HyClone, Belgium), 6 μg/L insulin (Sigma, USA), 64 mg/L hexamycin (Sandoz, Denmark), 4 mM glutamine (Sigma,USA), and 20 mM HEPES (Gibco, UK), and were left to incubate at 37 °C with 5% CO_2 overnight. The experimental medium consisted on phenol red-free DMEM (LONZA, Belgium) containing 0.5 % DCCS-FBS. Driedα-fractions were reconstituted in 44 μL EtOH:H_2O:DMSO (50:40:10, V/V/V) and from this reconstituted 44 μL, we used 20 μL that were subsequently diluted in experimental medium 55, 275, and 550 times. Successively, 100 μL of these diluted extracts were added into the well and analyzed in triplicate. The dilutions 55, 275 and 550 correspond to 1240 $mg_{placenta}$/mL, 248$mg_{placenta}$/mL and124 $mg_{placenta}$/mL, respectively (Table S2).

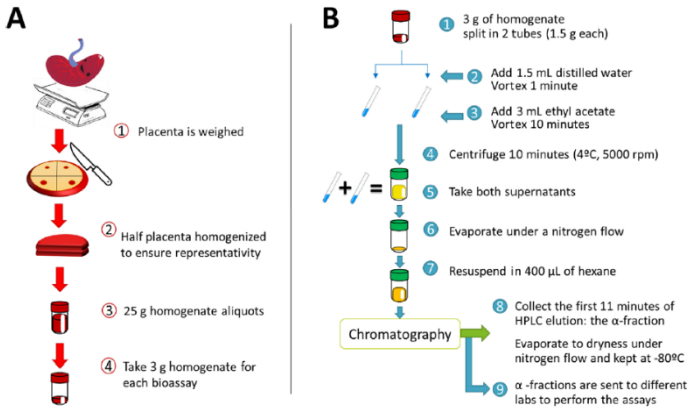


Fig. 1. A) Placental homogenization. After weighing the placenta (1), it was cut in half by using a template (2). One half was homogenized and the rest was kept at -80 °C. The homogenate was split into 25 g aliquots (3). From a 25 g homogenate, a 3 g aliquot was taken for the chemical extraction (4). B) Chemical extraction and chromatographic separation protocol. The α-fraction corresponds to the first 11 min of semi-preparative HPLC chromatographic elution, which has been previously shown to contain the most persistent and lipophilic compounds, while avoiding endogenous hormones.

After 24 h of exposure, the luciferase activity was measured with automatic injection of luciferase substrate (D-luciferin, free acid, Molecular Probes, L2911, Invitrogen) using a LUMIstar luminometer (BMG Labtech, RAMCON). Cell protein levels were quantified to correct for differences in cell numbers in the well. Protein was quantified by fluorometric measurements using a WALLAC Victor2 (Perkin Elmer). Cell viability was assessed visually at the microscope, and for cells with visual cytotoxicity, low protein levels were also observed. As a quality control, a dose-dependent 17β-estradiol curve at 1.5 pM-300 pM was tested in parallel.

1.2.3. *Androgen receptor (AR) reporter gene assay*

The AR reporter gene assay was performed as previously described [34] with few modifications, to evaluate the anti-androgenic activity of placental α-fractions. The AR-EcoScreen cell line (JCRB Cell Bank, cat.No. JCRB1328), cultured in DMEM-F12 without phenol red (Life Technologies, CA, USA) and supplemented with 5% DCCS-FBS (Life Technologies, CA, USA), 50 units/mL penicillin, 50 μg/mL streptomycin (Life Technologies, CA, USA), 200 μg/mL zeocin (Invivogen, CA, USA), and 100 μg/mL hygromycin (Invitrogen, CA, USA), was seeded at a density of 0.9 10^4 cells/well in 96-well plates (Costar, Corning, USA). The experimental medium consisted of DMEM-F12 without phenol red supplemented with penicillin/streptomicin (but without zeocin or hygromycin), and 5% DCCS-FBS (Sigma Aldrich, MO, USA). Before exposure was initiated, plates were left in the incubator for 24 h at 37 ◦C with 5% CO2. Dried α-fractions were reconstituted in 400 μL of hexane, which was split in four glass vials and left to evaporate until dryness. Evaporated α-fractions were reconstituted with 250 μL of experimental medium, left to rest for 30 min, filtered with a 0.22 μm filter and diluted60-, 180-, and 600 times. Successively, 50 μL of diluted fractions together with 150 μL of experimental medium were added into wells. Dilutions 60, 180 and 600 corresponded to 12.5 $mg_{placenta}$/mL, 4.2 $mg_{placenta}$/mL and 1.25 $mg_{placenta}$/mL (Table S2). All treatments, including controls were co-treated with 0.1 nM metribolone (R1881) (PerkinElmer, MA, USA), a known inductor of the AR activity. After ~20h of exposure, 100 μL Dual-Glo® Firefly Luciferase Reagent, prepared according to manufacturer's protocol (Dual-Glo® Luciferase Assay System, Promega, USA) was added to wells, and plates were left on a shaker table for 10 min. Then, the luminescence was measured using a BioOrbit, Galaxy luminometer to assess AR activity. Successively, 50 μLof Dual-Glo® Stop & Glo® reagent was added to wells, plates were left on shaker table for 15 min, and luminescence was measured (BioOrbit, Galaxy) to assess cell viability, measured by the stably transfected construct of Renilla luciferase. This assay provides a direct measure of cytotoxicity in the cells. The assay was performed in three independent experiments with technical triplicates for each treatment within the experiment. Hydroxyflutamide (OHF), a known antagonist of AR activity, was used as quality control and tested in concentrations ranging from 1 × 10^3-5000 × 10^3 pM in all experiments.

1.2.4. *Aryl hydrocarbon receptor (AhR-CALUX) reporter gene assay*

The induction of AhR is known for leading to the transcription of metabolizing enzymes [35]. To evaluate the activation of the AhR receptor, a stably transfected rat hepatoma (H4IIE-CALUX) cell line was used [36]. Briefly, 2.2 × 10^4 cells/well were seeded in 96-well plates and incubated for ~22 h at 37 ◦C with 5% CO2 in culture medium, which consisted of minimum essential medium α (MEM α) supplemented with 5% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL Gibco Amphotericin B (Life Technologies). The FBS content was reduced to 1% during experiments (experimental medium). Dried α-fractions were reconstituted in 400 μL of hexane, split into four glassvials, and left to evaporate until dryness. Evaporated α-fractions were reconstituted in 250 μL of experimental medium, left to rest for 30 min,filtered through a 0.22 μm filter, and diluted 100-, 300-, and 1000 times.Successively, 50 μL of diluted fractions together with 150 μL of experimental medium were added into wells and tested in two independent experiments. Dilutions corresponded to 7.5 $mg_{placenta}$/mL, 2.5 $mg_{placenta}$/mL and 0.75 $mg_{placenta}$/mL, respectively (Table S2). After ~22 h of exposure, cells were lysed and the luminescence was measured by the addition of 40 μL luciferin solution containing 0.5 mM luciferin and 0.5mM ATP in lysis buffer. Cell viability was tested following the protocol above, but seeding only 1.1 × 10^4 cells/well. At assay termination, medium was removed and 50 μL of fresh medium was added. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added leading to a final concentration of 0.45 mg/mL and incubated for 1.5 h. Afterwards, medium was removed and 50 μL/well isopropanolwas added. Plates were left on a shaker table for 5 min after which absorbance was measured. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was used as positive control and tested in concentrations ranging from 0.5 pM to 3000 pM.

1.2.5. *Xenopus eleutheroembryonic thyroid assay (XETA)*

The XETA is a miniaturized and relatively high throughput *in vivo* assay to detect disruption of thyroid hormone (TH) signaling through variations in the fluorescence emitted by transgenic tadpoles [37]. To assess whether placental α-fractions inhibit or activate TH signaling during embryonic stages, Nieuwkood and Faber (NF) stage 45 *Xenopuslaevis* tadpoles (1 week old) from the *Tg(thibz:eGFP)* line were used, as previously described [38]. The construct contains the promoter of the TH-sensitive TH/bZIP promoter coupled to a Green Fluorescent Protein (GFP) reporter gene to capture disruption of TH signaling that may involve multiple points of regulation of the thyroid axis. Briefly, dried α-fractions were reconstituted in 16 μL of DMSO and 400 μL of Evian water. Reconstituted α-fractions were then split into two parts of 208 μL each which were added to wells and tested in duplicates within four independent experiments. Each well was completed with experimental medium up to 8 mL, reaching a concentration equivalent to 187.5 $mg_{placenta}$/mL. Fifteen tadpoles per well were placed in 6-well-plates (TPP Switzerland) containing 8 mL of either control solvent (DMSO)in Evian water, thyroid hormone triiodothyronine T3 (5 nM), or α-fractions spiked with T3 (5 nM). DMSO (0.01 %) was present in all treatments and plates were placed at 23 °C. After 72 h of exposure, tadpoles were anesthetized and color images of each tadpole manually positioned in a well of a 96-well plate were acquired using an Olympus AX-70 binocular equipped with long pass GFP filters and a Q-Imaging Exi Aqa camera (25x objective, and 3 s exposure). QC Capture pro (QImaging) software was used for image acquisitions and quantifications were carried out using ImageJ. Quantifications were carried out ina region of interest (ROI) containing the whole head area while excluding the non-specific auto-fluorescence emitted from the gut area (Figure S1, Supplementary Material).

1.3. *Data handling and statistical analysis*

All bioassay data were normalized to the mean of solvent plate control(s). If more than one independent experiment was conducted, the means from independent experiments were pooled. Normal distribution analysis was conducted (D'Agostino & Pearsons omnibus test). XETA results were not normally distributed, and the difference between experimental samples and controls was performed using the Kruskal- Wallis test (Dunn's post hoc test). For *in vitro* data, and based on the number of replicates for each sample, the distribution could not be evaluated. Therefore, classification of samples as positive in the bioassays was based on cut-off values calculated from the limit of detection (LOD). For the E-Screen, LOD value has previously been standardized to >2.0 [39]; for the remaining agonist assays, LOD was defined as 1 plus 3 times the standard deviation (SD) of the vehicle control, and for the antagonist assays, the LOD was calculated as 1 minus 3 times the SD ofthe vehicle control. Positive controls were fitted to a four-parameter non-linear regression curve with the vehicle controls constrained to 1 and in the case of inhibitors, the lower limit constrained to >0. The maximum efficacy (E_{max}) obtained for the positive controls or α-fractions was defined as the observed maximum change compared to the vehicle control, and calculated as the percentage difference between thehighest fold change reached minus that of the vehicle control (set to 1). All data processing was performed in GraphPad Prism 8.1.

To assess correlation between bioassay responses, all samples were normalized to vehicle controls constrained to 1, and then fold-change values were expressed as percentages, in order to represent values for both agonist and antagonist effects. Firstly, Pearson correlation coefficients were calculated between the effect magnitude (percentage of biological activity) of the different bioassays. Secondly, and to assess the shape of correlations within relevant findings, we performed linearregression models categorizing the independent variable into tertiles. Thus, placentas eliciting the lowest signals in the bioassay of interest were categorized in the first tertile (T1), while placentas with the highest signal were located in the third tertile (T3). The coefficient of determination (r^2) was calculated for each regression to estimate the proportion of the variance in the dependent variable that is predicted by the independent variable. All correlations between biomarkers were conducted using the SPSS v24.0 (IBM, Chicago, IL), significance level was set at $p \leq 0.05$ and borderline significance was set at $p \leq 0.1$.

2. Results

2.1. *Study population*

In this study, 44 % of the newborns were boys and 56 % were girls; mean (SD) gestational age was 39 (1.2) weeks. Mean (SD) head circumference was 34.4 (1.7) cm, birth weight and birth length were 3.35 (0.4) kg and 50.94 (2.1) cm, respectively. No infant was born preterm (<37 week) or with low birth weight (<2.5 kg). Mean (SD) age, pre-pregnancy weight and height of mothers was 30 (4.9) years, 68.1 (15.9) kg and 1.65 (0.1) m, respectively; and with a mean (SD) pre- pregnancy BMI of 24.9 (4.5) kg/m^2. Half of mothers (48 %) received higher education (university studies/professional formation), 84 % did not consume tobacco and 52 % were primiparous.

2.2. *Positive controls and cut-off values*

Results obtained for positive controls known to induce or inhibit thetested endpoints are shown in Fig. 2. The half maximal effect concentration (EC_{50}) and the half maximal inhibitory concentration (IC_{50})- values were determined based on a 4-parameter curve fit. Briefly, EC_{50} values were 0.02 nM (17β-estradiol), 0.03 nM (17β-estradiol), 0.05 nM (metribolone) and 0.04 nM (2,3,7,8-tetrachlorodibenzo-p-dioXin) for the E-Screen, and the ER, AR, and AhR reporter gene assays, respectively. The IC_{50} value was 0.79 nM (hydroxyflutamide) in the AR reporter gene assay. Maximum efficacies for positive controls tested in each *in vitro* assay were as follows, E-Screen: 580 %, ER: 213 %, AR: 694 % metribolone and 66 % hydroxyflutamide and AhR: 904 % (Fig. 2). Cut-off values to classify a placenta extract as positive were based on LODs (illustrated with dotted line in Fig. 3). For E-screen, ER and AhR agonism, cut-off values were set to >2.0 [39], >1.5 and >1.8-fold change, respectively. For AR antagonism, the cut-off value was set to <0.8-fold change.

2.3. *Combined biological activities exerted by placental α-fractions*

2.3.1. *Estrogenic activity*

All twenty-four placental α-fractions elicited a concentration dependent proliferative effect (PE) at varying degrees in the E-Screen

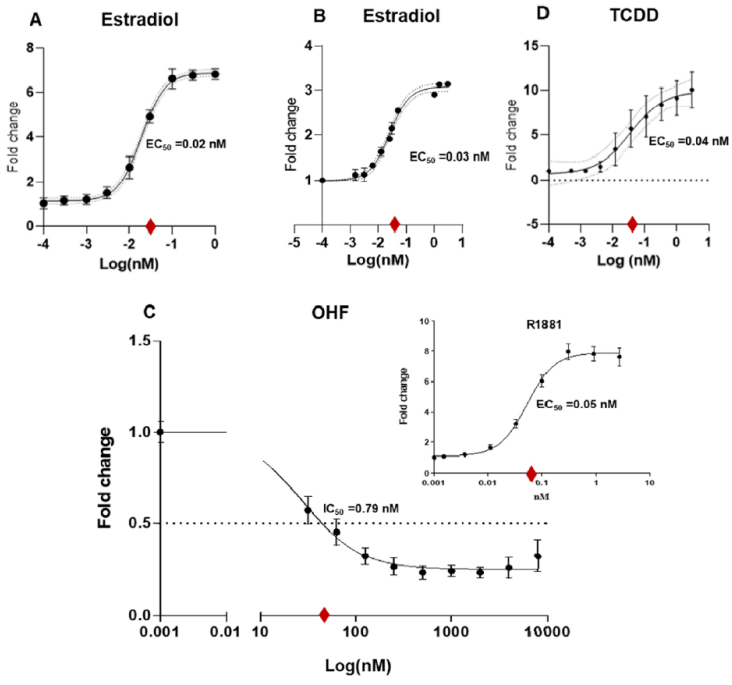


Fig. 2. Concentration-response curves and standard deviations (SD) for positive controls tested in *in vitro* bioassays. (A) 17β-estradiol (E_2) for the E-Screen assay. (B) E_2 for the ER transactivation assay. (C) Hydroxyflutamide (OHF) and metribolone (R1881) for the AR reporter gene assay. (D)2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for the AhR reporter gene assay. Data points represent means from 1 (Fig. A and, B), 2 (Fig. D) or 3 (Fig. C) independent experiments. Diamonds point out the EC_{50}/IC_{50} values.

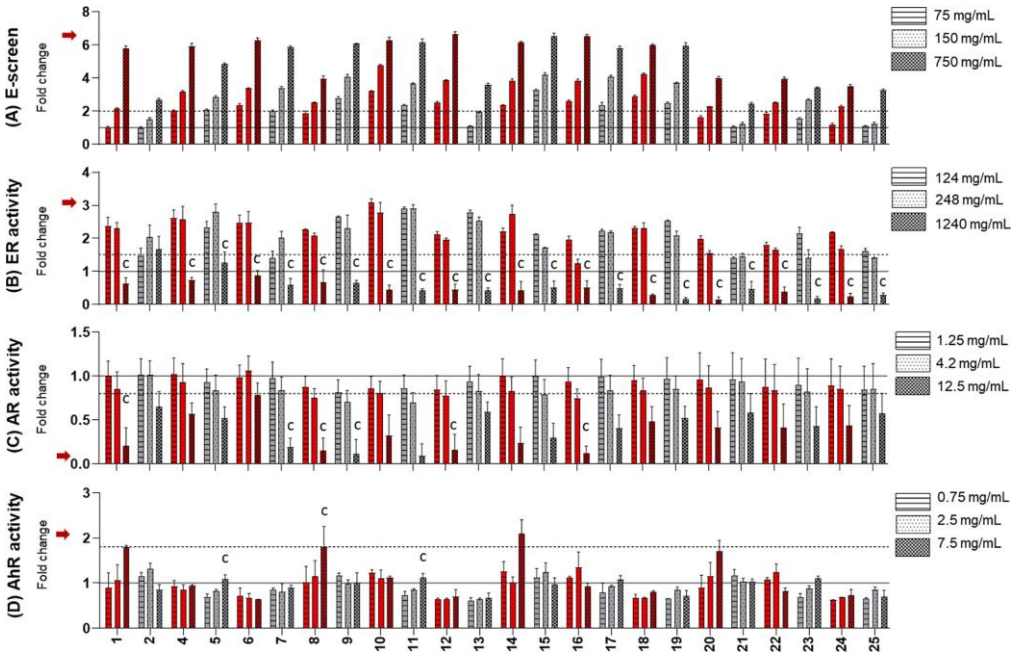


Fig. 3. *In vitro* activity exerted by placental α-fractions determined by the estrogenic- proliferative effect (E-Screen), estrogen receptor (ER) transactivity, androgen receptor (AR) antagonism, and aryl hydrocarbon receptor (AhR) activity gene assays. (A) E-Screen data for placental α-fractions tested at 75 $mg_{placenta}$/mL, 150 $mg_{placenta}$/mL and 750 $mg_{placenta}$/mL in duplicates in a single independent experiment. (B) ER transactivation assay data for placental α-fractions tested at 124 $mg_{placenta}$/mL, 248 $mg_{placenta}$/mL, and 1240 $mg_{placenta}$/mL in duplicates in a single independent experiment. (C) AR antagonism assay for placental α-fractions testedat 1.25 $mg_{placenta}$/mL, 4.2 $mg_{placenta}$/mL, and 12.5 $mg_{placenta}$/mL in triplicates in three independent experiments. (D) AhR reporter gene assay data for placental alpha-fractions tested at 0.75 $mg_{placenta}$/mL, 2.5 $mg_{placenta}$/mL, and 7.5 $mg_{placenta}$/mL in triplicates in two independent experiments. All graphs are presented as data normalized to vehicle controls (mean ± SD), set to 1 (continuous line). Graph C and D data were normalized in independent experiments and then mean values were pooled. Cut-off values are indicated with a dotted line. C = compromised cell viability/cell toxicity. Arrows highlight the maximum efficacy exerted by α-fractions.

assay (Fig. 3.A), and all α-fractions were above the cut-off value (dotted line, Fig. 3.A) at the highest concentration tested. Samples led to maximal effects at 750 $mg_{placenta}$/mL, with 14 out of 24 samples reaching a proliferation efficacy above 450 %.

In the ER transactivation assay, all α-fractions at both concentrationsof 124 and 248 $mg_{placenta}$/mL, were above the cut-off value, with the exception of placentas # 2, 21 and 25 (dotted line Fig. 3.B). Notwithstanding, at least one of the concentrations tested for placentas # 2 and25 were above the cut-off value and most placentas (23 out of 24) were thus deemed positive for ER activity (Table 2). Most placental α-fractions tested at the highest concentration (1240 $mg_{placenta}$/mL) compromised cell viability, except α-fraction # 2. Fractions tested at 124 and 248 $mg_{placenta}$/mL induced increased ER transactivity compared to the negative control, which appeared concentration dependent for placental α-fractions # 5, 7 and 14. More than half of the α-fractions led to an efficacy above 200 %, with the exception of fractions # 2, 7, 12, 15, 16, 20, 22, 23, 24, and 25.

2.3.2. *Anti-androgenic activity*

Most α-fractions showed an inhibitory effect on the AR activity at the highest concentration tested (12.5 $mg_{placenta}$/mL). However, six α-fractions compromised cell viability at this concentration (# 1, 7, 8, 9, 12 and 16), and three (# 2, 21 and 25) showed error bars slightly above the cut-off value. Consequently, 14 placentas were considered as positive forAR antagonism activity (Fig. 3.C) uniquely based on the cut-off criteria. Among the positives, about half of the placental fractions led to E_{max} values above 70 % of AR activity inhibition, with α-fractions # 10, 11, 14, and 15 being among the most efficacious. If the criteria were based on the presence of a concentration-dependent response together with the presence of an average response below the cut-off value, 22 out of the 24 placental fractions would be classified as antiandrogenic. No compromised cell viability was observed at the lowest (1.25 $mg_{placenta}$/mL) and medium (4.2 $mg_{placenta}$/mL) concentrations tested.

2.3.3. *Aryl hydrocarbon receptor (AhR) activity*

Although increases in AhR-transactivity were observed for placentalα-fractions # 1, 8, 14, and 20, only placenta 14 was above the cut-off value (Fig. 3.D). Cell viability was compromised in α-fractions # 5, 8 and 11 at the highest concentration tested (7.5 $mg_{placenta}$/mL).

2.3.4. *Thyroid activity*

Placental α-fractions # 5, 24 and 25 were not tested in this *in vivo* assay, due to lack of sample availability. The positive control group was exposed to triiodothyronine (T_3) hormone at 5 nM. All data was normalized to vehicle control, which was normalized to 1, shown in continuous line (Fig. 4).

The inhibition of the thyroid activity was consistent across all twenty-one α-fractions tested after 72 h of exposure (Fig. 4). All α-fractions, except # 8, 9, 15 and 23 induced a statistically significant decrease ($p < 0.05$) of the thyroid activity when compared with the positive control group. The highest antagonistic thyroid activity was shown for α-fractions # 1, 2, 12, 13, 14 and 20.

2.4. General overview of bioassay activities

A summary of the results obtained from the 5 bioassays is shown inTable 2.

2.5. Correlations among bioassays

Pearson correlation coefficients among bioassay activities are presented in Table 3. Additionally, the bioassay data taken as independent variable was categorized into tertiles to assess the shape of the correlation among biological activities (Fig. 5). Overall, α-fraction concentrations showing no cytotoxicity and with the highest magnituderesponses were selected to perform the correlation analyses. Thus, chosen concentrations were: 750 $mg_{placenta}$/mL for the E-Screen, 124 $mg_{placenta}$/mL for the ER reporter gene assay, 4.2 $mg_{placenta}$/mL for the AR reporter gene assay and 2.5 $mg_{placenta}$/mL for the AhR reporter gene assay. For XETA, only one concentration was tested and, therefore used for the analysis. Notwithstanding, a correlation matrix among all bioassay dilutions that showed no cytotoxicity can be consulted in TableS3 of the Supplementary Material.

The E-Screen assay activity showed a positive and significant correlation with the ER reporter gene assay activity, and a positive borderline-significant correlation with the AR reporter gene assay activity (Table 3). When the E-Screen was categorized into tertiles from lower to higher ER-mediated proliferative effect, a linear positive dose-response correlation was observed between responses in both estrogenic bioassays (Figs. 5.A and 5.C).

The ER reporter gene assay activity showed a positive and significantcorrelation with the E-screen assay activity and a positive borderline- significant correlation with the AR reporter gene assay activity(Table 3). After categorizing the ER reporter gene assay into tertiles from the lowest to highest ER transactivity, only the correlation with the E- Screen assay showed a linear dose-response shape (Figs. 5.C and 5.D).

The AR reporter gene assay activity showed a positive borderline-significant correlation with both, the E-Screen and the ER reporter assay activities (Table 3). When the AR reporter gene assay was categorized into tertiles from lower to higher AR antagonistic effect, results showed linear dose-response correlations for both the E-screen and the ER

Table 2

Effect of 24 placental α-fractions tested in the E-screen assay, the estrogen receptor (ER), androgen receptor (AR) and aryl hydrocarbon receptor (AhR) reporter gene assays, and the thyroid function assay (XETA). E-screen, ER and AhRassays were performed in agonist mode, whereas the AR reporter gene assaysand XETA were performed in the antagonist mode.

α-fractions	E-Screen	ER	AR	AhR	XETA
1	POS	POS	NEG	NEG	POS
2	POS	POS	NEG	NEG	POS
4	POS	POS	POS	NEG	POS
5	POS	POS	POS	NEG	--
6	POS	POS	NEG	NEG	POS
7	POS	POS	NEG	NEG	POS
8	POS	POS	NEG	NEG	NEG
9	POS	POS	NEG	NEG	NEG
10	POS	POS	POS	NEG	POS
11	POS	POS	POS	NEG	POS
12	POS	POS	NEG	NEG	POS
13	POS	POS	POS	NEG	POS
14	POS	POS	POS	POS	POS
15	POS	POS	POS	NEG	NEG
16	POS	POS	NEG	NEG	POS
17	POS	POS	POS	NEG	POS
18	POS	POS	POS	NEG	POS
19	POS	POS	POS	NEG	POS
20	POS	POS	POS	NEG	POS
21	POS	NEG	NEG	NEG	POS
22	POS	POS	POS	NEG	POS
23	POS	POS	POS	NEG	NEG
24	POS	POS	POS	NEG	--
25	POS	POS	NEG	NEG	--

(Red, POS): the placental α-fraction affects activity above the cut-off value and is deemed positive, in the case of XETA, it was statistically significant when compared to control. (Blue, NEG): the placental α-fraction affects activity below the cut-off value and is deemed negative, in the case of XETA, it was not statistically significant when compared to control. (White, –): the placental α-fraction was not tested.

Table 3

Pearson correlation coefficients assessing the relationships among bioassays when treated as continuous variables.

	E-Screen (%)	ER (%)	AR antagonism (%)	AhR (%)	XETA antagonism (%)
E-Screen (%)	–	0.492*	0.347†	−0.103	−0.058
ER (%)	0.492*	–	0.333†	−0.300	−0.230
AR antagonism (%)	0.347†	0.333†	–	0.086	−0.081
AhR (%)	−0.103	−0.300	0.086	–	0.009
XETA antagonism (%)	−0.058	−0.230	−0.081	0.009	–

Concentrations selected for the main analyses were: 750 $mg_{placenta}$/mL for the E-Screen; 124 $mg_{placenta}$/mL for the ER reporter gene assay; 4.2 $mg_{placenta}$/mL for the AR reporter gene assay; 2.5 $mg_{placenta}$/mL for the AhR reporter gene assay; and 1875 $mg_{placenta}$/mL for XETA.
*$p \leq 0.05$ † ≤ 0.10.

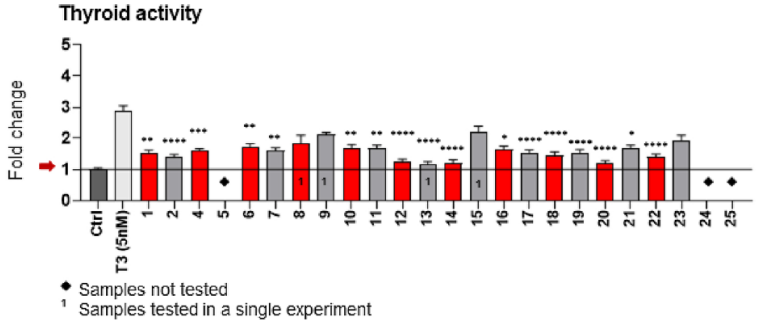


Fig. 4. Antagonistic thyroid activity exerted by 21 placental α-fractions tested in the Xenopus Embryo Thyroid-signaling Assay (XETA), after 72 h of exposure. Samples were tested at a concentration equivalent to 187.5 $mg_{placenta}$/mL in two independent experiments. Data was normalized to the negative control group (mean ± SE), set to 1 (continuous line). SE = standard error. Each α-fraction was tested in two experiments except for α-fractions 8, 9, 13 and 15. Samples 5, 24 and 25 were not tested due to lack of sample availability. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. The arrow highlights the maximum efficacy exerted by α-fractions.

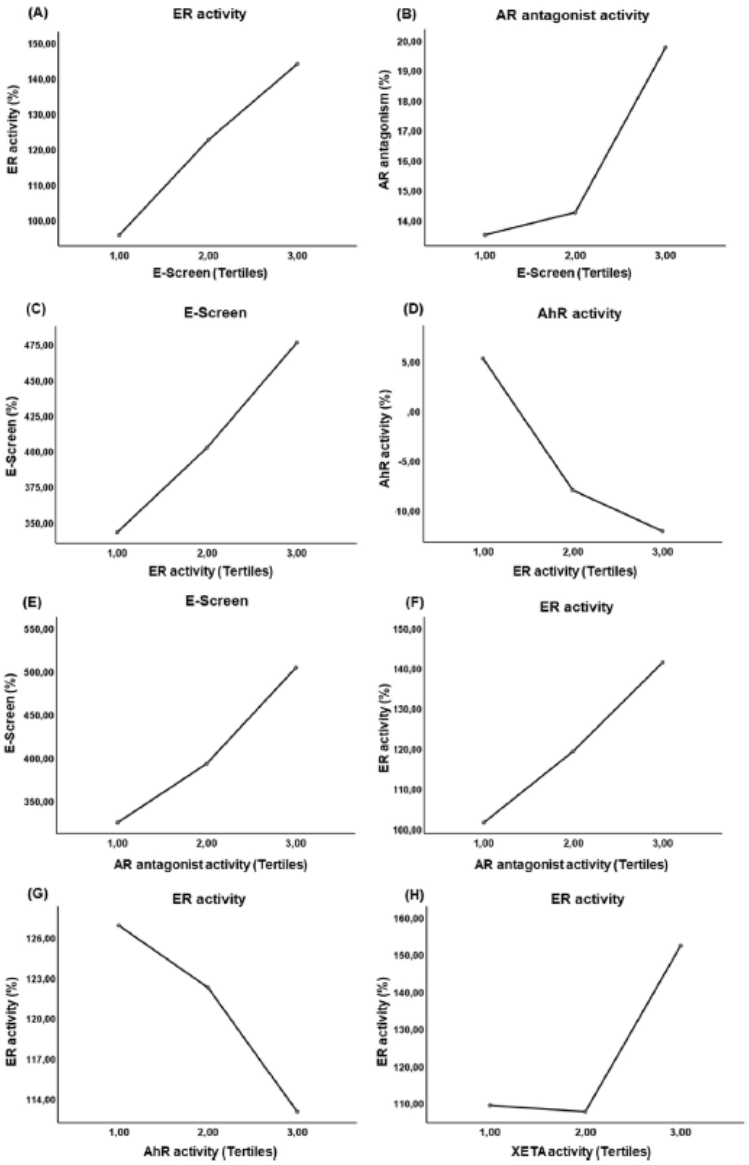


Fig. 5. Linear regression models categorizing the independent variable into tertiles from lower to higher signals obtained in each biomarker of combined activity. Data shown makes reference to significant or borderline-significant correlations found in Table 3, to test the functional shape of the correlations. (A) E-screen correlation with ER reporter gene assay; (B) E-Screen correlation with AR reporter gene assay; (C) ER gene reporter assay correlation with the E-Screen;(D) ER reporter gene assay correlation with AhR reporter gene assay; (E) AR reporter gene assay correlation with the E-Screen; (F) AR reporter gene assay correlation with the ER reporter gene assay; (G) AhR reporter gene assay correlation with the ER reporter gene assay;(H) XETA correlation with the ER reporter gene assay. Coefficients of determination (r^2): 5.A: $r^2 = 0.202$; 5.B: $r^2 = 0.095$; 5.C: $r^2 = 0.166$; 5.D: $r^2 = 0.228$; 5.E: $r^2 = 0.121$; 5.F: $r^2 = 0.105$; 5.G: $r^2 = 0.030$; 5.H: $r^2 = 0.151$.

reporter gene assay activities (Figs. 5.E, and 5.F). In other words, a higher xenoestrogenicity tended to be correlated with a higher anti- androgenicity.

The AhR reporter gene assay activity did not show any significant correlation with other bioassays (Table 3). After categorizing the AhR a reporter gene assay into tertiles from lower to highest AhR activity, a linear dose-response correlation between AhR and ER activity wasobserved (Fig. 5.G).

The XETA results were not correlated with any of the remaining bioassays (Table 3). Fig. 5.H shows a non-linear correlation between theER reporter gene assay and XETA values categorized into tertiles (Fig. 5.H). In general, coefficients of determination (r^2) in the regression models were modest (Fig. 5).

