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# Effects of the interaction between endocrine disruptor exposure and genetic polymorphisms on childhood obesity and neurodevelopmental disorders

Efectos de la interacción entre la exposición a disruptores endocrinos y polimorfismos genéticos en obesidad infantil y trastornos del neurodesarrollo

*Memoria presentada por*

**Viviana Gabriela Ramírez López**

Para optar al título de Doctora Internacional por la Universidad de Granada

*Directores*

Dra. Dña. Ana María Rivas Velasco

Dra. Dña. María Jesús Álvarez Cubero

**Granada, 2024**

# EFECTOS DE LA INTERACCIÓN ENTRE LA EXPOSICIÓN A DISRUPTORES ENDOCRINOS Y POLIMORFISMOS GENÉTICOS EN OBESIDAD INFANTIL Y TRASTORNOS DEL NEURODESARROLLO

Por

Viviana Gabriela Ramírez López

DEPARTAMENTO DE NUTRICIÓN Y BROMATOLOGÍA

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**Memoria presentada para aspirar al título de Doctora Internacional por la Universidad de Granada.**

Fdo. Dra. Dña. Ana Mª Rivas Velasco
Catedrática de Universidad del Departamento de Nutrición y Bromatología de la Universidad de Granada.

**Visada en Granada 11/11/2024.**

Fdo. Dra. Dña. Mª Jesús Álvarez Cubero
Profesora Titular de Universidad del Departamento de Bioquímica y Biología Molecular III e Inmunología.

Fdo. Dña. Viviana Gabriela Ramírez López. Graduada en Bioquímica

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Dra. Dña. Ana María Rivas Velasco, Catedrática del Departamento de Nutrición y Bromatología, Universidad de Granada.

Dra. Dña. María Jesús Álvarez Cubero, Profesora Titular de Universidad del Departamento de Bioquímica y Biología Molecular III e Inmunología.

Directores de la Memoria de Tesis Doctoral titulada «Efectos de la interacción entre la exposición a disruptores endocrinos y polimorfismos genéticos en obesidad infantil y trastornos del neurodesarrollo», realizada por Dña. Viviana Gabriela Ramírez López, autorizan su presentación ante el tribunal que en su día se designe.

Y para que así conste, y en cumplimiento de las disposiciones vigentes, firman la presente Tesis Doctoral el 11 de noviembre de 2024.

Fdo. Dra. Dña. Ana Mª Rivas Velasco  Fdo. Dra. Dña. Mª Jesús Álvarez Cubero

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Fdo. Dra. Dña. Ana Mª Rivas Velasco  Fdo. Dra. Dña. Mª Jesús Álvarez Cubero

Doctoranda*/ Doctoral candidate*

Fdo. Dña. Viviana Gabriela Ramírez López

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*Gracias a mi Madre, a mi Abuela,*
*simplemente por estar siempre*

*"Al final del día, podemos soportar mucho*
*más de lo que pensamos que podemos"*

*Frida Kahlo*

# Content

**ABBREVIATIONS**

| | |
|---|---|
| ABCs | ATP-binding cassette transporter superfamily |
| ADHD | Attention-deficit hyperactivity disorder |
| AESAN | Spanish Agency for Food Safety and Nutrition |
| ASD | Autism spectrum disorders |
| ATP7B | ATPase copper transporting beta |
| BDNF | Brain-derived neurotrophic factor |
| BMI | Body mass index |
| BPA | Bisphenol A |
| BPAF | Bisphenol AF |
| BPB | Bisphenol B |
| BPF | Bisphenol F |
| BPS | Bisphenol S |
| ButPB | Butylparaben |
| CAT | Catalase |
| CE1/CE2 | Carboxylesterases |
| CNVs | Copy number variations |
| COSI | Childhood Obesity Surveillance Initiative |
| CRP | C-reactive protein |
| CYP1A2 | Cytochrome P450 family 1 subfamily A member 2 |
| CYP2C9 | Cytochrome P450 family 2 subfamily C member 9 |
| DSM-5 | Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition |
| EDCs | Endocrine disrupting chemicals |
| EthPB | Ethylparaben |
| FCMs | Food contact materials |
| FTO | Fat mass and obesity-associated gene |
| GCLC | γ-Glutamyl-cysteine ligase catalytic subunit |
| GCLM | γ-Glutamyl-cysteine ligase modifier subunit |
| GLUT2/GLUT4 | Glucose transporters |
| GSH | Glutathione |
| GSTP1 | Glutathione S-transferase p1 |
| GWAS | Genome-wide association studies |

| | |
|---|---|
| HPT | Hypothalamic-pituitary-thyroid axis |
| HTR2C | Serotonin receptor |
| IL-6 | Interleukin-6 |
| ISCIII | Instituto de Salud Carlos III |
| LEP | Leptin |
| LEPR | Leptin receptor |
| MAF | Major allele frequency |
| MC4R | Melanocortin receptor |
| MetPB | Methylparaben |
| MSH | Melanocyte-stimulating hormones |
| MTs | Metallothioneins |
| MYT1L | Myelin transcription factor-1 like |
| NDDs | Neurodevelopmental disorders |
| PBDEs | Polybrominated flame retardants |
| PCBs | Polychlorinated biphenyls |
| PCSK1 | Preproconvertase 1 |
| PFASs | Perfluoroalkyl substances |
| PHBA | Para-hydroxybenzoic acid |
| POMC | Proopiomelanocortin |
| PPARγ | Proliferator-activator receptor gamma |
| PropPB | Propylparaben |
| SH2B1 | Src-homology-2 domain-containing putative adapter |
| SIM1 | Single minded 1 |
| SNPs | Single nucleotide polymorphisms |
| SNVs | Single nucleotide variants |
| SOD | Superoxide dismutase |
| SULTs | Sulfotransferases |
| TCF7L2 | Transcription factor 7-like 2 |
| TNF-α | Tumour necrosis factor-alpha |
| TrkB | Tyrosine kinase receptor tropomycin-related kinase B |
| UGT | Uridine 5'-diphospho-glucuronosyltransferase |
| WHO | World Health Organisation |

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**ABSTRACT**

The global prevalence of overweight and obesity - together known as **excess weight** - represents a major public health problem, given the marked increase observed in recent decades. The aetiology of obesity is complex and multifactorial, involving a combination of multiple risk factors, of which genetic and environmental factors are the main focus of this Doctoral Thesis. Polygenic obesity represents the most prevalent form of obesity. In these terms, genome wide association studies (GWAS) have uncovered hundreds of single nucleotide polymorphisms (SNPs) as the most common inherited genetic variations associated with body mass index (BMI) heritability, although they account for approximately 6 % of BMI variability. At the level of environmental exposure, the obesogenic activity of endocrine disrupting chemicals (EDCs) has been highlighted due to their capacity to disrupt normal developmental and homeostatic controls over adipogenesis and energy balance. The most widely recognised EDCs include metal(loid)s, bisphenols, and parabens.

A growing body of evidence has highlighted the strong link between obesity and neurodevelopmental disorders (NDDs), including attention-deficit hyperactivity disorder (ADHD), autism spectrum disorders (ASD), and intellectual disability, among others. The genetic component and EDCs-related neurodisruptive activity have emerged as key interconnected components in the bidirectional link between excess weight and NDDs. Therefore, the general objective of this Doctoral Thesis was to study the influence of different genetic polymorphisms on childhood excess weight and neurodevelopmental functioning according to the level of exposure to EDCs in the school-aged population.

A total of 351 Spanish children aged 3-12 years old were recruited from different elementary schools and primary care centres. Anthropometric data, neurodevelopment assessment tests, food frequency questionnaires (FFQs), buccal swabs, urine and hair samples were collected from each participant.

Firstly, a gene panel was designed that included multiple SNPs in hormone receptor genes, detoxification enzyme genes and genes associated with the excess

weight phenotype and neurodevelopmental disorders. Gene panel consisted of: 7 polymorphisms of genes involved in the detoxification (*GSTP1, GCLM*) and transport system of metal(loid)s (*ATP7B, ABCC2*); 13 polymorphisms of candidates genes responsible for obesity-related pathways (*FTO, TCF7L2, INSIG2, SH2B1, LEPR, SOD2, ADIPOQ, MC4R, IL6, ADRB2, BDNF*); 6 genetic variants of metabolising enzyme encoding genes (*COMT, GSTP1, CYP2C19, PON1, GPX1*); 11 SNPs within genes encoding hormone receptors and nuclear transcription factors (*PPARG, ESR1, AR, ESR2, TSHR, AHR, THRA, THRB*); and 10 genetic polymorphisms associated with neurodevelopmental processes (*BDNF/NTRK2, HTR2A, MTHFR, OXTR, SLC6A2, SNAP25*). SNP genotyping assays were performed through global screening array (GSA) microchip technology and quantitative PCRs (qPCRs) with Taqman® probes.

For chemical determination of EDCs in biological samples, ten metal(loid)s were analysed in urine samples through Inductively coupled plasma mass spectrometry (ICP-MS). Levels of bisphenols and parabens in urine and hair were used to assess short- and long-term exposure, respectively, via ultra high-performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry (UHPLC-MS/MS) system. And dietary exposure to bisphenols was estimated from FFQs and bisphenol content in food samples by UHPLC-MS/MS system.

In the first genetic association analysis, *GSTP1* rs1695 and *ATP7B* rs1061472 showed significant effects on excess weight increase in those children carrying two copies of the risk G allele and being highly exposed to chromium and lead. Conversely, *GCLM* rs3789453 and *ATP7B* rs1801243 appeared to play a protective role against excess weight in those exposed to copper and lead. These findings provide the first proof that SNP effect depended on the level of exposure.

SNP-by-exposure interactions on excess weight were also identified in models stratified by bisphenol and paraben exposure according to urinary and hair levels. Firstly, the *LEPR* rs9436303 emerged as a relevant risk variant for excess weight, and this effect persisted across exposure-stratified models. In long-term exposure

analyses, *GPX1* rs1050450 was associated with increased excess weight at low single exposure to parabens, whereas *LEPR* rs1137101 exhibited a protective function in those highly co-exposed to bisphenols and parabens. *ESR2* rs3020450 and CYP*2C19* rs4244285 were identified as predisposing variants at low and high co-exposure, respectively. In short-term exposure, a higher likelihood of overweight and obesity was observed for *INSIG2* rs7566605 at high bisphenol exposure and for *GSTP1* rs1695 and *GPX1* rs1050450 at low levels. Under situation of low and medium co-exposure, *SH2B1* rs7498665 and *MC4R* rs17782313 displayed a protective effect, whereas *ESR2* rs3020450 maintained its role in favour of excess weight. The findings reiterate the significance of considering the genetic susceptibility in the presence of exposure to environmental agents.

In the context of childhood cognitive functions assessed by the Weschler Intelligence Scale for Childre-Fifth Edition (WISC-V) Spanish form, *BDNF* rs11030101-T and *SNAP25* rs363039-A allele carriers scored better on the fluid reasoning domain, except for those inheriting the *BDNF* rs6265-A allele, who had lower scores. Secondly, consistent associations of *BDNF* rs11030101, *NTRK2* rs2289656/rs10868235, *MTHFR* rs1801133, *HTR2A* rs7997012, *OXTR* rs53576, and *SLC6A2* rs998424 with verbal comprehension, working memory and fluid reasoning domains were obtained in the presence of dietary bisphenol exposure, resulting in relevant SNP-bisphenol interactions.

Lastly, in order to assess the safety of dietary exposure to bisphenols, in particular to bisphenol A (BPA) in vulnerable populations, a comprehensive risk assessment was performed focusing on the provisional tolerable daily intake (TDI) of 200 ng/kg bw/day derived by the German Federal Institute for Risk Assessment (BfR). For this purpose, 213 children (3-9 years), 281 adolescents (10-17 years), and 122 adults (18-39 years) were included. In a probabilistic approach, exposure data were transferred to a log-normal distribution and combined with the data on hazard characterisation using the APROBA-Plus tool. The results demonstrated that children were higher exposed to BPA compared to adolescents and adults. About 50% of the children exceeded the BfR's TDI. Consequently, BPA exposure close to

the BfR's TDI may be of special concern for the child population and may serve as a basis for BPA risk assessment.

In conclusion, the works presented in this Doctoral Thesis emphasise the significance of investigating the genetic variability across pivotal mechanistic biological pathways related to exposure to EDCs and disease development, especially during critical periods such as childhood. The exploration of the genetic background and the environmental dynamics could help to fill the current knowledge gaps in the complex polygenic and multifactorial aetiology of excess weight and neurodevelopmental outcomes. In view of the lack of studies examining the impact of gene-EDCs interactions, it is argued here that genetics and EDCs exposure should be considered as interactive factors rather than individual modulators of excess weight and neurodevelopmental disabilities.

**RESUMEN**

La prevalencia mundial del sobrepeso y la obesidad – conocidos conjuntamente como **exceso de peso** – representa un importante problema de salud pública, dado el marcado aumento observado en las últimas décadas. La etiología de la obesidad es compleja y multifactorial, implicando una combinación de múltiples factores de riesgo, de los cuales los factores genéticos y ambientales son el foco principal de esta Tesis Doctoral. La obesidad poligénica representa la forma más prevalente de obesidad. En este sentido, los estudios de asociación del genoma completo (GWAS) han identificado cientos de polimorfismos de nucleótido único (SNPs) como las variaciones genéticas hereditarias más comunes asociadas a la heredabilidad del índice de masa corporal (IMC), aunque representan aproximadamente el 6 % de la variabilidad del IMC. A nivel de exposición ambiental, se ha destacado la actividad obesogénica de los disruptores endocrinos (EDCs) debido a su capacidad para alterar el normal desarrollo y el control homeostático sobre la adipogénesis y el balance energético. Entre los EDCs más conocidos se encuentran los metal(oide)s, los bisfenoles y los parabenos.

Es creciente la evidencia que ha destacado la estrecha vinculación de la obesidad con los trastornos del neurodesarrollo (TNDs), entre los que se incluyen el trastorno por déficit de atención e hiperactividad (TDAH), trastornos del espectro autista (TEA), y discapacidad intelectual, entre otros. El componente genético y la actividad neurotóxica asociada a los EDCs han emergido como componentes clave e interconectados en la relación bidireccional entre el exceso de peso y los TNDs. Por ello, el objetivo general de la presente Tesis Doctoral fue estudiar la influencia de diferentes polimorfismos genéticos sobre el exceso de peso infantil y el neurodesarrollo en función del nivel de exposición a EDCs en población en edad escolar.

Para ello, se han reclutado a un total de 351 niños/as españoles de entre 3 y 12 años de diferentes centros educativos y de salud de atención primaria. De cada participante se recogieron datos antropométricos, pruebas de evaluación del

neurodesarrollo, cuestionarios de frecuencia de consumo de alimentos (FFQs), así como muestras de hisopos bucales, orina y cabello.

En primer lugar, se diseñó un panel de genes que incluía múltiples SNPs en genes de receptores hormonales, genes de enzimas de detoxificación y genes asociados con el fenotipo de exceso de peso y desórdenes del neurodesarrollo. El panel de genes consistió en: 7 polimorfismos de genes implicados en la detoxificación (*GSTP1, GCLM*) y el sistema de transporte de metal(oide)s (*ATP7B, ABCC2*); 13 polimorfismos de genes candidatos responsables de vías relacionadas con la obesidad (*FTO, TCF7L2, INSIG2, SH2B1, LEPR, SOD2, ADIPOQ, MC4R, IL6, ADRB2, BDNF*); 6 variantes genéticas de genes que codifican para enzimas metabolizadoras (*COMT, GSTP1, CYP2C19, PON1, GPX1*); 11 SNPs en genes que codifican receptores hormonales y factores nucleares de transcripción (*PPARG, ESR1, AR, ESR2, TSHR, AHR, THRA, THRB*); y 10 polimorfismos genéticos asociados a procesos de neurodesarrollo (*BDNF/NTRK2, HTR2A, MTHFR, OXTR, SLC6A2, SNAP25*). Los ensayos de genotipado se realizaron mediante la tecnología de microchips de GSA (*global screening array*) y PCR cuantitativas (qPCRs) con sondas Taqman®.