3. Discussion

3.1. *Chemical mixtures interact with diverse signaling pathways*

In this screening study, HPLC α-fractions containing "real-world" mixtures of lipophilic and persistent chemicals were isolated from 24 human placentas and tested in 5 different bioassays, in an effort to comprehensively address the biological effect of chemical mixtures on different biological endpoints. Overall, most placental fractions elicited estrogenic activities in both the E-Screen and the ER reporter gene assay. While 14 out of 24 placental α-fractions were clearly positive for anti- androgenic activity based on the cut-off value, most fractions (22 out of 24) were considered positive for AR inhibition when both a

concentration-dependent response as well as average responses below the cut-off were accounted for. Additionally, most placental α-fractionsshowed a clear inhibition of thyroid activity in the *in vivo* XETA, while subtle induction effects were noticed in the AhR gene assay. Differences in sensitivity across bioassays were noticed, since effects were observed at placental concentrations < 12.5 $mg_{placenta}/mL$ in the AR reporter gene assay, whereas in the estrogen assays higher placental concentrations were needed to induce effects. However, the range of effects observed was higher in the E-Screen assay, which allowed to detect a wide variability among the α-fractions tested. Interestingly, when correlation analyses were conducted, the highest correlation was found between the two estrogenic bioassays, while a positive trend was observed between xeno-estrogenic and anti-androgenic activities. This is most likely due to the similar information reported by the E-Screen andthe ER-reporter gene assay, since both of them evaluate the same signaling pathway. Additionally, the positive correlation observed between both estrogenic assays and the anti-androgenic assay is not unexpected since compounds eliciting estrogenic activity often tend to behave as antiandrogens as previously shown, including a potential crosstalk between ER and AR signaling [40–43]. In addition, placentalfractions with the highest estrogenicity also tended to show the lowest AhR activity, which could be due to the inhibitory cross-talk reported between AhR and ER [44]. The XETA results hardly correlated with any other selected bioassays, suggesting that this assay is responsive to chemical families whose biological effects may not be captured by the remaining assays. Despite the observed correlations among bioassays, r^2 coefficients were modest (Fig. 5), suggesting that the variance in biological activity was only partially predicted by the remaining assays, and that consequently, various bioassays are needed to characterize the effects of complex chemical mixtures. To the best of our knowledge, this study represents the first attempt to comprehensively map the biological effects of chemical mixtures present in the same human placental samples.

3.2. *Bioassays as biomarkers of combined effect in epidemiologic studies*

One of the potential applications of these bioassays is their use as biomarkers for characterizing the combined effect of chemical mixturesin human samples. Previous epidemiologic studies have tested the cumulative estrogenic and anti-androgenic activities elicited by α-fractions extracted from placenta samples. For example, a nested case-control study within the Spanish INMA Granada mother-child cohort [27], reported for the first time that children with a higher placental xeno-estrogenicity, assessed by the E-Screen, had higher risk to develop urogenital malformations: cryptorchidism and/or hypospadias [27].Similarly, Arrebola et al. (2015), found that children with higher placental anti-androgenic activity had higher risk to develop the same urogenital malformations [20]. Interestingly, when comparing our results with the abovementioned studies that studied placenta samples collected years ago, the present screening study found similar estrogenic and anti-androgenic combined effects exerted by persistent chemical mixtures extracted from human placentas at similar magnitudes (Figs. 2.A and 2.C). Taken together, the observed biological activities suggest that unborn children are prenatally exposed to complex mixtures of chemicals that may exert potential harmful effects during this critical window of development. In a series of research studies from the Spanish INMA prospective birth cohort assessing placental α-fractions from different Spanish areas (Asturias, Gipuzkoa, Sabadell, Granada and Valencia), higher levels of xeno-estrogenicity were associated with increased birth weight [25], more behavioral problems in boys [24], as well as differences in genome-wide DNA methylation and repetitive elements [45,46]. Taken together, the combined estrogenic activity exerted by placental mixturesof lipophilic chemicals have been associated with several adverse health effects in children, supporting the utility of this approach for generating hypotheses between chemical mixtures and human health. Our current findings complement the abovementioned information, suggesting that bioassays related to different signaling pathways and specific receptor activities (e.g. XETA and AhR) may be needed to comprehensively characterize the whole biological fingerprint of exposure to complex mixtures. For instance, our results showed that placental α-fractions induced a weak AhR activity, which could be due tothe observed decreasing trends in exposure to dioxin and dioxin-like compounds in the Spanish population [47,48]. Moreover, dioxins present in placental α-fractions could exert an anti-estrogenic activity, thus potentially explaining the not significant but inverse dose-responsecorrelation observed between the AhR assay and the ER reporter gene assay, as previously suggested in other studies [49]. Although not in placenta samples, previous studies have used the AhR reporter gene assay in similar contexts (for a recent overview see [29]). In a case-control study using serum samples, higher POPs-induced AhR activity was found in breast cancer cases compared to controls [14]. Another case-control study showed that the activity of the AhR elicited by amniotic fluid extracts was not associated with the risk of autism spectrum disorder (ASD) in children [50].

3.3. *XETA as a potential novel biomarker of combined effect*

Few studies have tested the xenoactivity of mixtures on the thyroidaxis [51,52]; furthermore, the XETA has not been used before to assess the effect of chemical mixtures isolated from human samples. Moreover, there are no validated *in vitro* assays to assess thyroid disruption, and incontrast to the previous endocrine modalities tested, the XETA may detect endocrine disruption arising from multiple levels of regulation [53,54]. In the present screening study, placental α-fractions showed a strong inhibition of thyroid signaling, in line with previous anti-thyroid effects exerted by chemicals mixtures made *ad hoc* based on human amniotic fluid exposure levels [55]. While *ad hoc* mixtures can only test previously known chemicals, extraction protocols in human samples coupled to bioassays are able to assess biological activities of “real-world” mixtures with both known and unknown environmental chemicals. In the current study, the XETA was not correlated with any other implemented bioassays, suggesting that the observed anti-thyroid activity was not related to the remaining biological activities. On the one hand, this is in line with thyroid receptors not being structurally relatedto steroid hormone receptors. On the other hand, the XETA *in vivo* assay presents a higher level of biological complexity compared to *in vitro* models, and observed effects may be driven by diverse action mechanisms not limited to the receptor level of the hypothalamus-pituitary-thyroid axis. XETA results highlight that different biomarkers of combined activity, representing differentsignaling pathways, should be used to fully characterize the cumulativeeffects elicited by complex chemical mixtures in human populations. The XETA has been recently validated at the OECD level (TG248) as a Tier 3 assay [37], and our findings highlight it as a promising biomarkerof combined anti-thyroid effect which should be further investigated in population-based studies, particularly in relation to neurologicalendpoints.

3.4. *Identification of the chemicals responsible for biological effects*

Sample preparation and extraction is critically important, especially when investigating environmental chemicals in human samples. Asextraction methods are rarely optimized for bioassays but rather adopted from chemical analysis, this may result in a misrepresentation of the actual biological activity [15]. HPLC α-fractions obtained from human samples allows the assessment of the combined effects elicited by the most lipophilic and persistent compounds while avoiding the interference of endogenous hormones [26,56,57]. As previously reported, if theendogenous hormones are present in the mixture, they can saturate theendpoint of the *in vitro* or *in vivo* assay. Thus, the biological activity of chemical mixtures would be importantly masked, leading to a reduction in the variability of the signal elicited as well as an

underestimation of the combined effect [32,58]. This is not the case with the extraction protocol used. Several studies have demonstrated the absence of endogenous steroid hormones and identified some chemical families present in the α-fraction: mainly lipophilic and persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs), including lindane, hexachlorobenzene (HCB), dichlorodiphenyltrichloroethane (DDT) and dichlorodiphenyldichloroethylene (DDE), among others [14,27,57,59]. The chemical compounds detected by previous studies in the α-fraction are listed in Supplemental Table 1.

Although some chemical groups have been identified in α-fractions,its full chemical composition has not been elucidated yet, and the number of chemical families and metabolites contained is anticipated tobe much higher. A step forward for both environmental toxicology and epidemiology would be the identification of those chemical compoundsresponsible of such biological effects, including the characterization of both unknown and emerging contaminants. In this regard, it has been proposed that non-targeted chemical analyses can help to decipher the chemical composition of complex lipophilic mixtures isolated from human samples [60]. Indeed, suspect and non-target methodologies arebeing developed inside the HBM4EU project [61]. Interestingly, effect directed analysis (EDA) coupled with non-target chemical analysis can be used to overcome some limitations, allowing an efficient identification of biologically active chemicals through sub-fractionation processes, thus facilitating non-target chemical analysis [62]. Further research is ongoing using EDA coupled to non-targeted chemical analysis on these placental α-fractions in order to characterize the bioactive chemicals responsible for the observed combined biological activities.

3.5. *Strengths and limitations*

A major strength of this study is the application of five different biological assays on the same set of human placentas, mapping different signaling pathways and exploring their relationships. Additionally, this is the first study which has assessed human samples with the *in vivo* XETA, showing a clear anti-thyroid activity, which adds valuable information regarding the effect of lipophilic environmental contaminants on this important pathway. Finally, this is the first attempt to correlate various biological activities exerted by the same mixture of lipophilic compounds extracted from human samples in order to explore potential relationships among them. This study also presents some limitations. The relatively small number of placenta samples analyzed limited our ability to observe correlations among bioassays. Although the α-fractionprovides a good representation of the most lipophilic and persistent compounds, the full chemical exposome was not addressed. In addition to the known chemical families previously detected in the α-fraction, non-targeted chemical methodologies will be needed to unravel its complex chemical composition. Another limitation is that the tested biological assays do not measure the biological effects elicited by mixtures of lipophilic compounds at the same levels of biological complexity. For instance, the *in vivo* XETA evaluated the highest level ofbiological complexity; the E-screen assessed cell proliferation after 6 days of exposure; and the reporter gene assays tested the ability of the mixture to agonize or antagonize a particular nuclear receptor. These disparities and other methodological differences (e.g. different dilutions, medium for reconstitution of dried extracts, etc.) should be considered when interpreting the results of this screening study. Finally, the centralized isolation of α-fractions in the same laboratory under controlled conditions (e.g. glassware material, work inside flow-chambers) limited potential background contamination. These measures, together with the strong and variable biological activities observed for most -but not all- of the placentas in the selected bioassays (estrogenic, anti-androgenic, anti-thyroid and AhR activities), and at different dilutions, makes unlikely that results could be explained in terms of sporadic background contamination.

This work represents a multidisciplinary effort to evaluate human exposure to chemical mixtures by synergizing the toxicological and epidemiological fields. Isolation and assessment of chemical mixtures from human samples may help to identify bioactive chemicals responsible for a given biological activity, as well as identify novel bioactive chemicals through complementary methodologies. In parallel, a batteryof *in vitro* and *in vivo* bioassays should play a crucial role in the assessment of human exposure to complex mixtures, since they can determinethe joint activity elicited by complex mixtures through a specific mode of action [52]. The use of this combined approach together with the information provided by additional biomarkers of effect (e.g. hormone levels, biochemical parameters, etc.) would help to strengthen the weight of evidence in observational studies linking chemical exposures to health outcomes.

4. Conclusions

A panel of different bioassays is needed to provide a comprehensive assessment of "real-world" chemical mixtures. The biomarkers of combined activity investigated in this work enabled us to better characterizethe signaling pathways through which mixtures could elicit adverse health outcomes in humans. Epidemiological studies should also include these bioassays as a complementary tool to improve the causal inferenceof exposure-effects relationships in the context of complex mixtures. Further research is needed to characterize the full composition of complex chemical mixtures present in human samples.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.reprotox.2021.01.002.

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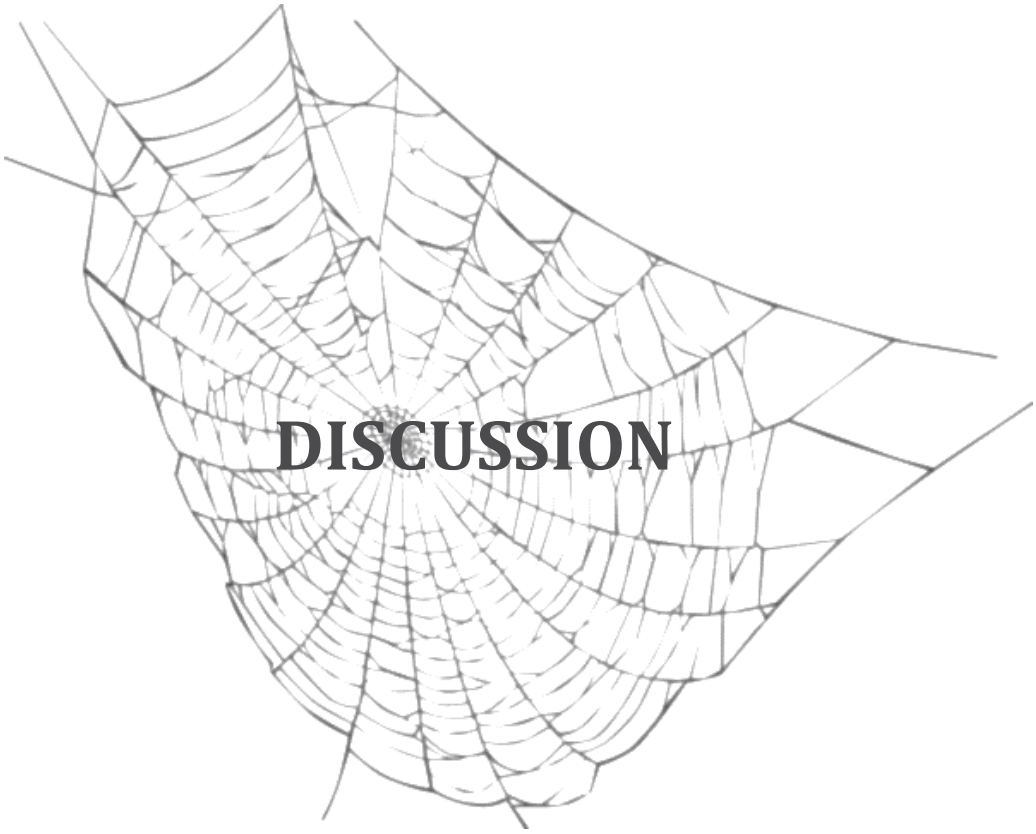


DISCUSSION

6 Discussion

Human biomonitoring (HBM) studies are trying to ascertain the internal exposure to environmental pollutants and their possible adverse effects on human health. Priority has been paid on critical windows of exposure, such as pregnancy, early-, and late childhood, but also adolescence, where little evidence is still available. In this PhD thesis, it has been shown that boys of the INMA-Granada birth cohort are exposed to BPA, non-persistent pesticides, and toxic metals from birth to adolescence. Thus, covering different developmental windows, and confirming previous studies that had already highlighted exposure to different environmental chemical compounds in this Spanish prospective cohort (Freire et al., 2018; Mustieles et al., 2018; Rocio Perez-Lobato et al., 2015).

Urinary BPA concentrations were measured during childhood (9-11 years) of the study subjects. Levels were similar to previous findings in children of the same age and younger populations (Braun et al., 2011; Calafat et al., 2008; Casas et al., 2011; Perera et al., 2012), although higher when compared to other children of the same age (Covaci et al., 2015; Findlay and Kohen, 2015; Hong et al., 2013). Moreover, urinary concentrations of some metals elements As, Cd, Hg, and Pb, measured during adolescence (15-17 years), were within ranges reported in the peripubertal/adolescence population, according to the National Health and Nutrition Examination Survey (NHANES, 2009–2014) and the German Human Biomonitoring Commission; except for of As, which were higher (Sanders et al., 2019; Schulz et al., 2011). Furthermore, some urinary non-persistent pesticides metabolite (also measured during adolescence) IMPy, DETP, and DCCA showed higher concentrations than those reported for children between 6-15 years from Spain and North America (Hernández et al., 2019; Hyland et al., 2019; Quirós-Alcalá et al., 2014; Roca et al., 2014). On the other hand, TCPy, MDA, and 1-N showed lower concentrations than those reported in children from Costa Rica, Spain, Germany, and the US (Hyland et al., 2019; Oulhote and Bouchard, 2013; Roca et al., 2014; van Wendel de Joode et al., 2016), while ETU was within ranges previously reported for French and Latin American children aged 5-9 years living near agricultural fields (Raherison et al., 2019; van Wendel de Joode et al., 2016).

Due to the increment of neurobehavioral alterations, such as autism spectrum disorder, or attention deficit hyperactivity disorders, neurodevelopment has been set as a prioritized health outcome (Grandjean and Landrigan, 2014). Environmental chemical exposure-neurodevelopment relationships have been explored across several epidemiological studies (Bjørling-Poulsen et al., 2008; González-Alzaga et al., 2014;

Perera et al., 2012; Rodríguez-Barranco et al., 2013). Some of them have revealed that exposure to BPA, toxics metals, and non-persistent pesticides is associated with more behavioral problems at different magnitudes (Dalsager et al., 2019b; Hu et al., 2006; Perera et al., 2012). In fact, in a previous study developed within the INMA-Granada cohort (when boys aged 9-11 years), BPA exposure was associated with a worse behavioral function (Perez-Lobato et al., 2015). However, within this doctoral thesis, results from the cross-sectional study evaluating childhood (9-11 years) BPA exposure and cognitive function with the same boys did not support the association between postnatal exposure to BPA and behavior, except for poorer working memory **(article #2)**. Childhood BPA exposure (9-11 years) was nevertheless longitudinally associated with more behavioral problems, concretely thought and somatic problems, later in life during adolescence, when boys were 15-17 yrs. **(article #3)**, confirming prospectively previous results (Mustieles and Fernández, 2020; Perez-Lobato et al., 2015).

Postnatal BPA was recently associated with emotional symptoms but not with cognitive function in 7-8 years old boys from the Polish Mother and Child cohort (Garí et al., 2021). Similarly, prenatal exposure to BPA was not longitudinally associated with cognitive function in boys aged 7 years from the Swedish Environmental Longitudinal Mother and Child, Asthma and Allergy (SELMA) study (Bornehag et al., 2021). Moreover, studies from the New York City (NYC) cohort confirmed a longitudinal relationship between BPA exposure and internalizing behavioral problems, including thought problems, that went from childhood to adolescence (Perera et al., 2016, 2012; Roen et al., 2015). Thus, it seems that BPA tends to be associated with behavior rather than cognitive function (Mustieles and Fernández, 2020).

The observed results of BPA associations with cognitive and behavioral functions could be due to the limited sample size, due to a very low attrition during the follow-up of the birth cohort (n=130). Additionally, sex-depended behavioral domains may be more sensitive to BPA exposure compared to cognitive function (Mustieles et al., 2015; Mustieles and Fernández, 2020). The brain is a sexually dimorphic organ, which morphological differences are permanently shaped under the influence of steroids hormones (estrogen and androgens) during prenatal development (Bao and Swaab, 2011). In experimental studies, BPA exposure increased, decreased, or even suppressed the dimorphic areas, altering sex-specific structural and behavioral patterns (Kundakovic et al., 2013; Nakagami et al., 2009; Negishi et al., 2014). The underlying mechanism by which BPA could exert these effects is through epigenetic alterations on estrogen-androgen balance since BPA is known to influence the gene expression of

estrogen receptors subtypes (ERs α, β, and γ) according to the sex, and the brain area (Bao and Swaab, 2011). A recent review of the Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA) confirmed the abrogation of sex-specific brain dimorphic areas at BPA doses below the considered ‘‘safe threshold’’ for human exposure (Patisaul, 2020). Nevertheless, some experimental studies reported that rodents exposed to BPA showed learning and memory impairments (Gore et al., 2018; Ni et al., 2021). Thus, more studies evaluating BPA exposure on cognitive function are warranted.

The observed pattern of BPA associations with cognitive and behavioral functions reported in this PhD thesis could also be related to methodological factors. For example, epidemiological studies addressing brain disruption due to chemical exposure typically use neuropsychological tests. With these tools, trained psychologists measure cognitive function at an isolated moment in time, and parents report their children’s behavior from a longitudinal perspective (*e.g.,* daily life). However, more sensitive tools are needed to address the effect of environmental exposure beyond the information provided by psychologists, teachers, or parents of the tested subjects. The hypothesis of the present PhD thesis was, therefore, to assess whether the use of novel biomarkers of effect, namely the brain-derived neurotrophic factor, would support the inference of causal relationships between chemical exposure and neurodevelopment.

For this purpose and under the scheme of WP14: effect biomarkers of the European project HBM4EU, we first made a comprehensive review in order to provide a framework of evidence regarding effect biomarkers and BPA exposure **(article #1).** Among the effect biomarkers selected and included in the review, brain-derived neurotrophic factor (BDNF) seemed one of the most promising for addressing neurodevelopmental alterations. The reasons could be because it is highly expressed in the hippocampus, its hippocampal DNA methylation patterns correlate with their peripheral levels in whole blood (useful for HBM purposes), and because impairments in BDNF secretion patterns (measured as DNA methylation, and serum BDNF protein levels) had been associated with cognitive and behavioral disorders (Kundakovic et al., 2015; Wang et al., 2016). Additionally, we were able to map an adverse outcome pathway (AOP) network, which highlighted BPA effects on neurodevelopment through glutamatergic and thyroid mechanisms, identifying three formed AOPs (#12, #13 and #54) correlated with ‘‘decreased BDNF synthesis’’.

According to **AOPs #12** and **#13**, BPA exposure would reduce glutamate intake through the inhibition of N-methyl-D-aspartate receptors (NMDAR). This would

decrease calcium influx, impairing Calcium/calmodulin-dependent kinase II (CaMKII), which plays an important role in BDNF-mediated neuroprotection, as reported *in vitro* (Fan et al., 2006). Additionally, lowered calcium influx also leads to the inactivation of cAMP response element-binding protein (CREB), which target was found to be BDNF in rat models (Wang et al., 2016). Moreover, reduced NMDAR subunits expression in the hippocampus and altered learning and memory cognitive domains were found in male pups mice and rats prenatally exposed to BPA (Tian et al., 2010; Wang et al., 2014; Xu et al., 2010), and in postnatally BPA-exposed female and male mice (Jardim et al., 2017). Finally, prenatal BPA exposure reduced CREB phosphorylation in the hippocampus, decreasing NMDAR2B and BDNF mRNA in male mice (Kundakovic et al., 2015).

On the other hand, **AOP 54** showed that BPA could inhibit the sodium/iodide symporter (NIS), as reported in a rat model (Wu et al., 2016), may by decreasing *Nis* and *tpo* mRNA levels (Silva et al., 2018); reducing the iodine uptake and impairing thyroperoxidase (TPO) activity, decreasing thyroid hormonal (TH) levels, and BDNF secretion. Several experimental studies have found that BPA decreased T4 in pregnant ewes, their offspring, and in aged mice showing impaired cognitive function (Jiang et al., 2016; Viguié et al., 2013). However, Silva et al., (2018), observed increased rather than decreased T4 levels in rats exposed to BPA.

Although the information provided by the AOP network is promising, other possible mechanisms should be highlighted. For example, it is known that NMDAR can be regulated by hippocampal nuclear ER in cells expressing BDNF (El-Bakri et al., 2004; Sohrabji and Lewis, 2006). Experimental evidence suggests that BPA exerts its effects in the hippocampus by estrogenic pathways (Chen et al., 2017; Leranth et al., 2008), altering NMDAR and overlapping with AOP 12 and 13. Thus, NMDAR would be a key event (KE) rather than a molecular initiating event (MIE) if the BPA-hippocampal ER effect is taken into account. Additionally, neuroinflammation caused by the misbalance of redox homeostasis due to BPA exposure is a feasible pathway for neurodevelopmental alteration. Indeed, prenatally BPA-exposed mice embryos showed increased microglia (brain-resident macrophages) and inflammatory markers (IL4 and TNF-α) (Takahashi et al., 2018); moreover, postnatal exposure to BPA increased malondialdehyde, nitric oxide (NO), glutathione peroxidase (GPX), and superoxide dismutase (SOD) in adult rats (Mohamed Eweda et al., 2021).

According to the above evidence framework, in the longitudinal association between BPA exposure and thought problems in adolescents from the INMA-Granada cohort (**article #3**), we found a possible mediating role of DNA methylation of the BDNF

gene in CpG6. BPA exposure increased DNA methylation at the promoter region IV of the BDNF gene (CpG1 and 2) in children (boys, not girls) highly exposed to BPA (Kundakovic et al., 2015). In the same study, offspring from BPA-orally treated pregnant rats also showed changes in Exon IV of hippocampal and blood BDNF gene. Although our results agree with those of Kundakovic et al., (2015), the associations between BPA and CpGs differ between studies. BDNF regulation changes according to developmental stages, which may explain why we found significant results with CpG6 and not with CpGs 1 and 2 (Kowiański et al., 2018). The longitudinal design of our **article #3**, would give greater reliability to the results obtained validating the use of BDNF as a biomarker of neurodevelopmental effect for BPA exposure.

In this doctoral thesis, we have also explored the relationships between exposure to toxics metals, As, Cd, Pb and/or Hg, and behavioral functioning in adolescent boys from the INMA-Granada birth cohort; together with the effect biomarker BDNF that was analyzed at two levels of biological organization (concentration of the protein in serum and urine, and DNA methylation of the BDNF gene). Results from **article #4** show that As and Cd exposure during adolescence may be associated with alterations in behavioral functioning through specific biological mechanisms. Thus, urinary total As concentration was associated with more somatic complaints and more attention problems, and it also tended to be associated with more internalizing problems. Meanwhile, urinary Cd was associated with more externalizing problems, such as social problems and aggressive behavioral problems.

Although neurotoxicity of metal elements has been widely studied, controversial results have been found when exploring postnatal As and Cd exposure on behavioral function. Thus, two epidemiological studies assessing urinary (total and inorganic) As exposure on the behavioral function of 6 to 16 years-old children and adolescents found significant associations with poorer attention and depressive problems (Rodríguez-Barranco et al., 2013; Tolins et al., 2014). Postnatal urinary Cd was also associated with prosocial behavior in children aged 10 years (Gustin et al., 2018), and postnatal new-born Cd hair with more social, withdrawal, and attention problems in children aged 7-to-16 years old (Bao et al., 2009), supporting our findings. However, two systematic reviews found no association between As exposure assessed in children and adolescents aged 5-15 years (Lin et al., 2017; Rodriguez-Barranco et al., 2016, 2013; Tolins et al., 2014); nor between Cd exposure analyzed in serum with behavioral function in children aged 2, 5, and 7 years (Cao et al., 2009). These patterns seem to point towards an association of As and Cd exposure with altered behavioral functioning in adolescents,

although available epidemiological evidence is still limited. Disparities among studies could be explained due to methodological differences, such as the neuropsychological tests used, time of exposure (prenatal *versus* postnatal), or the age of the study population. Additionally, another important source of heterogeneity among studies could be the matrix chosen for exposure biomarker assessment, since urine is the straightforward choice to measure As and Cd (Gil and Hernández, 2015), and not all studies measured them in urine samples.

Unexpectedly, urinary concentrations of Hg and Pb tended to be negatively associated with behavioral function. Concretely, higher levels of Hg were associated with fewer social problems, whereas Pb did not show any association. The neurotoxicity of Hg and Pb on cognitive function and intelligence has been well studied in humans, although little is known regarding their postnatal effects on behavior (Canfield et al., 2003; Cecil et al., 2008; Debes et al., 2006; Freire et al., 2018; Llop et al., 2012; Wright et al., 2008). Some epidemiological studies addressing this issue, found that postnatal blood Pb was associated with more anxiety, social problems, and ADHD in children (Liu et al., 2014; Renzetti et al., 2021; Roy et al., 2009), refuting our findings. Although postnatal hair mercury tended to be associated with fewer behavioral problems in Italian children (Renzetti et al., 2021). A plausible explanation would be the matrix where exposure biomarkers were measured. While urinary Cd and As levels appear to be a suitable choice to detect exposure-effect associations, it may not be the best matrix for Pb and Hg exposure. The short biological half-lives of urinary Hg and Pb make them suitable biomarkers for current or recent exposure (Gil and Hernández, 2015). It is known that blood is the most suitable matrix for Hg and Pb measurement, due to their long half-lives. Conversely, urinary Cd is a biomarker for long-term and lifetime exposure (Gil and Hernández, 2015; Järup and Åkesson, 2009), and although urinary As is considered as adequate short-term exposure biomarker, its concentrations remain relatively stable among individuals with consistent dietary patterns (Hughes, 2006; Marchiset-Ferlay et al., 2012). Consequently, results for Cd and As could be more reliable than Pb and Hg. Nevertheless, it is also possible that Hg and Pb associations could be due to dietary or lifestyle factors, not ruled out in the adjustment models.

Higher concentrations of urinary As tended to be associated with lower serum BDNF protein, as previously reported in an adult cohort (18-60 years old) chronically exposed to As (Karim et al., 2019). Additionally, intermediate concentrations of urinary Cd were associated with less serum BDNF protein and also showed suggestive associations with decreasing BDNF gene DNA methylation among adolescents with the

highest exposure. Both, As and Cd are known to cross the hemato-encephalic barrier, inhibiting hippocampal NMDAR receptors, according to some experimental studies (Karri et al., 2016), and with the AOP 12 and 13. Moreover, the hippocampus is in charge of the emotional response, learning, and memory, all associated with social behavior (Ciranna, 2006), which may explain the results found with As and Cd exposure on behavioral function. If hippocampal NMDAR are inhibited, so does BDNF levels, which may explain the association patterns found for urinary As and Cd concentrations. Indeed, prenatal As-exposed rats exhibited decreased BDNF expression and social isolation-like behavior (Htway et al., 2019), and the same results were also found in male rats pups prenatally exposed to Cd (Mimouna et al., 2018).

Other mechanisms affecting BDNF secretion and thereby mental health could be through alterations in DNA. Previous studies showed DNA methylation alterations after As exposure, possibly through its binding to transcription factors binding sites (TFBS), or blocking DNA repair mechanisms (Demanelis et al., 2019; Karim et al., 2019). High As concentrations were associated with increased percentage BDNF gene DNA methylation and moderate urinary Cd concentrations suggested associations with decreased BDNF gene DNA methylation percentages. Interestingly, increased serum BDNF levels were associated with fewer behavioral alterations (i.e., withdrawn and social, thought, and total problems). The suggestive association of As and Cd exposure with behavioral functioning might be explained by their binding to N-methyl-D-aspartate (NMDA) receptors in the hippocampus (Karri et al., 2016). This would lead to a reduction in BDNF concentrations and consequent behavioral and cognitive impairments, consistent with the adverse outcome pathways (AOPs) described in the revision **article #1, Figure 4** (Rodríguez-Carrillo et al., 2022).

Additionally, experimental studies also reported the imbalance of redox homeostasis exerted by As and Cd as a plausible neurotoxic mechanism of action, which has been reported previously at different levels of biological organization (Karri et al., 2016; Mimouna et al., 2018). No statistically significant relationships were found between Hg or Pb concentrations and percentage BDNF gene DNA methylation or serum BDNF protein concentrations. The absence of associations for Hg and Pb with behavioral function or with BDNF could be due to several reasons: neurotoxic mechanism of action

not involving BDNF secretion patterns, or most probable, the chosen matrix in which Hg and Pb were measured, as discussed previously.

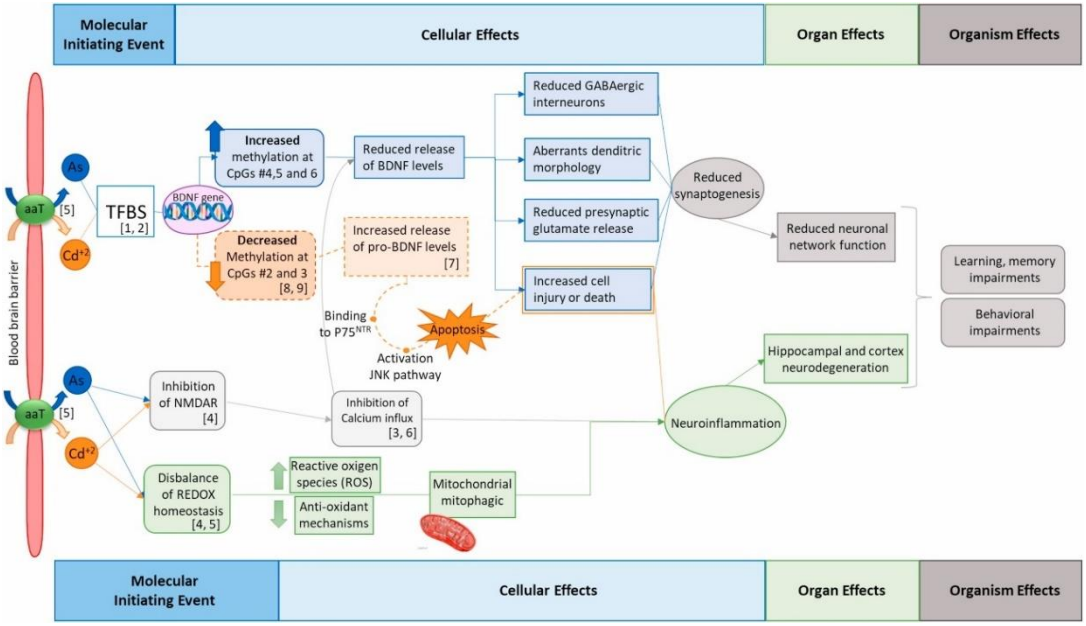


Figure 4. Hypothesized adverse outcome pathway (AOP) based on the AOPs published by Mustieles et al. (2020) and other specific toxicological references for As (Demanelis et al., 2019; Karim et al., 2019; Karri et al., 2016; C. Wang et al., 2016b) and Cd (Guan et al., 2019; Wang and Du, 2013; Xia et al., 2020; Zaletel et al., 2017)(Guan et al., 2019; Wang and Du, 2013; Xia et al., 2020; Xu et al., 2011; Zaletel et al., 2017).