Para la determinación de EDCs en matrices biológicas, se analizaron diez metal(oide)s en muestras de orina mediante espectrometría de masas con plasma acoplado inductivamente (ICP-MS). Los niveles de bisfenoles y parabenos en orina y cabello se utilizaron para evaluar la exposición a corto y largo plazo, respectivamente, mediante cromatografía de líquidos de ultra alta resolución acoplada a espectrometría de masas en tándem triple cuadrupolo (UHPLC-MS/MS). Y la exposición dietética a los bisfenoles se estimó a partir de encuestas alimentarias y su concentración en alimentos determinada mediante UHPLC-MS/MS.

En el primer análisis de asociación genética, las variantes *GSTP1* rs1695 y *ATP7B* rs1061472 mostraron efectos significativos sobre el aumento del exceso de peso en aquellos niños/as portadores de dos copias del alelo G de riesgo y altamente expuestos a cromo y plomo. Por el contrario, los polimorfismos *GCLM* rs3789453 y *ATP7B* rs1801243 mostraron un papel protector contra el exceso de peso en aquellos

expuestos a cobre y plomo. Estos hallazgos proporcionan la primera prueba de que el efecto de las variantes genéticas estudiadas podría depender del nivel de exposición.

De la misma manera, se identificaron interacciones gen-ambiente sobre el exceso de peso en modelos estratificados según la exposición a bisfenoles y parabenos a partir de los niveles en orina y pelo. El *LEPR* rs9436303 resultó ser la única variante de riesgo relevante para el exceso de peso, cuyo efecto se mantuvo en los modelos estratificados por exposición. En los análisis de exposición a largo plazo, la variante *GPX1* rs1050450 se asoció con un aumento del exceso de peso bajo una exposición baja a parabenos, mientras que el *LEPR* rs1137101 mostró una función protectora en el grupo con una alta coexposición a bisfenoles y parabenos. Los SNPs *ESR2* rs3020450 y *CYP2C19* rs4244285 se identificaron como variantes de susceptibilidad genética ante una baja y alta coexposición, respectivamente.

En la exposición a corto plazo, se observó una mayor probabilidad de sobrepeso y obesidad para *INSIG2* rs7566605 en situación de alta exposición a bisfenoles y para *GSTP1* rs1695 y *GPX1* rs1050450 en niveles bajos. Para una coexposición baja e intermedia, *SH2B1* rs7498665 y *MC4R* rs17782313 mostraron un efecto protector, mientras que *ESR2* rs3020450 mantuvo su papel a favor del exceso de peso. Tales resultados reiteran la importancia de considerar la susceptibilidad genética en presencia de la exposición a agentes ambientales.

En lo que concierne al neurodesarrollo infantil, particularmente al funcionamiento cognitivo evaluado mediante la Escala de Inteligencia de Wechsler para Niños-V (WISC-V), los escolares portadores de los alelos *BDNF* rs11030101-T y *SNAP25* rs363039-A obtuvieron mejores puntuaciones en el dominio del razonamiento fluido, excepto los que heredaron el alelo *BDNF* rs6265-A, quienes obtuvieron puntuaciones más bajas. En segundo lugar, se obtuvieron asociaciones consistentes de las variantes *BDNF* rs11030101, *NTRK2* rs2289656/rs10868235, *MTHFR* rs1801133, *HTR2A* rs7997012, *OXTR* rs53576, y *SLC6A2* rs998424 con las áreas de comprensión verbal, memoria de trabajo y razonamiento fluido en presencia de exposición dietética a bisfenoles, dando lugar a interacciones relevantes entre estos SNPs y los niveles de bisfenoles.

Por último, con el fin de evaluar la seguridad de la exposición alimentaria a los bisfenoles, en concreto al bisfenol A (BPA) en poblaciones vulnerables, se llevó a cabo una evaluación global de riesgo a raíz de la ingesta diaria tolerable (IDT) provisional de 200 ng/kg pc/día propuesta por el Instituto Federal Alemán de Evaluación de Riesgos (BfR). Para ello, se incluyeron 213 niños/as (3-9 años), 281 adolescentes (10-17 años) y 122 adultos (18-39 años). Con un enfoque probabilístico, los datos de exposición se transformaron atendiendo a una distribución log-normal y se combinaron con los datos de caracterización del peligro mediante la herramienta APROBA-Plus. Los resultados demostraron una exposición al BPA significativamente más alta en los niños/as que en los adolescentes y adultos. Alrededor del 50% de los niños/as superaron la IDT del BfR. En consecuencia, la exposición al BPA próxima a la IDT derivada por el BfR puede ser especialmente preocupante para la población infantil y puede servir de base para la evaluación de riesgo del BPA.

En conclusión, los trabajos de investigación presentados en esta Tesis Doctoral ponen de manifiesto la importancia de investigar la variabilidad genética en vías biológicas mecanicistas relacionadas con la exposición a los EDCs y el desarrollo de enfermedades, especialmente durante períodos críticos como la infancia. La exploración del trasfondo genético y de la dinámica ambiental podría ayudar a llenar las lagunas actuales en el conocimiento de la etiología compleja, poligénica y multifactorial del exceso de peso y los TNDs. En vista de la falta de estudios que examinen el impacto de las interacciones entre los genes y los EDCs, se argumenta aquí que la genética y la exposición a EDCs deberían considerarse como factores interactivos más que moduladores individuales del exceso de peso y los problemas del neurodesarrollo.

# 1. INTRODUCTION

## 1.1. Current situation of excess weight: overweight and obesity

Childhood overweight and obesity - together known as **excess weight** - are a major global public health problem (AESAN ISCIII, 2023). The World Health Organisation (WHO) defines overweight as, "a condition of excessive fat deposits", and obesity as, "a chronic complex disease defined by excessive fat deposits that can impair health" (WHO, 2024). According to data provided by the WHO in 2022, 2.5 billion adults over the age of 18 were overweight and obese, and more than 390 million children and adolescents aged 5-19 were overweight, of whom 160 million were obese. These data highlight the dramatic increase in the prevalence of excess weight in recent decades, from 25% in 1990 to 43% in 2022 in the adult population and from 8% to 20% in the child and adolescent population (WHO, 2024).

In the European Region, the highest prevalence rates are found in the Mediterranean and southern European countries, including Spain (Buoncristiano et al., 2021; López-Sobaler et al., 2018; Spinelli et al., 2021). Surveillance systems for overweight and obesity are of vital importance to monitor trends and provide the respective competent authorities with a sound basis for preventive action. In particular, the ALADINO (*ALimentación, Actividad física, Desarrollo INfantil, y Obesidad*) study is a reference study in the surveillance of childhood obesity in Spain developed within the Childhood Obesity Surveillance Initiative (COSI) promoted by the WHO, which provides periodic estimates of the national prevalence of overweight and obesity in a representative school-age population (6-9 years old) (AESAN, 2019). According to the latest study conducted in 2019, although the trends in overweight and obesity have decreased since the first edition in 2011, prevalence rates remain remarkably high. In 2011, the prevalence of overweight was 26.2% and of obesity 18.3%, compared to 23.3% and 17.3%, respectively in 2019 (AESAN, 2020). However, this study does not explore the other age groups, nor does it address estimates by region or province.

In 2020, the National Epidemiology Centre of the Instituto de Salud Carlos III (ISCIII) and the Spanish Agency for Food Safety and Nutrition (AESAN) conducted

the ENE-COVID study, involving more than 60000 children, adolescents and adults and collecting individual anthropometric, sociodemographic and socioeconomic data (AESAN ISCIII, 2023). The report shows that the prevalences of overweight, obesity and severe obesity were higher in boys aged 2-17 years (20.3%, 13.4%, and 2.9%, respectively) than in girls (18.1%, 7.9%, and 1.2%, respectively). Similarly, these prevalences were higher in households reporting low educational level, low income or at least one adult diagnosed with excess weight. In terms of geographical location, inter-provincial differences were observed, but with no obvious specific pattern. In general, some of the provinces or regions in the centre and north of the country had the lowest rates of excess weight, while the highest prevalences were distributed throughout Spain, with Huelva and Cordoba standing out with 41.3% and 43.6% respectively (see Figure 1) (AESAN ISCIII, 2023; Gutiérrez-González et al., 2024).

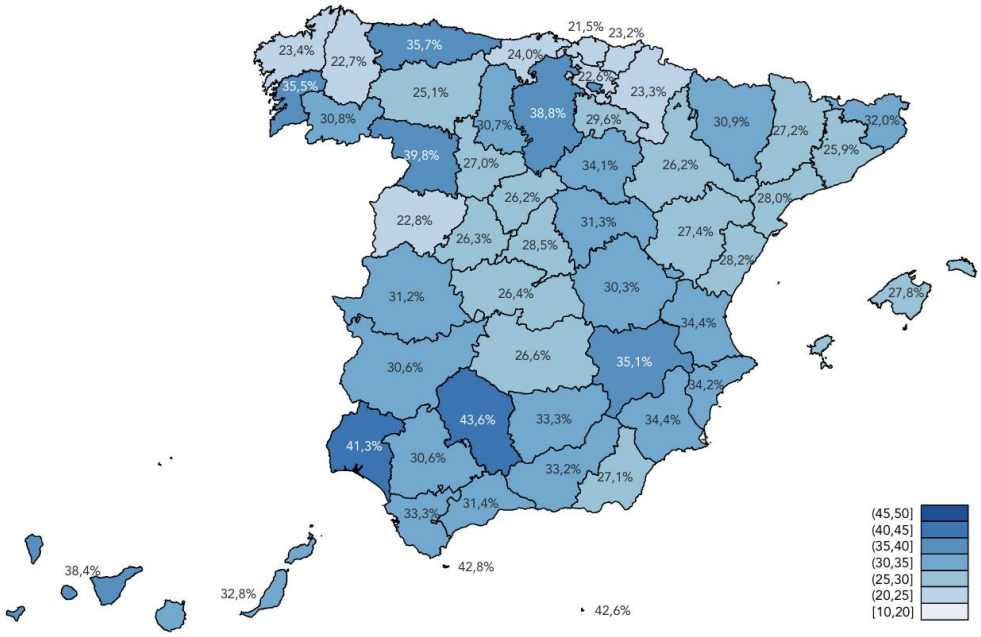


**Figure 1**. Crude prevalence (%) of excess weight (overweight and obesity) by province in the child and youth population of the ENE-COVID study.

## 1.2. Risk factors and health outcomes related to excess weight

The development of excess weight during childhood and adolescence has an immediate impact on physical and mental health, as an obese child is more likely to

become an obese adult, and this risk increases with age (Lancet, 2022). Childhood and adolescence are therefore key periods for the implementation of prevention systems, such as the establishment of healthy lifestyles (Lancet, 2022; Smith et al., 2020).

The aetiology of obesity is complex and multifactorial, involving a combination of multiple risk factors, such as: genetic (genetic predisposition), environmental (access to high-calorie foods and sedentary lifestyles encouraged by urban environments), lifestyle (high-fat and high-sugar diets, low physical activity and irregular sleep patterns), psychological (emotional and psychosocial stress), socioeconomic and cultural (low income, low educational level and cultural norms/practices), medical (underlying diseases and use of medications with side effects) and hormonal and metabolic (imbalance between appetite and satiety) (Safaei et al., 2021; Swinburn et al., 2019; World Obesity Federation, 2023). Among these, unhealthy eating habits and physical inactivity are the main causal factors due to an imbalance between caloric intake and energy expenditure (Di Cesare et al., 2019; Safaei et al., 2021).

Furthermore, childhood obesity can originate in utero through exposure to various prenatal factors, such as maternal obesity before conception or during the first trimester of pregnancy, malnutrition, recurrent tobacco and alcohol consumption, and exposure to toxins or compounds with endocrine disrupting and obesogenic effects (Deal et al., 2020). In addition to these factors at the prenatal level and their continuation to the postnatal window, among the modifiable risk factors in the first three months of life, breastfeeding has been shown to be a protective factor. It is rich in bioactive compounds associated with the development of a healthy gut microbiota (Porro et al., 2023; Rito et al., 2019).

The concern underlying the epidemic growth of overweight and obesity is that chronic low-grade systemic inflammation occurs as a direct result of adipose tissue dysfunction and the consequent impairment of the immune system (Figure 2) (Schleh et al., 2023; Taylor, 2021). Specifically, the body's adipocytes, or fat cells, increase in size and a microenvironment is created that favours the secretion of various pro-

inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), C-reactive protein (CRP), leptin, and others (Taylor, 2021). Additionally, other cytokines produced by adipocytes and immune cells, such as MCP-1, promote the infiltration of circulating monocytes and macrophages and other cells of the innate and adaptive immune system into adipose tissue, thereby exacerbating and perpetuating the inflammatory process (Li, X. et al., 2023; Taylor, 2021).

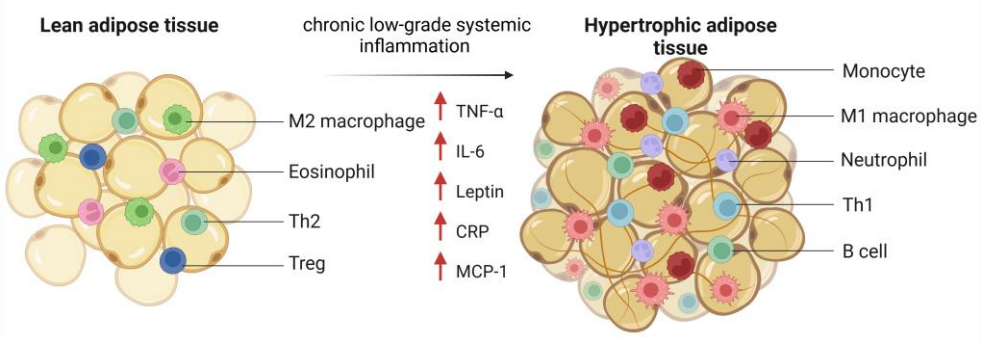


**Figure 2**. Chronic low-grade systemic inflammation in excess weight. Created with Biorender.

As a result of chronic inflammation, multiple tissues and organs are affected, contributing to the premature development of chronic non-communicable diseases such as type 2 diabetes, cancer, cardiovascular disease, respiratory problems or digestive disorders (Marcus et al., 2022; Molnár et al., 2022). Likewise, childhood excess weight has adverse psychosocial consequences, including social stigmatisation, bullying, and discrimination, leading to poor academic performance and self-esteem (Marcus et al., 2022). In the same way, excess weight has been closely linked to neurodevelopmental problems, and vice versa, as described below.

### 1.3. Neurodevelopmental disorders and their bidirectional relationship with excess weight: importance of gene-environment interactions

A growing body of evidence has highlighted the strong link between obesity and neurodevelopmental disorders (NDDs), including attention-deficit hyperactivity disorder (ADHD), autism spectrum disorders (ASD), and intellectual disability, among others (Braun, 2017; Flores-Dorantes et al., 2020). The DSM-5 (Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition) states that NDDs are a

heterogeneous group of mental conditions that begin in early brain development and adversely affect an individual's behaviour, learning ability, attention, memory, and/or psychomotor development (Morris-Rosendahl and Crocq, 2020). More than 3% of the children worldwide are affected by these disorders (Parenti et al., 2020).

On one hand, there is accumulating scientific evidence from studies in animal models and humans that the acquisition of inadequate dietary habits during pregnancy induces cognitive and behavioural alterations in offspring (Edlow, 2017; Hasebe et al., 2021; Tong and Kalish, 2020). Moreover, childhood overweight and especially obesity can lead to an increased risk of behavioural problems, ADHD symptoms, anxiety disorders and depression, as well as impaired executive function and working memory (Pérez-Bonaventura et al., 2015; Sheinbein et al., 2019; Wang, S. et al., 2019; Yang et al., 2018). Indeed, obesity-associated brain structure changes, specifically in the prefrontal cortex which plays a crucial role in the development of executive functions (e.g., planning, organisation, decision-making, emotion control and problem solving), may partially mediate the neurobiological association between weight gain and disruptive executive function (Laurent et al., 2020; Ronan et al., 2020).