In this PhD thesis, we have also investigated the relationship between exposure to various non-persistent organophosphate metabolites and behavioral functioning among male adolescents of the INMA-Granada birth cohort; including the role of the BDNF as biomarker of effect **(article #5).** Multivariable linear regression models based on tertiles of urinary concentrations of pesticide metabolites showed that adolescents with IMPy concentrations at the third tertile (T3), *versus* first (T1), were associated with more social problems, rule-breaking, aggressive behavior, externalizing, and total problems. An apparent dose-dependent association with more thought problems was also found. Detected *vs.* undetected urinary TCPy was significantly associated with more social and thought problems. When all OPs were considered (∑OPs), adolescent with higher concentrations (T3) were associated with more rule-breaking behavior and externalizing problems. Unexpectedly, DETP at T3 was associated with less withdrawn symptoms.

These findings are partially consistent with some previous epidemiological studies. For example, three epidemiological studies conducted among Egyptian and Latino American subjects (aged 12-21 years) working or living near plantations found

that urinary TCPy levels were associated with deficits in cumulative neurobehavioral performance and higher prevalence of depression, ADHD, irritability, and superficial sensation of abnormality (Ismail et al., 2017; Rohlman et al., 2016; van Wendel de Joode et al., 2016). However, as far as we know, no previous studies have assessed exposure to diazinon (IMPy) on child neurodevelopment. Some *in-vivo* studies found behavioral functioning alterations after diazinon exposure, supporting our data (Hawkey et al., 2020; Shin et al., 2001; Velki et al., 2017).

Pyrethroids' effects on neurodevelopment are controversial (Quirós-Alcalá et al., 2014; van Wendel de Joode et al., 2016). Among male adolescents of the INMA-Granada birth cohort, urinary DCCA suggested associations with more attention problems, rule-breaking, and aggressive behaviors, although without reaching statistical significance. Unexpectedly, detected *vs*. undetected urinary 3-BPA was associated with less somatic, attention, and internalizing problems. When these two pyrethroid metabolites were considered together, no association with behavioral function was found. Some studies performed in North America found that urinary DCCA concentrations were associated with more behavioral difficulties in children aged 6-11 years, and urinary 3-PBA with a higher risk of ADHD among boys aged 8-15 years (Oulhote and Bouchard, 2013; Wagner-Schuman et al., 2015). However, behavioral functioning of North and Latino American children aged 6-15 years old, were not associated with urine 3-PBA, nor with DCCA exposure (Oulhote and Bouchard, 2013; Quirós-Alcalá et al., 2014; van Wendel de Joode et al., 2016). As the neurotoxic potential of pyrethroids exposure is relatively a novel topic, there is a need for further epidemiological studies assessing their postnatal consequences on behavioral function.

Urinary ETU, primary metabolite of the fungicide mancozeb, at both T2 and T3 of exposure, was associated with more social problems and less anxiety problems. Adolescence exposure to 1-N, primary metabolite of the carbamate insecticide carbaryl, was not associated with the behavioral function. The observed associations between urinary ETU levels with more social and less anxiety problems do not agree with the results of a Costa Rican study that found no association between ETU and behavioral problems in children living near banana plantations and assessed at 6-9 years (van Wendel de Joode et al., 2016); although are partially supported by the results of a Mexican study that showed an association between prenatal ETU and more social problems in 1-year-old infants (Mora et al., 2018). Differences in windows of exposure (i.e., prenatal *versus* postnatal exposure) and age at behavioral assessment (infants *versus* adolescents), among others, impair the comparison of findings. Some

experimental studies exploring the effects of mancozeb have also found behavioral alterations among exposed mice, mechanistically supported by decreased relevant hippocampal proteins, such as calcium/calmodulin-dependent kinases II (CaMKII), glutamate receptor 1 (GluR1), and synaptophysin (Lee et al., 2015).

We used a WQS model to explore the mixture effect of the most detected (above 70 % of LOD) pesticide metabolites: IMPy, MDA, DCCA, and ETU. Results suggested associations with withdrawn, social, and thought problems, with MDA and IMPy presenting the greatest influence on the mixture effect (34% and 50%, respectively). The possible association between individual and/or combined exposure to non-persistent pesticides with behavioral could be partly explained by BDNF-mediated mechanisms of action. Thus, Urinary IMPy, MDA, DETP, and ΣOPs were associated with lower serum BDNF in a dose-response manner. We also observed a possible combined effect of non-persistent pesticides leading higher BDNF DNA methylation percentage at CpG 3, 6 and total CpGs methylation, from which MDA showed the highest weight. Interestingly, when tested individually, MDA did not show significant associations with behavioral outcomes or with DNA methylation.

A potential mechanism by which OPs could alter BDNF is based on alterations of the dopaminergic system. The induced inhibition of the tirosine hydrolase (TH) activity would decrease dopaminergic levels, thus down-regulating BDNF (Küppers and Beyer, 2001; Shin et al., 2001). Supporting this, *in vivo* studies reported TH inhibition after diazinon and malathion exposure together with increased behavioral alterations such as anxiety in *Japanese medaka* and rats (Ahmed et al., 2017; Shin et al., 2001). Additionally, exposure to chlorpyrifos decreased the cholinergic system, downregulated BDNF expression, and increased impulsive rates in zebrafish (Perez-Fernandez et al., 2020). Moreover, although 1-N was not associated with behavioral outcomes, it was associated with lower serum BDNF levels. Meanwhile, ETU tended to be linearly associated with lower BDNF levels, higher DNA methylation of the BDNF gene at CpGs 2 and 3, and non-linearly associated with CpGs 4, 5, and total CpGs.

According to experimental evidence, these results could be also explained based on additional adverse pathways. First, and according to AOPs 12 and 13, NMDAR inhibition after permethrin and deltamethrin exposure was shown in previous *in vivo*/*in vitro* models, which down-regulates CREB, involved in BDNF expression, leading to in hippocampal BDNF mRNA repression (Imamura et al., 2000; Zhang et al., 2018). Second, permethrin interacted with DNA methyltransferases (DNMT), altering DNA methylation patterns in vivo (Bordoni et al., 2015). Third, a compensatory mechanism for retardation

of sodium channels voltage-dependent (Nav) activation, down-regulating Nav expression, which has been linked to decreased BDNF expression in some *in vivo* studies with deltamethrin-exposed mice (Imamura et al., 2006; Magby and Richardson, 2017). Finally, increased ROS after cypermethrin and bifenthrin exposure leads to neuroinflammation and long-lasting behavioral impairments in murine models (Gargouri et al., 2018; Nasuti et al., 2007).

Summing up, the consistent results between BPA, As, Cd, IMPY, and TCPy with more somatic and/or thought problems in the INMA-Granada cohort, highlight the potential health implications of the exposure to environmental pollutants at critical windows of development, in this case, childhood and adolescence. The thought problems subscale refers to compulsive behavior and strange ideas, being linked to psychosis during adulthood (Morgan and Cauce, 1999; Paus et al., 2008). Additionally, mania has been linked to higher scores in both thought problems and somatic complaints (Salcedo et al., 2018), as observed in the **article #3** that investigated longitudinal consequences of BPA exposure. The occurrence of these two subscales may indicate higher odds to develop mental disorders during adulthood. Moreover, lower serum BDNF levels were associated with higher exposure to As, Cd, IMPy, MDA, DETP, 1-N, ETU and ΣOPs, in different magnitudes and alterations in the DNA methylation patterns of the BDNF gene were found in adolescents with higher exposure to BPA, As, Cd, MDA, 3-PBA, ETU and ΣOPs. In addition, the effect of exposure to the mixture of IMPy, MDA, DCCA and ETU, analyzed by weighted quintile sum (WQS), was associated with higher percentages of DNA methylation in CpGs 3, 6 and total CpGs.

The importance of assessing the effect of chemical mixtures was evidenced in this PhD. There is, however, scarce information available regarding human exposure to mixtures. Some epidemiological studies have shown that prenatal exposure to mixtures of environmental chemicals was associated with cognitive impairments and higher ADHD risk (Guo et al., 2020; Kalloo et al., 2021; Lenters et al., 2019; Vuong et al., 2020). A recent *in vivo* study found BDNF mRNA up-regulation after exposure to a mixture including chlorpyriphos, cyfluthrin, and deltamethrin (Özdemir et al., 2018). There is also a need for biomarkers of combined effect to understand in more extent the combined effect and biological activities exerted by real-world mixtures, since they are one of the main challenges for environmental toxicology and epidemiology due to their inherent complexity. Biomarkers of combined effect could help to detect and quantify the biological activity exerted by a given mixture and used in epidemiological studies. Moreover, these biomarkers could be used in effect-directed analyses to identify the

active components of a given mixture. Within this PhD thesis, we have comprehensively applied several biomarkers of combined effect to map the biological activities exerted by the same mixture of lipophilic compounds. Thus, to assess the biological effect of chemical mixtures on different biological endpoints, a screening study was performed **(article #6)**, extracting and testing a mixture of lipophilic compounds from 24 placental homogenate samples. Most placental lipophilic fractions (α-fractions) elicited estrogenic activities in the E-Screen and ER reporter gene assays. Additionally, 14 out of 24 placental α-fractions exerted anti-androgenic activities in the androgen receptor (AR) ECO assay. Moreover, most placental samples elicited anti-thyroid function activity in the in vivo XETA, whereas subtle induction of the aryl hydrocarbon receptor (AhR) was found in the AhR gene assay.

Interesting correlations between biomarkers of combined effect were found. The positive correlation between the E-Screen and the ER-reporter gene assay was within expected since both assays evaluated the same pathway. The positive correlation between estrogenic assays and the anti-androgenic assay was also within expectations. Estrogenic compounds often behave as anti-androgen, as previously reported (McLachlan, 2016; Molina-Molina et al., 2008; Panet-Raymond et al., 2000). Additionally, the reported inhibitory cross-talk between ER-AhR may explain that placental α-fractions with the highest estrogenic activities showed the lowest AhR activity (Safe et al., 2000). XETA assay hardly correlated with any other, suggesting that this biomarker could be evaluating biological activities from chemical families not captured by remaining assays. Additionally, it is in line with thyroid receptors not being structurally related to steroid hormone receptors. Overall, r^2 coefficients were modest, suggesting that the variance in biological activity can be only partially predicted by remaining assays. Thus, additional biomarkers are needed to characterize the combined effect of complex mixtures.

Previous epidemiological studies have tested the cumulative estrogenic and anti-androgenic activities elicited by chemical present in placenta extracts. Thus, a nested case-control found a higher risk for cryptorchidism and hypospadia among newborns with the highest placental estrogenicity (Fernandez et al., 2007b). Additionally, a study from the same cohort found a higher risk to develop the same urogenital malformation among children with the highest placental anti-androgenicity (Arrebola et al., 2015). Effects detected in our results exerted similar magnitudes to those reported in the abovementioned studies. Moreover, higher placental xeno-estrogenicity was associated with more behavioral problems in boys (Vilahur et al., 2014b), higher DNA methylation

(Vilahur et al., 2016, 2014a), and higher birth weight (Vilahur et al., 2013) in different subcohorts of INMA (Asturias, Gipuzkoa, Sabadell, Granada, and Valencia). Overall, results suggest that unborn children are prenatally exposed to complex mixtures of environmental chemicals which may be eliciting harmful effects on a critical period of development.

Our study complemented the aforementioned information by providing combined effects on AhR and thyroid function pathways. Our results showed that placental α-fractions elicited a weak induction of AhR activity, which may be due to decreased dioxin-like compounds in the Spanish population (Marquès and Domingo, 2019; Schuhmacher et al., 2019). Interestingly, dioxin-like compounds exert anti-estrogenic activities (Bonefeld-Jørgensen et al., 2014), explaining the non-significant inverse correlation between the ER and AhR reporter gene assays. Some epidemiological studies used this effect biomarker to test 'real-world' mixtures of persistent organic pollutants (POPs) extracted from serum and amniotic fluid. Thus, higher serum POPs-induced AhR activity was found in breast cancer cases compared to controls (Wielsøe et al., 2018). However, no association was found between higher amniotic fluid POPs-induced AhR activity and the risk of ASD in children (Long et al., 2019). On the other hand, there is no evidence of epidemiological studies using XETA for exploring the combined mixture effect on the thyroid axis. Moreover, thyroid function does not have any validated *in vitro* assay to assess its disruption (Couderq et al., 2020; OECD, 2019). Placentas α-fractions elicited strong inhibition of the thyroid function, according to a study using *ad hoc* mixtures of environmental chemicals (Fini et al., 2017). Our study design allows the testing of unknown chemicals substances, thus evaluating a closer real scenario than studies using *ad hoc* mixtures of previously known chemicals. Since XETA is an *in vivo* assay, it can detect biological effects not limited to receptor-level action mechanisms of the hypothalamus-pituitary axis. Thus, our findings highlight this assay as a promising biomarker of combined anti-thyroid effects which should be investigated in epidemiological studies addressing neuronal endpoints.

HPLC α-fractions extracted from human samples enhance the assessment of combined effects elicited by the most lipophilic and persistent compounds without the interference of endogenous hormones (Bonefeld-Jørgensen et al., 2014; Hjelmborg et al., 2006; Rivas et al., 2001). Although it may result in an underestimation of the combined effect, the potential saturation of the in *vivo*/*in vitro* assay due to the presence of endogenous hormones is avoided (Arrebola et al., 2015; Indiveri et al., 2014). Moreover, several studies have demonstrated the absence of endogenous hormones and identified

some chemical families, such as polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs), including lindane, hexachlorobenzene (HCB), dichlorodiphenyltrichloroethane (DDT), and dichlorodiphenyldichloroethylene (DDE), among others (Fernandez et al., 2007b, 2007a; Hjelmborg et al., 2006; Wielsøe et al., 2018). Since the full chemical composition of α-fractions was not elucidated, a step forward would be the identification of all chemical compounds present in the mixture. For this purpose, unknown compounds and emerging contaminants could be detected through non-targeted chemical analyses, as recently proposed (Vinggaard et al., 2021). Effect-directed analyses (EDA) coupled with non-target chemical analyses allow efficient identification of biologically active chemicals through sub-fractionation processes (Figure 5).

This doctoral work has several limitations, some of which are listed below. First, the small sample size of the epidemiological studies performed. Second, environmental chemicals were quantified in spot human samples (mainly urine samples), which may lead to exposure misclassification. However, this limitation would lead to an underestimation rather than an overestimation of the results. Moreover, the matrix chosen to measure some environmental chemicals, such as Hg and Pb, may not be suitable for establishing exposure-effect associations. Third, sex-dependent associations could not be ruled out, as all studies included only male children and adolescents. Fourth, although article #3 had a longitudinal design, articles #2, 4 and 5 are cross-sectional studies, which precludes inferring causality. Fifth, we cannot rule out spurious associations due to multiple comparisons, although several significant associations were supported by the robustness of the model and by both toxicological and epidemiological evidence. On the other hand, limitations of the screening study (**article #6**) were the small number of placentas and that the complete chemical exposome was not identified. Another limitation is that the combined effect biomarkers assessed biological activities at different levels of biological organization. In addition, methodological differences between the assays (e.g.,) should be taken into account when interpreting the results found.

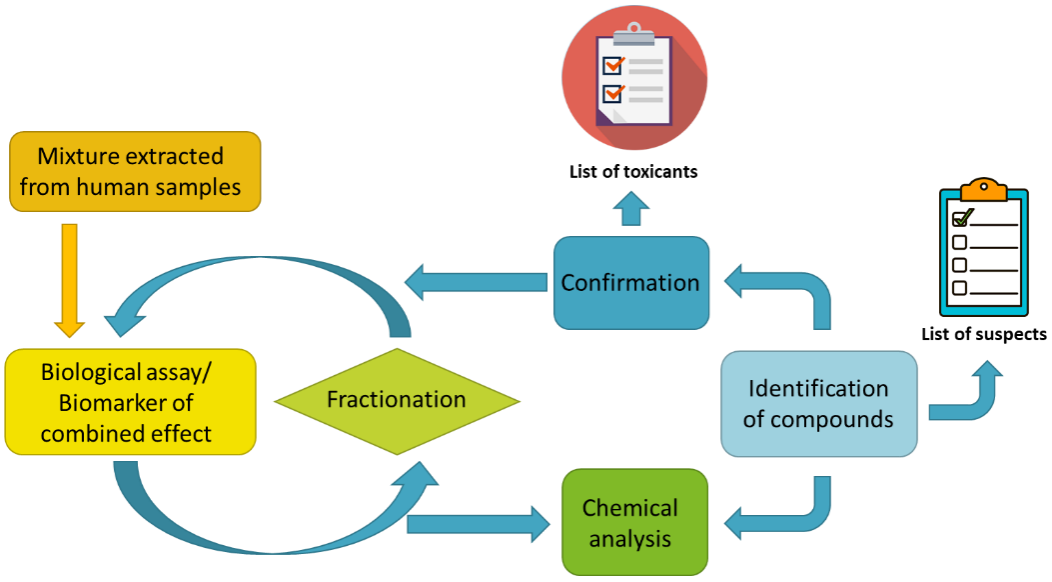
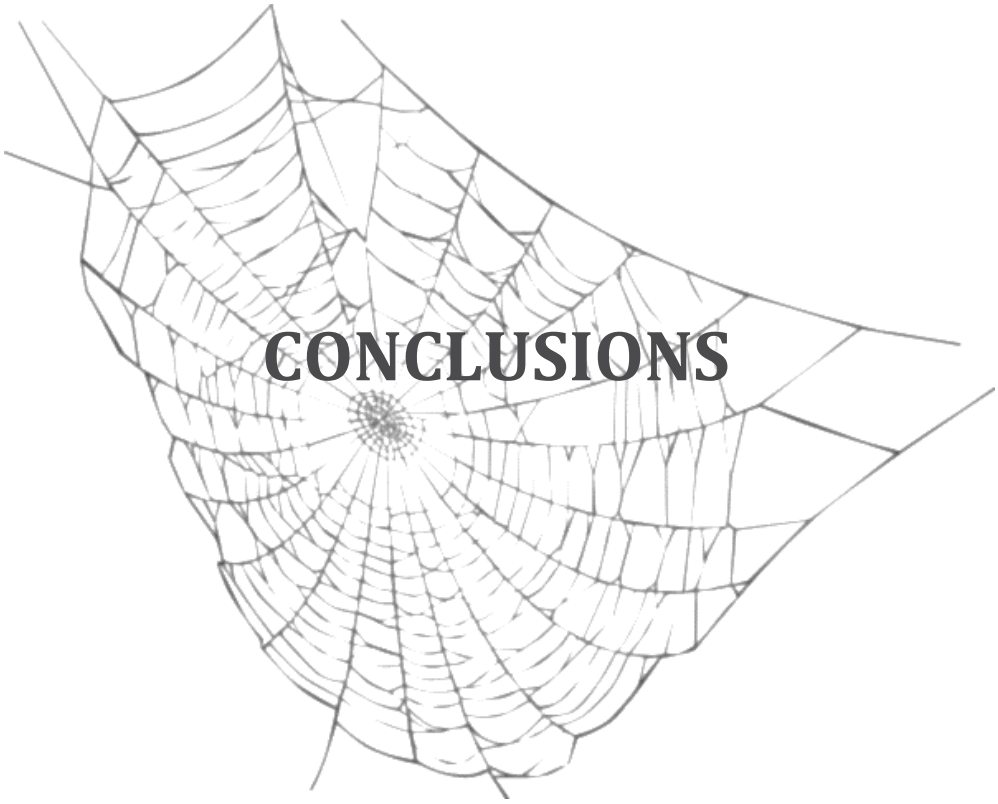


Figure 5. Scheme of effect-directed analysis building blocks. The α-fractions extracted from the placental/relevant matrix are tested in one or more selected in vitro/in vivo assays. The active extract is fractionated into several subfractions and re-tested in vitro. The active fraction undergoes chemical analysis, afterward, tentative identification of chemicals responsible for the activity is performed. When in vivo/in vitro activity of the tentatively identified chemicals is confirmed, a final identification and quantification are performed. The figure is taken from Vinggaard et al., (2021) with minor modifications.

This work also has some strengths, highlighting its contribution to the scarce data available on exposure to environmental pollutants and neurodevelopment through the application of a novel biomarker of effect, BDNF, measured at two levels of biological organization (protein and DNA methylation). Also noteworthy is the selection of three different windows of human susceptibility (prenatal, childhood, and adolescence), where adolescence is the least studied. It is also important to highlight the predefined hypothesis for BPA-neurodevelopmental-BDNF associations through the development of an AOP framework that supported our findings. The complete statistical evaluation of environmental chemical exposure on neurodevelopment and BDNF, as well as conducting mediation analyses highlighted the role of BDNF as a mediator of some exposure-effect associations. Finally, the main strengths of the exploratory study are the multidisciplinary effort to map the biological effects exerted by the same mixture of lipophilic compounds, including the use of XETA in human samples, not previously explored in human samples.



CONCLUSIONS

7 Conclusions

Conclusion 1

The comprehensive literature search has allowed us the creation of the first inventory of existing effect biomarkers for the environmental chemical group of bisphenols, and identified potential novel effect biomarkers for this environmental chemical group (bisphenols) that may be of utility in HBM studies. The implementation and assessment of these mechanistically-based effect biomarkers will help to improve the inference of causal relationships between bisphenols exposure and adverse health outcomes in future HBM and epidemiologic studies. This approach will also help to prioritize the selection of effect biomarkers for BPA substitutes, facilitating the evaluation of potential adverse effects in a timely manner.

Conclusion 2

BPA was found in all urine samples of children in the Spanish INMA-Granada cohort, collected when they were between 9 and 11 years of age. Urinary BPA concentrations were longitudinally and positively associated with thought problems and somatic complaints at adolescence (15-17 yrs.). BPA concentrations were also longitudinally associated with increased BDNF DNA methylation, supporting the biological plausibility of BPA-behavior relationships previously described in the epidemiological literature. Urinary BPA concentrations were not associated with their cognitive abilities, except with poorer working memory. Our results highlight the role of BDNF as a promising, toxicologically supported biomarker of effect on brain function that may help to improve the inference of causal relationships in observational studies addressing environmental exposures and neurodevelopment in children. However, given the modest sample size analyzed in the pilot study and the novelty of these findings, future studies should replicate this results in different settings, developmental windows, and in the context of chemical mixtures.

Conclusion 3

Results of the exploratory study among Spanish adolescent males (aged 15–17 years) aimed to investigate the relationships among exposure to environmental metals, BDNF and behavioral function, suggested a relationship between urinary As and Cd exposure and behavioral problems. Urinary As and Cd concentrations were associated with more internalizing and externalizing problems, respectively. Exposure to As and Cd was also associated with BDNF gene DNA methylation and with serum BDNF. Thus, the

percentage BDNF DNA methylation (at CPGs #5 and total CpG) increased across As tertiles, and 2nd tertile and 3rd tertile of Cd concentrations were associated with lower serum BDNF. Estimation of serum BDNF protein levels and BDNF gene DNA methylation profile might then serve as effect biomarkers to characterize the relationship of postnatal exposure to toxic metals, such as As and Cd, with behavioral problems in adolescents. Caution should be taken when interpreting the results relating postnatal Pb and Hg to behavioral functioning. Nevertheless, due to study limitations, our results need to be verified in future larger epidemiological studies on metal exposures during this and other critical windows of neurodevelopment.

Conclusion 4

We have provided relevant mechanistic and adverse outcome pathway (AOP) information in order to cover knowledge gaps and better interpretation of the complexity of exposure-human health associations. This was particularly evident when assessing the exposure to non-persistent pesticides with cognitive and behavioral function among Spanish adolescent males from the INMA-Granada cohort. Urinary concentrations of IMPy and ETU, metabolites of the (banned) insecticide diazinon and di-thiocarbamate fungicides, were associated with more behavioral problems among adolescents. Additionally, urinary TCPy was associated with more thought and social problems, while MDA and 3-PBA, metabolites of malathion and pyretroids, respectively, were associated with increasing BDNF gene DNA methylation percentages at several CpGs. A possible combined effect of IMPy, MDA, DCCA, and ETU on increasing behavioral problems was also found. Associations found were partly explained by alterations on BDNF at different levels of biological complexity. At the same time, serum BDNF levels were associated with more thought problems and rule breaking behavior, and BDNF DNA methylation percentage at CpG6 with more thought problems. However, longitudinal studies with larger sample sizes are needed to confirm these results.

Conclusion 5

Finally, the use of biomarkers of combined activity allowed us to better characterize the signalling pathways through which mixtures of lipophilic chemical compounds could elicit adverse health outcomes in humans. Thus, most placentas induced ER-mediated transactivation and ER-dependent cell proliferation, together with a strong inhibition of TH signalling and the AR transactivity; while the induction of the AhR was found in only one placental extract. The advantages of using receptor-based in vitro assays are: (i) that an integrated effect is measured, taking combined mixture

effects into account and (ii) that in vitro assays can reduce complexity in identification of toxic compounds and Chemicals of Emerging Concern (CECs) in human tissues. Their implement in epidemiological studies could therefore lead to a paradigm shift in the way we unravel adverse human health effects caused by “real-world” complex mixtures. Further research is needed, however, to characterize the full composition of complex chemical mixtures present in human samples as well as their interactions.

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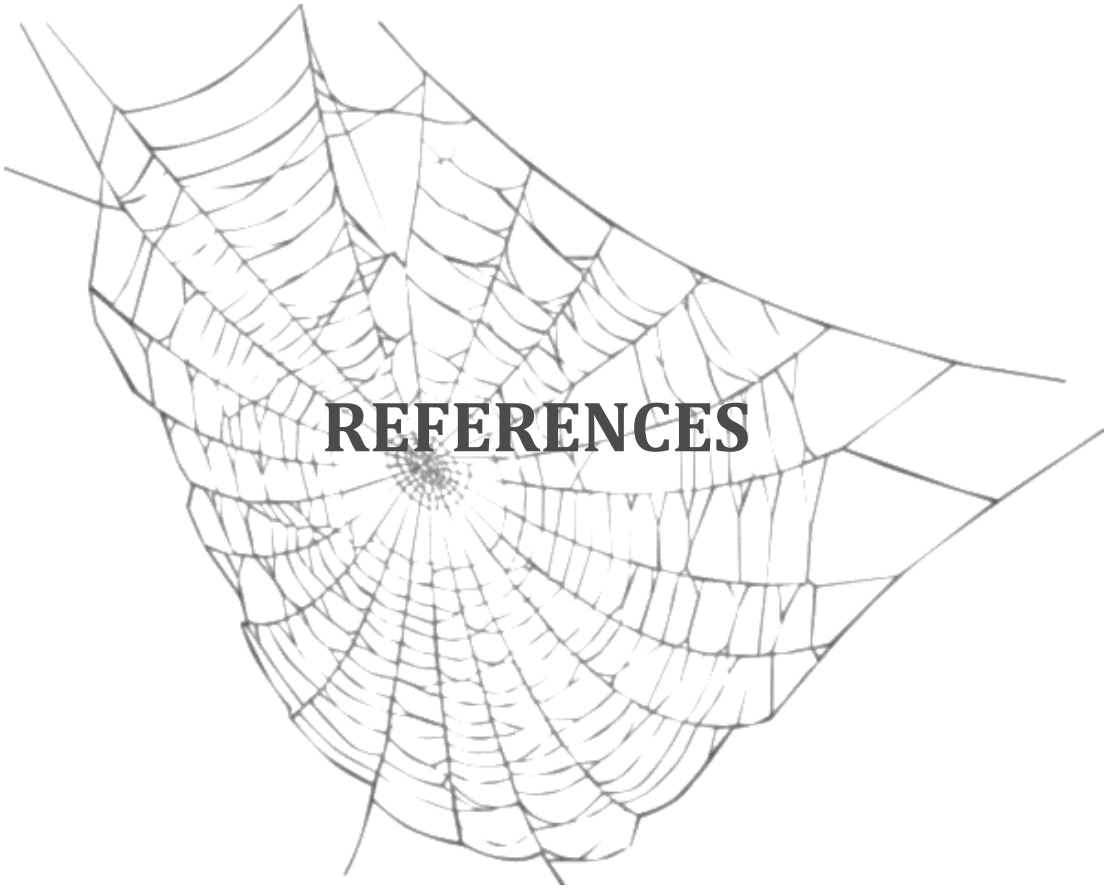
Muchas gracias,

Andrea



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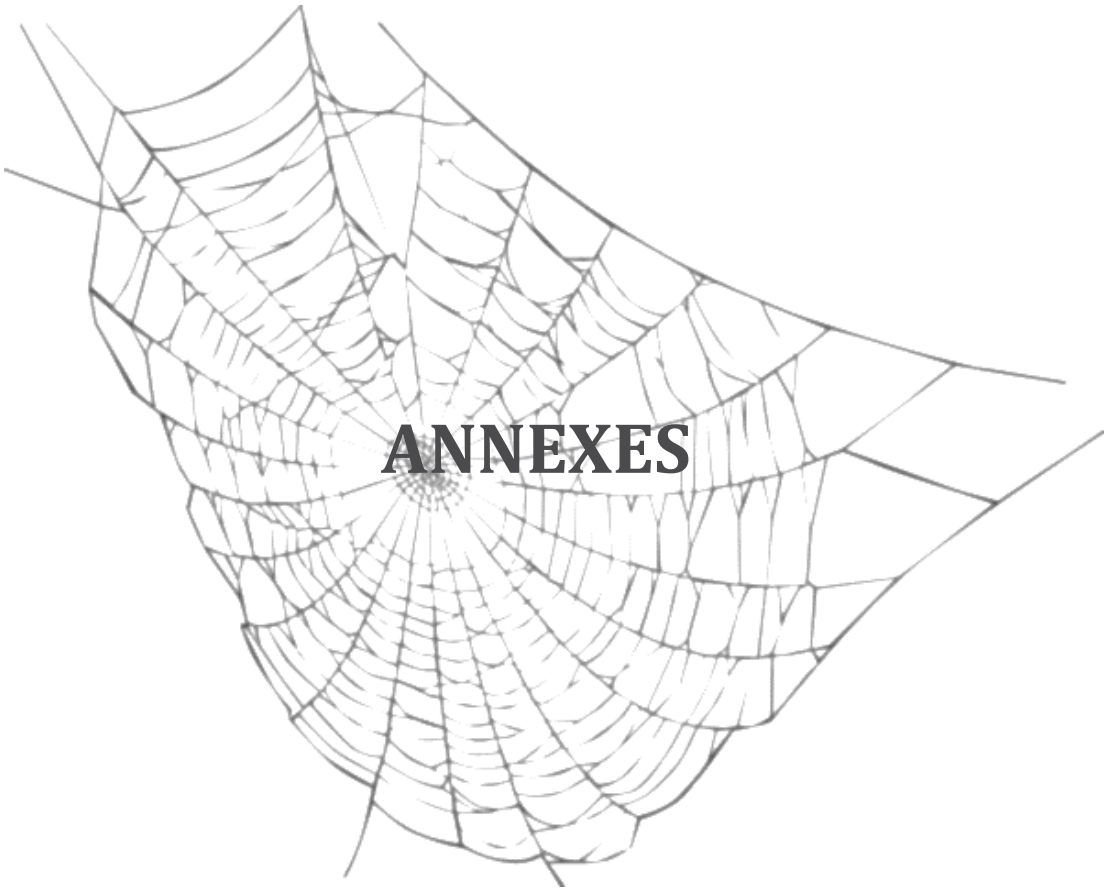
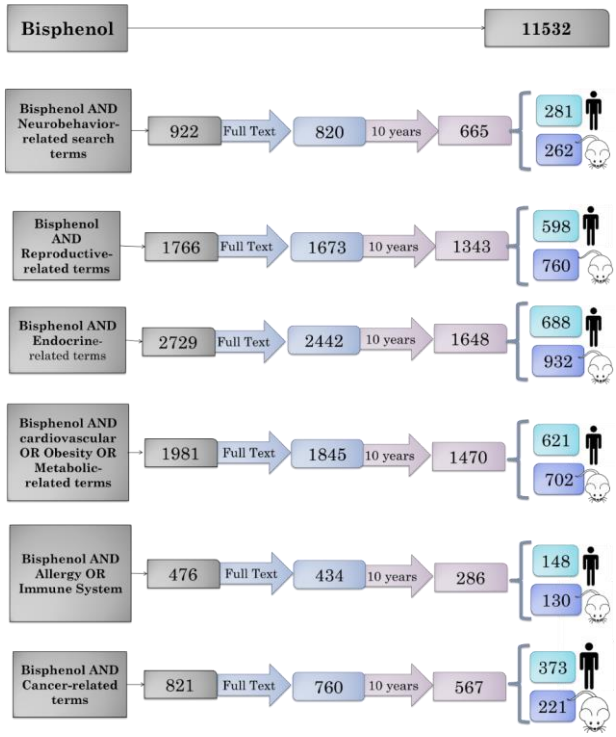


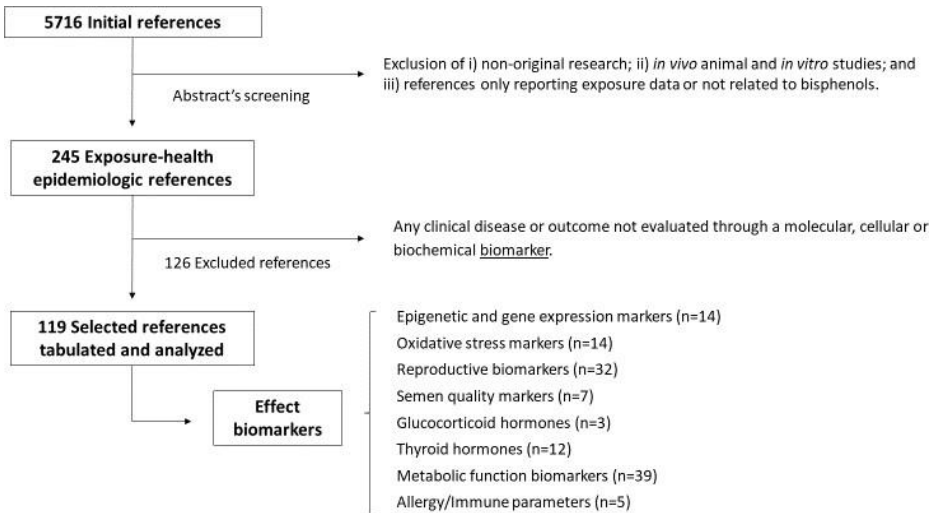
ANNEXES

9 Annexes

9.1 Supplementary Material. Article 1



Supplemental Figure 1. Exploratory search using 'bisphenol' as key exposure search term and search terms for each selected health endpoint. Below is displayed the number of references in either humans or experimental animals found for each health endpoint using MeSH and no MeSH terms, together with PubMed filters "Full text"/ "10 years"/ and "Humans" or "Other animals".



Supplemental Figure 2. Flow-chart showing the final number of references selected. Note: 126 effect biomarkers were retrieved from the selected 119 references

Supplemental Table 1. MeSH and non-MeS H search terms used for both the exposure (bisphenols) and effects (health endpoints), combined with boolean operators.

Search terms for Bisphenols and Health Endpoints	
Health Endpoint	**Bisphenol AND (MeSH Terms OR synonym)**
Behavior/ Neurobehavior	(Bisphenol) AND ((((((((((("Behavior"[Mesh]) OR ("Behavior and Behavior Mechanisms"[Mesh] OR "Reproductive Behavior"[Mesh])) OR "Social Behavior Disorders"[Mesh]) OR ("Child Behavior Disorders"[Mesh] OR "Adolescent Behavior"[Mesh])) OR "Antisocial Personality Disorder"[Mesh]) OR ("Infant Behavior"[Mesh] OR "Spatial Behavior"[Mesh])) OR "Sucking Behavior"[Mesh]) OR ("Sexual Behavior, Animal"[Mesh] OR "Sexual Behavior"[Mesh])) OR ("Paternal Behavior"[Mesh] OR "Maternal Behavior"[Mesh] OR "Impulsive Behavior"[Mesh] OR "Feeding Behavior"[Mesh] OR "Exploratory Behavior"[Mesh])) OR ("Compulsive Behavior"[Mesh] OR "Child Behavior"[Mesh] OR "Behavior, Animal"[Mesh])) OR "Mental Disorders"[Mesh])) OR (Behavior OR Neurobehavior OR Neurodevelopment OR Neurology OR Parkinson OR Alzheimer OR Autism OR Hyperactivity OR ASD OR ADHD OR mental retardation OR IQ loss OR internalizing OR externalizing))
Cancer	(Bisphenol) AND (((((((("Neoplasms"[Mesh] OR "Uterine Cervical Neoplasms"[Mesh] OR "Urologic Neoplasms"[Mesh] OR "Liver Neoplasms"[Mesh] OR "Hereditary Breast and Ovarian Cancer Syndrome"[Mesh] OR "Early Detection of Cancer"[Mesh]) OR ("Urogenital Neoplasms"[Mesh] OR "Testicular Neoplasms"[Mesh] OR "Endometrial Neoplasms"[Mesh] OR "Vaginal Neoplasms"[Mesh] OR "Uterine Neoplasms"[Mesh])) OR ("Prostatic Neoplasms"[Mesh] OR "Ovarian Neoplasms"[Mesh] OR "Endocrine Gland Neoplasms"[Mesh])) OR ("Breast Neoplasms"[Mesh] OR "Neoplasms, Germ Cell and Embryonal"[Mesh] OR "Tumor Microenvironment"[Mesh])) OR ("Thyroid Neoplasms"[Mesh] OR "Pituitary Neoplasms"[Mesh] OR "Brain Neoplasms"[Mesh]))) OR (Cancer OR hormone-dependent cancer OR neoplasm OR malignant tumor OR tumor OR tumour) OR (Colon neoplasms)))
Endocrine	(Bisphenol) AND ("Endocrine System"[Mesh] OR "Endocrine Glands"[Mesh] OR "Endocrine System Diseases"[Mesh] OR "Hormones"[Mesh] OR "Gonadal Hormones"[Mesh] OR "Placental Hormones"[Mesh] OR "Pituitary Hormones"[Mesh] OR "Growth Hormone"[Mesh] OR "Thyroid Hormones"[Mesh] OR "Gastrointestinal Hormones"[Mesh] OR "Sex Hormone-Binding Globulin"[Mesh] OR "Adrenocorticotropic Hormone"[Mesh] OR "Adrenal Cortex Hormones"[Mesh] OR Endocrine system OR hypothyroidism OR hyperthyroidism OR adrenal)
Immune System AND Allergy	(Bisphenol) AND ("Allergy and Immunology"[Mesh] OR "Hypersensitivity"[Mesh] OR "Rhinitis, Allergic, Seasonal"[Mesh] OR "Food Hypersensitivity"[Mesh] OR "Drug Hypersensitivity"[Mesh] OR "Shellfish Hypersensitivity"[Mesh] OR Allergy OR Hypersensitive OR respiratory allergy OR gastrointestinal allergy OR multiple chemical sensitivity OR allergic hypersensitivity disease OR contact allergy OR "Immune System"[Mesh] OR "Immune System Diseases"[Mesh] OR Immune system OR autoimmune disease OR cytokines OR white cells OR innate immune system OR adaptive immune system)
Obesity, Metabolic AND Cardiovascular	(Bisphenol) AND ("Metabolic Syndrome"[Mesh] OR "Nutritional and Metabolic Diseases"[Mesh] OR "Metabolic Diseases"[Mesh] OR "Metabolism"[Mesh] OR "Glucose Metabolism Disorders"[Mesh] OR "Acidosis"[Mesh] OR "Metabolome"[Mesh] OR "Metabolomics"[Mesh] OR "Receptor, Insulin"[Mesh] OR "Lipolysis"[Mesh] OR "Gout"[Mesh] OR "Diabetes Mellitus, Type 2"[Mesh] OR "Acidosis, Renal Tubular"[Mesh] OR "Homocysteinemia" [Supplementary Concept] OR "Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha"[Mesh] OR "Obesity"[Mesh] OR "Pediatric Obesity"[Mesh] OR "Obesity, Abdominal"[Mesh] OR "Abdominal obesity metabolic syndrome" [Supplementary Concept] OR "Cardiovascular System"[Mesh] OR "Cardiovascular Abnormalities"[Mesh] OR "Pregnancy Complications, Cardiovascular"[Mesh] OR "Cardiovascular Diseases"[Mesh] OR "Myocardial Infarction"[Mesh] OR obesity OR abdominal obesity OR waist hip ratio OR adipose tissue OR adypokine OR visceral fat OR body fat OR overweight OR Metabolic OR Metabolic disorder OR Metabolic syndrome OR glucose homeostasis OR Hyperlipidemia OR Dyslipidemia OR hypertriglyceridemia OR HOMA-IR OR insulin resistance OR pancreas OR liver OR kidney)

Reproductive	(Bisphenol) AND (reproductive OR puberty OR pregnancy OR infertility OR semen quality OR placenta OR anogenital distance OR hypospadia OR cryptorchidism OR "Reproductive Health"[Mesh] OR "Reproductive Medicine"[Mesh] OR "Reproduction"[Mesh] OR "Reproductive Techniques, Assisted"[Mesh] OR "Infertility"[Mesh])

Supplemental Table 2. Human biomonitoring studies addressing the relationship between BPA exposure and epigenetic and gene expression biomarkers (n=14).

Study design/Reference	Study population	Bisphenol exposure assessment[a]	Matrix/biomarker(s)	Analytical method	Main results
			Aborted Fetuses		
Fetal tissue biobank (USA) / Weinhouse et al. (2015)	Human fetal liver tissue at gestational day 74-120 (n=50)	Free and total BPA concentrations in liver tissue using HPLC-MS/MS following QA/QC procedures	DNA methylation in fetal liver	DNA methylation (*ESR1, IL-6, and STAT3*) in human liver samples were carried out by bisulfite sequencing method	*STAT3* DNA methylation displayed non-monotonic dose-responses to total and free BPA concentrations in human fetal liver samples
Fetal tissue biobank (USA) / Nahar et al. (2014)	Human fetal livers from elective abortion from 1st and 2nd trimester of pregnancy (gestational day 74-120)	Free and total BPA concentrations in liver tissue using HPLC-MS/MS following QA/QC procedures	- DNA methylation in fetal liver	DNA methylation by bisulfite conversion and Epityper Methylation Analysis	Higher BPA concentrations were associated with increased site-specific methylation at the catechol-O-methyltransferase gene
Fetal tissue biobank (USA) / Nahar et al. (2015)	Matched placenta, liver and kidney (n=12) from 2nd trimester human fetuses	Free and total BPA concentrations in placenta, liver and kidney using HPLC-MS/MS following QA/QC procedures	- DNA methylation in placenta, liver and kidney	Global methylation was analyzed by pyrosequencing	Positive association between BPA concentration and DNA methylation (LINE1 hypermethylation) in placenta

Fetal tissue biobank (USA) / Faulk et al. (2016)	Human fetal liver tissue (n=18)	Free and total BPA concentrations in liver tissue using HPLC-MS/MS following QA/QC procedures	-	DNA methylation in fetal liver	DNA samples enriched in methylated CpG sites using the MethylPlex kit	Hypomethylation of transposons and repetitive elements such as LINEs, LTR elements, DNA elements, and satellites in medium BPA exposure group when compared to low and high BPA groups
Perinatal/ Children/ Adolescent studies						
Mother-child prospective cohort (USA) / Kundakovic et al. (2015)	21 males and 22 females with maternal prenatal urinary BPA <1 µg/L and 19 males and 19 females with urinary BPA >4 µg/L	BPA measured in one maternal spot urine sample during the third trimester of gestation using HPLC-MS/MS following QA/QC procedures	DNA methylation in cord blood	DNA methylation by using pyrosequencing assay	Prenatal exposure to higher BPA concentrations was associated with altered BDNF IV DNA methylation at two CpG sites in the human cord blood. A significant effect of high BPA exposure on CpG1B (a site that lies exactly within the CREB-binding site) methylation levels was observed in boys when compared to girls
Pilot study (Egypt) / Kim et al. (2013)	Healthy females aged 10-13 years living in rural (n=30) and urban (n=30) areas	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC procedures	Genome-wide DNA methylation in Saliva	Genome-wide DNA methylation was assessed with the Infinium HumanMethylation27 BeadChip (Illumina)	High urinary BPA concentrations are associated with DNA hypomethylation of *BRCA1, BEX2, HOXA10* genes in prepuberscent girls
Adult studies						
Case-control occupational study (China) / Miao et al. (2014)	Control n=72, epoxy resin workers exposed to BPA n=77	Two spot urine (pre- and post-shift) analyses in exposed group and one spot urine in unexposed group. HPLC-MS/MS. No mention to QA/QC.	LINE-1 methylation in sperm and peripheral blood DNA	LINE-1 methylation was measured with a methylation-specific RT-PCR	A positive association between sperm LINE-1 methylation and urinary BPA concentrations. No association with urinary BPA concentrations and peripheral blood LINE-1 methylation
IVF Pilot study (USA) / Hanna et al. (2012)	58 women with mean age 36	Mercury, lead, cadmium and free BPA in one serum sample using HPLC with Coularray detection.	DNA methylation in peripheral blood	DNA methylation was assessed by using Illumina GoldenGate Cancer Panel I bead array	Free BPA concentration in serum of women undergoing IVF was found to have correlation with hypomethylation of promoter CpG site of *TSP50* gene

Case-control occupational study (China) / Zheng et al. (2017)	Control n=30, workers exposed to BPA n=26	Two spot urine (pre- and post-shift) analyses in exposed group and one spot urine in unexposed group. HPLC. No mention to QA/QC.	- 5-hydroxymethylcytosine (5-hmC) in sperm	- Hydroxymethylated DNA immunoprecipitation (hmeDIP) followed by sequencing	A genome-wide increase in 5hmc rate in the sperm from BPA-exposed men was observed
Case-control study (Italy) / De Felice et al. (2015)	n=40 placenta (subjected to therapeutic abortion) from women living in polluted and n=40 non-polluted area	Placental free BPA concentrations using GC-MS. Procedure fully explained.	- Genome-wide micro-RNA expression in placentas	MicroRNA profiles (1349 miRNAs) were measured by microarray technology	A strong association was observed between BPA concentrations in the placenta and miR-146a upregulation
Prospective cohort (USA) / Li et al. (2015)	n=110 placentas collected from term delivery from 13 counties across the USA	Free BPA concentrations in placenta using HPLC-MS/MS. Some quality measures described.	- miRNA expression profiling in placentas	miRNA profiling was done using the Counter human miRNA expression assay	No association was observed between the miRNA expression levels and BPA concentrations in placenta
			Gene-expression studies		
Case-control study (Italy) / La Rocca et al. (2015)	Infertile men from three different geographic areas - metropolitan (n=28), medium-sized urban area (n=19) and rural area (n=23). Control men from the same area (n=34 metropolitan, n=41 medium-urban and n=8 rural)	Total BPA in one serum sample using HPLC-MS/MS following QA/QC procedures	Gene expression of nuclear receptors (*ESR1, ESR2, AR, PXR, AhR, PPARγ*) in blood and semen -	- Gene expression analyses using RT-PCR	Serum BPA concentrations were positively correlated with *ESR1, ESR2, AR, AhR and PXR*. No correlation was found with *PPARγ*
Cross-sectional analysis in a prospective cohort (Italy) / Melzer et al. (2011)	InCHIANTI study (n= 100) men aged less than 76 years.	BPA in one spot urine sample using HPLC-MS/MS following detailed QA/QC procedures	- *ESR1*, *ESR2*, *ESRRA, ESRRB*, *ESRRG*, and *AR* genes in blood leukocytes	- Gene expression analyses using RT-PCR	Higher BPA concentrations induced higher expression of two estrogen-responsive genes, *ESR2* and *ESRRA*.
Prospective mother-infant cohort (China) / Xu et al. (2015)	192 mother-infant pair from e-waste recycling town and 70 from reference area	Free BPA measured in cord blood serum using GC-MS. No reference to QA/QC	- *KISS*, leptin and leptin receptor genes in placenta	- Gene expression analyses using RT-PCR	Higher BPA concentrations showed positive correlation with KISS1 gene expression. Additionally, BPA showed positive correlation with leptin and leptin receptor gene expression

[a] Total BPA (free plus conjugated) in urine, total and/or free BPA in blood, and total and/or free BPA in tissue. **Abbreviations:** Androgen receptor (*AR*); aryl hydrocarbon receptor (*AhR*); brain-derived neurotrophic factor (*BDNF*); brain expressed X-linked 2 (*BEX2*); breast cancer 1 (*BRCA1*); catechol O-methyltransferase (*COMT*); estrogen Receptor 1 and 2 (*ESR1 and 2*); estrogen related receptor alpha (*ESRRA*); gas chromatography tandem mass spectrometry (GC-MS/MS); homeobox A10 (*HOXA10*); high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS); *in vitro* fertilization (IVF); long interspersed element-1 (*LINE-1*); quality assurance and quality control (QA/QC); real-time polymerase chain reaction (RT-PCR); signal transducer and activator of transcription 3 (*STAT3*); sulfotransferase family 2A member 1 (*SULT2A1*); testis-specific protease-like protein 50 (*TSP50*).

Supplemental Table 3. Human biomonitoring studies addressing the relationship between BPA exposure and oxidative stress biomarkers (n=14).

Study design/Reference	Study population	Bisphenol exposure assessment[a]	Matrix/biomarker(s)	Analytical method	Main results
Perinatal and children studies					
Cross-sectional study on mother/fetus pairs (Taiwan) / Huang et al. (2018)	A total of 241 mother-fetus pairs	BPA measured in one spot urine sample (between weeks 27 and 38 of gestation) using HPLC-MS/MS. Methodological details described.	Third trimester maternal urine: 8-OHdG, 8-NO_2Gua, 8-isoprostane and HNE-MA levels Maternal and umbilical cord blood plasma: GPx, CRP, IL-6, TNF-α levels	8-OHdG, 8-NO_2Gua, 8-isoprostane and HNE-MA (LC-MS/MS). GPx, IL-6 and TNF-α (ELISA). CRP (infrared particle immunoassay rate methodology)	Positive associations between third trimester maternal BPA and 8-isoprostane levels, but no associations between BPA and 8-OHdG, HNE-MA or 8-NO_2Gua levels were found in pregnant women. BPA concentrations were inversely associated with maternal and cord blood plasma GPx levels
Cross-sectional study on children (China) / Lv et al. (2016)	96 children (59 boys, 37 girls) aged 3-6 years	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC procedures	Urinary 8-OHdG levels	8-OHdG (Q-Trap LC-MS/MS)	A significant positive association was found between BPA and 8-OHdG levels after log-transformation of creatine-adjusted data
Cross-sectional study during pregnancy (USA) / Veiga-Lopez et al. (2015)	24 women (12 having low and 12 high free BPA)	Free and conjugated BPA measured in maternal plasma at first trimester and pair-matched umbilical cord plasma at term. HPLC-MS/MS following QA/QC measures.	Plasma 3-NO_2Tyr, 3-ClTyr and diTyr levels that are specific for RNS, HOCl and ROS, respectively	3-NO_2Tyr, 3-ClTyr and diTyr (LC-MS/MS)	A positive association between BPA and 3-NO_2Tyr levels (but not 3-ClTyr and diTyr) was found in plasma from pregnant mothers and umbilical cords. High BPA was also associated with increased palmitic acid levels (a free fatty acid) in plasma, and palmitic acid correlated positively with 3-NO_2Tyr
Mother-child cohort (Puerto Rico) / Watkins et al. (2015)	106 pregnant women (aged 18-40 years) at less than 20 weeks gestation (105 had inflammatory and 54 had oxidative stress biomarkers analyzed)	Mean urinary BPA concentrations measured at three time points during pregnancy using HPLC-MS/MS following QA/QC procedures	Urinary 8-OHdG. Urinary 8-isoprostane. Blood plasma inflammation markers (IL-1β, IL-6, IL-10, TNF-α, or CRP)	8-OHdG (enzyme immunoassay), 8-isoprostane (affinity purified followed by enzyme immunoassay), CRP (ELISA), cytokines (Milliplex MAP high sensitivity human cytokine magnetic bead panel)	An interquartile range increase in BPA was associated with 29% higher 8-isoprostane levels and 21% higher 8-OHdG levels. No association was found between BPA and any of the inflammation markers (IL-1β, IL-6, IL-10, TNF-α, or CRP)

General population/ Occupational settings

Cross-sectional study (Saudi Arabia) / Asimakopoulos et al. (2016)	130 healthy persons (both genders) aged 1-87 years (median age 35 years)	Urinary bisphenols measured in one spot urine sample (BPA, BPAF, BPAP, BPS, BPF, BPP, BPZ, BPB) using HPLC-MS/MS following QA/QC procedures	Urinary 8-OHdG levels	8-OHdG (LC-MS/MS)	BPA and BPS metabolites were significantly associated with 8-OHdG levels. All 8 BPs together were positively associated with 8-OHdG levels
Cross-sectional study on urban residing adults (South Korea) / Hong et al. (2009)	960 adults (446 men, 514 women)	BPA measured in one spot urine sample using HPLC-MS/MS. No reference to QA/QC procedures	Urinary 8-OHdG and MDA levels	8-OHdG (ELISA), MDA (MDA-TBA adducts by HPLC-UV)	8-OHdG and MDA levels were not associated with BPA in Korean urban residing adults
Cross-sectional study on adults (South Korea) / Yang et al. (2009)	259 men, 92 pre- and 134 postmenopausal women	BPA measured in one spot urine sample using HPLC-MS/MS. Some quality aspects described.	Urinary 8-OHdG and MDA levels. Serum CRP levels	ELISA (8-OHdG), TBARS (MDA), LTIA (CRP)	BPA was positively associated with 8-OHdG, MDA, and CRP levels (only in one of three statistical models) in postmenopausal women, but not in men and premenopausal women
Cross-sectional study on people living in and around e-waste dismantling facilities (China) / Zhang et al. (2016)	116 residents in the e-waste recycling region Longtang Town, Qingyuan City (66 males, 50 females) aged 0.4-87 years. 22 in rural reference area (80 km northwest of Longtang) 20 in urban reference area in Guangzhou (60 km southeast of Longtang)	Urinary bisphenols (BPA, BPAF, BPAP, BPS, BPF, BPP, BPZ, BPB) using HPLC-MS/MS following QA/QC procedures	Urinary 8-OHdG levels	8-OHdG (LC-MS/MS)	In the e-waste dismantling location, urinary BPA and BPS, but not BPF, were positively associated with urinary 8-OHdG levels. A similar correlation was also observed for the combined data from the two reference areas

Case-control study – autistic children					
Case-control study on oxidant/antioxidant status in autistic children (Turkey) / Kondolot et al. (2016)	27 classic autism, 10 pervasive developmental disorder-not otherwise specified (PDD-NOS), and 35 control children. Mean age was ~5.7 ± 2.5 years Males: ~80% in study cases and ~78% in controls	Total blood plasma BPA concentrations were measured using HPLC-UV detector.	Plasma lipid peroxidation products. Plasma protein carbonyl levels. Erythrocyte antioxidant enzyme activities (GPx1, TrxR, CAT, SOD and GR), GSH, and Se levels (co-factor in some antioxidant enzymes)	Lipid peroxidation products (TBARS assay kit followed by spectrofluorometric assay), antioxidant enzyme activities, GSH (various kinetic assay kits) and Se levels (AAS). How plasma protein carbonyls were analyzed is not described	The group diagnosed PDD-NOS had higher BPA concentrations than the controls and classic autism groups and there were also other significant group differences. However, no association between BPA concentrations and TBARS or protein carbonyls was found. Also, no association between BPA concentrations and erythrocyte GPx1, TrxR, CAT, SOD, GR activities, GSH or Se levels, was found
Case-control study – COPD patients					
Case-control study on chronic obstructive pulmonary disease (COPD) (Turkey) / Erden et al. (2014b)	50 COPD patients (61.6 ± 11.2 years; 44 males/6 females) 33 controls (57.6 ± 11.1 years; 29 males/4 females)	Free serum BPA concentrations using HPLC-UV detector. Some details described. No mention to QA/QC.	Serum CRP, MDA and total thiol levels	CRP (nephelometric method), MDA (reaction with TBA followed by spectrophotometric analysis), total thiol levels (sulfhydryl groups react with DTNB followed by spectrophotometric analysis)	In serum of COPD patients, BPA concentrations were significantly higher, and total thiol levels significantly lower, than in controls. Serum MDA did not differ between the groups. A non-significant linear relationship between BPA and CRP was found
Intervention studies - Women					
Intervention study (single blinded randomized clinical trial) in young women investigated for gynecological complaints (South Korea) / Yang et al. (2014)	11 given Korean red ginseng (KRG, 22.91 ± 1.81 years) 11 given placebo (22.73 ± 1.68 years)	BPA measured in one spot urine sample before and after the intervention using GC-MS/MS. No reference to QA/QC	Urinary MDA levels	MDA (MDA-TBA adducts by HPLC-UV)	Urinary total BPA and MDA levels were positively associated. Intervention with KRG decreased both BPA and MDA levels
Intervention study - young women given wheat sprout juice for 14 days (South Korea) / Yi et al. (2011)	14 women, aged 24.4 ± 4.0 years	Urinary BPA measured before and after intervention using HPLC with fluorescence detector. Some details provided. No reference to QA/QC	Urinary 8-OHdG and MDA levels	8-OHdG (HPLC-ECD), MDA (MDA-TBA adducts by HPLC-UV)	BPA concentrations were positively associated with 8-OHdG (not significant) and MDA levels (significant). Wheat sprout juice intake lowered BPA concentrations

Genetic polymorphisms

Longitudinal panel study on elderly and their liver function (South Korea) / Kim et al. (2016)	471 elderly (≥60 years), both genders	Urinary BPA and oxidative markers were repeatedly assessed in spot urine samples up to 5 visits. HPLC-MS/MS following QA/QC procedures	SNPs in *COX2*, *EPHX1*, *CAT* and *SOD2* genes	Blood lymphocyte genomic DNA (PCR)	Significant associations (as significant odds ratios) of BPA with abnormal liver function was found in Koreans with certain polymorphisms in *COX2*, *CAT*, *EPHX1* and *SOD2* genes
Longitudinal panel study on elderly (South Korea) / Kim and Hong, (2017)	548 elderly (≥60 years, mean age 70.8 years), both genders	Urinary BPA and oxidative markers were assessed in spot urine samples up to 5 visits. HPLC-MS/MS following QA/QC procedures	Urinary MDA levels, SNPs in *COX2*, *EPHX1*, *CAT*, *SOD2*, *HSP70-hom*, *PON1*, *eNOS*, *DRD2* and *MPO* genes	MDA (MDA-TBA adducts by HPLC-UV), blood lymphocyte genomic DNA (PCR)	A positive association was found between urinary BPA with MDA levels in both males and females regardless of any genotype of the nine oxidative stress-related genes

[a] Total BPA (free plus conjugated) in urine, total and/or free BPA in blood, and total and/or free BPA in tissue.

Abbreviations: 2,2-dithiobisnitrobenzoic acid (DTNB or Ellman's reagent); 3-chloro-tyrosine (3-ClTyr); 3-nitro-tyrosine (3-NO_2Tyr); 4-hydroxy-2-nonenal-mercapturic acid (HNE-MA); 8-hydroxy-2'-deoxyguanosine (8-OHdG); 8-iso-prostaglandin F2α (8-isoprostane); 8-nitro-guanine (8-NO_2Gua); atomic absorption spectrometer (AAS); catalase (CAT); C-reactive protein (CRP); cyclooxygenase2 (COX2 or PTGS2); dopamine receptor D2 (DRD2); electrochemical detection (ECD); electronic waste (e-waste); endothelial nitric oxide synthase (eNOS); enzyme-linked immunosorbent assay (ELISA); epoxidehydrolase1 (EPHX1); gas chromatography tandem mass spectrometry (GC-MS/MS); glutathione (GSH, reduced form); glutathione peroxidase (GPx); glutathione reductase (GR); heat shock protein 70-hom (HSP70-hom); high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS); hypochlorous acid (HOCl); interleukin (IL); latex turbidimetric immuno assay (LTIA); malondialdehyde (MDA); myeloperoxidase (MPO); o,o'-di-tyrosine (diTyr); paraoxonase 1 (PON1); polymerase chain reaction (PCR); quality assurance and quality control (QA/QC); reactive nitrogen species (RNS); reactive oxygen species (ROS); selenium (Se); single nucleotide polymorphism (SNP); superoxide dismutase (SOD); thiobarbituric reactive substance (TBARS); ultraviolet radiation (UV); thioredoxin reductase (TrxR); tumor necrosis factor alpha (TNF-α).

Supplemental Table 4. Human biomonitoring studies addressing the relationship between BPA exposure and reproductive hormone biomarkers (n=32).

Study design/Reference	Study population	Bisphenol exposure assessment[a]	Matrix/biomarker(s)	Analytical method	Main results
Perinatal and children studies					
Ongoing prospective cohort of pregnant women (Puerto Rico) / Aker et al. (2016)	106 pregnant women, Recruitment: 2010-2012	Maternal BPA concentration in spot urine samples at each trimester of gestation using HPLC-MS/MS following QA/QC procedures	Serum E2, P4 and SHBG levels at first and third trimester of gestation	Chemiluminescence immunoassay	No significant associations were observed
Mother-child study (China)/ Liu et al. (2016)	137 mother-infant pairs. Recruitment: 2010-2011	Maternal BPA concentration in one spot urine sample collected during the third trimester of gestation using GC-MS. Some quality aspects described.	Cord blood sex hormones: TT, E2 levels. The T/E2 ratio was estimated. Anogenital distance (AGD). Anogenital index (AGI) = AGD (mm)/neonate weight (kg)	Radioimmunoassay (RIA)	Higher maternal urinary BPA concentrations were associated with decreased TT and T/E2 ratio at birth among male neonates No association between BPA and AGD or AGI
Prospective cryptorchidism case-control study (France) / Chevalier et al. (2015)	52 boys with cryptorchidism. 128 control boys. Recruitment: 2002-2005	Free BPA concentration was measured in cord blood at birth using RIA and following some quality controls including comparison with HPLC-MS/MS method.	Cord blood INSL3 and TT levels	INSL3 level was measured by ELISA. TT was measured by LC/MS-MS	Among all boys, higher cord blood BPA concentrations were associated with reduced cord blood INSL3 levels, but not TT levels Cord blood BPA concentrations did not differ between cases and controls
Mother-child cohort (Mexico) / Ferguson et al. (2014)	118 mother-son pairs	Maternal BPA concentration in spot urine samples during the third trimester of gestation using HPLC-MS/MS following QA/QC procedures BPA was also analyzed in one spot urine sample from boys at 8-14 years of age	Serum E2, TT, INHB, SHBG and DHEA-S levels in boys. fT was estimated using TT and SHBG	DHEA-S, E2, SHBG, and TT levels were measured using an automated chemiluminescent immunoassay Active INHB was assayed using ELISA	Prenatal BPA exposure was not clearly associated with any of the hormones measured Childhood BPA exposure tended to be positively associated with SHBG and inversely associated with TT and fT levels
Mother-child cohort (Mexico) / Watkins et al. (2017)	109 mother-son pairs	Maternal BPA concentration in spot urine samples from first, second and third trimester of gestation using HPLC-MS/MS following QA/QC procedures	Peripubertal boys (8-14 years of age) provided fasting blood samples. Serum E2, TT, INHB and SHBG as biomarkers of puberty.	DHEA-S, E2, SHBG, and TT levels were measured using an automated chemiluminescent immunoassay Active INHB was assayed using ELISA	*In utero* BPA exposure was not associated with hormone levels in boys, except for a positive association between BPA concentrations during the second trimester and INHB levels

			DHEA-S as a biomarker of adrenarche		
Mother-child cohort (Mexico) / Watkins et al. (2014)	129 mother-daughter pairs	BPA was measured in one spot urine sample from daughters at 8-13 years of age using HPLC-MS/MS following QA/QC procedures	Serum E2, TT, INHB, SHBG and DHEA-S levels in daughters	DHEA-S, E2, SHBG, and TT levels were measured using an automated chemiluminescent immunoassay Active INHB was assayed using ELISA	Peripubertal BPA concentrations were not associated with any concurrent serum hormone concentrations
Mother-child cohort (Mexico) / Watkins et al. (2017)	120 mother-daughter pairs	BPA measured in three spot urine samples collected during the first, second, and third trimesters of pregnancy using HPLC-MS/MS following QA/QC	Serum E2, TT, INHB, SHBG and DHEA-S in daughters. fT level was estimated using TT and SHBG.	DHEA-S, E2, SHBG, and TT levels were measured using an automated chemiluminescent immunoassay Active INHB was assayed using ELISA	BPA concentrations during the second trimester of gestation were associated with higher TT levels among 8-13 years old peripubertal girls
Cross-sectional, nationally representative survey (USA) / Scinicariello and Buser, (2016)	588 children (aged 6-11) and adolescents (aged 12-19) (NHANES) 2011–2012	BPA concentrations measured in one spot urine sample using HPLC-MS/MS following QA/QC	Serum TT level in children and adolescents	LC-MS/MS	Higher BPA concentrations were associated with reduced TT levels among adolescent males, and with increased TT levels among adolescent females BPA was not associated with TT concentrations in male or female children
Cross-sectional study / Mustieles et al. (2018)	172 peripubertal boys from the INMA-Granada cohort	BPA concentrations measured in one spot urine sample using HPLC-MS/MS following QA/QC	Serum TT, LH and FSH levels in boys	Chemiluminescent immunoassay	Urinary BPA concentrations were associated with increased serum TT levels and the TT:LH ratio
			Case-control studies – Precocious puberty		

Case-control study precocious puberty (Korea) / Lee et al. (2014)	80 girls with central and peripheral precocious puberty, and age-matched healthy control girls	BPA measured in one spot urine sample using GC-MS. Some quality measures described.	Urinary steroid and metabolic parameters were assessed in urine	GC-MS	Higher urinary BPA concentrations correlated with higher urinary concentrations of TT, E2 and PREG BPA concentrations did not significantly differ among controls, peripheral or central precocious cases
Case-control study precocious puberty (Turkey) / Özgen et al. (2016)	28 girls with central precocious puberty (CPP) 22 prepubertal control girls	BPA measured in one spot urine sample from peripubertal girls using HPLC-MS/MS following a previously published method	Serum kisspeptin and E2 levels	ELISA	No association between BPA and kisspeptin or estradiol. Kisspeptin was significantly higher in girls with CPP compared to controls
Case-control studies – PCOS patients					
Case-control PCOS study (United Kingdom) / Kandaraki et al. (2011)	71 PCOS cases and 100 age- and BMI-matched controls	Total BPA was assessed in one serum sample using a commercial ELISA kit and duplicate measurements.	Serum TT, SHBG, AD, LH, FSH, 17-OH-PROG, and DHEA-S levels	-	Serum BPA concentrations were significantly higher in the PCOS group compared with controls, and were associated with higher TT and AD levels
Case-control PCOS study (Italy) / Tarantino et al. (2013)	40 women with PCOS and 20 healthy age-matched controls	Total BPA was measured in one serum sample using a commercial ELISA kit and duplicate measurements.	Serum TT and SHBG. FAI was calculated	Chemiluminescent enzyme immunoassay	Serum BPA concentrations positively correlated with the FAI in premenopausal women with PCOS