On the other hand, it is common for people with psychiatric disorders and/or impulsive personality traits to turn to over-consumption of calories as a method of self-medication to alleviate emotional distress. Compulsive intake of palatable foods initially produces a pleasurable effect by activating the reward circuits of the mesolimbic dopaminergic system, but in the long term, it can develop into a food addiction that predisposes to excess weight (Brunault et al., 2019; Leigh and Morris, 2018). Available epidemiological research in this field indicates that children, adolescents and adults diagnosed with intellectual disabilities, ADHD or ASD develop inadequate eating behaviours marked by physical inactivity, becoming overweight and/or obese (Cortese et al., 2016; Kahathuduwa et al., 2019; Li, Y. et al., 2020; Maiano et al., 2016).

Beyond these unidirectional relationships, there may be a bidirectional link between obesity and neurodevelopmental disorders through different biological

mechanisms and common risk factors. Here, genetic factors are an important component, as obesity-related genes are highly expressed in brain regions responsible for appetite, energy metabolism, mood regulation, and neural development (see section 1.4.2) (Flores-Dorantes et al., 2020; Milaneschi et al., 2019). Early exposure to environmental factors, such as exogenous compounds with hormonal activity, may also increase the risk of excess weight and NDDs through disruption of shared neuroendocrine pathways (Braun, 2017). Thus, the study of the interlinkage of the genetic and environmental component in the bidirectional relationship could provide a more complete answer to the complex and multifactorial aetiology of excess weight and NDDs. In this line, gene-environment interaction studies have emerged as an essential tool to explore the additive or synergistic effect between genetic variants and environmental risk factors on human health (Virolainen et al., 2023).

## 1.4. Genetic predisposition to excess weight and neurodevelopmental disorders

Excess weight and NDDs are highly heritable, underlining the importance of genetic influence. In genetics, the term "heritability" describes "the proportion of observed variation in a particular trait that can be attributed to inherited genetic factors" (Flores-Dorantes et al., 2020).

### *1.4.1. Genetics of excess weight*

There is a strong genetic component to the interindividual variability in body mass index (BMI) that determines the response to an obesogenic environment. Genetic factors are estimated to account for 40-80% of the variation in BMI (Loos and Yeo, 2021; Rohde et al., 2019). Obesity can be classified into two broad categories, monogenic and polygenic obesity.

**Monogenic obesity**, which is inherited in a Mendelian pattern, is caused by mutations in a single gene (Loos and Yeo, 2021). It is typically rare and about 5% of severe cases of obesity occur at an early age due to highly penetrant genetic variants (Serra-Juhé et al., 2020). Most of the genes associated with severe monogenic obesity are those involved in the leptin-melanocortin signalling pathway, which is crucial in

the control of energy balance (Figure 3): leptin (*LEP*), leptin receptor (*LEPR*), Src-homology-2 domain-containing putative adapter (*SH2B1*), proopiomelanocortin (*POMC*), melanocortin receptor (*MC4R)*, preproconvertase 1 (*PCSK1*), single minded 1 (*SIM1*), brain-derived neurotrophic factor (*BDNF*), and its receptor tyrosine kinase receptor tropomycin-related kinase B (*TrkB*) (Kleinendorst et al., 2018; Littleton et al., 2020).

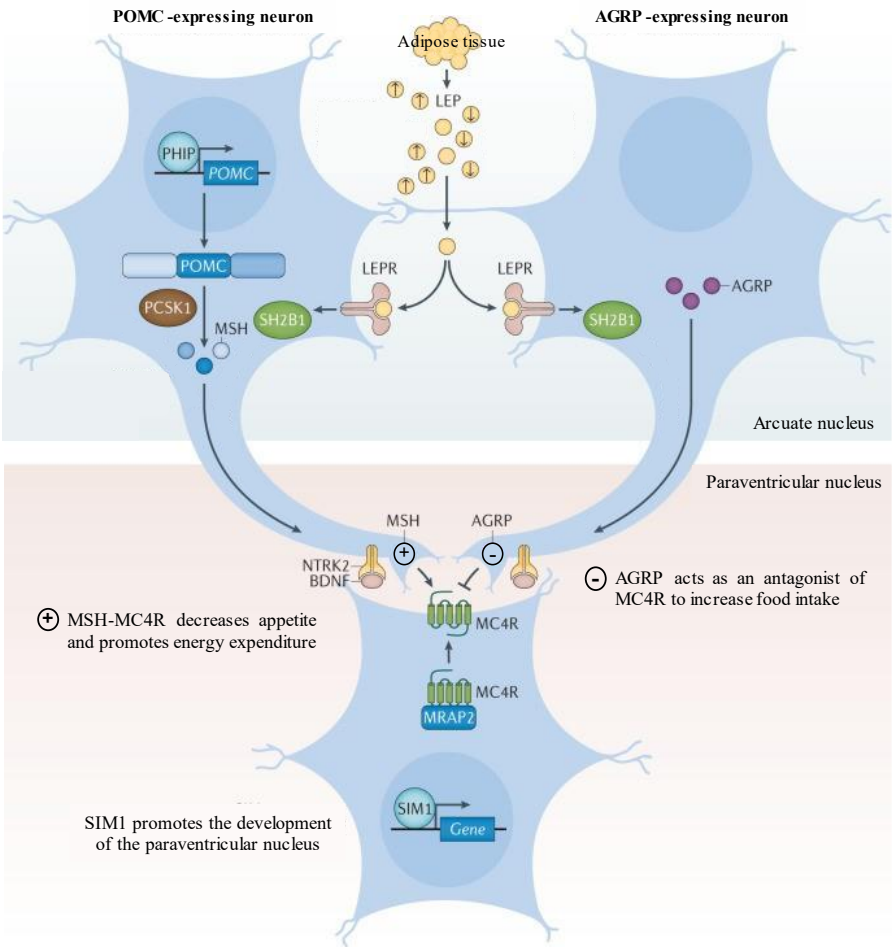


**Figure 3**. Leptin-melanocortin signalling pathway. Adapted from Loos and Yeo (2021).

Leptin is an adipokine produced by adipose tissue, which by binding to its hypothalamic receptors (LEPR), decreases appetite and stimulates energy expenditure (Landecho et al., 2019). This adipokine stimulates POMC-expressing neurons, which produces the activated form of melanocyte-stimulating hormones

(MSH). MSH binds to its MC4R receptors on neurons in the paraventricular nucleus, coordinating energy intake and expenditure (Flores-Dorantes et al., 2020; Mera-Charria et al., 2023). Throughout this process, the cytoplasmic protein SH2B1 acts as a positive endogenous regulator of energy homeostasis and body weight maintenance (Aerts et al., 2015). Leptin gene mutations and defects in protein synthesis or secretion lead to congenital leptin deficiency, which has described as a significance cause of a rare monogenic severe early-onset obesity with an autosomal recessive pattern. Children are born with a normal weight but develop severe hyperphagia at 4 months of age (ElSaeed et al., 2020).

On the other hand, **polygenic obesity** is the most common form of childhood obesity (Pigeyre et al., 2016). The term "polygenic" refers to the combined influence of genetic variants in multiple genes, each of which has a small but significant effect on the overall risk of developing a disease (Littleton et al., 2020; Pigeyre et al., 2016). Genome-wide association studies (GWAS) are the global tool for the identifying new genetic variants associated with disease or specific traits, such as single nucleotide polymorphisms (SNPs) (Littleton et al., 2020). A SNP is a single nucleotide substitution at a specific position in the DNA sequence. SNPs are found throughout the genome, in both coding and non-coding regions (introns, regulatory elements, etc.) (Figure 4) (Ensembl, 2016). The importance of SNPs in clinical diagnosis and genetic research is that they are the most common source of interindividual genetic variation, with a major allele frequency (MAF) greater than 1% (Wu et al., 2023).

Fat mass and obesity-associated gene (*FTO*) rs9939609 was the first locus to be associated with overweight and obesity in the childhood and adulthood (Frayling et al., 2007). *FTO* is highly expressed in the brain and interferes negatively with appetite and satiety signals, lipid metabolism, energy balance and adipogenesis (Ramírez et al., 2021c). Since 2007, many more GWAS have followed, and to date, large-scale GWAS meta-analyses have found more than 1000 independent loci associated with different aspects of obesity (Keller et al., 2023; Yengo et al., 2018).

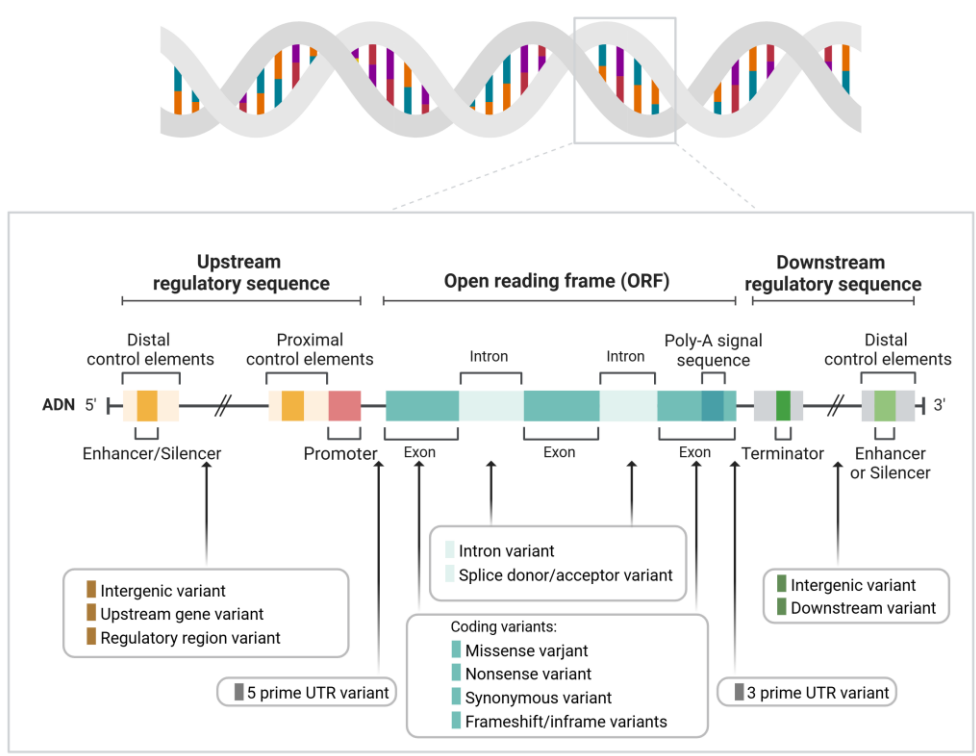


**Figure 4**. Location of SNPs in the DNA sequence. Created with Biorender.

While GWAS studies have contributed extensively to understanding the genetic architecture of common obesity, a major limitation in understanding the genetic contribution is that much of the BMI heritability remains unexplained. SNPs only appear to account for about 6% of the observed variability in BMI (Rohde et al., 2019; Yengo et al., 2018). Then, how can the rest of the heritability be explained? This has been defined as "**missing heritability**", which refers to the fact that a single gene or genetic variant is unable to reliably explain the heritability of a disease and may therefore be partly explained by the influence of the environment on genes (Flores-Dorantes et al., 2020).

### *1.4.2. Genetics of neurodevelopmental disorders and common genetic profile with excess weight*

Advances in biomolecular knowledge (e.g. genotyping or whole genome sequencing tools) have enable the identification of hundreds of candidate genes in neurodevelopment, highlighting the importance of genetic contribution (Leblond et

al., 2021; Stefanski et al., 2021). Nonetheless, the clinical heterogeneity in NDDs is reflected in the extreme genetic diversity, which makes molecular-genetic diagnosis difficult in many cases (Morris-Rosendahl and Crocq, 2020). Besides, phenotype-genotype correlation studies have yielded evidence that the number and severity of clinical signs can vary substantially between patients with similar genetic profile. Hence, missing heritability and phenotypical variability point to a complex, multifactorial, and/or polygenic nature of NDDs (Parenti et al., 2020).

Apart from the genetic correlation between neurodevelopmental disorders, many of the key genes associated with excess weight risk are expressed in brain regions involved in energy homeostasis and brain development (Ronan et al., 2020). **Figure 5** presents a Venn diagram illustrating the set of genes shared between excess weight and NDDs.

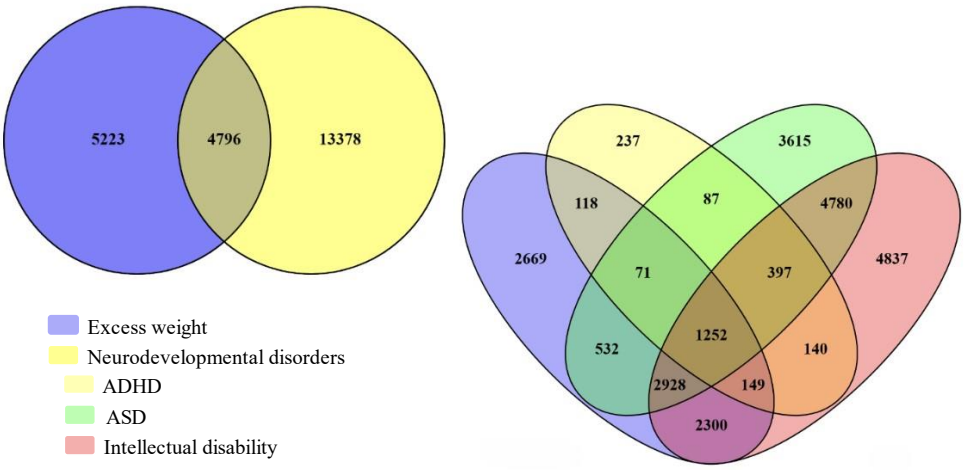


**Figure 5**. Venn diagram of genes associated with excess weight and NDDs. Created with the Venny 2.1.0 tool (https://bioinfogp.cnb.csic.es/tools/venny/).

Alterations in these genes could therefore be modulating genetic susceptibility to both pathologies. A substantial number of investigations have identified genetic variants (copy number variations (CNVs), single nucleotide variants (SNVs) and SNPs) in patients suffering from cognitive-behavioural impairment and excess weight. These genetic changes are in genes implicated in both brain development and metabolic processes, such as myelin transcription factor-1 like (*MYT1L*) (Blanchet et al., 2017), *SH2B1* (Bachmann-Gagescu et al., 2010; Gimeno-Ferrer et

al., 2019), *BDNF* and its receptor *TrkB* (Sonoyama et al., 2020), serotonin receptor (*HTR2C*) (Vimaleswaran et al., 2010), *FTO* (Rivera et al., 2017), and transcription factor 7-like 2 (*TCF7L2*) (Winham et al., 2014).

It is important to emphasise that in the presence of environmental stress, such as an obesogenic environment, the individual's specific genotype determines the manifestation of the observed phenotype (Goodarzi, 2018). And given the multifactorial nature of overweight/obesity and NDDs, it is necessary to consider genetics and environment as interactive factors, rather than studying their effects separately. In this way, exposure to endocrine disrupting environmental pollutants has become important in the prevalence of excess weight and NDDs in recent decades, as described in the next section (Heindel et al., 2022; Nesan and Kurrasch, 2020).

### 1.5. Endocrine disrupting chemicals

The concept of "endocrine disruptor" was first introduced at the Wingspread Conference in 1991, where a group of scientific experts met to discuss the emerging evidence on the adverse effects of certain chemicals on the endocrine system (Soto et al., 2021). Subsequently, in 1996, the concept gained wider acceptance with the publication of Theo Colborn's book *Our Stolen Future*. Since then, the term "endocrine disrupting chemicals (EDCs)" has been widely used in the scientific community, with several definitions by different public health agencies (Langlois et al., 2022). The Endocrine Society defines EDC as "an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action" (Gore et al., 2015).

Disruption can occur by mimicking or antagonising endogenous hormones; altering their production, transport and distribution; interfering with cell signalling after binding to hormone receptors; and altering receptor expression (Heindel et al., 2022). Although there are around 1000 environmental chemicals with EDC activity, the best-known EDCs are polychlorinated biphenyls (PCBs), polybrominated flame retardants (PBDEs), pesticides, perfluoroalkyl substances (PFASs), heavy metals, phthalates, bisphenols, and parabens (Nowak et al., 2018; Ullah et al., 2022). They

are present in a broad range of everyday products, such as toys, electronic devices, food containers, water bottles, personal care products, cosmetics, clothing, cleaning products, and many more (Kasonga et al., 2021; Yilmaz et al., 2020).