Case-control PCOS study (Turkey) / Akın et al. (2015)	180 adolescent girls aged 13-19 112 girls with polycystic ovary syndrome (PCOS) and 68 healthy controls	Free serum BPA concentrations were measured using HPLC and a UV detector	Serum FSH, LH, E2, 17-OH-PROG, PROG, DHEA-S, AD, TT, fT and SHBG levels	Chemiluminiscence immunoassay and radioimmunoassay	Among the entire study group, higher serum BPA concentrations significantly correlated with higher serum TT, fT and DHEA-S levels Serum BPA concentrations were significantly higher in adolescent girls with PCOS than their age-matched healthy controls
Women studies – Fertility Clinics / Occupational settings					
Fertility clinic (USA) / Mok-Lin et al. (2010)	84 women undergoing fertility treatment	BPA measured in two spot urine samples using HPLC-MS/MS following QA/QC	Serum FSH and E2 levels and other fertilization outcomes (total oocytes, oocyte maturation, quality embryos, etc.)	Electrochemiluminescence immunoassay	Higher urinary BPA concentrations were associated with a decrease in the number of oocytes retrieved and a decrease in peak E2 levels
Fertility clinic (USA) / Follow-up of Mok-Lin et al. (2010) / Ehrlich et al. (2012)	174 women undergoing fertility treatment (recruited between 2004-2010)	BPA measured in two spot urine samples using HPLC-MS/MS following QA/QC	Serum FSH and E2 levels and other fertilization outcomes (total oocytes, oocyte maturation, quality embryos, etc.)	Electrochemiluminescence immunoassay	Higher BPA concentrations were associated with a decreased number of oocytes and decreased E2 levels. Additionally, higher BPA exposure was associated with decreased blastocyst formation
Fertility clinic (USA) / Follow-up of Ehrlich et al. (2012) / Mínguez-Alarcón et al. (2015)	256 women undergoing fertility treatment (recruited between 2004-2012)	BPA measured in two spot urine samples using HPLC-MS/MS following QA/QC	Serum FSH and E2 levels and other fertilization outcomes (endometrial wall thickness, quality embryos, etc.)	Electrochemiluminescence immunoassay	No associations were observed
Fertility setting (USA) / Bloom et al. (2011)	44 women undergoing IVF	Free BPA measured in one serum sample using HPLC with Coularray detection. Some quality measures described.	Serum FSH and E2 peak levels. Number of oocytes retrieved	Chemiluminescent enzyme immunoassay	BPA concentrations were inversely associated with E2 levels. No association was observed between BPA and oocyte total number

Occupational cross-sectional study (China) / Miao et al. (2015)	106 occupationally exposed females working with epoxy resins 250 unexposed female workers from other sectors	BPA measured in one spot urine sample using HPLC with fluorescence detection. Some quality measures.	Serum FSH, LH, E2, PRL and P4 levels	Radioimmunoassay	Higher urinary BPA concentrations were associated with higher serum PRL and P4 levels among all females BPA was associated with higher serum E2 levels among exposed workers, and with lower FSH levels in the non-occupationally exposed group
Men studies – General population / Fertility Clinics / Occupational settings					
Cross-sectional study (Denmark) / Lassen et al. (2014)	308 young men	BPA measured in one spot urine sample using TurboFlow-LC-MS/MS following QA/QC procedures	Serum LH, FSH, TT, E2 and INHB levels fT was estimated based on TT and SHBG levels Semen volume, sperm concentration, total sperm count, motility and morphology	FSH, LH and SHBG were measured by immunofluorometric assays. TT and E2 levels were measured by fluoroimmunoassay. INHB was determined by a double antibody enzyme–immunometric assay	Higher urinary BPA concentrations were associated with increased serum TT, E2, LH and fT levels, as well as with reduced percentage of progressive motile spermatozoa
InCHIANTI Adult cohort (Italy) / Galloway et al. (2010)	715 adults (20-74 years old)	BPA measured in one 24-hour urine sample using HPLC-MS/MS following QA/QC	Serum TT, E2 and SHBG levels. fT level was estimated	Commercial radioimmunological assay (RIA)	Higher BPA concentrations were associated with higher TT levels in men. Among premenopausal women, BPA was associated with SHBG
Cross-sectional study (China) / Liang et al. (2017)	560 men	BPA assessed in one spot urine sample using HPLC-MS/MS. Some quality measures but not exhaustive description	Serum FSH, LH and TT	Electrochemiluminescence immunoassay	Urinary BPA concentrations were positively associated with LH and FSH levels among male smokers, and negatively associated with TT levels among men with a BMI ≤25
Cohort study (USA) / Mendiola et al. (2010)	375 men	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC	Serum FSH, LH, SHBG, TT, E2 and INHB levels. FAI and fT levels were estimated	Immunofluorometric assays Radioimmunoassay	Urinary BPA concentrations were negatively associated with FAI levels and the FAI/LH ratio, and positively associated with SHBG levels

Fertility clinic setting (Prague, CZ) / Vitku et al. (2016)	191 men (2012-2015)	Free BPA measured in both samples of plasma and seminal plasma collected from each patient using HPLC-MS/MS following QA/QC	Plasma and seminal plasma: P4, 17-OH-PREG, cortisol, cortisone, DHEA, 7α-OH-DHEA, 7β-OH-DHEA, 7-oxo-DHEA, TT and AD levels. Estrogens (E1, E2 and E3) levels. LH, FSH and SHBG. Plasma DHT levels	Most steroids were assessed using LC–MS/MS. LH, FSH, SHBG and DHT levels were assessed using an immunoradiometric assay	Plasma BPA concentrations were positively correlated with E1, E2, PREG, 17-OH-PREG and DHEA levels, and negatively associated with DHT levels Seminal BPA and seminal hormone concentrations revealed different results from that in plasma. Contrary to plasma associations, seminal BPA was negatively associated with P4, 17-OH-P4 and DHEA levels. Similarly, seminal BPA concentrations were positively correlated with E2 and E3 levels
Fertility clinic setting (USA) / Meeker et al. (2010a)	167 men	BPA measured in one spot urine sample (except for a subsample with repeated urines) using HPLC-MS/MS following QA/QC	Serum TT, E2, SHBG, INHB, FSH, LH, PRL, fT4, TT3 and TSH levels. FAI and fT were estimated TT:LH and FSH:INHB ratios were calculated as measures of Leydig and Sertoli function, respectively	Immunoassay	Urinary BPA concentrations were inversely associated with serum INHB levels and the E2:TT ratio, while positively associated with FSH and the FSH:INHB ratio
Fertility clinic setting (Belgium) / Den Hond et al. (2015)	163 men	BPA measured in one spot urine sample using GC-MS following QA/QC measures	Serum TT, LH, FSH, SHBG, E2 and INHB levels. fT and E2 levels were estimated using SHBG, assuming a fixed albumin concentration	Commercial immunoassays	Urinary BPA concentrations were associated with reduced serum TT levels
Cross-sectional study (China) / Zhou et al. (2013)	290 men with and without BPA exposure in the workplace	BPA measured in one serum sample using HPLC with a fluorescence detector	Sex hormones were assessed: TT, E2, SHBG, INHB, FSH, PRL, AD, fT	Magnetic microparticle immune radiation analysis (TT, E2, INHB, FSH, and PRL). ELISA (SHBG, AD, and FT)	Serum BPA concentrations were associated with decreased serum AD and fT levels and the androgen index, while positively associated with SHBG levels

Occupational cross-sectional study (China)/ Liu et al. (2015)	592 male workers. 165 were occupationally exposed (epoxy resin manufacturers) and 427 were from unexposed factories. Recruitment: 2004-2008	BPA measured in one spot urine sample using HPLC-MS/MS. Some quality measures but not exhaustive description	Serum TT, fT, SHBG, INHB, AD, FSH, E2 and PRL levels.	FSH, PRL, TT and E2 levels were measured by radioimmunoassay fT, SHBG, AD and INHB levels were measured by enzyme-linked immunosorbent assay	Higher BPA concentrations were associated with increased levels of PRL, SHBG and E2 levels; as well as with reduced levels of FSH, AD and FAI levels. No associations were observed with TT, fT or INHB levels
Occupational cross-sectional study (China) / Zhuang et al. (2015)	281 male workers occupationally exposed to BPA 278 male workers non-occupationally exposed	Serum BPA concentrations were measured using GC-MS. Some experimental methods described, but no reference to QA/QC.	Serum TT, SHBG, AD and INHB levels	TT and SHBG levels were assessed by radioimmunoassay. AD and INHB levels were measured by enzyme-linked immunosorbent assay	Increased serum BPA concentrations were associated with decreased serum AD and increased SHBG levels
			Other studies		
Korean Biomonitoring Program (Korea) / Kim et al. (2014)	1904 Korean adults	BPA measured in one spot urine sample using GC-MS. Some details provided, although no direct reference to QA/QC. A subsample of 100 adults highly-exposed to BPA were age- and sex-matched with 100 adults low-exposed to BPA	Urinary estrogen levels were assessed, including: E1, E2, and their hydroxylated metabolites	Urinary estrogens were assessed with GC-MS	Concentrations of E1, E2 and their hydroxylated metabolites were higher in the high-exposed BPA (BPA-H) than in the low-exposed group (BPA-L). In the BPA-H group, estrogen metabolism to 4-hydroxy-E1 and 4-hydroxy-E2 was more active than that to 2-hydroxy-E1 and 2-hydroxy-E2, with possible implications for breast cancer and other endocrine disorders
Case-control study (China)/ Xiong et al. (2015)	88 patients with dilated cardiomyopathy (DCM) 88 age- and sex-matched healthy controls	Total BPA was measured in one serum sample using a commercial ELISA kit	Serum TT, E2 and SHBG levels. FAI = TT in nmol/L × 100/SHBG in nmol/L	ELISA	Increasing serum BPA concentrations were associated with increased SHBG levels. BPA concentrations in the DCM group were significantly higher compared with that in the controls
Nested preterm birth case-control	457 mother-child pairs (103 cases and	BPA measured in urine from up to four visits during pregnancy (median 10, 18, 26 and 35	Placental growth factor (PlGF) and soluble fms-like tyrosine kinase-1 (sFlt-1) were measured in plasma samples	Immunoassay	BPA concentrations were associated with increased sFlt-1 levels, as well as the sFlt-1 to PlGF ratio, suggesting a potential effect of BPA affecting these

(USA) / Ferguson et al. (2015)	weeks) using HPLC-MS/MS following QA/QC procedures	from up to four visits during pregnancy (median 10, 18, 26, and 35 weeks)	placental angiogenesis markers predictive of preeclampsia and preterm birth

[a] Total BPA (free plus conjugated) in urine, total and/or free BPA in blood, and total and/or free BPA in tissue.

Abbreviations: androstenedione (AD); dehydroepiandrosterone sulfate (DHEA-S); dihydrotestosterone (DHT); enzyme-linked immunosorbent assay (ELISA); estrone (E1); 17β-estradiol (E2); estriol (E3); free androgen index (FAI); free testosterone (fT); follicle stimulating hormone (FSH); gas chromatography tandem mass spectrometry (GC-MS/MS); high-performance liquid chromatography tandem mass spectrometry (HPLC-MS); inhibin B (INHB); insulin-like peptide 3 (INSL3); luteinizing hormone (LH); pregnenolone (PREG); prolactin (PRL); progesterone (PROG); quality assurance and quality control (QA/QC); sex hormone-binding globulin (SHBG); ultraviolet radiation (UV); total testosterone (TT).

Supplemental Table 5. Human biomonitoring studies addressing the relationship between BPA exposure and semen quality markers (n=7).

Study design/Reference	Study population	Bisphenol exposure assessment[a]	Matrix/biomarker (s)	Analytical method	Main results
Cross-sectional study in a fertily center (Czech Republic) / Vitku et al. (2016)	191 Czech men attending the Prenatal Center of Assisted Reproduction	BPA measured in samples of blood plasma and seminal plasma collected from each patient using HPLC-MS/MS following QA/QC	Semen sample	Semen quality parameters Spermiogram was conducted following the WHO 2010 criteria	Seminal plasma BPA concentrations, but not blood plasmatic BPA, were negatively correlated with sperm count, concentration and morphology
Fertility center (Slovenia) / Knez et al. (2014)	149 couples undergoing assisted reproduction techniques	BPA measured in one spot urine sample using GC-MS. The procedure is fully described.	Semen sample	Semen quality parameters	Urinary BPA concentrations was associated with lower sperm counts, concentration and vitality
Multi-country study (USA) / Goldstone et al. (2015)	501 men	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC procedures	Semen sample	Comprehensive assessment of semen quantity, quality, motility and other measures	No associations were observed, except for an inverse association between BPA concentrations and sperm DNA damage
Cross-sectional adult study (China) / Li et al. (2011)	218 men with and without BPA exposure in the workplace	BPA measured in one spot urine sample using HPLC-MS/MS. Some quality aspects described	Semen sample	Semen quality parameters	Urinary BPA concentrations were associated with decreased sperm concentration, counts, vitality and motility, but not with semen volume or abnormal morphology
Fertility setting (USA) / Meeker et al. (2010b)	190 men	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC procedures	Semen sample	Computer-aided semen analyzer. WHO guidelines were followed Comet assay to assess sperm DNA damage	Urinary BPA concentrations were associated with lower sperm concentrations and motility, as well as higher sperm DNA damage
Cohort study (USA) / Mendiola et al. (2010)	375 men	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC procedures	Semen sample	Semen quality parameters WHO criteria were followed	No associations observed between BPA concentrations and any semen parameter

Cross-sectional study (Denmark)/ Lassen et al. (2014)	308 young men	BPA measured in one spot urine sample using TurboFlow-LC-MS/MS following QA/QC procedures	Semen volume, sperm concentration, total sperm count, motility and morphology	WHO guidelines	Higher urinary BPA concentrations were associated with reduced percentage of progressive motile spermatozoa

[a] Total BPA (free plus conjugated) in urine, total and/or free BPA in blood, and total and/or free BPA in tissue.

Abbreviations: gas chromatography tandem mass spectrometry (GC-MS/MS); high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS); quality assurance and quality control (QA/QC); World Health Organization (WHO).

Supplemental Table 6. Human biomonitoring studies addressing the relationship between BPA exposure and glucocorticoid biomarkers (n=3).

Study design/Reference	Study population	Bisphenol exposure assessment[a]	Matrix/biomarker(s)	Analytical method	Main results
			Perinatal and children studies		
Longitudinal birth cohort (Canada) / Giesbrecht et al. (2016)	174 pregnant women	Maternal BPA measured in one spot urine sample during the second trimester of gestation using HPLC-MS/MS following QA/QC procedures	Diurnal salivary cortisol levels (measured at waking and different time points in the day)	Enzyme immunoassay	Higher urinary BPA concentrations were associated with dysregulation of the daytime cortisol pattern, including reduced cortisol at waking and a flatter daytime pattern
Longitudinal birth cohort (Canada) / Giesbrecht et al. (2017)	132 pregnant women	Maternal BPA measured in one spot urine sample during the second trimester of gestation using HPLC-MS/MS following QA/QC procedures	Cortisol levels in infant saliva samples collected before and after an infant stressor (blood draw) at 3 months of age	Enzyme immunoassay	Higher maternal BPA concentrations were associated with increases in baseline cortisol levels among females but decreases among males. In contrast, after the blood draw, maternal BPA concentrations were associated with an increased reactivity among males but decrease reactivity among females
Cross-sectional study / Mustieles et al. (2018)	172 peripubertal boys from the INMA-Granada cohort	Peripubertal BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC	Serum cortisol levels.	Chemiluminescent immunoassay	An increase in BPA concentrations was associated with decreased cortisol levels and decreased TT:cortisol ratio

[a] Total BPA (free plus conjugated) in urine, total and/or free BPA in blood, and total and/or free BPA in tissue.

Abbreviations: Environment and childhood cohort (INMA Cohort); high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS); total testosterone (TT)

Supplemental Table 7. Human biomonitoring studies addressing the relationship between BPA exposure and thyroid function biomarkers (n=12).

Study design/Reference	Study population	Bisphenol exposure assessment[a]	Matrix/biomarker(s)	Analytical method	Main results
			Perinatal studies		
Ongoing prospective cohort of pregnant women (Puerto Rico) / Aker et al. (2016)	106 pregnant women	BPA measured in spot urine samples at each trimester of gestation using HPLC-MS/MS following QA/QC procedures	Serum TSH, FT3 and FT4 levels at two time points (18 weeks and 26 weeks)	FT3 and FT4: LC-MS TSH: Immunochemilumi-nometric assay	Higher maternal BPA concentrations were associated with increased FT4 levels
Nested case-control study during pregnancy (USA) / Aung et al. (2018)	439 pregnant women including 116 preterm births	BPA measured in spot urine samples at four times during gestation using HPLC-MS/MS following QA/QC procedures	Plasma TSH, FT3, FT4 and TT4 levels (9, 18, 26 and 35 weeks)	TSH, TT3 and TT4: chemiluminescence immunoassay FT4: radioimmunoassay	Higher maternal BPA concentrations were associated with decreased TSH levels and increased FT4 levels
Mother-child cohort (USA) / Romano et al. (2015)	181 pregnant women	BPA measured in two spot urine samples (16 and 26 weeks of gestation) using HPLC-MS/MS following QA/QC procedures	Serum TSH, FT3, TT3, FT4, TT4 levels and TPOAb and TgAb levels (16 weeks and in cord serum)	TSH, FT3, TT3, FT4, TT4, TPOAb and TgAb: immunoassay	Higher maternal BPA concentrations were associated with decreased TSH levels in female newborns
Mother-child cohort from the CHAMACOS study (USA) / Chevrier et al. (2013)	364 pregnant immigrant Mexican-American women of low socioeconomic status	BPA measured in two spot urine samples (12 and 26 weeks of pregnancy) using HPLC-MS/MS following QA/QC procedures	Serum TSH, TT4 and FT4 levels (~ 26 weeks) and neonatal TSH	FT4: radioimmunoassay TSH and TT4: immunochemiluminometric assay Neonatal TSH: by heel stick and fluoroimmunoassay	Higher maternal BPA concentrations were associated with decreased TT4 levels at 26 weeks and decreased TSH levels in male newborns
			Children studies		

Case-control study on cryptorchidism (France) / Brucker-davis et al. (2011)	60 cryptorchid boys and 76 control boys (age 3)	BPA measured in cord blood using RIA and following some quality controls including comparison with HPLC-MS/MS method.	TSH, FT3, FT4 levels in cord blood (and TPOAb when TSH levels were high)	Chemiluminescence	A trend for a negative correlation between cord blood BPA and TSH
			Adult studies		
Pilot case-control study on thyroid nodular disease (Cyprus and Romania) / Andrianou et al. (2016)	In non-pregnant women, 106 cases and 106 controls (~age 50)	BPA measured in one spot urine sample using GC-MS/MS. Some quality aspects are described.	Serum TSH, FT4, TPOAb and TgAb levels	Immunoassay in Cyprus & electro-chemiluminescence in Romania	Higher BPA concentrations were associated with increased TSH levels
Fertility clinic (USA) / Meeker et al. (2010a)	167 men	BPA measured in spot urine samples (2 samples for a subset of 75 men) using HPLC-MS/MS following QA/QC procedures	Serum TSH, FT4 and TT3 levels	Immunoassay	Higher BPA concentrations were associated with decreased TSH levels
Case-control study on weight-loss (Belgium) / Geens et al. (2015)	151 overweight adults and 43 lean adults	BPA measured in 24-hour urine samples (4 times a year) using GC-MS/MS following QA/QC procedures	Serum TSH and FT4 levels	Chemiluminescence	Higher BPA concentrations were associated with increased TSH in the lean population
Cross-sectional analysis from the Thai National Health Examination Survey IV (Thailand) / Sriphrapradang et al. (2013)	2340 adults	Total BPA in one serum sample using commercial ELISA kits	Serum TSH, FT4 levels and TgAb and TPOAb levels	Electrochemiluminescence	Higher BPA concentrations were associated with decreased FT4 levels in men
Cross-sectional analysis from the Thai National Health Examination Survey IV (Thailand) / Chailurkit et al. (2016)	2361 adults with normal TSH and Free T4	Total BPA in one serum sample using commercial ELISA kits	Serum TPOAb, TgAb, TRAb	Electrochemiluminescence immunoassay	A positive association between BPA and TPOAb positivity was observed in both men and women

Cross-sectional study (China) / Wang et al. (2013)	3394 adults (age > 40)	BPA measured in one spot urine sample using HPLC-MS/MS. Quality control measures are described in detail.	Serum TSH, FT3, FT4, TgAb and TPOAb levels	Chemiluminescent microparticle immunoassay	Higher BPA concentrations were associated with decreased TSH levels and increased FT3 levels in both men and women
Cross-sectional analysis from the NHANES survey (USA)/ Meeker and Ferguson, (2011)	1348 adults (age > 20) and 329 adolescents (age 12-19)	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC procedures	Serum TSH, FT3, FT4, TT3, TT4 and TgAb levels	Immunoassay	A trend for a negative correlation between BPA concentrations and TT4 and TSH levels was observed in adults

[a] Total BPA (free plus conjugated) in urine, total and/or free BPA in blood, and total and/or free BPA in tissue.

Abbreviations: enzyme-linked immunosorbent assay (ELISA); free triiodothyronine (FT3); free thyroxine (FT4); gas chromatography tandem mass spectrometry (GC-MS/MS); high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS); quality assurance and quality control (QA/QC); thyroid-stimulating hormone (TSH); TSH receptor autoantibodies (TRAb); thyroglobulin autoantibodies (TgAb); thyroperoxydase autoantibodies (TPOAb); total triiodothyronine (TT3); total thyroxine (TT4).

Supplemental Table 8. Human biomonitoring studies addressing the relationship between BPA exposure and metabolic function biomarkers (n=39).

Study design/Reference	Study population	Bisphenol exposure assessment[a]	Matrix/biomarker(s)	Analytical method	Main results
		Perinatal and children studies			
Ongoing prospective cohort (USA) / Chiu et al. (2017)	245 subfertile pregnant women	BPA measured in at least one urine sample during the first or second trimester using HPLC-MS/MS following QA/QC measures	Blood glucose levels, 1-hour after a 50-g glucose challenge test between 24-28 weeks of gestation	-	Second-trimester - but not first trimester - urinary BPA concentrations were positively associated with blood glucose
Case-control pilot study (USA) / Robledo et al. (2013)	22 GDM cases cases and 72 controls	BPA measured in one maternal spot urine sample (at enrollment) using HPLC-MS/MS following QA/QC measures	Blood glucose levels, 1-hour after a 50-g glucose challenge test (around 26 weeks of gestation)	-	BPA concentrations were not associated with fasting blood glucose levels or GDM
Mother-child cohort (Canada) / Shapiro et al. (2015)	1274 pregnant women	BPA measured in one maternal spot urine sample at first trimester using GC-MS/MS following QA/QC measures	After a glucose-challenge or tolerance test, subjects were assigned a diagnosis of impaired glucose tolerance (IGT) or GDM	-	No associations were observed between BPA concentrations and IGT or GDM
Mother-child cohort (Mexico) / Volberg et al. (2013)	188 mother-child pairs	BPA measured in spot urine samples at early (12 weeks) and late (26 weeks) pregnancy using HPLC-MS/MS following QA/QC measures BPA measured in one spot urine sample in offspring at 9 years of age	Plasma leptin and adiponectin in 9 years-old boys and girls	ELISA	Higher late pregnancy BPA concentrations were associated with greater leptin levels in boys Higher early pregnancy BPA concentrations were associated with greater adiponectin levels in girls No associations between childhood BPA concentrations and adipokines were observed
Mother-child cohort (USA)/ Ashley-Martin et al. (2014)	1363 mother-child pairs	BPA measured in one first-trimester spot urine sample using GC-MS/MS following QA/QC measures	Cord blood leptin and adiponectin levels	ELISA	Higher BPA concentrations were associated with decreased fetal adiponectin levels among males

Mother-child cohort (Taiwan) / Chou et al. (2011)	97 mother-child pairs	Free BPA measured in maternal plasma and neonates umbilical cord blood using HPLC coupled to a UV detector. Some QA/QC details described.	Plasma leptin and adiponectin levels in cord blood	ELISA	Maternal serum BPA concentrations were associated with increased risk of low-adiponectin and high-leptin cord blood levels among male neonates
Cross-sectional study (Italy) / Menale et al. (2017)	141 prepubertal obese children	BPA measured in one spot urine sample using HPLC-MS/MS. Quality details described.	Insulin resistance was assessed using the homeostasis model assessment (HOMA) as fasting insulin (pmol L−1) × fasting glucose (mmol L−1)/135. Serum triglycerides and HDL cholesterol levels. Serum adiponectin and resistin levels	ELISA	Higher BPA concentrations were associated with increased HOMA values and decreased adiponectin levels, showing an association with insulin resistance These results were experimentally confirmed *in vitro* in adipocytes obtained from 8 normal weight prepubertal boys
Mother-child cohort (Mexico) / Watkins et al. (2016)	250 pregnant women and their peripubertal children (8-14 years)	BPA measured in one maternal spot urine sample during the third trimester of gestation using HPLC-MS/MS following QA/QC BPA was also analyzed in one spot urine sample from boys at 8-14 years of age	Fasting serum c-peptide, glucose, IGF-1, leptin, and glucose concentrations among children at follow-up A measure of insulin resistance similar to HOMA-IR (c-peptide-based measure of insulin resistance [CP-IR]): [fasting serum c-peptide × fasting serum glucose/405]	Chemiluminescent enzyme immunoassay RIA	Higher childhood BPA concentrations were associated with increased serum leptin levels among peripubertal boys Maternal prenatal BPA exposure was not associated with peripubertal markers of metabolic function
Mother-child cohort (Mexico) / Perng et al. (2017)	248 pregnant women and their peripubertal children (8-14 years)	BPA measured in three maternal spot urines (each trimester of gestation), and the average concentration was used. HPLC-MS/MS with QA/QC. BPA was also analyzed in one spot urine sample from boys and girls at 8-14 years of age	TC, HDL-C, LDL-C and TG levels	Automated biochemical analyzer	Maternal prenatal or peripubertal BPA concentrations were not associated with any disruption of levels of lipids studied in serum

Cross-sectional analysis from the NHANES survey 2003-2010 (USA) / Eng et al. (2013)	Around 800 Children aged 6-18 years	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC	Fasting TC, HDL-C, LDL-C, TG, insulin and glucose levels. HOMA-IR was calculated	Automated analyzer	BPA concentrations were not associated with any of these cardiometabolic markers in children
Prospective study (Korea) / Lee et al. (2013)	80 preadolescent girls (7-8 years). 48 came back one year later for re-evaluation	BPA measured in two spot urine samples, one at baseline and the other one-year later using HPLC coupled to a fluorescence detector. No reference to QA/QC	Serum insulin and plasma glucose levels. HOMA-IR was calculated. LH, fT, androstendione and DHEA, free E2 levels	Immunoradiometric assay ELISA	At baseline, girls in third tertile of BPA concentrations tended to present increased levels of all metabolic and hormonal parameters At 1 year later, there were significant differences for HOMA-IR between girls in third tertile compared to those in the lowest exposed tertile, in addition to altered hormonal levels
Cross-sectional analysis (USA) / Khalil et al. (2014)	39 overweight and obese male and female children aged 3-8 years	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC procedures	Fasting insulin, glucose and HbA1C. HOMA-IR was calculated. LDL-C, HDL-C, TC, TG levels Liver profile: ALT, AST. Thyroid profile: TSH and FT4. levels	Automated analyzer (Beckman Coulter)	BPA concentrations were associated with increased insulin and HOMA-IR values, in addition to elevated FT4 and serum AST levels
			Endothelial dysfunction		
Cross-sectional analysis from the NHANES survey 2009-2010 (USA) / Trasande et al. (2013)	710 Children aged 6-19 years	BPA measured in one spot urine samples using HPLC-MS/MS following QA/QC measures	First morning urinary albumin to creatinine ratio (ACR)	Solid-phase fluorescentimmunoassay	BPA concentrations were associated with higher ACR levels in children, a marker of early endothelial dysfunction

Cross-sectional analysis (China) / Li et al. (2012)	3055 adults (mean age: 60)	BPA measured in one spot urine sample using HPLC-MS/MS. Some quality measures described.	Urinary albumin to creatinine ratio (ACR)	Immunoturbidimetry	BPA concentrations were associated with a higher risk of low-grade albuminuria among Chinese adults
Cross-sectional pilot study (USA) / Kataria et al. (2017)	41 children (aged 10-13 years)	BPA and BPS measured in one spot urine sample using HPLC-MS/MS following QA/QC measures	HOMA-IR Urinary ACR 8OHdG and 8-isoprostane	Automated analyzer and ELISA kits	BPS concentrations were associated with an increased ACR BPA concentrations were associated with increased oxidant stress (8-isoprostane levels)
Case-control studies - PCOS women					
Case-control PCOS study (Italy) / Tarantino et al. (2013)	40 women with PCOS and 20 healthy age-matched controls	Total BPA measured in one serum sample using ELISA kits. All samples were measured in duplicate.	Serum fasting glucose and insulin (HOMA-IR), liver enzymes, CRP, IL-6	Chemiluminescent enzyme immunoassay Ultrasonography	Serum BPA concentrations positively correlated with insulin resistance, hepatic steatosis and markers of low-grade inflammation in premenopausal women with PCOS
Adult Studies – Insulin resistance and type 2 diabetes					
Cross-sectional analysis from the CHMS (2009-2011) / Tai and Chen, (2016)	2405 participants (aged 3-79 years)	BPA measured in one spot urine sample using GC-MS/MS following QA/QC	Fasting blood glucose (n=2405) and glycated hemoglobin (HbA1c) (n=1915)	Chemiluminescence immunoassay	Higher BPA concentrations were associated with higher HbA1c levels suggesting a deleterious effect on glucose homeostasis among adult men, but not in women and children
Cross-sectional analysis from the NHANES (USA) (2003-2008) / Silver et al. (2011)	4389 adult men and women participants	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC measures	Fasting serum HbA1c levels	HPLC	Higher BPA concentrations were associated with increased HbA1c levels, especially on the 2003-2004 NHANES cycle

Cross-sectional analysis from the NHANES (USA) (2005-2008) / Beydoun et al. (2014)	1586 adult men and women (mean age of 45 years)	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC measures	Fasting blood and insulin levels HOMA was calculated	–	Higher BPA concentrations were associated with greater insulin levels and insulin resistance, especially among men
Cross-sectional analysis from the NHANES (USA) (2003-2008) / (Sabanayagam et al. 2013)	3516 adult participants (mean age: 44 years)	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC measures	Plasma glucose levels HbA1c levels Prediabetes was defined as fasting plasma glucose concentration of 100–125 mg/dL or an A1C value of 5.7–6.4 % among those without diabetes	Modified hexokinase method HPLC	BPA concentrations were associated with a higher risk of prediabetes, which was stronger among women and obese subjects
Cross-sectional analysis from the NHANES (USA) (2003-2008) / Shankar and Teppala, (2011)	3967 adult men and women (mean age: 45 years)	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC measures	Fasting serum glucose levels Diabetes was defined as a fasting serum glucose ≥126 mg/dL, non-fasting serum glucose ≥200 mg/dL, and/or HbA1c >6.5% or self-reported use of diabetic medication	Modified hexokinase method	BPA concentrations were associated with the risk of diabetes mellitus independent of other risk factors
Cross-sectional analysis (China) / Ning et al., (2011)	3423 adults (median age 59 years)	BPA in one spot urine sample using HPLC-MS/MS following quality control measures	Plasma glucose, plasma glucose 2-hours after a glucose tolerance test, serum insulin	Glucose oxidase method on an autoanalyzer and serum insulin measured using an electrochemiluminescence assay	The second and fourth quartile of BPA concentrations, but not the third, were associated with an increased risk of type 2 diabetes
Cross-sectional analysis (China) / Wang et al., (2012)	3390 adults (mean age: 60)	BPA measured in one spot urine sample using HPLC-MS/MS. Quality control measures are described in detail	Plasma glucose and serum insulin levels Insulin resistance was defined as a HOMA-IR index > 2.5	Glucose was measured using the glucose oxidase method on an autoanalyzer. Insulin was measured by electrochemiluminescence assay	In non-overweight participants, BPA concentrations were associated with an increased prevalence of insulin resistance
Cross-sectional analysis from the Thai National Health Examination Survey (2009) / Aekplakorn et al. (2015)	2581 participants	Total BPA measured in one serum sample using ELISA kits	Fasting plasma glucose. Impaired fasting glucose (IFG) was defined as ≥100 and < 126 mg/dL Diabetes was defined as >126 mg/dL	-	BPA concentrations were not associated with impaired fasting glucose, but with type 2 diabetes

Cross-sectional analysis (Korea) / Hong et al. (2017)	296 healthy women (aged 30-49 years)	BPA measured in one spot urine sample using HPLC-MS/MS. No reference to QA/QC	Fasting plasma glucose and serum insulin levels. Calculation of HOMA-IR	Glucose oxidase method. Insulin was measured using a radioimmunoassay	BPA concentrations were positively associated with fasting serum insulin levels and HOMA-IR
Prospective study (China) / Bi et al. (2016)	2209 nondiabetic middle-age and elderly subjects followed during 4 years	BPA measured in one sport urine sample at baseline using HPLC-MS/MS. Some quality details reported.	Genetic risk score (GRS) based on 34 common genetic variants for Type 2 Diabetes. Fasting plasma glucose (FPG) levels at baseline and follow-up	Glucose oxidase method on an autoanalyzer	The GRS modified the effect of BPA exposure on 4-year changes in FPG levels. In the highest quartile of GRS, BPA concentrations were associated with increases in FPG levels, while no associations were found in the lower three quartiles of the GRS
Cross-sectional analysis (Italy) / Savastano et al. (2015)	76 adult men	Total BPA measured in one plasma sample using commercial ELISA kits. All samples were measured in duplicate.	TC, HDL-C, LDL-C, TG Plasma glucose and insulin. HOMA was calculated. Plasma MCP-1, IL-6 and TNF-α	ELISA and commercial kits	BPA concentrations correlated with insulin resistance, TG and inflammatory markers IL-6 and TNF-α but not MCP-1
Cross-sectional study (Korea) / Choi et al. (2017)	200 healthy adults (96 men and 104 women, 30-64 years of age)	BPA measured in one spot urine sample using HPLC-MS/MS. Some quality aspects were reported.	Fasting serum hs-CRP levels	Turbido-immunometric assay (TIA)	Higher BPA concentrations were associated with higher hs-CRP levels, independently of other cardiometabolic risk factors
Cross-sectional analysis from the NHANES (USA) (2003-2004) / Lang et al. (2008)	1455 adult men and women (range of age: 18-74 years)	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC measures	CRP GGT, lactate dehydrogenase (LD), and alkaline phosphatase (AP) TG LDL-C Fasting glucose and insulin	Latex-enhanced nephelometry (CRP); enzymatic method (GGT, LD, AP, TG); immunoenzymometric assay (insulin and glucose); estimation from TC, TG and HDL-C values (LDL-C)	Higher BPA concentrations were associated with clinically abnormal levels of GGT, LD and AP BPA was also positively associated with CRP, insulin and glucose levels in unadjusted analysis, but these associations were attenuated in adjusted models No associations with levels of LDL-C or triglycerides were observed
Cross-sectional NHANES (USA) (2003-2006) / Melzer et al. (2010)	2780 adult participants (18-74 years)	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC measures	Liver enzymes: GGT, AP, LD levels	Enzymatic method (GGT, LD, AP)	BPA concentrations were still associated with clinically abnormal levels of LD and AP levels in the 2003-2004 and 2005-2006 pooled surveys, but the previously observed association with GGT did not remain

Prospective analysis (Korea) / Lee et al. (2014)	560 elderly participants	BPA measured in spot urine samples up to 5 visits using HPLC-MS/MS following QA/QC procedures	Liver enzymes: AST, ALT, GGT levels	Serum AST, ALT and γ-GTP measured using an autobiochemical analyser	BPA concentrations were associated with the three liver markers. Subjects above the median BPA concentrations showed increased abnormal liver function
Longitudinal cohort of elders / Rönn et al. (2014)	890 men and women around 70 years of age	Total BPA measured in serum using HPLC-MS/MS. Some quality aspects were reported.	Fasting serum leptin, adiponectin and ghrelin levels	RIA and ELISA	Higher BPA concentrations were positively associated with leptin and adiponectin levels and negatively associated with the gut-hormone ghrelin
Cross-sectional analysis (China) / Zhao et al. (2012)	246 healthy premenopausal women with regular menstrual cycles	BPA measured in one spot urine sample using HPLC-MS/MS. Some quality details described.	Fasting serum leptin, estradiol, osteocalcin and N-terminal telopeptides of type I collagen (NTx)	Immunofluorescence (E2), RIA (leptin), Immunoradiometry (osteocalcin), ELISA (NTxs)	Higher urinary BPA concentrations were associated with higher serum leptin levels
Cross-sectional analysis (Serbia) / Milošević et al., (2017)	103 healthy women in reproductive age	BPA measured in one spot urine sample using GC-MS/MS. Some quality aspects mentioned.	Fasting serum TG, HDL-C and LDL-C levels	Standard automated laboratory methods	BPA concentrations were associated with increased obesity but not with TG, HDL or LDL cholesterol levels
			Nephropathy studies		
Clinical prospective study / Hu et al. (2015)	121 patients with T2D and eGFR ≥60 mL/min/1.73^2	Total BPA measured in one serum sample at recruitment (ELISA kit)	eGFR calculated as the serum creatinine-cystatin equation CKD was defined as an eGFR < 30 mL/min/1.73^2	Automatic biochemical analyzer	BPA concentrations were negatively associated with the annual change in eGFR and percentage change in eGFR. Patients with higher BPA concentrations had about a sevenfold increased risk of developing CKD through the study follow-up

Clinical prospective study (China) / Hu et al. (2016)	302 patients with primary hypertension (195 men and 107 women, mean age 65 years) were followed during 6 years	Total BPA measured in one serum sample at baseline (ELISA kit)	eGFR calculated as the serum creatinine-cystatin equation Development of CKD stage 3 or greater was defined as baseline eGFR ≥60 ml/min per 1.73 m2, and eGFR<60 ml/min per 1.73 m2 at last follow-up	Automatic biochemical analyzer	BPA concentrations were negatively associated with the annual change in eGFR. Patients with higher BPA concentrations at baseline had a five-fold risk of developing CKD throughout the follow-up
			Vitamin D		
Cross-sectional analysis NHANES (USA) (2005-2010) / Johns et al. (2016)	4724 adult men and women	BPA in one spot urine sample using HPLC-MS/MS following QA/QC measures	Serum total 25-OH-vitamin D levels	LC-MS	BPA concentrations were associated with reduced vitamin D levels in women but not men
Mother-child cohort (USA) / Johns et al. (2017)	447 mother-child pairs	BPA measured in two spot urine samples at 10 and 26 weeks of gestation using HPLC-MS/MS following QA/QC	Serum 25-OH-vitamin D levels at 10 and 26 weeks of gestation	Chemiluminescence immunoassay Total 25(OH)D was measured	Participants with greater BPA concentrations presented lower vitamin D levels. Additionally, BPA concentrations were associated with a higher risk of vitamin D deficiency
Case-control study (Turkey) / Erden et al. (2014)	128 adults 43 control subjects and 85 subjects with obstructive sleep apnea	Free serum BPA concentrations using HPLC-UV detector. Some details described. No mention to QA/QC.	Serum 25-OH-vitamin D and PTH levels	Chemiluminescence immunoassay	BPA concentrations were negatively associated with serum vitamin D levels in all the participants. BPA was not associated with PTH levels

[a] Total BPA (free plus conjugated) in urine, total and/or free BPA in blood, and total and/or free BPA in tissue.

Abbreviations: Alanine aminotransferase (ALT); albumin to creatinine ratio (ACR); alkaline phosphatase (AP); aspartate transferase (AST); C-reactive protein (CRP); estradiol (E2); high-sensitivity CRP (hs-CRP); gestational diabetes mellitus (GDM); high-density lipoprotein cholesterol (HDL-C); homeostasis model assessment for insulin resistance (HOMA-IR); impaired glucose tolerance (IGT); dehydroepiandrosterone (DHEA); estimated glomerular filtration rate (eGFR); enzyme-linked immunoassay (ELISA); fasting plasma glucose (FPG); free thyroxine (FT4); gamma-glutamyl transpeptidase (GGT); gas chromatography tandem mass spectrometry (GC-MS/MS); genetic risk score (GRS); glycated hemoglobin (HbA1c); high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS); interleukin (IL); insulin-like growth factor 1 (IGF-1); lactate dehydrogenase (LD); low-density lipoprotein cholesterol (LDL-C); luteinizing hormone (LH); macrophage chemoattractant protein 1 (MCP-1); national health and nutrition examination survey (NHANES); parathyroid hormone (PTH); quality assurance and quality control (QA/QC); radioimmunoassay (RIA); tumor necrosis factor-alpha (TNF-α); thyroid stimulating hormone (TSH); testosterone (T); total cholesterol (TC); triglycerides (TG); ultraviolet radiation (UV); 8-hydroxydeoxy-guanosine (8OHdG).

Supplemental Table 9. Human biomonitoring studies addressing the relationship between BPA exposure and immune, inflammatory and allergy biomarkers (n=5).

Study design/Reference	Study population	Bisphenol exposure assessment[a]	Matrix/biomarker(s)	Analytical method	Main results
			Immunoglobulin E		
Mother-child cohort (Canada) / Ashley-Martin et al. (2015)	1137 mother-child pairs	BPA measured in one first-trimester spot urine sample using GC-MS/MS following QA/QC measures	Umbilical cord plasma IgE, TSLP and IL-33 levels	ELISA kits	BPA concentrations were non-linearly associated with IL-33 and TSLP levels. No associations with IgE levels were observed
Prospective analysis (Taiwan) / Wang et al. (2016)	453 children	BPA measured in one spot urine sample at 3 and 6 years of age using HPLC-MS/MS following a previously validated methodology.	Serum IgE levels at ages 3 and 6	ELISA kit	BPA concentrations at age 3 were associated with higher IgE levels, particularly in girls. Similar significant results were also found at age 6 BPA concentrations at age 3 were prospectively associated with IgE levels. IgE levels mediated 70% of the total effect of BPA concentrations on asthma risk
Cross-sectional analysis from NHANES (USA) (2005-2006) / Vaidya and Kulkarni, (2012)	2548 adults (mean age 39 years)	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC measures	Total IgE and 19 allergen-specific IgE levels Asthma diagnosis	ImmunoCAP 1000 System.	BPA was associated with allergic asthma in females, and was significantly associated with sensitization to various specific allergens in a dose–response manner
			Other markers		
Cross-sectional analysis (Korea) / Song et al. (2017)	141 elder participants (>60 years) with cardiovascular problems	BPA measured in one spot urine sample using HPLC-MS/MS-	Blood WBC and serum CRP and IL-10 levels	Cell counter and ELISA kits	BPA concentrations were associated with a higher WBC. Non-linear associations were observed for serum CRP and IL-10 levels

Cross-sectional analysis from NHANES (USA) (2003-2006) / Clayton et al. (2011)	787 participants (335 children aged 6-18 years, and 452 adults)	BPA measured in one spot urine sample using HPLC-MS/MS following QA/QC measures	Serum cytomegalovirus (CMV) antibody levels	ELISA kit	In adults (≥18 years), higher BPA concentrations were associated with higher CMV antibody levels In children (6-18 years), higher BPA concentrations were associated with lower CMV antibody levels

[a] Total BPA (free plus conjugated) in urine, total and/or free BPA in blood, and total and/or free BPA in tissue.

Abbreviations: Cytomegalovirus (CMV); C-reactive protein (CRP); enzyme-linked immunosorbent assay (ELISA); gas chromatography tandem mass spectrometry (GC-MS/MS); high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS); interleukin (IL); immunoglobulin E (IgE); national health and nutrition examination survey (NHANES); thymic stromal lymphopoietin (TSLP); white blood cells (WBC).

9.2 Supplementary Material. Article 2

Supplemental Table 1. Summary of epidemiological studies that have analyzed BPA exposure in relation to both children's behavior and cognitive function.

Authorship/ Study design	Study population	BPA exposure assessment	Neurodevelopmental evaluation	Behavior	Cognitive	Main Results
Maserejian et al. (2012a) NECAT Study Randomized Clinical Safety Trial (USA)	434 multiethnic children. Recruited between 1997-2005	Estimation of cumulative exposure from dental treatment (composite vs. amalgam or urethane material), establishing surface-years categories; at 4-5 years of follow-up.	BASC-SR (self-reported) at 8 years. CBCL, by parents, at 6 to 10 years.	X		Higher exposure to bisphenol A-glycidyl methacrylate (bisGMA)-based composite was associated with worse scores on three out of four BASC-SR scales: Emotional symptoms (β= 0.8; p= 0.003), clinical maladjustment (β= 0.7; p= 0.02) and personal adjustment (β= 0.8; SE=0.2; p= 0.002).
Maserejian et al. (2012b)	Secondary analysis with 444 children	Idem	WISC-III WIAT WRAML WRAVMA COWAT		X	Higher exposure to dental composite materials containing bisGMA was not statistically associated with cognitive function scores at the 4-5 years follow-up.
Casas et al., 2015 INMA-Sabadell prospective mother-child cohort (Spain)	438 children at 1, 4 and 7 years of age	Two spot maternal urine samples, at the first and third trimester of pregnancy.	BSID at 1 year MSCA at 4 years		X	**Psychomotor development:** At 1 year of age, highest BPA exposure was associated with a reduction of psychomotor scores (T3 *vs.*T1: β= -4.28, 95%CI: -8.15, -0.41). The association disappeared at 4 and 7 years. **Cognitive development:** No associations were found between BPA and cognitive development (age 1 and 4 years). **Behavior development:** BPA exposure was associated with increased risk of ADHD-hyperactivity symptoms [(IRR) per $\log_{10}$ µg BPA/g creatinine increase= 1.72; 95%CI: 1.08, 2.73), which was stronger in boys than in girls at 4 years of age. At 7 years, all these associations were no longer present
			ADHD-DSM-IV at 4 years (by teachers) CPRS and SDQ at 7 years (by parents)	X		

Philippat et al., 2017 EDEN mother-child cohort (France)	529 mother-son pairs. Recruited between 2003-2006	Phenols and phthalate metabolites in maternal spot urine samples collected between 22 and 29 gestational weeks.	SDQ at 3 and 5 years of age (by mothers)	X		Maternal prenatal urinary BPA was positively associated with relationship problems at 3 years [Incidence Rate Ratio (IRR): 1.11; 95% CI: 1.03, 1.20] and hyperactivity–inattention at 5 years (IRR: 1.08; 95% CI: 1.01, 1.14)].
Nakiwala et al., 2018 EDEN cohort	452 mother-son dyads	Idem	WPPS-III at 5–6 years (by psychologists)		X	BPA exposure was not associated with cognitive development (boys' verbal or performance IQ).
Braun et al., 2017a MIREC prospective mother-child cohort, Canada.	812 mother-child pairs	Spot maternal urine sample at an average of 12.1 weeks of gestation	Cognitive abilities at 3 years: WPPSI-III (by trained study staff)		X	BPA was not associated with cognitive development (any WPPSI-III scale). BPA was associated with diverse behavior problems such as more internalizing and somatic problems, among boys, but not in girls. Several associations were found between BPA and BRIEF-P scores in boys (such as poorer working memory). BPA was associated with poorer social skills among all the children.
			Behavior BASC-2 at 3 years (by parents) Executive function BRIEF-P (by parents) SRS (Social competence) (by parents)	X		
Hong et al., 2013 Cross-sectional study (Korea)	1008 children Ethnicity: Asiatic	One spot urine sample	CBCL at 8-11 years (by parents)	X		BPA levels were positively associated with behavior [CBCL total score (β= 0.85; 95%CI: 0.26, 1.44)] and learning problems [LDES total score (β= -1.90; 95%CI: -3.5, -0.30)]. Further, anxiety-depression score (β= 1.07; 95%CI: 0.57, 1.58) and listening score (β= -0.81; 95%CI: -1.27, -0.34) remained after correction for multiple comparisons.
			LDES at 8–11 years (by parents)		X	
Braun et al., 2011 HOME Study. Prospective mother-child cohort (USA).	244 mother-child pairs	Average of BPA concentrations from three spot urine samples collected throughout pregnancy	Behavior (at 3 years). BASC-2 (by parents)	X		Gestational BPA exposure was associated with higher scores of anxiety, hyperactivity, emotional control, and behavioral inhibition. Each 10-fold increase in BPA concentrations was associated with more anxious and depressed behavior and poorer emotional control and inhibition. The effect differed according to child sex (larger among girls than boys).
			Executive function (at 3 years). BRIEF-P (by parents)		X	
Stacy et al., 2017 HOME Study (follow-up)		Average of BPA concentrations from two maternal spot urine samples.	Behavior (at 8 years)BASC-2	X		Prenatal urinary BPA concentrations were associated with more maladaptive behaviors, specifically externalizing problems among girls. In boys, postnatal urinary BPA concentrations at 8 years of age were

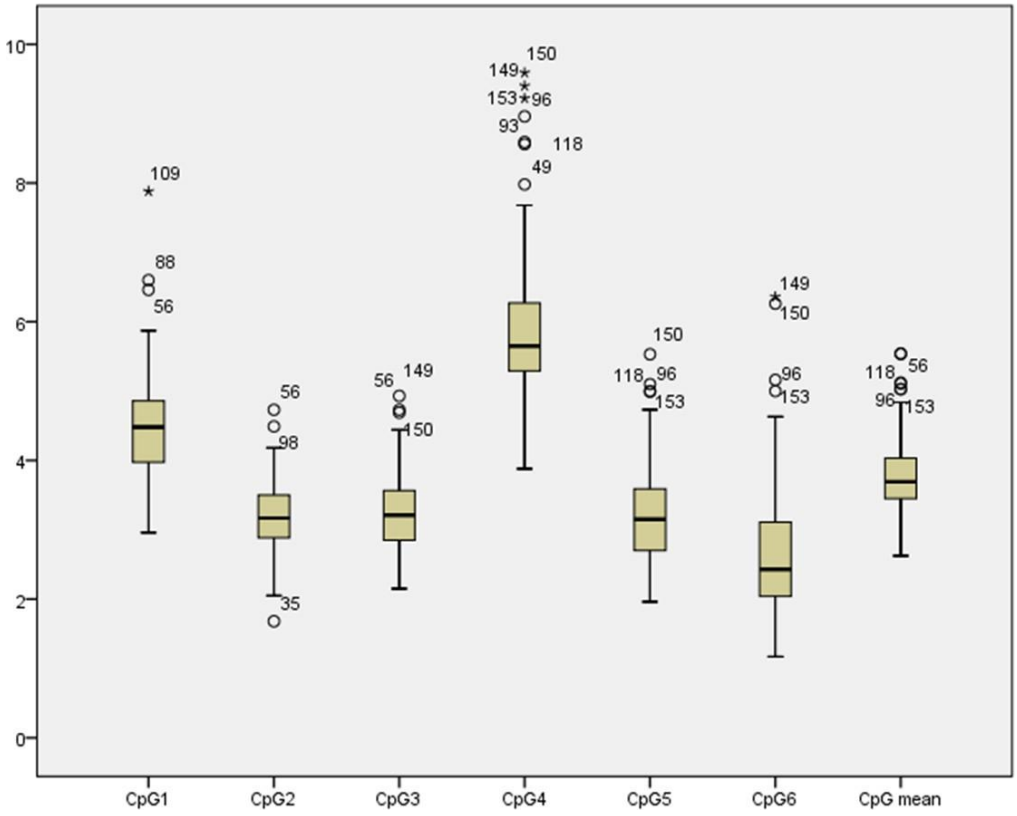
	228 mother-child pairs	BPA measured in spot urine samples at 1, 2, 3, 4, 5 and 8 years of age.	BRIEF WISC-IV		X	associated with more behavioral, executive function, and cognitive impairments. Children's urinary BPA concentrations at most ages were not associated with worse WISC-IV scores, except at 8-years of age where BPA was inversely associated with overall cognition, verbal abilities, and speed of mental processing.
Braun et al., 2017b HOME Study (another analysis)	346 mother-child pairs	BPA measured in spot urine samples at 16 and 16 weeks of gestation.	BASC-2 (at 2,3,4,5 and 8 years)	X		Prenatal BPA concentrations were persistently associated with more externalizing behaviors among girls from ages 2-8 years, enduring to school-age. In contrast, BPA concentrations were not associated with child cognitive and language abilities, gross and fine motor skills, or children's overall cognitive ability.
			BSID-II (at 1,2,3 years) WPPSI-III and WISC-IV (at 5 and 8 years)		X	
Braun et al., 2017c HOME Study (a new computerized test)	198 mother-child dyads	BPA measured in spot urine samples at 16 and 16 weeks of gestation.	Visual-spatial abilities (at 8 years) VMWM Computerized test		X	Prenatal BPA exposures were not associated with children's visual-spatial abilities at 8 years of age assessed with VMWM performance. Sex did not consistently modify these associations.
Jensen et al., 2019 Odense Child Cohort (Denmark)	796 mother-child pairs	BPA measured in one spot maternal urine sample at gestational week 28	535 parents completed the MB-CDI test (between 18 and 36 months of age)		X	Higher urinary BPA levels were associated with lower language ability score in boys, but no associations were found in girls. No associations were observed between BPA and ADHD, or other behavioral outcomes.
			658 parents completed the CBCL 1½-5 (1.5 to 5 years of age)	X		
Pérez-Lobato et al., 2016 INMA-Granada cohort (Spain)	269 children	Single spot urine sample at 9-12 years old	CBCL/6-18 completed by parents (9 to 12 years of age)	X		Among boys between 9 to 12 years of age, BPA was associated with somatic complaints and thought problems. Higher BPA concentration were associated with higher risk of a T-score above 60 in thought problems, rule-breaking problems and total problems than those with low urinary BPA concentrations.
Present study INMA-Granada cohort (Spain)			K-BIT; CPT; TAVECI; TMT; WISC-IV; FAS; STROOP (by a trained phycologist)		X	No consistent associations were found between urinary BPA concentrations and impulsivity, interference and processing speed. Only a possible association towards poorer working memory was observed.

EDEN: Study of pre- and early postnatal determinants of the child's development and health; **HOME:** Health Outcomes and Measures of the Environment; **INMA:** Infancia y Medioambiente (Childhood and Environment); **MIREC:** Maternal-Infant Research on Environmental Chemicals; **NECAT:** New England Children's Amalgam Trial; **ADHD-DSM-IV:** Attention deficit hyperactivity disorder-Criteria of Diagnostic and Statistical Manual of Mental Disorders-4th Edition; **BASC-2:** Behavior Assessment System for Children–2; **BRIEF-P:** Behavior Rating Inventory of Executive Function–Preschool; **BSID-II:** Bayley Scales of Infant Development; **CBCL 1½-5:** Child Behavior Checklist for ages 1½-5; **COWAT:** Controlled Oral Word Association Test; **CPRS:** Conner's Parent Rating Scales; **CPT:** Continuous Performance Test; **FAS:** Verbal Fluency Test; **K-BIT:** Kaufman Brief Intelligence Test; **LDES:** Learning Disability Evaluation Scale; **MB-CDI:** MacArthur-Bates Communicative Development Inventories ("Words and Sentences"); **MSCA:** McCarthy Scales of Children's Abilities; **SDQ:** Mothers completed the Strength and Difficulties Questionnaire; **SRS-2:** Social Responsiveness Scale TM–2; **STROOP:** Stroop Color and Word Test; **TAVECI:** Complutense-Spain Madrid verbal learning test; **TMT A and B:** Trail Making Test; **VMWM:** Virtual Morris Water Maze; **WIAT:** Wechsler Individual Achievement Test; **WISC-III:** Wechsler Intelligence Scale for Children-Third Edition; **WISC-IV:** Wechsler

Intelligence Scale for Children-Fourth Edition; **WPPSI-III:** Wechsler Primary and Preschool Scale of Intelligence ™-III; **WRAML:** Wide Range Assessment of Memory and Learning; **WRAVMA:** Wide Range Test of Visual-Motor Ability. *Many different neurobehavioral tests have been used to assess behavior and cognition in relation to BPA exposure, among many other psychological dimensions (learning, social interaction, specific abilities). For reasons of simplicity, we decided to group those tests assessing behavioral components (BASC-2, CBCL, SDQ, ADHD-DSM-IV) in the "behavior" group while tests assessing cognitive abilities, IQ, executive function, psychomotor development learning and language development where grouped in the "cognitive" column. However, it should be noted that executive function, learning and language development go beyond specific cognitive domains.

9.3 Supplementary Material. Article 3

Figure S1. Boxplot of BDNF DNA methylation at Exon IV of six CpG islands and the meanmethylation of CpGs. The Y-axis presents the percentage of DNA methylation.



Note: Circles and stars represent outliers.

Figure S2. Tertile analysis of selected associations among BPA, BDNF DNA methylation and thought problems t-scores. Brown color (BPA-thoughtproblems); blue color (BPA-BDNF methylation); and green color (BDNF methylation-thought problems).

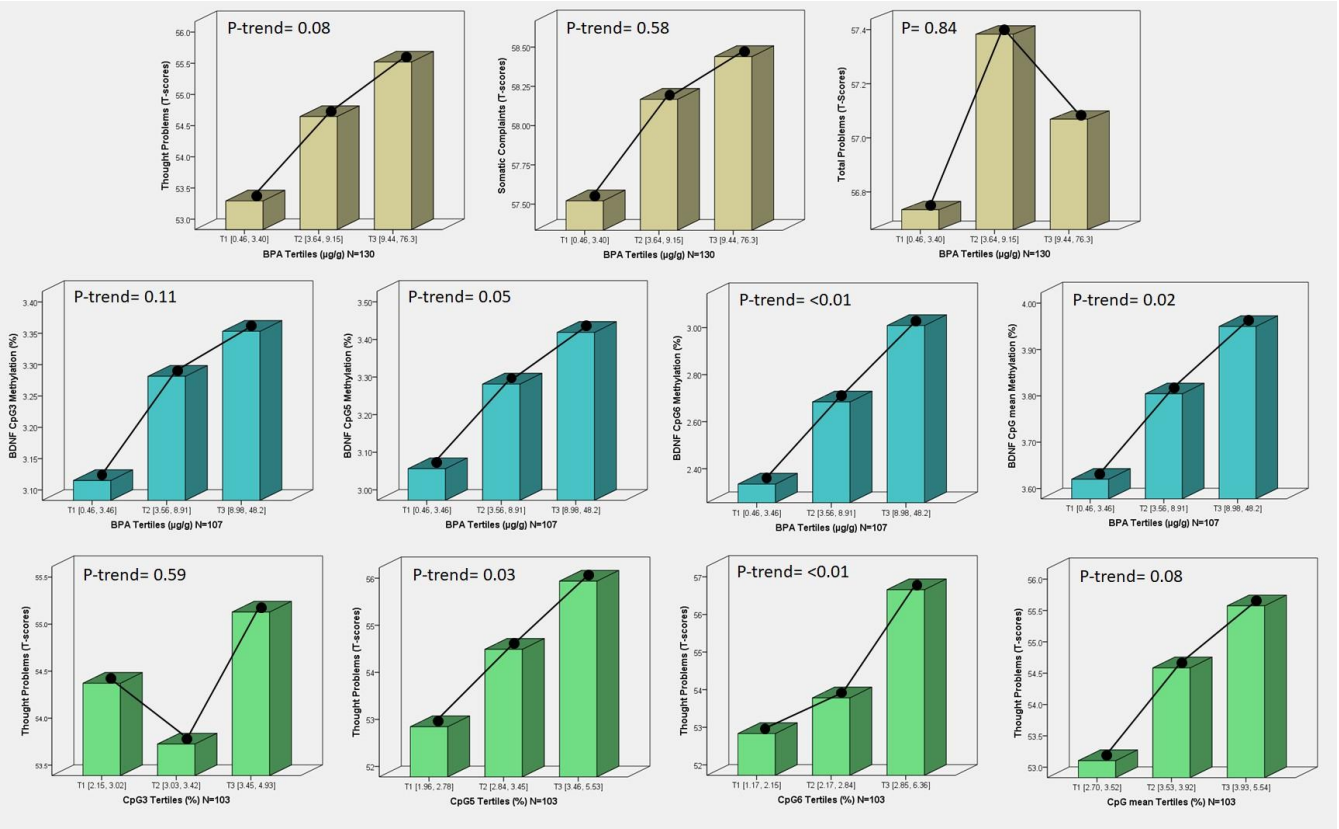


Table S1. Comparison of characteristics between the final study population and boys thatparticipated in the previous follow-up at 9-11 years of age.

Characteristics	Study population with BPA at9-11 y. and behavior data at 15-17yrs. (n=130)	Rest of sample with BPA at 9-11 yrs. that did not attend the latest follow-up (n=139)	P-value[a]
Child BPA concentrations (μg/g)	5.41 (3.05, 10.6)	4.41 (2.46, 9.06)	0.072
Child age (yrs)	9.82 (9.67, 10.0)	9.82 (9.67, 10.1)	0.861
Child cotinine (ng/mL)	7.38 (2.00, 18.7)	9.15 (2.00, 29.9)	0.255
Child BMI	18.6 (16.3, 21.4)	18.4 (16.6, 21.7)	0.720
Maternal education, n(%)			0.066
Primary	34 (26.1)	29 (20.9)	
Secondary	47 (36.2)	38 (27.3)	
University	49 (37.7)	72 (51.8)	
Child somatic problems(t-scores)	55 (50, 61)	57 (50, 61)	0.549
Child thought problems(t-scores)	51 (50, 58)	51 (50, 58)	0.571
Child total behavior problems (t-scores)	51.5 (47, 58)	53 (49, 61)	0.517

Data are presented as medians and interquartile ranges (IQRs) for continuous variables or n (%) forcategorical variables.

[a] *Value of hypothesis testing calculated using the Kruskal-Wallis test for continuous variables and theChi-square test for categorical variables.*

Table S2. Bisulfite-converted primers used for PCR amplification.

Gene	Forward primer	Reverse primer
BDNF	GATTTTGGTAATTAGTGTATTAGAGTGTT	/5Biosg/ATCAACCAAAAACTCCATTTAATCTC

Table S3. Pearson correlations coefficients among BDNF biomarkers.

Correlations

		Serum BDNF protein levels	Urinary BDNF protein levels	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG mean
Serum BDNF protein levels	Pearson Correlation	1	-.080	.111	-.203*	-.189	-.240*	-.161	-.091	-.178
	Sig. (2-tailed)		.389	.264	.040	.056	.015	.104	.359	.073
	N	120	119	103	103	103	103	103	103	103
Urinary BDNF protein levels	Pearson Correlation	-.080	1	.183	.006	-.043	-.010	-.076	-.066	-.004
	Sig. (2-tailed)	.389		.062	.950	.667	.918	.444	.506	.969
	N	119	121	104	104	104	104	104	104	104
CpG1	Pearson Correlation	.111	.183	1	.285**	.201*	-.211*	-.070	-.210*	.157
	Sig. (2-tailed)	.264	.062		.003	.038	.030	.474	.030	.106
	N	103	104	107	107	107	107	107	107	107
CpG2	Pearson Correlation	-.203*	.006	.285**	1	.624**	.359**	.487**	.331**	.636**
	Sig. (2-tailed)	.040	.950	.003		.000	.000	.000	.000	.000
	N	103	104	107	107	107	107	107	107	107
CpG3	Pearson Correlation	-.189	-.043	.201*	.624**	1	.608**	.708**	.654**	.851**
	Sig. (2-tailed)	.056	.667	.038	.000		.000	.000	.000	.000
	N	103	104	107	107	107	107	107	107	107
CpG4	Pearson Correlation	-.240*	-.010	-.211*	.359**	.608**	1	.807**	.814**	.848**
	Sig. (2-tailed)	.015	.918	.030	.000	.000		.000	.000	.000
	N	103	104	107	107	107	107	107	107	107
CpG5	Pearson Correlation	-.161	-.076	-.070	.487**	.708**	.807**	1	.868**	.911**
	Sig. (2-tailed)	.104	.444	.474	.000	.000	.000		.000	.000
	N	103	104	107	107	107	107	107	107	107
CpG6	Pearson Correlation	-.091	-.066	-.210*	.331**	.654**	.814**	.868**	1	.860**
	Sig. (2-tailed)	.359	.506	.030	.000	.000	.000	.000		.000
	N	103	104	107	107	107	107	107	107	107
CpG mean	Pearson Correlation	-.178	-.004	.157	.636**	.851**	.848**	.911**	.860**	1
	Sig. (2-tailed)	.073	.969	.106	.000	.000	.000	.000	.000	
	N	103	104	107	107	107	107	107	107	107

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Urinary BDNF protein concentrations were normalized by urinary creatinine levels

Table S4. Distribution of CBCL behavior t-scores in INMA-Granada adolescent boys (n=130).

Behavioral functions				
Syndrome scores	Score range	Mean (SD)	Median (IQR)	N (%) borderline /clinical*
Anxious/Depressed	50-78	54.7 (5.8)	51 (50, 57)	30 (23.1)
Withdrawn	50-81	56.9 (6.2)	54 (53, 60)	43 (33.1)
Somatic complaints	50-79	58.0 (7.3)	58 (50, 61)	46 (35.4)
Social problems	50-75	54.6 (5.7)	51 (50, 58)	28 (21.5)
Thought problems	50-82	54.5 (5.7)	51 (50, 56)	17 (13.1)
Attention problems	50-75	55.4 (6.1)	53 (50, 59)	24 (18.5)
Rule-breaking problems	50-89	53.5 (5.5)	51 (50, 54)	17 (13.1)
Aggressive behavior	50-81	54.8 (5.5)	52 (50, 58)	27 (20.8)
Composite scores				
Internalizing problems	50-80	56.7 (6.7)	55 (51, 61)	39 (30.0)
Externalizing problems	50-69	53.8 (4.4)	51 (51, 57)	18 (13.8)
Total problems	50-80	57.1 (7.3)	50 (56, 60)	30 (23.1)

CBCL: Child behavior checklist (6–18 years). Higher t-scores mean more problems in all scales.