During embryonic development, organogenesis and tissue differentiation take place through a series of tightly regulated and coordinated unidirectional events (Gore et al., 2015). Infancy and puberty are also periods of intense change in organs and endocrine-dependent systems. As a result, pregnancy, childhood and adolescence are critical windows of susceptibility to the effects of EDCs, which can lead to permanent damage in adulthood (Kahn et al., 2020; Lucaccioni et al., 2020). In addition to prenatal exposure through placental transport, neonates and infants are exposed to EDCs through breast milk, and later through the diet, which is the main source of exposure, followed by inhalation of contaminated air and dermal absorption. This demonstrates the ubiquity and continuous exposure to these compounds (Ghassabian and Trasande, 2018; Mathiesen et al., 2021).

### *1.5.1. EDCs as multitarget compounds with multi-organ system effects: obesogen hypothesis*

EDCs have the potential to act at different levels in multiple organs and systems, interfering with diverse biological pathways to exert their adverse health effects (Figure 6) (Ahn and Jeung, 2023; Maqbool et al., 2016; Midya et al., 2022; Toni et al., 2020). They can therefore be considered as multitarget compounds with multi-organ system effects. Because of this complexity, the identification of a specific underlying biological mechanism by which they contribute to disease development is rather difficult and not yet fully understood in humans.

In 2002, Baillie-Hamilton hypothesised the obesogenic activity of EDCs based on the parallel increase of exposure to pollutants and the incidence of obesity (Baillie-Hamilton, 2002). In 2006, Grun and Blumberg coined the term "obesogens" to refer to "xenobiotic chemicals that can disrupt normal developmental and homeostatic controls over adipogenesis and energy balance" (Grün and Blumberg, 2006). Approximately 50 obesogens have been identified so far, but there is a gap in knowledge of how most of function (Heindel and Blumberg, 2019).

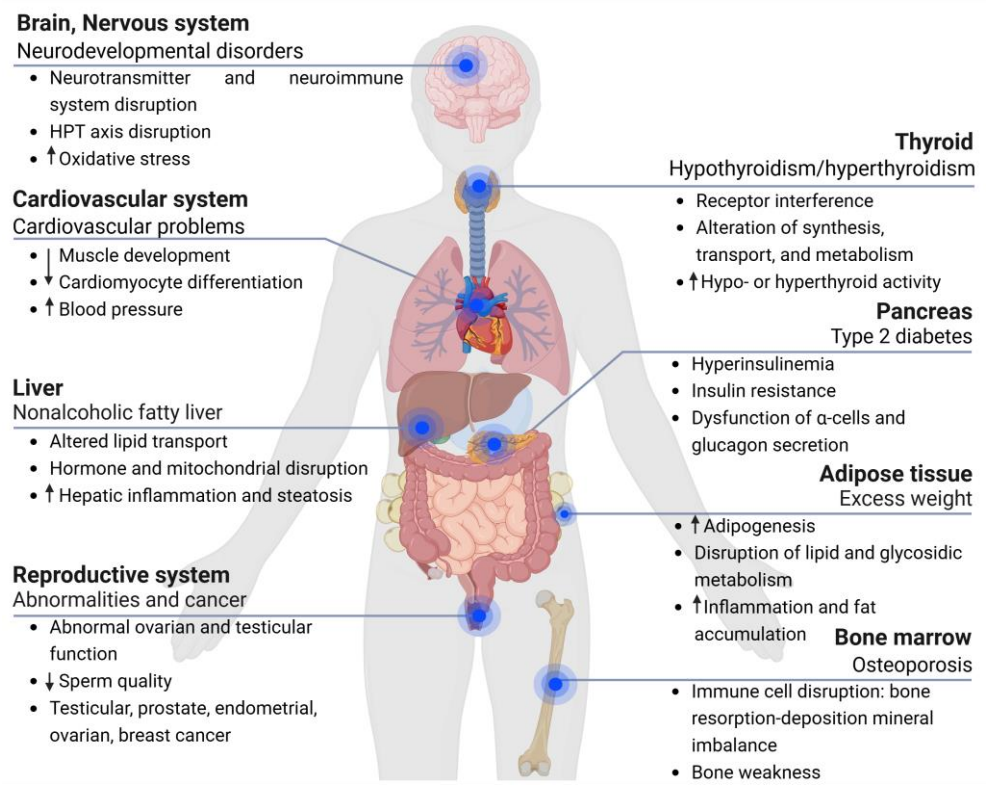


**Figure 6**. Multi-organ-system effects of EDCs. Created with Biorender.

The mechanisms of action of obesogens can be grouped into: 1) long-term mechanisms, including epigenetic modifications that account for intergenerational and transgenerational effects, 2) proximal mechanisms through interaction with steroid hormone receptors and transcription factors, 3) intermediate events such as inflammation, oxidative stress or changes in the gut microbiota, and 4) organ-dependent mechanisms with specific changes in different critical organs and tissues such as liver, adipose tissue, pancreas, muscle and brain (Figure 7) (Heindel et al., 2022).

Peroxisome proliferator-activator receptor gamma (PPARγ) is the master regulator of adipogenesis, and its direct activation is the most common mechanism by which EDCs promote adipogenesis, adipose tissue inflammation through the production of inflammatory cytokines and lipogenesis (Egusquiza and Blumberg, 2020; van der Meer et al., 2021). Moreover, adipose tissue is the body's main energy reservoir (Taylor, 2021). It is also an endocrine organ in charge of the secretion of

numerous adipokines (leptin, adiponectin, angiotensin, among others), which regulate various physiological processes, such as feeding behaviour, glucose and lipid metabolism, and immunity (Tinkov et al., 2021; Veiga-Lopez et al., 2018). In vitro studies have shown that obesogens can induce preadipocyte differentiation and increase adipose tissue mass, either by increasing the size of adipocytes (hypertrophy) or by increasing the number of adipocytes (hyperplasia) (Egusquiza and Blumberg, 2020; Veiga-Lopez et al., 2018). Consequently, adipose tissue dysfunction mediated by exposure to obesogens results in an overall energy imbalance, conferring susceptibility to weight gain.

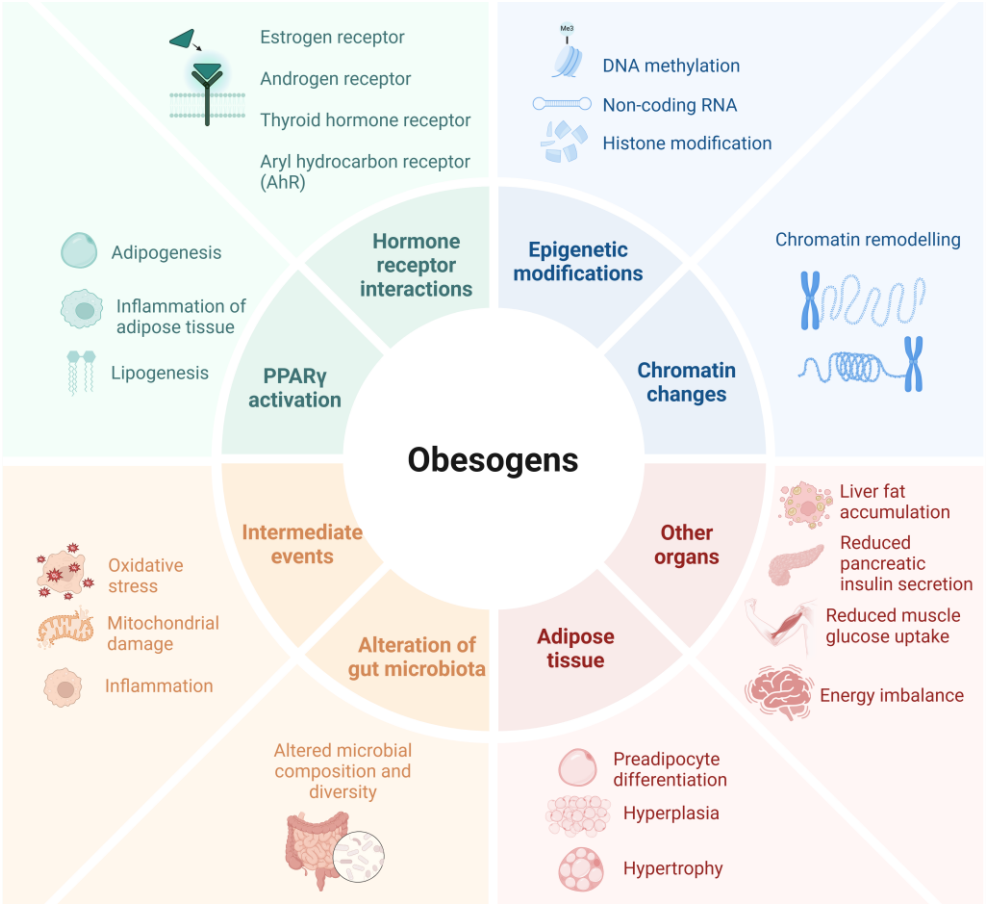


**Figure 7**. Mechanisms of action of obesogens. Created with Biorender.

### *1.5.2. Possible common mechanisms of action of EDCs in excess weight and neurodevelopment*

The role of EDCs as neuroendocrine disruptors was recognised in the First International Symposium on the "Neuroendocrine Effects of Endocrine Disruptors" (Trudeau et al., 2011). León-Olea et al. (2014) modified the definition of neuroendocrine disruptors of Waye and Trudeau (2011) to "neuroendocrine disruptors are exogenous substances found in the environment that alter normal neuroendocrine function and result in an adverse effect on the organism or population".

Due to its complex neuronal organisation, the developing brain is more sensitive than other organs to the disruptive effects of chemicals. EDCs cross the blood-brain barrier and exert their neuroendocrine disrupting effects by 1) changing the levels, transport, release and function of neurotransmitters, 2) altering the bioavailability, function and metabolism of thyroid hormones, compromising the functioning of the hypothalamic-pituitary-thyroid (HPT) axis, 3) disrupting the neuroimmune system, 4) increasing oxidative stress with neuronal death, and 5) causing epigenetic modifications (Dórea, 2021; Morris-Rosendahl and Crocq, 2020; Nesan and Kurrasch, 2020; Vuong et al., 2018).

Once EDCs are conceptualised as obesogens and neuroendocrine disruptors, they could modulate susceptibility to excess weight and NDDs through shared biological pathways (Braun, 2017). At the level of the neuroendocrine system, EDCs could impair the development and function of hypothalamic circuits responsible for neuroendocrine control of food intake and energy homeostasis (Street et al., 2018). Specifically, EDCs alter the signalling of peripheral molecules, such as insulin, ghrelin, leptin and adiponectin, which reach the brain where they monitor the body's energy status. This issue drives a dysregulation of glycosidic and lipid metabolism, together with a decrease in insulin sensitivity (Figure 8) (Braun, 2017; Marraudino et al., 2019; Street et al., 2018).

In addition to their metabolic functions, leptin and insulin are thought to influence synaptic plasticity, memory, learning and cognition, suggesting that

leptin/insulin resistance caused by EDCs may be associated with possible neuronal damage (Edlow, 2017; Hasebe et al., 2021).

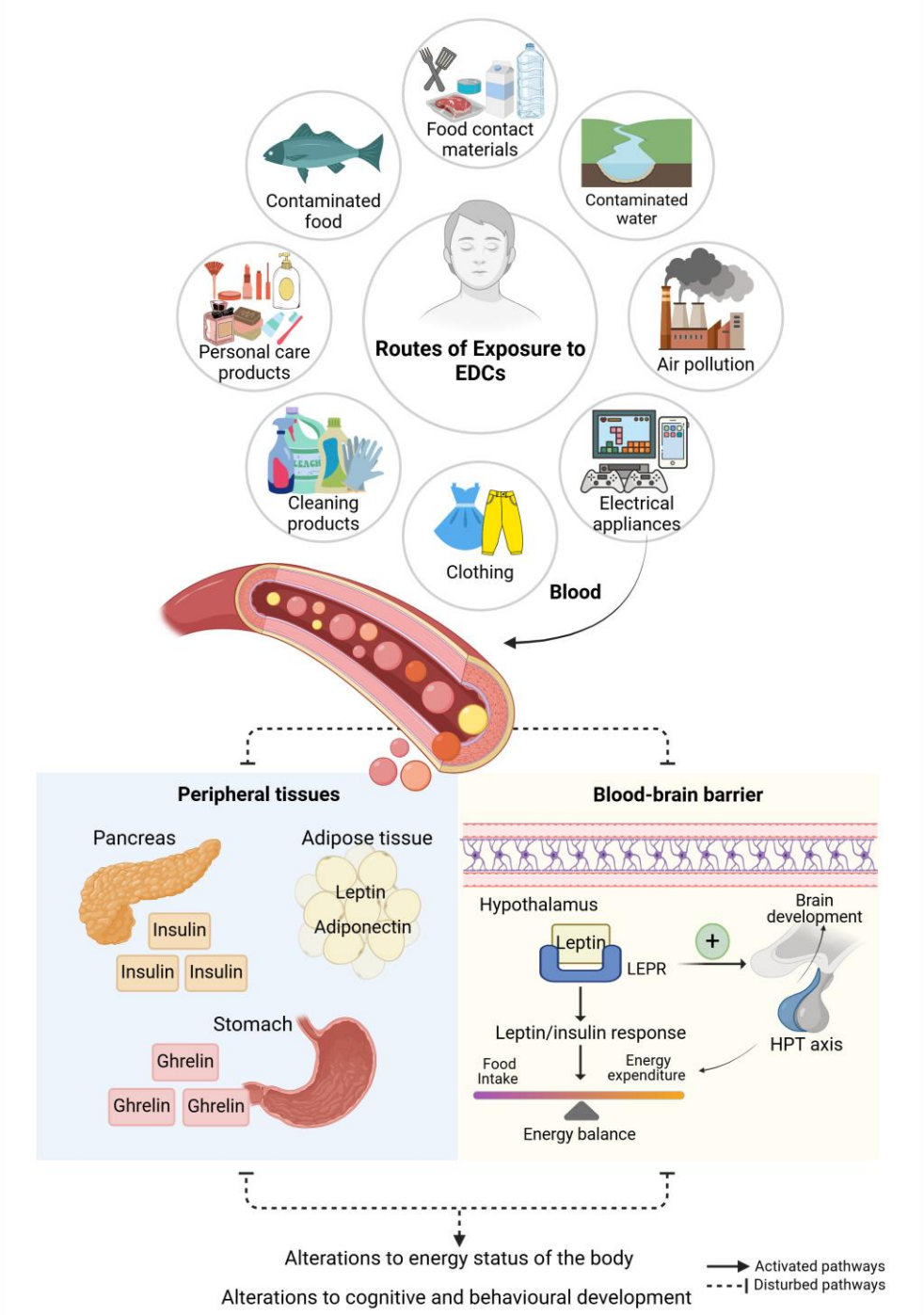


**Figure 8**. Possible common mechanisms of action of EDCs in excess weight and neurodevelopmental disorders. Taken from Ramírez et al. (2022).

Accordingly, neuroendocrine disruption provides a more comprehensive mechanistic view of how EDCs jointly impact on excess weight and neurodevelopment.

In the following sections, we will focus on metal(loid)s, bisphenols and parabens as well-known EDCs, highlighting the evidence available up to now on the interaction with genetics in both pathological scenarios.

### 1.6. Exposure to metal(loid)s

The group of metal(loid)s includes heavy metals (lead, manganese, mercury, cadmium, chromium, molybdenum, zinc, copper, iron, cobalt, etc.) and metalloids (such as arsenic). Heavy metals are characterised by their high density and, frequently, their ability to bioaccumulate in the body and to be toxic at low concentrations. Among them, mercury, lead, cadmium and nickel are known to cause serious health problems (DalCorso et al., 2019). Others are essential in small amounts but can be harmful in larger doses. For example, copper is present in virtually all tissues and is essential for several metabolic reactions; iron is crucial for oxygen transport; molybdenum acts as a cofactor for several enzymes; and cobalt and chromium are involved in vitamin B12 synthesis and carbohydrate metabolism, respectively (Paithankar et al., 2021).