** T-scores categorized as borderline or clinical (≥60), or normal (<60).*

Table S5. Mediation analysis showing associations between childhood urinary BPA concentrations, BDNF DNA methylation and thought/somatic problems t-scores during adolescence (n=103).

BDNF methylation marker	CBCL scale	Indirect effectβ (95% CI)	Direct effectβ (95% CI)	Total effectβ (95% CI)	Estimate dpercent mediated (%)*
Log-2 CpG5	Thought problems	0.13 (-0.04, 0.40)	0.55 (-0.33, 1.43)	0.68 (-0.19, 1.55)	19%
	Somatic Complaints	0.09 (-0.18, 0.45)	0.81 (-0.37, 1.99)	0.90 (-0.26, 2.06)	10%
Log-2 CpG6	**Thought problems**	**0.23 (0.01, 0.57)**	0.45 (-0.45, 1.34)	0.68 (-0.19, 1.55)	**34%**
	Somatic Complaints	0.17 (-0.22, 0.62)	0.72 (-0.48, 1.93)	0.90 (-0.26, 2.06)	19%
Log-2 CpG mean	Thought problems	0.09 (-0.08, 0.34)	0.60 (-0.30, 1.49)	0.68 (-0.19, 1.55)	13%
	Somatic Complaints	-0.00 (-0.34, 0.30)	0.90 (-0.29, 2.09)	0.90 (-0.26, 2.06)	0%

Continuous log2-transformed BPA concentrations and CpG methylation percentage, and continuous thought problems t-scores were used. Models were adjusted for age and BMI z-scores (continuous) at neuropsychological evaluation (15-17 years), maternal education (primary, secondary or university), andurinary cotinine at 9-11y. (continuous).

[a] *The direct effect, indirect effect, and total effect reflect the natural beta coefficients (β) and 95%Confidence Intervals (95% CI). The indirect effect represents the mediated effect.*

[b] *Percent mediated = indirect effect/ (direct effect + indirect effect) × 100.*

9.4 Supplementary Material. Article 4

Table S1. Limits of detection (LOD) and limits of quantification (LOQ) of metals and Bisulfite-converted primers used for PCR amplification

Metals	As	Cd	Hg	Pb
LOD (µg/L)	0.60	0.01	0.05	0.16
LOQ (µg/L)	1.81	0.04	0.14	0.48
Gene	**Forward primer**		**Reverse primer**	
BDNF	GATTTTGGTAATTAGTGTATTAGAGTGTT		/5Biosg/ATCAACCAAAAACTCCATTTAATCTC	

Table S2. Correlation between urinary metal concentrations (µg/L)

		As	Cd	Hg	Pb
As	Spearman's rho	1	0.22	0.52	0.19
	P		0.01	<0.01	0.03
Cd	Spearman's rho	0.22	1	0.52	0.36
	P	0.01		<0.01	<0.01
Hg	Spearman's rho	0.52	0.52	1	0.10
	P	<0.01	<0.01		0.26
Pb	Spearman's rho	0.19	0.36	0.10	1
	P	0.03	<0.01	0.26	

Table S3. Distribution of CBCL outcome scores (n=125)

CBCL scores	Range	Mean[a]	SD	Median	n (%) in borderline or clinical range[b]
Syndrome scores					
Anxious depressed	50-79	55.1	6.1	54.0	37 (25.7)
Withdrawn	50-89	57.5	7.3	54.0	50 (34.7)
Somatic complaints	50-79	58.1	7.2	58.0	53 (36.8)
Social problems	50-79	55.1	6.4	51.0	33 (22.9)
Thought problems	50-82	54.9	6.0	51.0	23 (15.1)
Attention problems	50-86	56.1	7.0	53.0	31 (20.4)
Rule-breaking behavior	50-89	54.0	6.2	51.0	22 (15.3)
Aggressive behavior	50-81	55.2	6.4	52.0	33 (21.7)
Composite scores					
Internalizing problems	34-79	55.6	9.2	55.0	46 (31.9)
Externalizing problems	34-79	51.0	9.2	51.0	23 (16.0)
Total problems	31-84	54.0	9.2	54.0	38 (26.4)

[a]For all scales, raw scores were transformed to T-scores to compare each subject to a normative sample of adolescents.
[b]Adolescents were grouped in two groups according to the T-score obtained in each scale: clinical or subclinical (≥60) and normal (<60) T-score.
SD: Standard deviation.

Table S4. Linear regression for the association between metal exposure and CBCL scores (n=125)

CBCL scores	Metals			
	As	Cd	Hg	Pb
Syndrome scores	**β (95% CI)**	**β (95% CI)**	**β (95% CI)**	**β (95% CI)**
Anxious depressed	0.02 (-1.10,1.14)	0.44 (-1.88,2.76)	0.04 (-1.48,1.56)	-0.58 (-2.23,1.07)
Withdrawn	0.07 (-1.21,1.34)	0.05 (-2.59,2.70)	-0.82 (-2.55,0.92)	-0.29 (-2.17,1.59)
Somatic complaints	0.79 (-0.63,2.21)	-2.08 (-5.02,0.86)	0.35 (-1.58,2.28)	-0.81 (-2.90,1.28)
Social problems	0.36 (-0.76,1.47)	1.82 (-0.48,4.12)	-1.62 (-3.13,-0.11)*	-0.19 (-1.83,1.45)
Thought problems	0.02 (-1.03,1.06)	1.31 (-0.85,3.47)	0.32 (-1.10,1.74)	-0.58 (-2.12,0.97)
Attention problems	-0.09 (-1.14,0.97)	0.84 (-1.34,3.02)	0.27 (-1.16,1.70)	-0.74 (-2.29,0.81)
Rule-breaking behavior	-0.70 (-1.65,0.25)	-0.57 (-2.54,1.39)	0.04 (-1.25,1.32)	1.11 (-0.29,2.51)
Aggressive behavior	-0.09 (-1.22,1.03)	2.11 (-0.22,4.44)†	-0.74 (-2.27,0.79)	-0.33 (-1.98,1.33)
Composite scores				
Internalizing problems	0.99 (-0.87,2.86)	-0.71 (-4.57,3.16)	-0.25 (-2.78, 2.29)	-1.36 (-4.11,1.39)
Externalizing problems	-0.53 (-2.26,1.20)	1.86 (-1.71,5.43)	-0.51 (-2.85,1.83)	-0.35 (-2.89,2.20)
Total problems	0.20 (-1.52,1.93)	0.78 (-2.78,4.35)	-0.53 (-2.87,1.81)	-0.98 (-3.52,1.56)

Model was adjusted for adolescent's age (months), BMI (kg/m^2), and creatinine (mg/dL), maternal schooling (primary/secondary/university) and intelligence, and for all metals simultaneously (continuous).

[a]For all scales, raw scores were transformed to T-scores to compare each subject to a normative sample of adolescents.

SD: Standard deviation.

*p<0.05; †p< 0.1

Table S5. Linear regression for the association between metal exposure and effect biomarkers (serum BDNF; n=125; BDNF gene DNA methylation, n=113)

Effect biomarkers	Metals			
	As	Cd	Hg	Pb
	β (95% CI)	**β (95% CI)**	**β (95% CI)**	**β (95% CI)**
Serum BDNF	-0.64 (-2.36,1.08)	-2.02 (-2.36,1.55)	-0.44 (-2.80,1.91)	1.65 (-0.84,4.15)
BDNF methylation				
CpG 1	0.02 (-0.15,0.19)	-0.11 (-0.48,0.27)	-0.02 (-0.26,0.22)	0.22 (-0.04,0.48)†
CpG 2	0.08 (-0.02,0.19)	-0.06 (-0.29,0.18)	-0.04 (-0.19,0.11)	-0.02 (-0.17,0.03)
CpG 3	0.08 (-0.04,0.20)	-0.20 (-0.47,0.06)	-0.02 (-0.19,0.14)	0.06 (-0.11,0.24)
CpG 4	0.15 (-0.09,0.39)	-0.17 (-0.73,0.38)	-0.07 (-0.43,0.30)	0.18 (-0.18,0.54)
CpG 5	0.12 (-0.04,0.28)	-0.06 (-0.42,0.31)	-0.04 (-0.27,0.20)	0.03 (-0.21,0.28)
CpG 6	0.16 (-0.09,0.40)	-0.01 (-0.56,0.55)	-0.05 (-0.41,0.31)	0.10 (-0.25,0.46)
CpG total	0.12 (-0.00,0.25)†	-0.11 (-0.39,0.18)	-0.03 (-0.20,0.15)	0.08 (-0.10,0.27)

Model was adjusted for adolescent's age (months), BMI (kg/m^2), and creatinine (mg/dL), maternal schooling (primary/secondary/university) and intelligence, and for all metals simultaneously (continuous).

*p<0.05; †p< 0.1

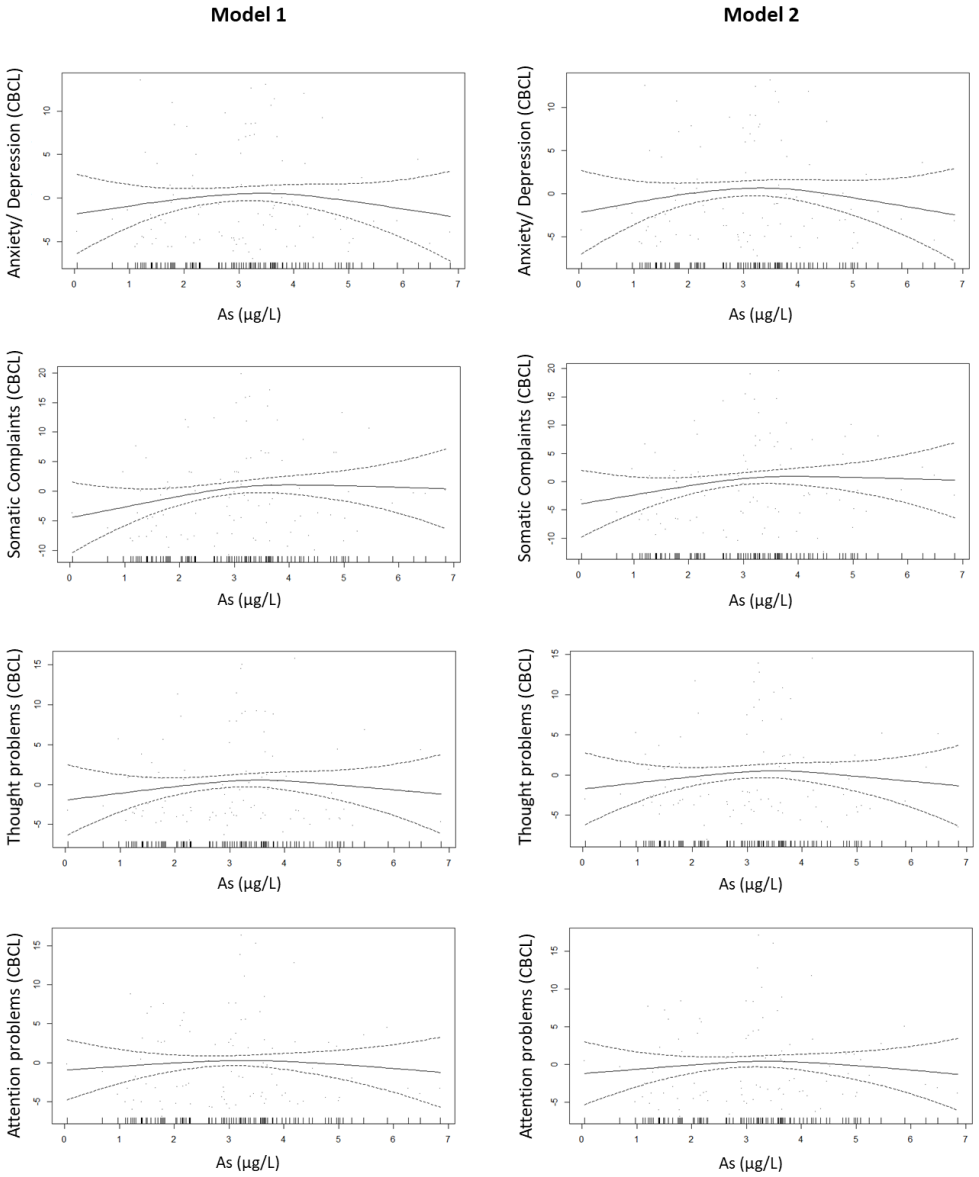


Figure S1. Generalized additive models (GAM) for the relationship between urinary As concentrations (natural log-transformed concentrations) and adolescents' behavior. Model 1: adjusted for adolescents' age, BMI, and urinary creatinine (mg/dL), maternal education and maternal intelligence, and for all metals simultaneously; Model 2: additionally adjusted for passive tobacco smoking and total fish consumption.

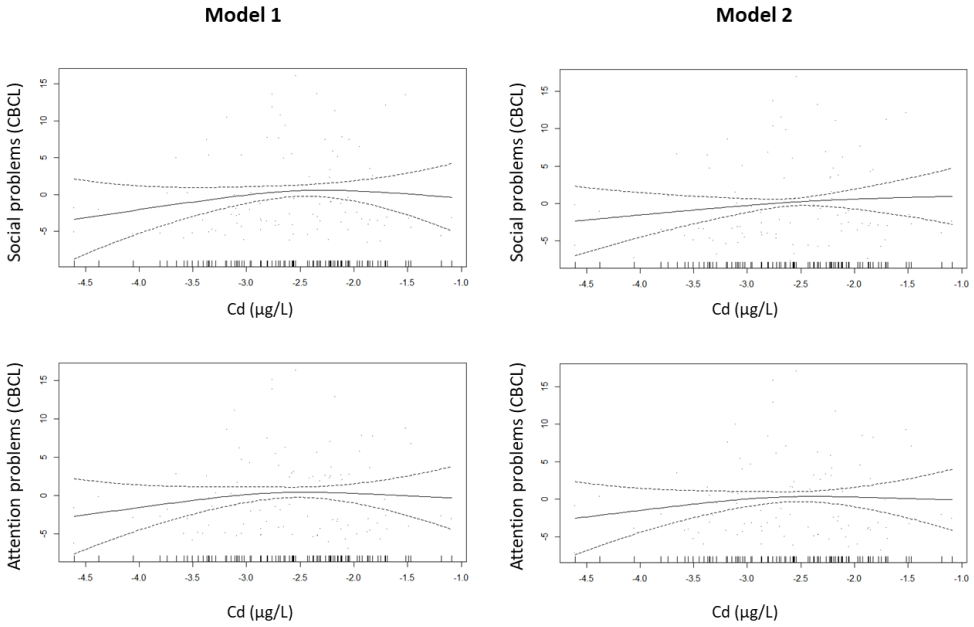


Figure S2. Generalized additive models (GAM) for the relationship between urinary Cd concentrations (natural log-transformed concentrations) and adolescents' behavior. Model 1: adjusted for adolescents' age, BMI, and urinary creatinine (mg/dL), maternal education and maternal intelligence, and for all metals simultaneously; Model 2: additionally adjusted for passive tobacco smoking and total fish consumption.

Table S6. Association between serum BDNF protein levels and CBCL outcome scores (n=113).

CBCL scores	Serum BDNF protein tertiles (ng/mL)			p-trend
	1st (16.0-28.0)	2nd (28.3-36.7)	3rd (36.8-56.0)	
Syndrome scores	**Mean (SD)**	**β (95% CI)**	**β (95% CI)**	
Anxious depressed	55.2 (5.1)	-0.52 (-3.48,2.44)	-0.22 (-3.39,2.95)	0.88
Withdrawn	59.2 (8.0)	-3.77 (-7.00,-0.53)*	-3.49 (-6.95,-0.02)*	0.04
Somatic complaints	59.0 (8.7)	-0.76 (-4.60,3.09)	-1.73 (-5.85,2.39)	0.41
Social problems	55.9 (6.3)	-1.46 (-4.42,1.50)	-2.52 (-5.69,0.65)	0.12
Thought problems	56.0 (6.3)	-1.72 (-4.42,0.98)	-2.88 (-5.78,0.01)†	0.04
Attention problems	55.8 (5.4)	-1.21 (-3.86,1.62)	-0.80 (-3.74,2.14)	0.58
Rule-breaking behavior	54.7 (5.7)	-1.35 (-3.85,1.14)	-1.89 (-4.57,0.78)	0.16
Aggressive behavior	56.2 (6.2)	-1.55 (-4.51,1.41)	-1.50 (-4.67,1.68)	0.34
Composite scores				
Internalizing problems	57.3 (8.8)	-4.13(-9.01,0.74)†	-2.65 (-7.87,2.58)	0.30
Externalizing problems	53.3 (9.1)	-2.99 (-7.51,1.53)	-2.26 (-7.11,2.58)	0.34
Total problems	55.3 (8.3)	-3.85 (-8.28,0.58)†	-2.87 (-7.62,1.88)	0.22

CI: Confidence Interval; SD: Standard deviation.
[a]For all scales, raw scores were transformed to T-scores to compare each subject to a normative sample of adolescents. Higher scoring indicated worse behavioral outcomes.
All models were adjusted for adolescent's age and BMI, and maternal education and intelligence.
*p≤ 0.05; †p< 0.10

Sensitivity Analyses

Table S7. Adjusted models for the association between tertiles of urinary metal concentrations and CBCL scores (n=125).

CBCL scores	Model 1				Model 2			
	As tertiles (µg/L)							
	1st (LOD-2.29) Mean (SD)	2nd (2.31-3.56) β (95% CI)	3rd (3.57-6.85) β (95% CI)	p-trend	1st (LOD-2.29) Mean (SD)	2nd (2.31-3.56) β (95% CI)	3rd (3.57-6.85) β (95% CI)	p-trend
Syndrome scores								
Anxious depressed	54.2 (5.1)	1.18 (-1.91,4.28)	0.44 (-2.65,3.52)	0.80	54.5 (5.2)	1.40 (-1.88,4.67)	0.36 (-2.84,3.56)	0.88
Withdrawn	57.1 (6.0)	-0.62 (-4.17,2.94)	-0.77 (-4.31,2.78)	0.67	57.2 (6.2)	-0.50 (-4.23,3.24)	-0.44 (-4.08,3.21)	0.82
Somatic complaints	55.7 (6.0)	3.76 (-0.20,7.71)†	258 (-1.36,6.52)	0.22	55.7 (6.1)	3.86 (-0.28,7.99)†	2.68 (-1.35,6.72)	0.24
Social problems	55.0 (5.7)	-0.06 (-3.22,3.10)	-1.27 (-4.42,1.88)	0.41	54.7 (5.6)	1.26 (-2.01,4.53)	-0.30 (-3.49,2.89)	0.79
Thought problems	53.3 (4.2)	2.08 (-0.81,4.98)	0.74 (-2.15,3.62)	0.66	53.3 (4.3)	2.08 (-0.98,5.14)	0.79 (-2.20,3.77)	0.68
Attention problems	54.8 (4.7)	1.91 (-0.97,4.80)	-0.36 (-3.23,2.52)	0.75	54.6 (4.7)	2.67 (-0.37,5.70)†	0.15 (-2.81,3.12)	0.95
Rule-breaking behavior	54.6 (5.6)	-1.10 (-3.77,1.57)	-1.60 (-4.26,1.06)	0.24	54.8 (5.8)	-0.79 (-3.50,1.92)	-1.39 (-4.03,1.26)	0.30
Aggressive behavior	55.9 (6.0)	-1.26 (-4.43,1.90)	-0.93 (-4.08,2.22)	0.58	55.9 (6.0)	-0.40 (-3.68,2.88)	-0.40 (-3.61,2.80)	0.81
Composite scores								
Internalizing problems	52.9 (10.1)	1.76 (-3.42,6.95)	3.15 (-2.02,8.32)	0.23	53.1 (10.5)	2.15 (-3.37,7.67)	3.53 (-1.86,8.92)	0.20
Externalizing problems	52.8 (9.2)	-2.24 (-7.03,2.56)	-1.01 (-5.79,3.77)	0.70	53.0 (9.1)	-1.16 (-6.06,3.74)	-0.31 (-5.10,4.47)	0.93
Total problems	52.9 (9.0)	0.15 (-4.63,4.93)	0.42 (-4.35,5.18)	0.86	52.8 (9.2)	1.24 (-3.75,6.23)	1.25 (-3.62,6.12)	0.63
CBCL scores	Cd tertiles (µg/L)							
	1st (LOD-0.06) Mean (SD)	2nd (0.06-0.10) β (95% CI)	3rd (0.11-0.34) β (95% CI)	p-trend	1st (LOD-0.06) Mean (SD)	2nd (0.06-0.10) β (95% CI)	3rd (0.11-0.34) β (95% CI)	p-trend
Syndrome scores								
Anxious depressed	54.3 (5.1)	0.33 (-2.65,3.30)	1.15 (-2.27,4.58)	0.51	54.3 (5.1)	0.64 (-2.41,3.70)	1.52 (-1.98,5.02)	0.39
Withdrawn	56.3 (5.4)	1.47 (-1.92,4.87)	0.26 (-3.66,4.18)	0.83	56.3 (5.4)	1.15 (-2.33,4.63)	0.40 (-3.58,4.38)	0.79
Somatic complaints	58.7 (7.5)	-1.78 (-5.57,2.01)	-4.35 (-8.72,0.0)†	0.06	58.7 (7.5)	-1.74 (-5.60,2.11)	-4.34 (-8.74,0.07)	0.06
Social problems	53.1 (4.0)	3.88 (0.94,6.81)**	2.28 (-1.11,5.66)	0.14	53.1 (4.0)	3.77 (0.81,6.72)*	1.98 (-1.41,5.36)	0.18
Thought problems	52.9 (3.9)	1.81 (-0.95,4.56)	2.91 (-0.27,6.08)†	0.06	52.9 (3.9)	1.92 (-0.91,4.75)	2.90 (-0.34,6.13)†	0.07
Attention problems	54.1 (4.5)	3.13 (0.40,5.86)*	0.75 (-2.39,3.90)	0.50	54.1 (4.5)	3.23 (0.43,6.03)*	0.58 (-2.62,3.78)	0.56
Rule-breaking behavior	53.7 (4.9)	0.43 (-2.15,3.01)	-0.01 (-2.99,2.97)	0.98	53.7 (4.9)	0.55 (-2.00,3.09)	0.13 (-2.78,3.04)	0.90
Aggressive behavior	53.9 (4.8)	2.85 (-0.13,5.83)†	2.79 (-0.65,6.22)	0.09	53.9 (4.8)	3.19 (0.22,6.17)*	2.71 (-0.69,6.11)	0.09
Composite scores								
Internalizing problems	54.4 (10.2)	0.25 (-4.76,5.27)	-0.78 (-6.56,5.0)	0.81	54.4 (10.2)	0.19 (-5.01,5.39)	-0.47 (-6.42,5.48)	0.89
Externalizing problems	50.5 (8.6)	2.64 (-1.94,7.22)	3.09 (-2.19,8.37)	0.23	50.5 (8.6)	3.09 (-1.42,7.61)	3.17 (-2.00,8.34)	0.20
Total problems	52.0 (8.2)	2.81 (-1.74,7.36)	1.11 (-4.13,6.36)	0.60	52.0 (8.2)	2.96 (-1.66,7.58)	1.07 (-4.21,6.35)	0.60
CBCL scores	Hg tertiles (µg/L)							
	1st (LOD-0.40) Mean (SD)	2nd (0.41-0.86) β (95% CI)	3rd (0.86-4.57) β (95% CI)	p-trend	1st (LOD-0.40) Mean (SD)	2nd (0.41-0.86) β (95% CI)	3rd (0.86-4.57) β (95% CI)	p-trend
Syndrome scores								
Anxious depressed	55.4 (5.9)	-1.28 (-4.27,1.72)	-0.52 (-3.69,2.64)	0.71	55.8 (5.9)	-1.50 (-4.61,1.61)	-0.55 (-3.89,2.80)	0.72
Withdrawn	57.4 (6.1)	-0.67 (-4.11,2.76)	-1.61 (-5.24,2.02)	0.38	57.5 (6.3)	-0.52 (-4.06,3.01)	-1.76 (-5.56,2.05)	0.36
Somatic complaints	58.1 (7.6)	-0.92 (-4.82,2.99)	-0.15 (-4.28,3.97)	0.92	58.2 (7.8)	-1.73 (-5.72,2.26)	-1.28 (-5.58,3.02)	0.54

Social problems	56.3 (6.7)	-2.46 (-5.46,0.54)	-3.24 (-6.41,-0.07)*	0.04	56.0 (6.9)	-1.91 (-4.99,1.16)	-2.55 (-5.86,0.76)	0.12
Thought problems	53.7 (4.6)	0.14 (-2.69,2.97)	1.11 (-1.88,4.10)	0.47	53.8 (4.7)	-0.29 (-3.22,2.65)	0.50 (-2.66,3.66)	0.76
Attention problems	55.4 (5.2)	0.50 (-2.34,3.34)	-0.39 (-3.39,2.60)	0.82	55.2 (5.3)	0.56 (-2.39,3.50)	-0.31 (-3.48,2.86)	0.86
Rule-breaking behavior	54.3 (5.4)	-1.58 (-4.17,1.01)	-0.30 (-3.03,2.43)	0.77	54.5 (5.5)	-1.98 (-4.53,0.58)	-0.77 (-3.52,1.98)	0.54
Aggressive behavior	55.4 (5.5)	-0.06 (-3.13,3.02)	-0.37 (-3.62,2.88)	0.82	55.4 (5.5)	0.04 (-3.08,3.16)	-0.04 (-3.40,3.32)	0.98
Composite scores								
Internalizing problems	55.1 (10.9)	-1.29 (-6.35,3.76)	0.27 (-5.07,5.61)	0.95	55.3 (11.2)	-1.46 (-6.74,3.82)	-0.03 (-5.72,5.66)	0.97
Externalizing problems	52.1 (9.1)	-1.01 (-5.67,3.65)	0.53 (-4.40,5.45)	0.86	52.3 (9.1)	-1.12 (-5.76,3.53)	0.65 (-4.35,5.66)	0.82
Total problems	53.7 (9.0)	-1.58 (-6.21,3.04)	-0.58 (-5.46,4.30)	0.79	53.8 (9.2)	-1.65 (-6.39,3.08)	-0.63 (-5.73,4.47)	0.79

	Pb tertiles (µg/L)							
CBCL scores	**1st (LOD-0.34) Mean (SD)**	**2nd (0.34-0.58) β (95% CI)**	**3rd (0.59-4.45) β (95% CI)**	**p-trend**	**1st (LOD-0.34) Mean (SD)**	**2nd (0.34-0.58) β (95% CI)**	**3rd (0.59-4.45) β (95% CI)**	**p-trend**
Syndrome scores								
Anxious depressed	53.7 (9.0)	0.74 (-2.25,3.74)	-1.61 (4.73,1.51)	0.29	55.1 (6.0)	0.58 (-2.58,3.74)	-1.55 (-4.87,1.77)	0.33
Withdrawn	57.0 (5.2)	0.57 (-2.90,4.04)	-0.88 (-4.49,2.74)	0.61	57.0 (5.3)	0.67 (-2.95,4.28)	-0.96 (-4.76,2.83)	0.59
Somatic complaints	59.2 (7.1)	-2.73 (-6.61,1.16)	-3.19 (-7.24,0.86)	0.13	59.3 (7.2)	-2.14 (-6.18,1.91)	-3.36 (-7.60,0.89)	0.12
Social problems	54.4 (5.4)	1.52 (-1.56,4.61)	0.15 (-3.07,3.36)	0.96	54.2 (5.4)	2.06 (-1.11,5.22)	0.67 (-2.65,3.99)	0.73
Thought problems	54.2 (4.7)	0.31 (-2.56,3.17)	-0.24 (-3.22,2.75)	0.87	54.2 (4.8)	1.02 (-1.97,4.02)	0.12 (-3.02,3.27)	0.97
Attention problems	55.6 (5.6)	-0.50 (-3.35,2.36)	-1.51 (-4.48,1.47)	0.31	55.5 (5.7)	0.02 (-2.98,3.02)	-1.27 (-4.42,1.89)	0.41
Rule-breaking behavior	53.8 (4.5)	-0.54 (-3.16,2.08)	0.75 (-1.98,3.48)	0.57	53.8 (4.6)	0.30 (-2.33,2.93)	1.52 (-1.23,4.28)	0.26
Aggressive behavior	55.1 (5.5)	0.45 (-2.65,3.56)	0.07 (-3.16,3.30)	0.98	54.9 (5.5)	1.32 (-1.86,4.49)	1.31 (-2.02,4.64)	0.45
Composite scores								
Internalizing problems	56.3 (8.7)	-2.50 (-7.56,2.56)	-3.63 (-8.90,1.64)	0.18	56.4 (8.9)	-2.46 (-7.82,2.90)	-3.75 (-9.37,1.88)	0.19
Externalizing problems	52.7 (7.2)	-1.75 (-6.45,2.95)	-1.40 (-6.30,3.49)	0.58	52.5 (7.2)	-0.49 (-5.25,4.28)	0.38 (-4.62,5.37)	0.87
Total problems	54.4 (7.3)	-1.73 (-6.38,2.91)	-2.69 (-7.53,2.14)	0.27	54.3 (7.4)	-0.72 (-5.56,4.12)	-1.74 (-6.83,3.34)	0.49

Model 1: adjusted for adolescent's age, BMI, and creatinine (mg/dL), maternal schooling and intelligence.

Model 2: additionally adjusted for passive tobacco smoking and total fish intake of adolescents.

For all subscales, higher score indicates more behavioral problems.

**p<0.0; *p<0.05; †p<0.10

Table S8. Adjusted models for the association of tertiles of urinary metal concentration with serum BDNF (n=125) and BDNF gene methylation (n=113).