Although metal(loid)s occur naturally through processes such as rock weathering, soil erosion, forest fires and volcanic eruptions, their environmental concentration has increased dramatically due to their widespread industrial and agricultural applicability (Nguyen et al., 2022; Paithankar et al., 2021). Thus, human exposure to these metallic elements often comes from consumption of cultivated products, seafood and contaminated drinking water, air inhalation, as well as from dermal absorption, resulting in ubiquitous and continuous contact (Astolfi et al., 2020; Vogel et al., 2021).

In vitro and in vivo studies have evidenced that mercury, cadmium, lead, arsenic and copper are involved in obesity-related biological processes such as oxidative stress, inflammation, leptin/insulin resistance, as well as adipose tissue dysfunction (Gu et al., 2020; Hernández-Mendoza et al., 2022; Zhong et al., 2021). Nonetheless,

epidemiological evidence on childhood and adolescent excess weight remains scarce.

On the other side, the influence of prenatal and postnatal exposure to heavy metals on neurodevelopmental changes has been extensively documented in children and adolescents (Ramírez et al., 2021a). Exposure to arsenic, lead, manganese, mercury and cadmium in early childhood and adolescence has shown to be mainly related to reduced intellectual performance and marked hyperactivity and impulsivity (Gustin et al., 2018; Lin et al., 2019; Menezes-Filho et al., 2018; Reuben et al., 2020; Vahter et al., 2020).

### *1.6.1. Influence of genetic variability in the detoxification and transport system of metal(loid)s*

Genetic variants are considered to be important modulators of metal body burden. These internal factors have been shown to directly or indirectly control the adsorption, distribution, detoxification and excretion of metal(loid)s (Joneidi et al., 2019). Consequently, genetic variations could influence the body's response to metal(loid)s, thereby modulating individual susceptibility to their adverse health effects (Joneidi et al., 2019; Rahbar et al., 2020).

Glutathione (GSH)-related genes (*GSTP1*, *GCLC/GCLM*), metallothioneins (MTs), selenoproteins, as well as genes encoding transporters (ATP-binding cassette transporter superfamily (ABCs) and ATPase copper transporting beta (*ATP7B*)) are some of the genetic elements involved in detoxification, storage and transport of metal(loid) group (Figure 9) (Andreoli and Sprovieri, 2017; Joneidi et al., 2019; Parajuli et al., 2016).

Regarding evidence for gene-environment interactions on overweight/obesity and neurodevelopment, numerous interactions have been described between gene polymorphisms within the detoxifying system (*GCLC* rs761142, *GSTP1* rs1695, *MT1M* rs2270837, *MT2A* rs10636, *ATP7B* rs1061472 and rs1801243) and certain heavy metals (mercury, lead, manganese and copper) in childhood cognitive dysfunction and inattention, as detailed elsewhere (Ramírez et al., 2022). However,

to date, no studies have investigated this multifactorial role in the aetiology of obesity.

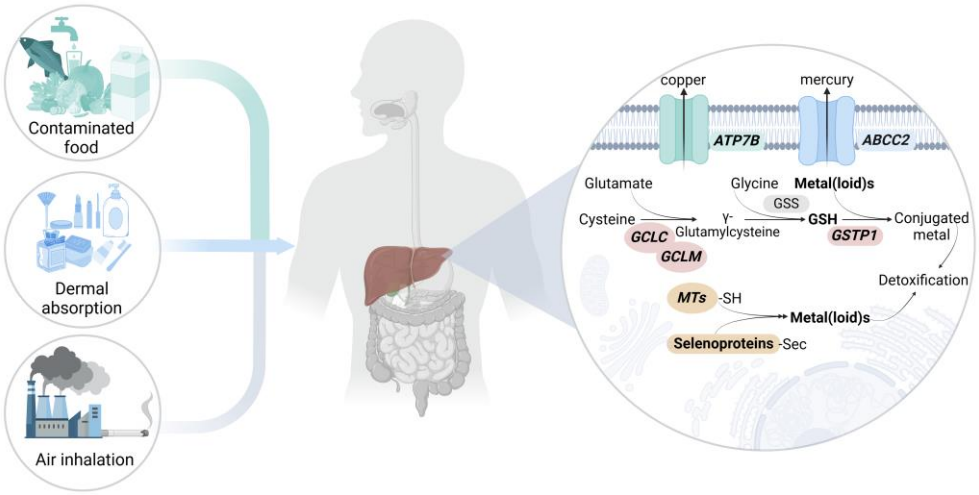


**Figure 9.** Routes of exposure and detoxification system of metal(loid)s. Created with Biorender.

Therefore, genetic variability in the detoxification system could regulate the physiological response to external exposure to heavy metals and the subsequent disease risk.

### 1.7. Exposure to bisphenol A and analogues

Bisphenol A (BPA) is a synthetic chemical compound commonly used as a basic monomer for the industrial production of polycarbonate plastics and epoxy resins, which are found in a wide range of everyday consumer products, such as food and beverage storage containers, personal care products, kitchenware, toys, clothing, thermal paper, dental composites and electronic devices, as well as in the inner lining of canned products and jar lids (Andujar et al., 2019; Garcia-Corcoles et al., 2018).

The synthesis of BPA was first achieved in 1891 by the Russian chemist Aleksandr Dianin. From the 1950s onwards, it began to be mass-produced by the plastics manufacturing industry (Akash et al., 2020). Subsequently, the growing demand for BPA has resulted in its ubiquitous and continuous presence in environmental and biological matrices, including urine, saliva, blood, breast milk, placenta, and umbilical cord (Akash et al., 2020; Lee et al., 2018; Wang, X. et al.,

2022). BPA is one of the most produced synthetic compounds globally, with an annual production of more than 3.8 million tonnes and an atmospheric release of 100 tonnes (Costa and Cairrao, 2024).

Under European Commission Regulation (EU) No 10/2011, BPA was approved for use as a base monomer in food contact materials (FCMs) (European Commission, 2018). BPA migration from FCMs into foodstuffs is a significant contamination source by which BPA enters the food chain, and for this reason dietary consumption has been considered the primary contributor to BPA exposure (EFSA, 2015; Wang, X. et al., 2023). To protect the most vulnerable populations, the European Commission has taken preventive measures by banning the use of BPA in infant feeding bottles and restricting its use in the production of recipients intended for infants and young children (European Commission, 2018).

Importantly, BPA is structurally similar to estrogens and its estrogenic activity was first demonstrated in 1936 (Dodds, 1936). Since then, BPA exposure has been associated with a wide spectrum of adverse effects on human health, including obesogenic and neurodevelopmental disrupting activities (EFSA, 2023). BPA could trigger weight gain through disruption of adipogenesis and lipid metabolism via PPARγ activation (Legeay and Faure, 2017). In an experimental study in rats, BPA exposure led to significant disruptions in metabolic pathways: increased lipid biomarkers; impaired glucose uptake due to decreased expression of glucose transporters (GLUT2, GLUT4); decreased serum levels of oxidative stress biomarkers (CAT, GSH and SOD); and increased serum levels of pro-inflammatory components (TNFα, IL-6 and leptin), while adiponectin levels were decreased. These perturbations resulted in accelerated inflammatory processes, insulin resistance and impaired glucose and lipid metabolism (ul Haq et al., 2020).

Concerning neurodevelopment, influence of BPA on the nervous system is still poorly understood, but it is recognised that BPA exposure can affect brain development and physiology (Costa and Cairrao, 2024). The neurotoxicity of BPA is complex and multifaceted, involving a number of pathological mechanisms. These include induction of oxidative stress, neuronal apoptosis, altered neurotransmission,

neuroinflammation, damage to blood-brain barrier integrity, reduced axonal length, microglial DNA damage and reduced myelination (Costa and Cairrao, 2024; Rebolledo-Solleiro et al., 2021).

Alternatives to BPA have been developed for its gradual replacement, such as bisphenol S (BPS), bisphenol F (BPF), bisphenol B (BPB), and bisphenol AF (BPAF) (Barboza et al., 2020). However, these analogues have a similar chemical structure to BPA and have demonstrated similar or even worse effects than BPA in both animals and humans (Barboza et al., 2020; Costa and Cairrao, 2024).

### *1.7.1. Metabolism of bisphenols and related genetic variations*

After exposure, BPA is rapidly conjugated to glucuronides by the hepatic phase II uridine 5'-diphospho-glucuronosyltransferase (UGT), increasing its water solubility and facilitating urinary excretion (Figure 10) (Hanioka et al., 2022). In humans, BPA lacks enterohepatic circulation, which reduces the half-life to less than 6 hours (Ramírez et al., 2021b). UGT2B15 is the major isoform involved in the detoxification and elimination of BPA. *UGT2B15* genetic variants, such as the D95Y polymorphism (rs1902023), have been shown to reduce its enzymatic activity in vitro, thereby impairing the metabolic efficiency of the organism to properly eliminate BPA (Hanioka et al., 2011).

Other minority metabolic pathways are those catalysed by phase II sulfotransferases (SULTs) and phase I microsomal cytochrome P450 enzymes (CYP1A2, CYP2C9) (Ramírez et al., 2021b; Skledar et al., 2016; Wang, W. et al., 2020). The latter produces highly reactive metabolites, such as the BPA quinone, which has proven to form DNA adducts and cause genotoxicity through the formation of reactive oxygen species (Pandit et al., 2022). BPA analogues follow similar metabolic pathways and their metabolites, particularly the BPF quinones, have demonstrated genotoxic properties (Pandit et al., 2022; Ramírez et al., 2021b).

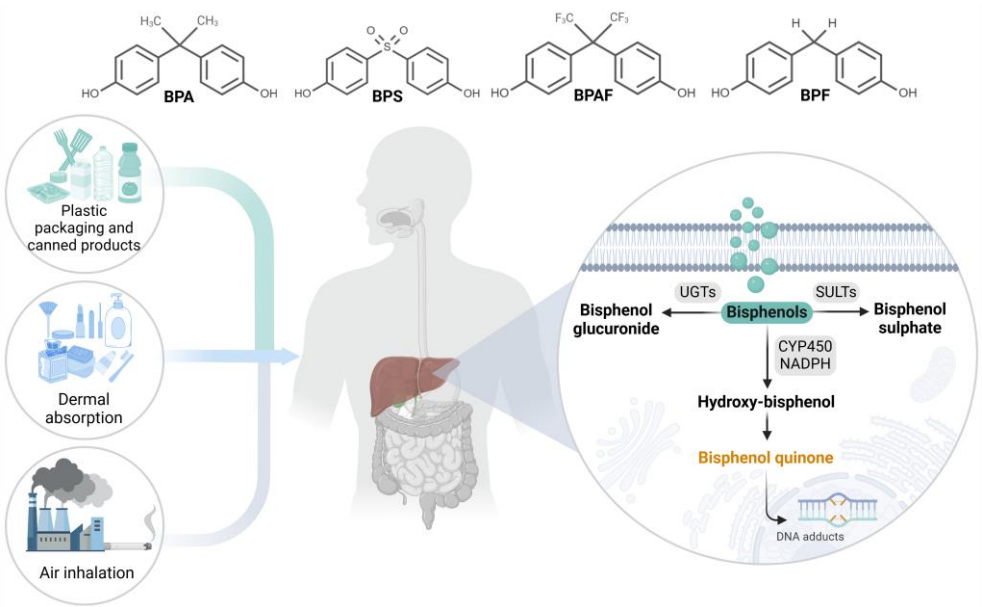


**Figure 10**. Routes of exposure and metabolism of bisphenols. Created with Biorender.

So far, no studies have assessed the SNP-bisphenol interactions on excess weight and neurodevelopment. Although much of bisphenols that enter the bloodstream are excreted in the urine, the rest tends to bioaccumulate in adipose tissue, brain, placenta, liver, and kidneys (Cimmino et al., 2020; Costa and Cairrao, 2024). Human exposure to bisphenols is persistent due to their ubiquity, and if the detoxification machinery is additionally altered by any genetic polymorphism, bioaccumulation would be enhanced and could lead to an aggravation of their obesogenic and neurotoxic effects.

## 1.8. Exposure to parabens

Parabens are a broad group of alkyl esters of para-hydroxybenzoic acid (PHBA) used as preservatives in the cosmetic, pharmaceutical and food products because of their antimicrobial, antifungal and low allergenic properties (Heindel et al., 2022; Moscoso-Ruiz et al., 2023). Among the most industrially used parabens are methyl (MetPB), ethyl (EthPB), propyl (PropPB), and butylparaben (ButPB) (Nowak et al., 2018). All four are allowed in cosmetics, but according to Commission Regulation (EU) No 1004/2014, the maximum recommended concentration is 0.14% when added individually, and 0.8% when mixed in the same cosmetic product (European

Commission, 2014). In contrast, only MetPB and EthPB are authorised as food additives (E218 and E214, respectively), with a maximum permitted level of 300 mg/kg (European Commission, 2011).

Hence, unlike metal(loid)s and bisphenols, the main route of exposure to parabens is dermal absorption, followed by dietary intake and inhalation (aerosols) (Moos et al., 2016).

The disruptive capacity of parabens increases with the length of the alkyl chain, with PropPB and ButPB being of greatest concern (Moscoso-Ruiz et al., 2023). Parabens display their obesogenic activity through changes in adipocyte morphology, that is, they promote lipid accumulation in addition to promoting adipocyte differentiation (Heindel et al., 2022; Nowak et al., 2018). They act as PPARγ agonists and have been shown to decrease adiponectin expression while increasing leptin expression (Hu et al., 2016). A study of 2- to 8-year-old children reported that those prenatally exposed to ButPB had an increased risk of becoming overweight. It was also revealed that fetal exposure to ButPB resulted in increased food intake and significant weight gain in female mice (Leppert et al., 2020). These effects were attributed to epigenetic silencing and reduced hypothalamic expression of *POMC*, suggesting that parabens may contribute to the development of childhood excess weight via neuronal regulation of appetite (Leppert et al., 2020).

Additionally, the mechanisms of action by which parabens alter neurodevelopment include hormone disruption, oxidative stress, neuroinflammation, neurotransmitter dysfunction, etc. Of these, interference with thyroid function has been considered the main biological driving mechanism (Oskar et al., 2024).

### *1.8.1. Metabolism of parabens and genetic variations*

Once in the human body, parabens are metabolised by carboxylesterases (CE1, CE2) to PHBA acid and alcohol. This biotransformation takes place in liver microsomes, the small intestine as well as in the epidermis and dermis (Nowak et al., 2018). They are then conjugated in the liver to their corresponding sulphate and glucuronide derivatives and are excreted mainly in urine and to a lesser extent in bile and faeces (Figure 11) (Nowak et al., 2018; Wei et al., 2021).

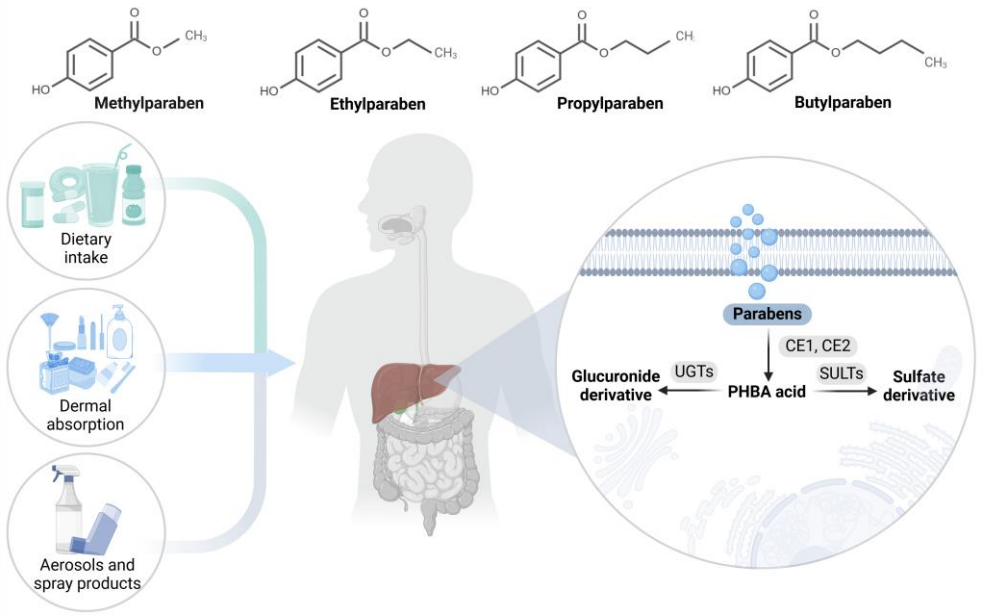


**Figure 11**. Routes of exposure and metabolism of parabens. Created with Biorender.

The metabolic efficiency along with the pattern of hydrolysis of parabens depends on the route of exposure and the length of alkyl chain (Moos et al., 2016). In an intervention study in which 30 young volunteers were given paraben-free hygiene products with all meals of the day, the half-life of parabens ranged from 7.7 to 10.8 hours (Nguyen et al., 2024). In another study of 5 volunteers exposed to parabens after topical application of a cream, the half-life ranged from 9.3 to 12.2 hours (Shin et al., 2023). These results suggest that dermal exposure to parabens may prolong their half-life compared to dietary exposure.

UGTs are responsible for the glucuronidation of parabens, a process in which the UGT2B15 isoform is remarkably involved. In 246 children and adolescents from Slovenia, it was shown for the first time that those carrying two copies of the variant C allele of rs1902023 had lower urinary concentrations of methyl and ethyl parabens than those with one or no copies of this allele (Tkalec et al., 2021). This indicates that the *UGT2B15* polymorphism could be a biomarker of susceptibility to the adverse effects of parabens. However, the role of this genetic variant in childhood excess weight and neurodevelopment has not yet been assessed.

## 2. HYPOTHESIS AND JUSTIFICATION

Considering that genetic polymorphisms represent the most prevalent source of genetic variability in the genome and that exposure to EDCs is constant and ubiquitous, exploring the synergistic or additive effect of these two factors allows addressing excess weight and NDDs from a **more holistic approach** (Virolainen et al., 2023). This could facilitate the understanding of their multifactorial, complex and polygenic aetiology. The interaction between genetics and a highly dynamic environment determines the response to that environment. Therefore, gene-environment or genotype-environment interaction studies could be considered as a promising tool to shed light on the reasons for the rapid increase in childhood excess weight and its bidirectional relationship with neurodevelopmental problems.

So far, available research on neurodevelopment has pointed to significant interactions between exposure to EDCs (mainly pesticides and heavy metals) and polymorphisms of genes involved in detoxifying system, neurotransmission, and metal homeostasis. However, the evidence for overweight and obesity is more limited, with only the role of pesticide levels and polymorphisms reported in children (Ramírez et al., 2022). To the best of our knowledge, there are no studies assessing the interactive effects of genetic polymorphisms with exposure level of bisphenols and parabens on excess weight and childhood neurodevelopment. This raises the need for further research into the co-influence of genetic and environmental factors, rather than studying their contribution separately in both scenarios.

Hence, the hypothesis proposed is the possible relationship between excess weight, neurodevelopment, and exposure to EDCs with known hormonal activity, taking into account the effect of genetic variability. To this end, it is considered: 1) the level of exposure in childhood, and 2) whether children genetically predisposed to excess weight and neurodevelopmental dysfunction might be more susceptible to the adverse effects of an obesogenic and neurodisruptive environment.

# 3. OBJECTIVES & OBJETIVOS

## 3.1. Objectives

The **general objective** of this work is to study the influence of different genetic polymorphisms on childhood overweight and/or obesity and neurodevelopmental disorders according to the level of exposure to EDCs in the school-aged population.

The **specific objectives** are:

1. To elaborate a gene panel comprising multiple SNPs in hormone receptor genes, detoxification enzyme genes and genes associated with the excess weight phenotype and neurodevelopmental disorders.
2. To estimate the risk of these diseases for each polymorphism as a biomarker of individual susceptibility.
3. To study the common genetic profile in excess weight and neurodevelopment.
4. To investigate whether there is an interaction between genetic variants and EDC levels in excess weight and neurodevelopment.
5. To assess the risk of BPA in the school-aged population and compare their exposure with that of adolescents and young adults, with the aim of confirming that children are the most vulnerable population.

## 3.2. Objetivos

El **objetivo principal** de este trabajo es estudiar la influencia de diversos polimorfismos genéticos en el sobrepeso y/u obesidad infantil y trastornos del neurodesarrollo según el nivel de exposición a EDCs en la población escolar.

Los **objetivos específicos** son:

1. Elaborar un panel de genes en el que se recojan varios SNPs de genes de receptores hormonales, de enzimas de detoxificación, y de genes relacionados con el fenotipo de exceso de peso y desórdenes del neurodesarrollo.
2. Estimar el riesgo de estas enfermedades para cada polimorfismo como biomarcador de susceptibilidad individual.
3. Estudiar la genética compartida entre el exceso de peso y el neurodesarrollo.
4. Examinar si existe interacción entre las variantes genéticas estudiadas y los niveles de EDCs en el exceso de peso y neurodesarrollo.
5. Evaluar el riesgo de BPA en la población en edad escolar y comparar su exposición con la estimada en adolescentes y adultos jóvenes, con el objetivo de comprobar que los niños/as son la población más vulnerable.

## 4. CHAPTERS

In order to achieve the objectives set out in this Doctoral Thesis, four research studies have been conducted and are presented in three chapters (Figure 12). Chapters I and II address the first four objectives, while the fifth objective was achieved with the chapter III.

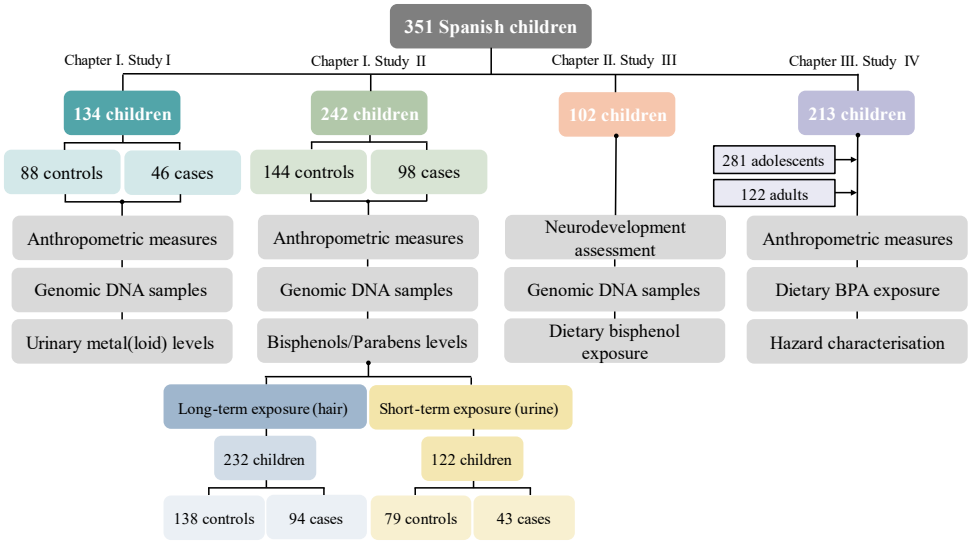


**Figure 12**. Flow diagram of population selection for each study.

**Chapter I** presents the associations of several genetic polymorphisms and levels of EDCs measured in biological matrices with childhood excess body weight. Firstly, a proof-of-concept study was performed to address the associative and interactive role between urinary metal(loid) exposure and certain gene polymorphisms involved in body metal level variability on excess weight among Spanish children. To further investigate other EDCs with known obesogenic activity, the influence of polymorphisms of obesity-related genes, and those coding for metabolising enzymes and hormone receptors, was examined according to a short- and long-term exposure to total bisphenols and parabens, combining individual approach with the joint effect of them.

**Chapter II** provides the first evidence of gene-environment interactions in the context of neurodevelopment. Here, the purpose was to assess the impact of genetic polymorphisms (associated with brain development, synaptic plasticity, and

neurotransmission) on cognitive function according to dietary exposure to bisphenols during childhood.

**Chapter III** present a comprehensive risk assessment of BPA, combining the data from the probabilistic hazard characterisation with the probabilistic exposure estimation. The estimated dietary exposure to BPA in the child population was compared to that estimated in other populations, namely adolescents and young adults who had participated in previous research projects. Thereafter, the estimates were compared to the tolerable daily intake (TDI) derived by European regulatory agencies, such as the German Federal Institute for Risk Assessment (BfR).

**Chapter I.** Relationship between genetic polymorphisms and levels of exposure to EDCs in childhood excess weight

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# Association of genetic polymorphisms in detoxifying systems and urinary metal(loid) levels with excess body weight among Spanish children: A proof-of-concept study

**Viviana Ramírez[a,b,c,d,1], Inmaculada Salcedo-Bellido[c,e,f,1], Lourdes Rodrigo[g], Fernando Gil Hernández[g], Pablo Olmedo Palma[g], Luis Javier Martínez-González[b, *], María Jesús Álvarez-Cubero[b,c,h,1], Ana Rivas[a,c,d,1]**

[a] Department of Nutrition and Food Science, Faculty of Pharmacy, University of Granada, Granada, Spain

[b] GENYO. Centre for Genomics and Oncological Research: Pfizer / University of Granada / Andalusian Regional Government PTS Granada - Avenida de la Ilustración, 114, 18016 Granada, Spain

[c] Instituto de Investigación Biosanitaria ibs.GRANADA, Granada, Spain.

[d] Institute of Nutrition and Food Technology "Jose Mataix Verdú," Biomedical Research Center, University of Granada, Granada, Spain

[e] Department of Preventive Medicine and Public Health, Faculty of Pharmacy, University of Granada, Granada, Spain

[f] Consortium for Biomedical Research in Epidemiology & Public Health (CIBER en Epidemiología y Salud Pública—CIBERESP), Monforte de Lemos 5, 2809, Madrid, Spain

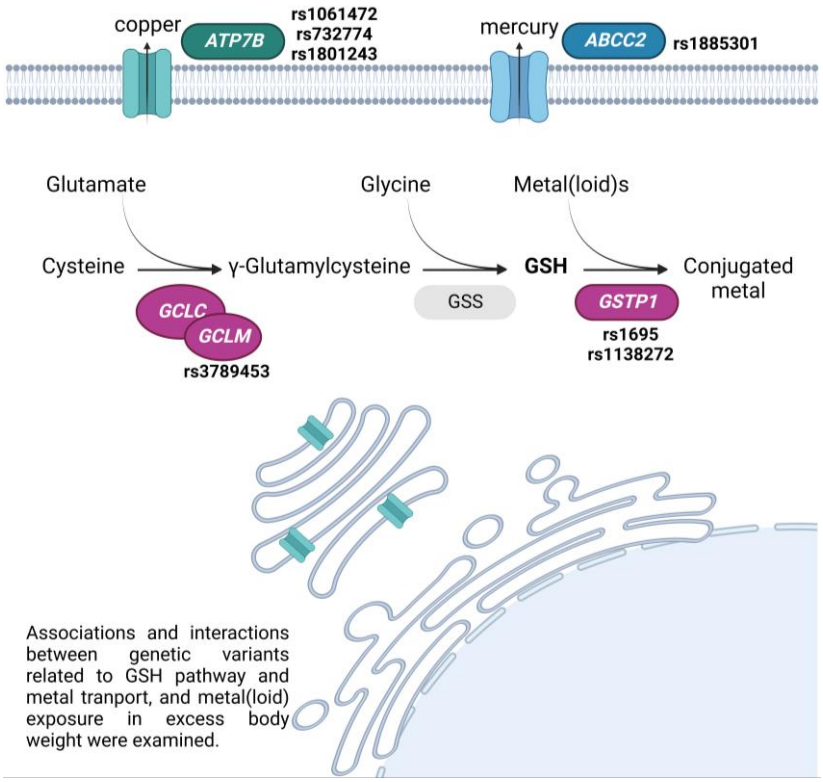
[g] Department of Legal Medicine, Toxicology and Physical Anthropology, Faculty of Medicine, University of Granada, Granada, Spain

[h] Department of Biochemistry and Molecular Biology III, Faculty of Medicine, University of Granada, Granada, Spain

[1] These authors contributed equally to the work.

*Corresponding author. Email: luisjavier.martinez@genyo.es (L.J. González-Martínez)

**Graphical abstract**




**Abstract**

Exposure to metal(loid)s during critical developmental windows could result in permanent damage to the target organ system, increasing susceptibility to disease later in life. In view of the fact that metals(loid)s have been shown to work as obesogens, the aim of the present case-control study was to evaluate the modification effect of exposure to metal(loid)s on the association between SNPs in genes involved in metal(loid) detoxification and excess body weight among children. A total of 134 Spanish children aged 6-12 years old were included (88 controls and 46 cases). Seven SNPs (*GSTP1* rs1695 and rs1138272; *GCLM* rs3789453, *ATP7B* rs1061472, rs732774 and rs1801243; and *ABCC2* rs1885301) were genotyped on GSA microchips, and ten metal(loid)s were analysed in urine samples through Inductively coupled plasma mass spectrometry (ICP-MS). Multivariable logistic regressions were conducted to assess the genetic and metal exposures' main association and interaction effects. *GSTP1* rs1695 and *ATP7B* rs1061472 showed significant effects

on excess weight increase in those children carrying two copies of the risk G allele and being highly exposed to chromium (ORa = 5.38, $p = 0.042$, $p$ interaction = 0.028 for rs1695; and ORa = 4.20, $p = 0.035$, $p$ interaction = 0.012 for rs1061472) and lead (ORa = 7.18, $p = 0.027$, $p$ interaction = 0.031 for rs1695, and ORa = 3.42, $p = 0.062$, $p$ interaction = 0.010 for rs1061472). Conversely, *GCLM* rs3789453 and *ATP7B* rs1801243 appeared to play a protective role against excess weight in those exposed to copper (ORa = 0.20, $p = 0.025$, $p$ interaction = 0.074 for rs3789453) and lead (ORa = 0.22, $p = 0.092$, $p$ interaction = 0.089 for rs1801243). Our findings provide the first proof that interaction effects could exist between genetic variants within GSH and metal transporting systems and exposure to metal(loid)s, on excess body weight among Spanish children.

**Highlights**

- *GSTP1* rs1695 and *ATP7B* rs1061472 contributed to excess weight in the presence of chromium and lead.
- *GCLM* rs3789453 and *ATP7B* rs1801243 showed the opposite effect for copper and lead exposures.
- First gene – metal(loid) interactions reported in excess body weight among children.

**Keywords:** obesity, overweight, children, genetic polymorphism, metal(loid)s

## 1. Introduction

Environmental exposure to metal(loid)s has increased dramatically as a consequence of accelerated urbanization and industrialization processes (Ahmad et al., 2021; Nguyen et al., 2022). Metal(loid)s including metals (lead, manganese, mercury, chromium and cadmium) and metalloids (e.g. arsenic) are used in a wide range of sectors, such as industry, agriculture, healthcare, and cosmetics, as well as both pharmaceutical and household applications (Paithankar et al., 2021). Human exposure to these metal elements frequently comes from marine food consumption and contaminated drinking water, air inhalation through active smoking, as well as dermal absorption, resulting in ubiquitous and continuous contact (Astolfi et al.,

2020; Vogel et al., 2021). Epidemiological evidence related to the contribution of metal(loid)s to the epidemic growth of obesity prevalence is quite limited in children and adolescents. Higher blood levels of mercury, copper, manganese, cadmium (Cho, 2021; Fan et al., 2017; Green et al., 2018), as well as higher urinary concentrations of arsenic, lead and chromium have been associated with increased obesity risk (Nasab et al., 2022). In contrast, negative associations were found between cadmium, cobalt and lead levels in urine and obesity in children and adolescents aged 6–19 years old (Shao et al., 2017). Nonetheless, to the best of our knowledge, there is no data on childhood obesity and metal(loid) exposure in Spanish children.