	Model 1				Model 2			
	As tertiles (µg/L)							
	1st (LOD-2.29) Mean (SD)	**2nd (2.31-3.56) β (95% CI)**	**3rd (3.57-6.85) β (95% CI)**	**p-trend**	**1st (LOD-2.29) Mean (SD)**	**2nd (2.31-3.56) β (95% CI)**	**3rd (3.57-6.85) β (95% CI)**	**p-trend**
sBDNF	35.8 (10.3)	-2.22 (-7.02,2.58)	-3.57 (-8.38,1.24)	0.14	35.7 (10.6)	-1.60 (-6.75,3.54)	-3.21 (-8.26,1.83)	0.21
metBDNF								
CpG 1	4.6 (0.8)	-0.13 (-0.60,0.34)	0.09 (-0.38,0.57)	0.67	4.6 (0.9)	-0.03 (-0.55,0.43)	0.14 (-0.34,0.63)	0.53
CpG 2	3.1 (0.5)	0.17 (-0.12,0.46)	0.19 (-0.10,0.49)	0.20	3.09 (0.5)	0.15 (-0.15,0.46)	0.19 (-0.11,0.49)	0.23
CpG 3	3.2 (0.5)	0.10 (-0.23,0.43)	0.16 (-0.17,0.50)	0.33	3.2 (0.5)	0.05 (-0.28,0.39)	0.12 (-0.21,0.45)	0.46
CpG 4	5.8 (0.9)	0.24 (-0.44,0.91)	0.45 (-0.24,1.14)	0.20	5.8 (0.9)	0.18 (-0.52,0.87)	0.42 (-0.28,1.12)	0.23
CpG 5	3.1 (0.7)	0.29 (-0.17,0.75)	0.37 (-0.09,0.84)	0.12	3.1 (0.7)	0.22 (-0.23,0.67)	0.33 (-0.13,0.78)	0.16
CpG 6	2.5 (0.9)	0.36 (-0.31,1.04)	0.46 (-0.25,1.16)	0.20	2.5 (0.8)	0.28 (-0.37,0.93)	0.38 (-0.29,1.06)	0.26
CpG t	3.7 (0.5)	0.21 (-0.13,0.55)	0.34 (0.00,0.68)*	0.05	3.7 (0.5)	0.16 (-0.18,0.50)	0.30 (-0.04,0.64)†	0.08
	Cd tertiles (µg/L)							
	1st (LOD-0.06) Mean (SD)	**2nd (0.06-0.10) β (95% CI)**	**3rd (0.11-0.34) β (95% CI)**	**p-trend**	**1st (LOD-0.06) Mean (SD)**	**2nd (0.06-0.10) β (95% CI)**	**3rd (0.11-0.34) β (95% CI)**	**p-trend**
sBDNF	37.3 (10.1)	-5.65 (-10.21,-1.09)*	-4.36 (-9.59,0.87)	0.08	37.3 (10.1)	-5.80 (-10.51,-1.08)*	-4.86 (-10.23,0.50)†	0.06
metBDNF								
CpG 1	4.6 (1.0)	-0.10 (-0.56,0.36)	-0.01 (-0.55,0.52)	0.93	4.6 (1.0)	-0.12 (-0.59,0.35)	-0.01 (-0.55,0.53)	0.93
CpG 2	3.3 (0.5)	-0.07 (-0.36,0.21)	-0.24 (-0.57,0.09)	0.16	3.3 (0.5)	-0.09 (-0.38,0.20)	-0.25 (-0.59,0.08)	0.14
CpG 3	3.4 (0.6)	-0.26 (-0.57,0.05)†	-0.37 (-0.73,-0.00)*	0.04	3.4 (0.6)	-0.27 (-0.58,0.03)†	-0.38 (-0.73,-.02)*	0.03
CpG 4	6.0 (1.1)	0.01 (-0.65,0.68)	-0.01 (-0.81,0.79)	0.98	6.0 (1.1)	0.01 (-0.66,0.69)	-0.04 (-0.84,0.77)	0.94
CpG 5	3.3 (0.8)	-0.06 (-0.51,0.40)	-0.11 (-0.64,0.41)	0.67	3.3 (0.8)	-0.05 (-0.49,0.39)	-0.12 (-0.62,0.39)	0.64
CpG 6	2.7 (1.1)	0.10 (-0.58,0.78)	-0.04 (-0.82,0.73)	0.93	2.7 (1.1)	0.14 (-0.52,0.79)	-0.05 (-0.78,0.68)	0.92
CpG t	3.9 (0.6)	-0.07 (-0.41,0.27)	-0.14 (-0.55,0.27)	0.49	3.9 (0.6)	-0.09 (-0.42,0.24)	-0.19 (-0.59,0.21)	0.34
	Hg tertiles (µg/L)							
	1st (LOD-0.40) Mean (SD)	**2nd (0.41-0.86) β (95% CI)**	**3rd (0.86-4.57) β (95% CI)**	**p-trend**	**1st (LOD-0.40) Mean (SD)**	**2nd (0.41-0.86) β (95% CI)**	**3rd (0.86-4.57) β (95% CI)**	**p-trend**
sBDNF	35.2 (9.9)	-1.60 (-6.34,3.15)	-2.85 (-7.72,2.02)	0.25	35.1 (10.2)	-1.17 (-6.14,3.80)	-2.17 (-7.36,3.02)	0.41
metBDNF								
CpG 1	4.6 (0.9)	-0.33 (-0.76,0.11)	0.32 (-0.14,0.78)	0.25	4.6 (1.0)	-0.25 (-0.70,0.19)	0.41 (-0.07,0.89)†	0.13
CpG 2	3.2 (0.5)	0.10 (-0.19,0.38)	-0.03 (-0.33,0.27)	0.88	3.2 (0.5)	0.07 (-0.22,0.36)	-0.07 (-0.39,0.24)	0.69
CpG 3	3.3 (0.6)	-0.05 (-0.36,0.27)	-0.22 (-0.55,0.11)	0.20	3.3 (0.6)	-0.06 (-0.37,0.26)	-0.21 (-0.55,0.13)	0.23
CpG 4	5.9 (1.1)	0.38 (-0.25,1.00)	-0.23 (-0.94,0.48)	0.66	5.9 (1.1)	0.35 (-0.30,0.99)	-0.26 (-1.01,0.49)	0.59
CpG 5	3.3 (0.8)	0.08 (-0.37,0.52)	-0.20 (-0.68,0.28)	0.44	3.2 (0.8)	0.06 (-0.37,0.50)	-0.20 (-0.68,0.28)	0.44
CpG 6	2.7 (1.0)	0.09 (-0.56,0.74)	-0.30 (-1.03,0.42)	0.45	2.7 (1.1)	0.09 (-0.54,0.73)	-0.22 (-0.93,0.49)	0.58
CpG t	3.8 (0.6)	0.11 (-0.23,0.45)	-0.03 (-0.39,0.33)	0.94	3.8 (0.6)	0.09 (-0.24,0.43)	-0.05 (-0.41,0.32)	0.84
	Pb tertiles (µg/L)							

	1st (LOD-0.34) Mean (SD)	2nd (0.34-0.58) β (95% CI)	3rd (0.59-4.45) β (95% CI)	p-trend	1st (LOD-0.34) Mean (SD)	2nd (0.34-0.58) β (95% CI)	3rd (0.59-4.45) β (95% CI)	p-trend
sBDNF	34.2 (9.7)	-0.34 (-5.19,4.51)	1.37 (-3.59,6.32)	0.56	34.0 (9.8)	-0.17 (-5.28,4.95)	2.01 (-3.26,7.27)	0.42
metBDNF								
CpG 1	4.5 (1.0)	0.05 (-0.42,0.53)	0.06 (-0.43,0.55)	0.81	4.5 (1.0)	0.02 (-0.47,0.52)	0.06 (-0.44,0.56)	0.81
CpG 2	3.2 (0.5)	0.05 (-0.25,0.35)	-0.05 (-0.35,0.25)	0.68	3.19 (0.5)	0.08 (-0.22,0.39)	-0.08 (-0.39,0.23)	0.55
CpG 3	3.2 (0.5)	0.10 (-0.24,0.43)	-0.00 (-0.34,0.34)	0.94	3.2 (0.5))	0.07 (-0.27,0.41)	-0.07 (-0.41,0.26)	0.61
CpG 4	5.8 (1.1)	0.36 (-0.32,1.04)	0.35 (-0.34,1.03)	0.35	5.8 (1.1)	0.41 (-0.28,1.11)	0.29 (-0.41,0.99)	0.44
CpG 5	3.0 (0.7)	0.13 (-0.33,0.60)	0.03 (-0.43,0.49)	0.93	3.2 (0.7)	0.14 (-0.31,0.59)	-0.07 (-0.52,0.38)	0.69
CpG 6	2.6 (0.9)	0.27 (-0.41,0.95)	0.25 (-0.43,0.93)	0.49	2.6 (0.9)	0.24 (-0.44,0.91)	0.10 (-0.56,0.75)	0.82
CpG t	3.8 (0.5)	0.17 (-0.19,0.52)	0.07 (-0.29,0.43)	0.75	3.8 (0.5)	0.17 (-0.18,0.53)	0.01 (-0.34,0.36)	0.96

Model 1: adjusted for adolescent's age, BMI, and creatinine (mg/dL), maternal schooling and intelligence.
Model 2: additionally adjusted for passive tobacco smoking and total fish intake of adolescents.
For all subscales, a higher score indicates more behavioral problems.
sBDNF: serum BDNF; metBDNF: BDNF gene methylation.
*p<0.05; †p<0.10

9.5 Supplementary Material. Article 5

Table S1. Urinary metabolites of non-persistent pesticides: classification, abbreviation, possible parent compound, and expiration of approval.

Classification		Urinary metabolites	Abbreviation	Possible parent compounds[a]		
				Approved in the EU[b]	Expiration of approval (year)	Not approved in the EU[b]
Organophosphate insecticides	Non-specific metabolites	Diethyl thiophosphate	DETP	Chlorpyrifos-ethyl	2020	Chlorethoxyphos, coumaphos, diazinon, disulfoton, ethion, parathion, phorate, phosalone, phoxim, sulfotep, terbufos
		Diethyl dithiophosphate	DEDTP			
	Specific metabolites	3,5,6-trichloro-2-pyridinol	TCPy	Chlorpyrifos-ethyl, chlorpyrifos-methyl, triclopyr	2020, 2020, 2019	-
		2-isopropyl-4-methyl-6-hydroxypyrimidine	IMPy	-	-	Diazinon
		Malathion dicarboxilic acid	MDA	Malathion	2022	-
Pyrethroid insecticides	Non-specific metabolites	3-Phenoxybenzoic acid	3-PBA	Alpha-cypermethrin, deltamethrin, beta-cyfluthrin, lambda-cyhalothrin	2019, 2019, 2019, 2023	Cyhalothrin, cyfluthrin, fenpropathrin, permethrin, tralomethrin
	Specific metabolites	2,2,dichlorovinyl-2,2-dimethylcyclopropane-1-carboxilic acid	DCCA	Alpha-cypermethrin, beta-cyfluthrin	2019, 2019	Permethrin
Carbamates	Specific metabolite	1-Naphthol	1-N	-	-	Carbaryl
Dithiocarbamate fungicides	Non-specific metabolite	Ethylene thiourea	ETU	-	-	Mancozeb

Table S2. Distribution of LOD-LOQ values of pesticide metabolites and CBCL score outcomes in adolescent males from the INMA-Granada cohort (n=140)

Pesticides	**IMPy**	**MDA**	**DETP**	**DEDTP**	**TCPy**	**DCCA**	**1-N**	**3-PBA**	**ETU**
LOD (ng/mL)	0.117	0.052	0.116	0.142	0.039	0.055	0.156	0.117	0.072
LOQ (ng/mL)	0.391	0.172	0.387	0.474	0.130	0.184	0.527	0.389	0.241
Q-SRM	151.09	273.0	169.01	184.98	197.91	113.00	143.04	213.05	103.03
tR (min)	1.74	6.38	3.14	2.29	3.21	2.61	1.45	3.36	1.73
a	-0.80231	3.2986	-1.22351	-1.53302	-0.76826	4.7924	-1.06224	-0.58052	-2.43736
b	0.60619	0.9203	0.84444	0.84401	0.91352	1.2798	0.88090	0.83928	0.97101
R^2	0.994	0.996	0.996	0.996	0.997	0.999	0.997	0.994	0.999
Mean accuracy (%)	92.77	98.64	101.98	99.46	88.29	90.49	101.68	93.58	94.64
% RSD	10.99	11.51	14.74	8.58	13.03	10.47	9.11	14.19	13.28

Adolescents' behavioral functioning

CBCL	Score range	Mean of scores[a]	SD	Median	n (%) in borderline or clinical range[b]
Syndrome scores					
Anxious depressed	50-79	55.1	6.1	54.0	37 (25.7)
Withdrawn	50-89	57.5	7.3	54.0	50 (34.7)
Somatic complaints	50-79	58.1	7.2	58.0	53 (36.8)
Social problems	50-79	55.1	6.4	51.0	33 (22.9)
Thought problems	50-82	54.9	6.0	51.0	23 (15.1)
Attention problems	50-86	56.1	7.0	53.0	31 (20.4)
Rule-breaking behavior	50-89	54.0	6.2	51.0	22 (15.3)
Aggressive behavior	50-81	55.2	6.4	52.0	33 (21.7)
Composite scores					
Internalizing problems	34-79	55.6	9.2	55.0	46 (31.9)
Externalizing problems	34-79	51.0	9.2	51.0	23 (16.0)
Total problems	31-84	54.0	9.2	54.0	38 (26.4)

[a]For all scales, raw scores were transformed to T-scores to compare each subject to a normative sample of adolescents. BDNF= Brain-derived neurotrophic factor; IMPy= 2-isopropyl-4-methyl-6-hydroxypyrimidine; MDA= malathion dicarboxylic acid; TCPy=3,5,6-trichloro-2-pyridinol; DETP= diethyl thiophosphate; ΣOPs= sum of organophosphates; DCCA= 2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxiclic acid; 3.PBA= 3-phenoxybenzoic acid; ΣPYR= sum of pyrethroids; 1-N= 1-naphthol; ETU= ethylene thiourea. Q-SRM: Selected reaction monitoring; tR: Retention time; LOD: Limit of detection; LOQ: Limit of quantification; SD= standard deviation

Table S3. Spearman's correlation among urinary pesticides metabolites

		IMPy	MDA	TCPy	DETP	DCCA	3-PBA	1-N	ETU
IMPy	Rho	1.00	0.10	0.08	0.09	0.07	**0.20**	0.10	0.08
	p	.	0.25	0.31	0.27	0.37	**0.02**	0.22	0.34
MDA	Rho	0.10	1.00	0.11	**0.15**	-0.03	0.13	-0.03	0.04
	p	0.25	.	0.20	**0.07**	0.76	0.12	0.69	0.60
TCPy	Rho	0.08	0.11	1.00	0.06	0.08	**0.17**	0.07	0.00
	p	0.31	0.20	.	0.44	0.32	**0.04**	0.42	0.99
DETP	Rho	0.09	**0.15**	0.06	1.00	**0.20**	0.09	**0.21**	0.08
	p	0.27	**0.07**	0.44	.	**0.02**	0.29	**0.01**	0.31
DCCA	Rho	0.07	-0.03	0.08	**0.20**	1.00	-0.02	-0.01	0.03
	p	0.37	0.76	0.32	**0.02**	.	0.86	0.90	0.72
3-PBA	Rho	**0.20**	0.13	**0.17**	0.09	-0.02	1.00	0.04	0.04
	p	**0.02**	0.12	**0.04**	0.29	0.86	.	0.66	0.65
1-N	Rho	0.10	-0.03	0.07	**0.21**	0.08	0.04	1.00	0.03
	p	0.22	0.69	0.42	**0.01**	0.31	0.66	.	0.71
ETU	Rho	0.08	0.04	0.00	0.08	0.03	0.04	0.03	1.00
	p	0.34	0.60	0.99	0.31	0.72	0.65	0.71	.

Urinary OP metabolites (ng/mL).

Table S4. Beta regression coefficients (β, 95% CI) of adjusted associations between urinary pesticides metabolite concentrations and CBCL typical behavior score outcomes (n= 140 adolescent males).

	Syndrome scores								Composite scores		
	Anxious depressed	**Withdrawn**	**Somatic complaints**	**Social problems**	**Thought problems**	**Attention problems**	**Rule-breaking behavior**	**Aggressive behavior**	**Internalizing problems**	**Externalizing problems**	**Total problems**
IMPy[a]	0.35 (-0.49,1.20)	0.76 (-0.24,1.75)	-0.38 (-1.38,0.63)	**0.86 (0.00,1.71)***	0.56 (-0.27,1.39)	0.71 (-0.24,1.66)	**1.31 (0.46,2.16)****	**1.04 (0.19,1.90)***	0.31 (-0.97,1.60)	**1.67 (0.43,2.92)****	**1.40 (0.16,2.64)***
MDA[a]	0.68 (-0.51,1.87)	0.22 (-1.19,1.63)	-0.41 (-1.83,1.01)	0.09 (-1.14,1.31)	0.14 (-1.03,1.32)	0.11 (-1.24,1.45)	0.26 (-0.98,1.49)	-0.06 (-1.29,1.18)	0.41 (-1.40,2.22)	0.12 (-1.68,1.91)	0.49 (-1.29,2.26)
DETP[b]	0.33 (-0.54,1.19)	**-0.88 (-1.89,0.12)†**	0.46 (-0.56,1.48)	-0.21 (-1.09,0.68)	0.09 (-0.76,0.94)	0.11 (-0.86,1.09)	0.24 (-0.65,1.13)	-0.21 (-1.10,0.68)	0.03 (-1.28,1.34)	-0.14 (-1.44,1.16)	-0.11 (-1.39,1.18)
DCCA[a]	-0.20 (-0.92,0.52)	-0.23 (-1.08,0.62)	0.21 (-0.65,1.06)	0.49 (-0.25,1.22)	0.32 (-0.39,1.02)	0.36 (-0.45,1.17)	0.42 (-0.33,1.16)	0.40 (-0.34,1.15)	0.08 (-1.02,1.17)	0.66 (-0.41,1.74)	0.54 (-0.53,1.61)
ETU[a]	**-0.73 (-1.50,0.04)†**	0.07 (-0.85,0.99)	-0.36 (-1.28,0.56)	0.26 (-0.54,1.06)	-0.08 (-0.85,0.69)	0.03 (-0.85,0.91)	-0.17 (-0.98,0.63)	-0.04 (-0.85,0.77)	-0.89 (-2.06,0.29)	-0.41 (-1.58,0.76)	-0.57 (-1.72,0.59)
ΣOPs	0.81 (-0.40,2.03)	-0.16 (-1.60,1.28)	0.64 (-0.81,2.09)	0.65 (-0.59,1.89)	0.89 (-0.31,2.09)	0.48 (-0.89,1.85)	**1.31 (0.07,2.55)***	0.36 (-0.90,1.62)	0.80 (-1.06,2.65)	0.99 (-0.84,2.81)	1.12 (-0.69,2.93)
ΣPYR	-0.37 (-1.25,0.51)	-0.35 (-1.38,0.69)	0.01 (-1.03,1.06)	0.49 (-0.40,1.39)	0.31 (-0.55,1.18)	0.31 (-0.68,1.30)	0.48 (-0.42,1.38)	0.48 (-0.42,1.38)	-0.15 (-1.49,1.18)	0.78 (-0.53,2.09)	0.47 (-0.83,1.78)

Regression estimates reflect (average) change in behavioral score for each one-log unit increase in urinary metabolite concentration (a) or detected versus undetected concentrations (b). For all scales, raw scores were transformed to T-scores to compare each subject to a normative sample of adolescents.
Models were adjusted for adolescents' age (months), BMI, alcohol consumption (>1 beverage/month; <1 beverage/month), season of urine collection (spring/summer/autumn/winter), creatinine concentrations (mg/dL), and maternal education (primary/secondary/university). * p≤0.05; ** p≤ 0.01; † p<0.1

Table S5. Weighted Quantile Sum (WQS) association to assess the mixture effect of urinary IMPy, MDA, DCCA and ETU metabolites on adolescents' behavior (n=140) and serum BDNF protein levels (n=130) and BDNF DNA methylation (n=118).

CBCL scoring	**WQS**	
	β (SE)	p
Syndrome scores		
Anxious depressed	0.84 (0.98)	0.39
Withdrawn	**2.71 (1.38)**	**0.05**
Somatic complaints	0.43 (1.31)	0.75
Social problems	**4.27 (1.33)**	**0.002**
Thought problems	**2.24 (1.34)**	**0.10**
Attention problems	1.81 (1.48)	0.22
Rule-breaking behavior	2.10 (1.28)	0.11
Aggressive behavior	2.15 (1.40)	0.12
Composite scores		
Internalizing problems	0.76 (1.63)	0.64
Externalizing problems	3.03 (2.01)	0.14
Total problems	2.81 (1.88)	0.14
BDNF biomarkers	**WQS**	
	β (SE)	p
Serum BDNF	-1.98 (2.10)	0.35
CpG1	0.18 (0.17)	0.30
CpG2	0.07 (0.07)	0.34
CpG3	**0.26 (0.12)**	**0.03**
CpG4	0.41 (0.25)	0.11
CpG5	0.25 (0.16)	0.12
CpG6	**0.49 (0.27)**	**0.07**
CpGt	**0.27 (0.12)**	**0.03**

SE= Standard error; [a]For all scales, raw scores were transformed to T-scores to compare each subject to a normative sample of adolescents. Higher scoring indicated worse neurobehavior outcomes. Model was adjusted for adolescents' age (months), BMI, alcohol consumption (>1 beverage/month; <1 beverage/month), season of urine collection (spring/summer/autumn/winter), creatinine concentrations (mg/dL), and maternal education (primary/secondary/university).

Table S6. Beta regression coefficients (β, 95% CI) of adjusted association between urinary pesticides metabolites and serum BDNF protein levels (n=130) and DNA methylation of the BDNF gene (n=118).

	sBDNF	CpG 1	CpG 2	CpG 3	CpG 4	CpG 5	CpG 6	CpG total
IMPy	**-1.16 (-2.53,0.22)†**	0.02 (-0.10,0.14)	0.04 (-0.04,0.11)	0.05 (-0.04,0.14)	0.01 (-0.19,0.21)	0.05 (-0.07,0.18)	0.08 (-0.09,0.26)	0.04 (-0.05,0.14)
MDA	**-1.86 (-3.81,0.10)†**	**0.17 (0.00,0.33)***	**0.10 (-0.01,0.20)†**	0.10 (-0.03,0.22)	0.04 (-0.23,0.31)	0.06 (-0.11,0.23)	-0.03 (-0.27,0.22)	0.07 (-0.06,0.20)
DETP	**-3.59 (-7.60,0.29)†**	-0.08 (-0.42,0.25)	0.09 (-0.12,0.31)	0.12 (-0.13,0.38)	0.21 (-0.35,0.76)	0.05 (-0.30,0.40)	0.34 (-0.16,0.83)	0.12 (-0.15,0.39)
DCCA	0.10 (-1.06,1.26)	0.04 (-0.06,0.14)	-0.01 (-0.08,0.05)	-0.01 (-0.08,0.07)	-0.05 (-0.21,0.12)	-0.02 (-0.13,0.08)	-0.02 (-0.17,0.13)	-0.01 (-0.09,0.07)
ETU	-0.85 (-2.06,0.36)	0.03 (-0.07,0.14)	**0.06 (-0.01,0.13)†**	**0.10 (0.03,0.18)****	0.14 (-0.03,0.31)	0.06 (-0.05,0.17)	0.13 (-0.03,0.28)	**0.09 (0.01,0.17)***
ΣOPs	**-2.79 (-4.75,-0.83)***	0.06 (-0.11,0.23)	**0.09 (-0.01,0.20)†**	**0.12 (-0.01,0.25)†**	0.05 (-0.23,0.33)	0.09 (-0.09,0.26)	0.17 (-0.07,0.42)	**0.10 (-0.04,0.23)**
ΣPYR	-0.13 (-1.55,1.29)	0.06 (-0.06,0.18)	**-0.01 (-0.09,0.06)**	**-0.01 (-0.10,0.08)**	-0.04 (-0.24,0.16)	-0.02 (-0.14,0.11)	-0.01 (-0.19,0.17)	-0.00 (-0.10,0.09)

Regression estimates reflect (average) change in BDNF for each one-log unit increase in urinary metabolite concentration (a) or detection versus non-detection of concentrations (b). For all scales, raw scores were transformed to T-scores to compare each subject to a normative sample of adolescents. Model was adjusted for adolescents' age (months), BMI, alcohol consumption (>1 beverage/month; <1 beverage/month), season of urine collection (spring/summer/autumn/winter), creatinine concentrations (mg/dL), and maternal education (primary/secondary/university).
* p≤0.05; ** p≤0.01; † p<0.10

Table S7. Beta regression coefficients (β, 95% CI) of association between serum BDNF protein levels (ng/mL) with behavioral function of adolescent males (n= 130).

		Syndrome scores								Composite scores		
		Anxious depressed	**Withdrawn**	**Somatic complaints**	**Social problems**	**Thought problems**	**Attention problems**	**Rule-breaking behavior**	**Aggressive behavior**	**Internalizing problems**	**Externalizing problems**	**Total problems**
BDNF protein	Continuous	0.01 (-0.11,0.12)	-0.04 (-0.17,0.09)	-0.04 (-0.19,0.11)	-0.03 (-0.15,0.10)	**-0.10 (-0.21,0.01)†**	-0.05 (-0.18,0.08)	-0.08 (-0.19,0.04)	-0.03 (-0.14,0.09)	-0.05 (-0.24,0.14)	-0.04 (-0.21,0.14)	-0.06 (-0.24,0.12)
	T2	-0.12 (-2.74,2.50)	-2.10 (-5.11,0.92)	-1.33 (-4.78,2.12)	-1.95 (-4.72,0.83)	**-2.93 (-5.47,-0.38)***	-1.53 (-4.57,1.50)	**-2.71 (-5.39,-0.02)***	-1.89 (-4.58,0.79)	-3.00 (-7.37,1.37)	-2.87 (-6.99,1.26)	**-3.57 (-7.72,0.58)†**
	T3	0.60 (-2.10,2.30)	-1.71 (-4.82,1.40)	-0.95 (-4.51,2.60)	-1.03 (-3.89,1.82)	**-3.03 (-5.65,-0.40)***	-0.23 (-3.36,2.89)	**-2.35 (-5.11,0.42)†**	-1.28 (-4.04,1.49)	-1.16 (-5.66,3.34)	-1.64 (-5.88,2.61)	-1.70 (-5.98,2.58)
	p-trend	0.65	0.71	0.61	0.49	**0.03**	0.90	**0.10**	0.37	0.63	0.46	0.46

[a]For all scales, raw scores were transformed to T-scores to compare each subject to a normative sample of adolescents. Higher scoring indicated worse neurobehavior outcomes. Model was adjusted for the following covariates: adolescent's age, BMI, alcohol consumption and maternal education. * p≤ 0.05; † p< 0.10

Figure S1. Forest Box Plot showing the linear regression estimates (95%CI) of the association between categorized BDNF gene DNA methylation percentages and adolescents' behavior. Model was adjusted for adoelscent's age, bmi, alcohol consumption, season of urinary collection and maternal education. Red diamonds show statistical associations with p-values below 0.05; Green diamonds show p-values below 0.1; Black diamonds show p-values above 0.1.

9.6Supplementary Material. Article 6

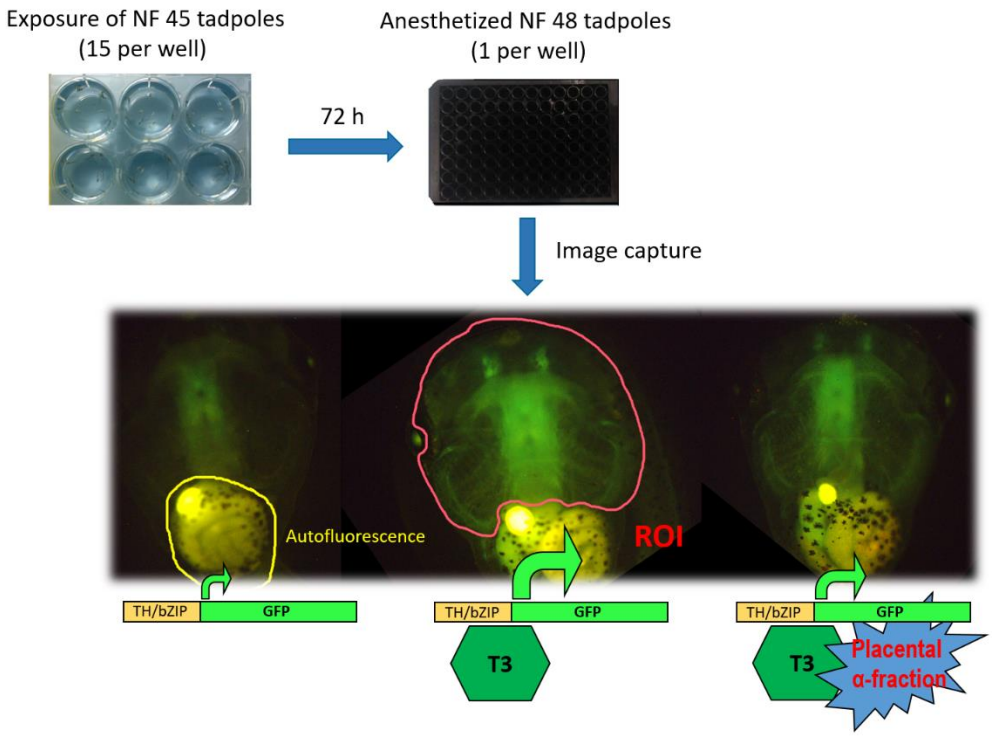


Figure S1: Graphical description of the XETA. Quantifications were carried out in a region of interest (ROI) containing the whole head area (red) to exclude the non-specific auto-fluorescence emitted from the gut area (yellow). Eye(s) were also excluded as some images do not capture both eyes.

Table S1. Xenobiotics and endogenous hormones present in fractions obtained through semi-preparative HPLC separation procedures in previous studies. In this study we investigated the α-fraction, representing the first 11 minutes of chromatographic elution.

Author	**Human matrix**	**Minute**	**Bioaccumulated xenobiotic**
(Fernández et al., 2004)	Adipose tissue	2	*p,p*'-DDT
		2.35	Lindane
		3.6	Aldrin
		4	Methoxychlor
		4.4	Dieldrin
		4.8	*p,p*'-DDE
		5	Endosulfan II
		5.4	*o,p*'-DDD
		5.7	*o,p*'-DDT
		6	Endrin
		7.8	Endosulfan-diol
		7.9	Endosulfan I
		10.0	Endosulfan-ether
		10.5	Endosulfan-lactone
		12.5	Endosulfan-sulfate
		12.96	Progesterone
		12.99	Estradiol esters
		15.30	Testosterone
		16.16	17 β-Estradiol
(Indiveri et al., 2014)	Placenta	2	Benzophenone
		4	2-Phenylphenol
			Triclosan
		6	Triphenylphosphate
			7,9-Di-tert-butyl-1-oxaspiro (4,5)
			Deca-6,9-diene-2,8-dione
			2,4,7,9-Tetramethyl-5-decyne-4,7,diol
			Estratetraenol
		12-13	Progesterone
		14	Methylparaben
		15	Estrone
		17	Hydroxypregn-4-en-3-one
		20-21	17-β-Estradiol

DDD= Dichlorodiphenyldichloroethane; DDE= Dichlorodiphenyldichloroethylene; DDT= Dichlorodiphenyltrichloroethane

Table S2. Procedure followed to calculate the mg of placenta extracted per mL of experimental medium tested in bioassays:

		E-Screen			ER reporter gene assay			Antagonist AR reporter gene assay			AhR assay			XETA
1. Calculate the mg in 3g of placenta		3000 mg			3000 mg			3000 mg [1]			3000 mg [1]			3000 mg
1.1. Calculate mg of placenta after split α-fraction in 4 vials		-			-			750			750			-
2. Divide the mass of placenta into reconstitution volume (µL)		1000			44 [2]			250			250			416
3. Divide the concentration after resuspension by the dilution performed		x1	x5	x10	x55	x275	x550	x60	x180	x600	x100	x300	x1000	x1
4. Calculate the final concentration (mg/µL) according to formula (2)	Co	3	0.6	0.3	1.240	0.248	0.124	0.05	0.0167	0.005	0.03	0.01	0.003	7.21
	Vo	50	50	50	100	100	100	50	50	50	50	50	50	208
	Vf	200	200	200	100	100	100	200	200	200	200	200	200	8000
5. Convert final concentration from mg/µL to mg/mL		750	150	75	1240	248	124	12.5	4.2	1.25	7.5	2.5	0.75	187.5

[1] α-fractions were split in 4 vials, corresponding to 750 mg of placenta extract per vial. [2] Only 20 µL of the 44 µL were used for dilutions.

General Formula:

$$(1)\ 3g\ placenta \times \frac{1000\ mg}{1\ g} \times \frac{1}{\frac{Reconstitution\ volume\ (\mu L)}{}} \times \frac{1}{\frac{Dilution}{(X)}} = Co$$

$$(2)\ \frac{Co \times Vo}{Vf} \times \frac{1000 \mu L}{1\ mL} = Final\ concentration\ in\ well\ (\frac{mg}{mL})$$

Co= Initial concentration (mg/µL); Vo= Initial volume (µL); Cf= Final concentration (mg/mL); Vf= Final volume (µL)

Table S3. Pearson correlation coefficients assessing bivariate relationships among bioassays. Values corresponding to the highest α-fraction concentrations tested for the ER, AR and AhR reporter gene assays were excluded due to the presence of cytotoxicity.

		E-screen (75 mg placenta/mL)	E-Screen (150 mg placenta/mL)	E-Screen (750 mg placenta/mL)	ER (124 mg placenta/mL)	ER (248 mg placenta/mL)	AR antagonism (1.25 mg placenta/mL)	AR antagonism (4.2 mg placenta/mL)	AhR (0.75 mg placenta/mL)	AhR (2.5 mg placenta/mL)	XETA antagonism (187.5 mg placenta/mL)
E-screen (75 mg placenta/mL)	Pearson 's Correlation	-	,949**	,822**	,449*	,310	,096	,408*	,205	,046	-,109
	p		,000	,000	,028	,140	,655	,048	,337	,829	,639
	N		24	24	24	24	24	24	24	24	21
E-Screen (150 mg placenta/mL)	Pearson 's Correlation	,949**	-	,881**	,528**	,397	,047	,430*	,117	-,061	-,089
	p	,000		,000	,008	,054	,829	,036	,586	,777	,701
	N	24		24	24	24	24	24	24	24	21
E-Screen (750 mg placenta/mL)	Pearson 's Correlation	,822**	,881**	-	,492*	,439*	-,097	,347	,055	-,103	-,058
	p	,000	,000		,015	,032	,652	,097	,799	,633	,802
	N	24	24		24	24	24	24	24	24	21
ER (124 mg placenta/mL)	Pearson 's Correlation	,449*	,528**	,492*	-	,700**	,195	,333	-,167	-,300	-,230
	p	,028	,008	,015		,000	,361	,112	,436	,154	,317
	N	24	24	24		24	24	24	24	24	21
ER (248 mg placenta/mL)	Pearson 's Correlation	,310	,397	,439*	,700**	-	-,096	,036	-,041	-,325	,066
	p	,140	,054	,032	,000		,655	,867	,850	,122	,777
	N	24	24	24	24		24	24	24	24	21
AR antagonism (1.25 mg placenta/mL)	Pearson 's Correlation	,096	,047	-,097	,195	-,096	-	,594**	-,634**	-,133	-,175
	p	,655	,829	,652	,361	,655		,002	,001	,535	,448
	N	24	24	24	24	24		24	25	24	21
AR antagonism (4.2 mg placenta/mL)	Pearson 's Correlation	,408*	,430*	,347	,333	,036	,594**	-	,048	,086	-,081
	p	,048	,036	,097	,112	,867	,002		,823	,689	,728
	N	24	24	24	24	24	24		24	24	21
AhR (0.75 mg placenta/mL)	Pearson 's Correlation	,205	,117	,055	-,167	-,041	-,634**	,048	-	,819**	,054
	p	,337	,586	,799	,436	,850	,001	,823		,000	,817
	N	24	24	24	24	24	25	24		24	21
AhR (2.5 mg placenta/mL)	Pearson 's Correlation	,046	-,061	-,103	-,300	-,325	-,133	,086	,819**	-	,009
	p	,829	,777	,633	,154	,122	,535	,689	,000		,968
	N	24	24	24	24	24	24	24	24		21
XETA antagonism (187.5 mg placenta/mL)	Pearson 's Correlation	-,109	-,089	-,058	-,230	,066	-,175	-,081	,054	,009	-
	p	,639	,701	,802	,317	,777	,448	,728	,817	,968	
	N	21	21	21	21	21	21	21	21	21	

*p≤ 0,05; **p≤ 0.01