Metal(loid)s have been shown to interfere with the normal functioning of the endocrine system, playing principal roles as endocrine disrupting chemicals (EDC) (Kasonga et al., 2021; Onat et al., 2021). The mechanisms of action by which metal(loid)s induce their endocrine disruption in human obesity remain inconclusive. *In vitro* and *in vivo* studies have revealed that metals are involved in obesity-related biological processes, such as oxidative stress, inflammation, leptin and insulin resistance, as well as the tissue adipose function (Gu et al., 2020; Hernández-Mendoza et al., 2022; Zhong et al., 2021). Adipose tissue, which is an endocrine organ in charge of adipokine secretion, has been proposed as a potential target of heavy metal toxicity (Tinkov et al., 2021). In this respect, for example, mercury has been shown to alter functions of adipose tissue, such as leptin secretion and peroxisome proliferator-activated receptor gamma (PPARγ) signalling, causing disturbances in nutrient metabolism and energy homeostasis (Jeon et al., 2021).

Two considerations are important to mention. Firstly, childhood obesity is currently a major concern worldwide, since obese children are more likely to develop adulthood obesity and, consequently, are more susceptible to obesity-associated chronic comorbidities (Smith et al., 2020). Secondly, exposure to metallic elements during critical developmental windows could result in permanent damage to the target organ system, increasing susceptibility to disease later in life (Pesce, et al. 2021; Ramírez et al., 2022a). Therefore, intervention during prenatal and postnatal

exposure to metallic metals could prove to be an effective therapeutic choice to mitigate an obesogenic environment.

In addition to environmental factors, genetic variants like single nucleotide polymorphisms (SNPs) in genes controlling metal body burden could influence susceptibility to the adverse health effects of metals. Enzymes belonging to the GSH system, such as glutathione S-transferases (GSTs), protect against oxidative stress by the conjugation of GSH to xenobiotic compounds, including metal(loid)s (Joneidi et al., 2019; Rahbar et al., 2020). GST genes (*GSTP1, GSTM1,* and *GSTT1*) are highly polymorphic, so their genetic variants could interfere with the absorption, distribution, metabolism and excretion of metal(loid)s (Andreoli and Sprovieri, 2017; Rahbar et al., 2021). Genes encoding γ-Glutamyl-cysteine ligase catalytic and modifier subunits (*GCLC* and *GCLM*, respectively) are key components of GSH synthesis, and genetic polymorphisms within GCL complex have been related to modifications of metal concentrations (Barcelos et al., 2015; Chan et al., 2020; Parajuli et al., 2016; Wahlberg et al., 2018). Xenobiotic transporter gene variants could also compromise uptake, distribution, and elimination of metals at a molecular level. ATPase copper transporting beta (*ATP7B*) and multidrug resistance-associated proteins, codified by *ABCC1*-ABCC2 genes, have been associated with cellular copper and mercury efflux (Andreoli and Sprovieri, 2017; Parajuli et al., 2016).

There are epidemiological studies reporting gene-metal interactions (Chan et al., 2020; Liu et al., 2021; Rahbar et al., 2020; Rahbar et al., 2021), but their deleterious effects on obesity have not yet been examined. Therefore, we aim to address the associative and interactive role between urinary metal(loid) exposure and certain gene polymorphisms involved in body metal level variability, on excess body weight (overweight and obesity) among Spanish children aged 6-12 years old.

## 2. Materials and Methods

### *2.1. Study design and population*

The present case-control study was carried out in different elementary schools and primary care centres in Granada, Spain. Participants were recruited from January 2020 through January 2022. Cases and controls met the following inclusion criteria:

(1) prepuberal children aged between 6 and 12 years old, (2) having lived in the study area continuously for at least 6 months and (3) overweight or obesity diagnosis (only for cases). Children with obesity as a result of a pathological side-effect or pharmacological treatment were excluded from the study. All parents or legal guardians of the children were informed of the study objectives and provided a written informed consent. The study protocol was approved by Ethics Committee of Provincial Biomedical Research of Granada (CEI).

A total of 134 children (88 controls and 46 cases) had available urinary metal(loid) levels and genomic DNA samples of adequate concentrations for the study. There were no statistically significant differences between controls with and without information on urinary metal(loid) concentrations and genomic DNA, and cases with or without these data (supplementary **Table S1**).

*2.2. Data collection*

Trained interviewers conducted face-to-face interviews with the participants' parents or legal tutors using a structured questionnaire. The same interviewers surveyed the parents or legal tutors of both cases and controls. Sociodemographic information (gender and age of the children; educational, occupational and marital status of parents), lifestyles (smoking habits of parents, physical activity and dietary patterns) and anthropometric data were obtained for both cases and controls.

The anthropometric measurements were collected by qualified personnel. Body weight (kg) was measured with children barefooted and only wearing underwear using a portable Tanita scale (model MC 780-S MA). Height (m) was measured with a stadiometer (model SECA 214 (20 – 207 cm)) in a standing position with the back, buttocks and heels in contact with the wall. Body mass index (BMI) was calculated with the formula weight (kg)/(height ($m^2$). Sex- and age-specific cut-off points established by Cole et al. (2000, 2007) were used to define childhood underweight, normal weight, overweight and obesity. These international BMI cut-off values cover the age range of 2-18 years old in 6-month intervals.

### *2.3. SNPs selection and genotyping assays*

Polymorphisms of genes involved directly or indirectly in metal(loid) metabolism were selected from Ensembl (https://www.ensembl.org/index.html) and The National Centre for Biotechnology Information SNP website (https://www.ncbi.nlm.nih.gov/). These included SNPs from GSH-related genes (*GSTP1* rs1695 and rs1138272; and *GCLM* rs3789453) and metal transporters (*ATP7B* rs1061472, rs732774 and rs1801243; and ATP binding cassette subfamily C member 2 (*ABCC2*) rs1885301). Characteristics of the selected SNPs are detailed in **Table 1**.

**Table 1.** Summary aspects of selected SNPs in the Spanish reference population (n = 107) and in our study population (n=134).

| Gene name | rs ID | Chr position (GRCh38/ hg38) | Reference/ alternate allele | Genetic variation | MAF[a] | | HWE *p*[c] |
|---|---|---|---|---|---|---|---|
| | | | | | IBS[b] | Our cohort | |
| *GSTP1* | rs1695 | chr11: 67585218 | A/G | Missense variant (Ile105Val) | 0.364 (G) | 0.354 (G) | 0.414 |
| *GSTP1* | rs1138272 | chr11: 67586108 | C/T or G/A | Missense variant (Ala114Val) | 0.056 (T) | 0.041 (A) | 0.620 |
| *GCLM* | rs3789453 | chr1: 93908470 | C/T or G/A | Intron variant | 0.308 (C) | 0.302 (G) | 0.258 |
| *ABCC2* | rs1885301 | chr10: 99781296 | A/G | Intergenic variant | 0.467 (A) | 0.429 (G) | 0.412 |
| *ATP7B* | rs1061472 | chr13: 51950352 | T/C or A/G | Missense variant | 0.327 (T) | 0.410 (A) | 0.386 |
| *ATP7B* | rs732774 | chr13: 51949672 | C/T or G/A | Missense variant | 0.318 (C) | 0.399 (G) | 0.342 |
| *ATP7B* | rs1801243 | chr13: 51974004 | A/C | Missense variant | 0.416 (A) | 0.485 (A) | 0.230 |

[a]MAF: minor allele frequency; [b]IBS: MAF values established for the Iberian population from the Ensembl database (https://www.ensembl.org/index.html); [c]HWE: Hardy-Weinberg equilibrium by chi-square test.

Two buccal swabs were taken from each participant and stored at –20ºC prior to genomic DNA extraction. DNA was extracted using an organic protocol based on proteinase K and saline purification (Ramírez et al., 2022c). DNA concentration was quantified using the Qubit™ 4.0 fluorometer with the Qubit dsDNA BR Assay Kit

(Invitrogen™). DNA samples were stored at –20ºC until genotyping assays. SNP genotyping was developed using the Global Screening Array (GSA) on the iScan platform by llumina® Infinium® HTS Assay support according to the manufacturer's recommendations. This genotyping method by microarray technology enables the whole genome genotyping (WGG), allowing the DNA analysis of up to 750000 SNPs and CNV per sample. 300 ng of DNA from each sample was dispensed into 96-deepwell plates. Then, samples were denatured and isothermally amplified in a single overnight step, then enzymatically fragmented, precipitated with isopropanol, resuspended, hybridized to the BeadChips overnight, single-base extended, stained, and scanned on an iScan high-resolution optical imaging system (Illumina, Inc.). Additionally, 300 DNA samples were genotyped to ensure quality control performance. GSA data were read and processed with llumina® GenomeStudio V2010.3 software.

SNPs were excluded if they (i) had a minor allele frequency (MAF) < 0.01, (ii) had a call rate < 95%, or (iii) deviated from Hardy–Weinberg equilibrium (HWE, $p < 0.05$). Samples were excluded from the final analysis if they had overall call rates less than 95%.

### *2.4. Sampling and metal(loid) analysis*

A spot urine sample was collected from each participant in a sterile polyethylene container. The samples were stored at -80ºC until analysis. For this study, nine metals (cadmium, chromium, cobalt, copper, lead, manganese, mercury, molybdenum and nickel) and one metalloid (arsenic) were analysed.

A calibration curve was prepared in ultrapure water (Milli-Q, Merck, Darmstadt, Germany) with 2% HNO3 (Merck, Darmstadt, Germany) and 1% HCl (Merck) using appropriate metal standard solutions (Agilent Technologies, Santa Clara, CA, USA). Urine samples were diluted 1:10 in ultrapure water (Milli-Q) with 2% $HNO_3$ (Merck) and 1% HCl (Merck). Appropriate blanks were analysed to correct for the results.

The multielement analyses were performed on an Agilent 8900 triple quadrupole Inductively coupled plasma mass spectrometry (ICP-MS) (Agilent Technologies). The instrument was tuned and performance parameters were checked prior to analysis. To ensure the quality of the results, a multielement 400 µg/L internal standard solution with Sc, Ge, Ir and Rh was added online to the samples. Furthermore, the suitable certified reference material [Seronorm (Sero, Billingstad, Norway) Trace Elements Urine L2 (reference 210705)] was reanalysed along with a blank and an intermediate calibration standard every 12 samples. National Institute of Standards and Technology NIST (Gaithersburg, MD, USA) Trace Elements in Natural Water Standard Reference Material SRM 1640a was also used as certified reference material and analysed at the beginning and end of each sequence. Additionally, one in every 12 samples was reanalysed at the end of each session. Limits of detection (LOD) (µg/L) for the different studied metal(loid)s were: arsenic (0.07), cadmium (0.04), cobalt (0.01), chromium (0.2), copper (0.5), mercury (0.08), manganese (0.08), molybdenum (0.03), nickel (0.1), and lead (0.1). The determination of urine creatinine levels was performed by the Ángel Méndez Soto Clinical Analysis Laboratory (Granada, Spain).

Concentrations below the limit of LOD were replaced by LOD/√2 (CDC 2015), except for Cd and Mn (% ≥ LOD= 54.5% and 25% for cases, 60.9% and 19.6% for controls, respectively) that were dichotomized (<LOD/≥ LOD) by their lower number of samples below the LOD. The low and high exposures for the rest of the metal(loid)s were assessed taking the median values into account.

### *2.5. Statistical analysis*

Participant characteristics and urinary metal(loid) levels were assessed using mean, standard deviation (SD), median, 25th and 75th percentiles (interquartile range) for quantitative variables, frequency and percentages for categorical variables. Chi-square tests or Fisher Exact tests (when expected frequency was < 0.05) were used to examine the level of significance of the differences in categorical variables, while Student's t-tests or Wilcoxon Mann-Whitney tests were used for continuous variables. Additionally, chi-square tests ($p > 0.05$) were applied in order

to verify whether genotypic frequencies were distributed following Hardy-Weinberg equilibrium (HWE). Linkage disequilibrium (LD) analyses were performed with the SNPStats software available online (https://snpstats.net/start.htm). SNPs were in LD if they had an $r^2$ value > 0.5.

Firstly, multivariable logistic regression models were used to analyse the association between genetic variants and excess weight, estimating odds ratios (OR) and 95% confidence intervals (95% CI). A variety of genetic models were also tested, including the codominant model (contribution of each genotype to disease risk), dominant model (one copy of the variant allele being sufficient to influence disease risk) and recessive model (two copies of the variant allele being necessary to influence disease risk). The total number of each allele was also taken into account. The potential modifying effect of metal(loid) exposure in the association between genetic variants and excess body weight was tested by incorporating in each model the interaction term (metal(loid) concentrations x genetic variants) and by stratified analysis (low and high exposure to metal(loid)s). Models were adjusted for age and gender as potential confounders, as per other relevant studies (da Fonseca et al., 2019; Olza, et al. 2017). When exposure to metal(loid)s was considered, creatinine levels were also included in the models to reduce inter-individual variation of urinary element levels. This approach is less likely to produce a biased effect estimate than when using creatinine-adjusted urinary metal(loid)s (creatinine standardisation) (Barr et al., 2005). The significance level was set at $p$ value $\leq 0.050$, and borderline significance at $p \leq 0.100$. Statistical analyses were performed using Stata v.15 (Stata Corp., 2017; College Station, Tx, U.S.).

## 3. Results

### *3.1. General characteristics of the study population*

**Table 2** shows the primary characteristics of the cases (52.2% boys and 47.8% girls) and controls (50% boys and 50% girls). Statistically significant differences were observed for mean age values (7.8 years old for controls and 9.2 years old for cases, $p = 0.002$). In relation to urinary metal(loid) levels, non-significant differences were found in both study groups, except for mercury, where the cases exhibited

lower median concentrations than the controls (0.3 µg/L cases vs 0.6 µg/L in controls, $p < 0.001$).

**Table 2.** Characteristic features of cases and controls.

| | **Controls (N=88)** | **Cases (N=46)** | ***p*** |
|---|---|---|---|
| Age (years), mean (SD) | 7.8 (2.6) | 9.2 (1.8) | **0.002**[b] |
| Gender, n (%) | | | 0.811[a] |
| Boys | 44 (50.0) | 24 (52.2) | |
| Girls | 44 (50.0) | 22 (47.8) | |
| Creatinine (g/L), median (P25-P75) | 0.9 (0.6-1.2) | 0.9 (0.6-1.3) | 0.612[c] |
| Metal(loid)s (µg/L), median (P25-P75), detected (%) | | | |
| Chromium | 0.3 (<LOD-0.5) 67.0 | 0.3 (<LOD-0.3) 69.6 | 0.649[c] |
| Manganese | <LOD (<LOD-0.1) 25.0 | <LOD (<LOD-<LOD) 19.6 | 0.372[c] |
| Cobalt | 0.5 (0.3-1.0) 100 | 0.5 (0.3-0.8) 100 | 0.180[c] |
| Nickel | 1.5 (1.0-2.8) 100 | 1.5 (0.8-2.2) 100 | 0.509[c] |
| Copper | 5.1 (2.9-7.8) 92.4 | 4.8 (2.2-7.9) 86.9 | 0.713[c] |
| Arsenic | 25.6 (9.6-51.0) 100 | 19.3 (8.9-66.0) 100 | 0.623[c] |
| Molybdenum | 58.1 (33.2-84.9) 100 | 58.2 (28.3-86.6) 100 | 0.833[c] |
| Cadmium | 0.1 (<LOD-0.1) 54.5 | 0.1 (<LOD-0.1) 60.9 | 0.752[c] |
| Mercury | 0.6 (0.3-1.0) 97.7 | 0.3 (0.2-0.5) 89.1 | **<0.001**[c] |
| Lead | 0.2 (0.1-0.4) 88.6 | 0.2 (0.1-0.3) 82.6 | 0.387[c] |

[a]Chi-square test; [b]Student's t-test; [c]Wilcoxon Mann Whitney test; LOD: limit of detection; P25-P75: 25$^{th}$ percentile – 75$^{th}$ percentile; SD: standard deviation. The bold indicates significant *p* values lower than 0.05.

### *3.2. Association of SNPs and excess body weight*

Each SNP was characterized by a reference allele and an alternate or variant allele (**Table 1**). From the seven analysed genetic variants, the minor allele frequencies (MAFs) coincided with available genotyping data for the Iberian population (*GSTP1* rs1695 G, rs1138272 A; *GCLM* rs3789453 G; *ATP7B* rs1061472 A, rs732774 G and rs1801243 A), except for *ABCC2* rs1885301, whose minor allele

was G in our study, instead of the previously reported A allele. All SNPs were in HWE ($p > 0.05$ by chi-square test). Linkage analyses of the three polymorphisms located within the *ATP7B* gene showed a strong linkage: between rs1061472 and rs732774 ($r^2 = 0.95$), followed by rs1061472/rs1801243 ($r^2 = 0.68$), and rs732774/rs1801243 pairs ($r^2 = 0.65$). rs732774 results are not shown here (available in supplementary material), because rs1061472 and rs1801243 demonstrated stronger different associations in gene association and gene-environment interaction studies (discussed below).

We did not find statistically significant differences in the distribution of genotype and allele frequencies among controls and cases (**Table 3**).

**Table 3.** Distribution of genotypes and alleles for controls and cases.

| | **Controls N=88** | **Cases N=46** | ***p*** |
|---|---|---|---|
| | N (%) | N (%) | |
| ***GSTP1* rs1695** | | | |
| AA | 42 (47.7) | 16 (34.8) | 0.343[a] |
| AG | 35 (39.8) | 22 (47.8) | |
| GG | 11 (12.5) | 8 (17.4) | |
| **Dominant model** | | | |
| AA | 42 (47.7) | 16 (34.8) | 0.151[a] |
| AG+GG | 46 (52.3) | 30 (65.2) | |
| **Recessive model** | | | |
| AA+AG | 77 (87.5) | 38 (82.6) | 0.441[a] |
| GG | 11 (12.5) | 8 (17.4) | |
| A | 119 (67.6) | 54 (58.7) | 0.147[a] |
| G | 57 (32.4) | 38 (41.3) | |
| ***GSTP1* rs1138272** | | | |
| GG | 82 (93.2) | 41 (89.1) | 0.510[b] |
| AG | 6 (6.8) | 5 (10.9) | |
| G | 170 (96.6) | 87 (94.6) | 0.519[b] |
| A | 6 (3.4) | 5 (5.4) | |
| ***GCLM* rs3789453** | | | |
| GG | 11 (12.5) | 4 (8.7) | 0.592[a] |
| AG | 31 (35.2) | 20 (43.5) | |
| AA | 46 (52.3) | 22 (47.8) | |
| **Dominant model** | | | |
| GG | 11 (12.5) | 4 (8.7) | 0.507[a] |
| AG + AA | 77 (87.5) | 42 (91.3) | |
| **Recessive model** | | | |
| GG + AG | 42 (47.7) | 24 (52.2) | 0.625[a] |
| AA | 46 (52.3) | 22 (47.8) | |
| G | 53 (30.1) | 28 (30.4) | 0.957[a] |
| A | 123 (69.9) | 64 (69.6) | |
| ***ABCC2* rs1885301** | | | |
| AA | 33 (37.5) | 13 (28.3) | 0.563[a] |
| AG | 38 (43.2) | 23 (50.0) | |
| GG | 17 (19.3) | 10 (21.7) | |

**Table 3** (*continued*)

| | Controls N=88 | Cases N=46 | *p* |
|---|---|---|---|
| | N (%) | N (%) | |
| **Dominant model** | | | |
| AA | 33 (37.5) | 13 (28.3) | 0.285[a] |
| GG+AG | 55 (62.5) | 33 (71.7) | |
| **Recessive model** | | | |
| AA+AG | 71 (80.7) | 36 (78.3) | 0.740[a] |
| GG | 17 (19.3) | 10 (21.7) | |
| A | 104 (59.1) | 49 (53.3) | 0.360[a] |
| G | 72 (40.9) | 43 (46.7) | |
| ***ATP7B* rs1061472** | | | |
| AA | 16 (18.2) | 9 (19.6) | 0.788[a] |
| AG | 38 (43.2) | 22 (47.8) | |
| GG | 34 (38.6) | 15 (32.6) | |
| **Dominant model** | | | |
| AA | 16 (18.2) | 9 (19.6) | 0.845[a] |
| GG+AG | 72 (81.8) | 37 (80.4) | |
| **Recessive model** | | | |
| AA+AG | 54 (61.4) | 31 (67.4) | 0.492[a] |
| GG | 34 (38.6) | 15 (32.6) | |
| A | 70 (39.8) | 40 (43.5) | 0.558[a] |
| G | 106 (60.2) | 52 (56.5) | |
| ***ATP7B* rs732774** | | | |
| GG | 15 (17.1) | 9 (19.6) | 0.935[a] |
| AG | 39 (44.3) | 20 (43.5) | |
| AA | 34 (38.6) | 17 (37) | |
| **Dominant model** | | | |
| GG | 15 (17) | 9 (19.6) | 0.718[a] |
| AG + AA | 73 (83.0) | 37 (80.4) | |
| **Recessive model** | | | |
| GG + AG | 54 (61.4) | 29 (63.0) | 0.849[a] |
| AA | 34 (38.6) | 17 (37.0) | |
| G | 69 (39.2) | 38 (41.3) | 0.739[a] |
| A | 107 (60.8) | 54 (58.7) | |
| ***ATP7B* rs1801243** | | | |
| AA | 20 (22.7) | 15 (32.6) | 0.464[a] |
| AC | 41 (46.6) | 19 (41.3) | |
| CC | 27 (30.7) | 12 (26.1) | |
| **Dominant model** | | | |
| AA | 20 (22.7) | 15 (32.6) | 0.216[a] |
| AC+CC | 68 (77.3) | 31 (67.4) | |
| **Recessive model** | | | |
| AA+AC | 61 (69.3) | 34 (73.9) | 0.578[a] |
| CC | 27 (30.7) | 12 (26.1) | |
| A | 81 (46.0) | 49 (53.3) | 0.260[a] |
| C | 95 (54.0) | 43 (46.7) | |

[a]Chi-square test; [b]Fisher exact test.

The association between the genotype and the allelic contribution of each of the SNPs with overweight/obesity was analysed by logistic regression models (**Table 4**).

**Table 4.** Associations between genetic variants and overweight/obesity.

| | ORa | 95% CI | *p* |
|---|---|---|---|
| **GSTP1 rs1695 (Ref. AA)** | | | |
| AG | 2.13 | 0.92-4.93 | *0.078** |
| GG | 1.86 | 0.61-5.72 | 0.276 |
| AA vs AG+GG (Dom) | 2.05 | 0.94-4.48 | *0.071** |
| AA+AG vs GG (Rec) | 1.29 | 0.46-3.59 | 0.631 |
| Ref. A vs G | 1.55 | 0.90-2.67 | 0.115 |
| **GSTP1 rs1138272 (Ref. GG)** | | | |
| AG | 1.32 | 0.37-4.75 | 0.674 |
| Ref G vs A | 1.30 | 0.37-4.50 | 0.683 |
| ***GCLM* rs3789453 (Ref. GG)** | | | |
| AA | 1.44 | 0.39-5.30 | 0.582 |
| AG | 2.07 | 0.55-7.81 | 0.582 |
| GG vs AA+GG (Dom) | 1.69 | 0.48-5.90 | 0.413 |
| GG+AG vs AA (Rec) | 0.81 | 0.39-1.71 | 0.588 |
| Ref. G vs A | 1.00 | 0.56-1.76 | 0.988 |
| ***ABCC2* rs1885301 (Ref. AA)** | | | |
| AG | 1.58 | 0.67-3.75 | 0.296 |
| GG | 1.42 | 0.50-4.05 | 0.514 |
| AA vs GG+AG (Dom) | 1.53 | 0.68-3.43 | 0.302 |
| AA+AG vs GG (Rec) | 1.08 | 0.44-2.69 | 0.865 |
| Ref. A vs G | 1.24 | 0.73-2.10 | 0.427 |
| ***ATP7B* rs1061472 (Ref. AA)** | | | |
| AG | 0.78 | 0.28-2.17 | 0.634 |
| GG | 0.70 | 0.24-2.01 | 0.506 |
| AA vs GG+AG (Dom) | 0.74 | 0.29-1.92 | 0.538 |
| AA+AG vs GG (Rec) | 0.83 | 0.38-1.82 | 0.643 |
| Ref. A vs G | 0.84 | 0.49-1.42 | 0.511 |
| ***ATP7B* rs732774 (Ref. GG)** | | | |
| AA | 0.72 | 0.25-2.07 | 0.547 |
| AG | 0.59 | 0.21-1.69 | 0.327 |
| GG vs AA+GG (Dom) | 0.65 | 0.25-1.71 | 0.386 |
| GG+AG vs AA (Rec) | 1.04 | 0.48-2.25 | 0.915 |
| Ref. G vs A | 0.90 | 0.53-1.43 | 0.692 |
| ***ATP7B* rs1801243 (Ref. AA)** | | | |
| AC | 0.45 | 0.18-1.13 | *0.090** |
| CC | 0.51 | 0.19-1.39 | 0.186 |
| AA vs CC+AC (Dom) | 0.47 | 0.20-1.10 | *0.082** |
| AA+AC vs CC (Rec) | 0.83 | 0.36-1.91 | 0.661 |
| Ref. A vs C | 0.69 | 0.41-1.18 | 0.175 |

ORa: Odds Ratio adjusted for age and sex; CI: confidence interval; Ref: reference category; Dom: dominant model; Rec: recessive model. The italics indicates (*) borderline *p* values lower than 0.1.

In the codominant model, children with heterozygote genotype for *GSTP1* rs1695 displayed borderline increased odds of overweight/obesity (ORa = 2.13, $p$ = 0.078). This suggested that a positive association held true in the dominant model

(ORa = 2.05, $p$ = 0.071). However, we observed opposite associations for *ATP7B* rs1801243 with the codominant and dominant genetic models. Although no significant results were reached, carriers of rs1801243 AC genotype were 55% less likely to develop overweight/obesity (ORa = 0.45, $p$ = 0.090) compared to those homozygous for the wild A allele, suggesting a protective effect. This borderline trend remained towards the dominant model (ORa = 0.47, $p$ = 0.082). Non-significant differences were observed for the other SNPs.

### *3.3. Association between genetic variants and urinary metal(loid) levels in excess body weight*

We evaluated the contribution of each genetic variant on excess weight by testing the different genetic models in children exposed to low and high median concentrations of meta(loid)s. **Tables 5** and **6** show the significant associations between four SNPs (rs1695, rs3789453, rs1061472, and rs1801243) and exposure to certain metals. The full results are in the supplementary material (**Tables S2-S8**).

#### *3.3.1. GSH-related gene polymorphisms*

For *GSTP1* rs1695 A/G variant (**Table 5**), subjects homozygous for the high-risk G allele and highly exposed to chromium (ORa = 6.28, 95% CI: $p$ = 0.040) and lead levels (ORa = 9.64, $p$ = 0.019) displayed significantly higher odds of developing excess weight compared to individuals inheriting two copies of the low-risk A allele. Under the recessive model (AA+AG vs GG) this significance was also observed (ORa = 5.38, $p$ = 0.042, and ORa = 7.18, $p$ = 0.027, respectively). Considering contribution per allele, each copy of the G allele contributed significantly to increased odds of excess weight in children with high exposure to both chromium and lead (ORa = 2.45, $p$ = 0.042, and ORa = 3.42, $p$ = 0.009, respectively). Additionally, the interactions were statistically significant for chromium exposure in the recessive model ($p$ interaction = 0.028); and for lead exposure in both the codominant ($p$ interaction = 0.045) and recessive models ($p$ interaction = 0.031).

**Table 5.** Main effects between metal(loid) exposure and *GSTP1* rs1695 and *GCLM* rs3789453 polymorphisms on excess body weight.

| | **Low exposure (<median)** | | | **High exposure (≥ median)** | | | ***p*-int** |
|---|---|---|---|---|---|---|---|
| | ORa | *p* | SE | ORa | *p* | SE | |
| *GSTP1* rs1695 | | | | | | | |
| **Chromium (**Ref. AA) | | | | | | | |
| AG | 2.16 | 0.270 | 1.50 | 1.39 | 0.631 | 0.97 | 0.114 |
| GG | 0.44 | 0.402 | 0.43 | 6.28 | **0.040** | 5.61 | |
| AA+AG vs GG (Rec) | 0.34 | 0.252 | 0.32 | 5.38 | **0.042** | 4.45 | **0.028** |
| A vs G | 0.85 | 0.721 | 0.38 | 2.45 | **0.042** | 1.07 | *0.096** |
| **Lead** (Ref. AA) | | | | | | | |
| AG | 1.52 | 0.526 | 1.01 | 2.03 | 0.352 | 1.55 | **0.045** |
| GG | 0.52 | 0.488 | 0.49 | 9.64 | **0.019** | 9.28 | |
| AA+AG vs GG (Rec) | 0.42 | 0.333 | 0.38 | 7.18 | **0.027** | 6.40 | **0.031** |
| A vs G | 0.84 | 0.684 | 0.37 | 3.42 | **0.009** | 1.62 | **0.025** |
| **Mercury** (Ref. AA) | | | | | | | |
| AG | 0.87 | 0.823 | 0.55 | 6.63 | *0.053** | 6.49 | 0.165 |
| GG | 0.97 | 0.972 | 0.82 | 3.93 | 0.171 | 3.94 | |
| AA vs AG+GG (Dom) | 0.90 | 0.851 | 0.52 | 5.15 | **0.046** | 4.23 | 0.109 |
| A vs G | 0.95 | 0.909 | 0.40 | 2.74 | **0.048** | 1.40 | 0.114 |
| **Molybdenum** (Ref. AA) | | | | | | | |
| AG | 2.45 | 0.198 | 1.71 | 1.10 | 0.886 | 0.76 | 0.380 |
| GG | 1.04 | 0.962 | 0.86 | 8.12 | *0.090** | 10.1 | |
| AA+AG vs GG (Rec) | 0.70 | 0638 | 0.53 | 7.78 | *0.088** | 9.35 | *0.066** |
| *GCLM* rs3789453 | | | | | | | |
| **Nickel** | | | | | | | |
| GG+AG vs AA (Rec) | 0.29 | *0.066** | 0.20 | 0.74 | 0.631 | 0.46 | 0.407 |
| **Arsenic** | | | | | | | |
| GG+AG vs AA (Rec) | 0.32 | *0.071** | 0.20 | 0.69 | 0.558 | 0.44 | 0.337 |
| **Molybdenum** | | | | | | | |
| GG+AG vs AA (Rec) | 0.35 | *0.097** | 0.22 | 0.88 | 0.840 | 0.56 | 0.270 |
| **Copper** | | | | | | | |
| GG+AG vs AA (Rec) | 0.20 | **0.025** | 0.14 | 1.14 | 0.827 | 0.72 | *0.074** |

ORa: Odds Ratio adjusted for age, sex, and creatinine; SE: standard error; *p*-int: *p* for interaction; Ref: reference category; Dom: dominant model; Rec: recessive model. Chromium (median: 0.260 µg/L); Lead (median: 0.196 µg/L); Mercury (median: 0.439 µg/L); Molybdenum (median 58.201 µg/L); Nickel (median 1.531 µg/L); Arsenic (median: 24.019 µg/L); Copper (median: 5.052 µg/L). The bold indicates significant *p* values lower than 0.05, and italics (*) means *p* values lower than 0.1.

In the case of increased mercury exposure, those with the AG genotype had a 6.6-fold higher chance of exhibiting excess weight at the limit of significance ($p$ = 0.053) compared to individuals with the AA genotype. Statistical significance was reached in the dominant model (AA vs AG+GG, ORa = 5.15, p = 0.046), and each

G allele was significantly related to greater odds of overweight/obesity (ORa = 2.74, $p = 0.048$).

Borderline associations and interactions were found with elevated molybdenum levels for GG carriers (ORa = 7.78, $p = 0.088$, $p$ interaction = 0.066).

For the other genetic variant in the GSH pathway (**Table 5**), *GCLM* rs3789453 in the recessive model (GG+AG vs AA) contributed to decreased obesity odds at near statistical significance for lower levels of nickel, arsenic and molybdenum ($p \leq 0.100$). For lower copper exposure, individuals with AA genotype in comparison to those with GG or AG genotypes had a significantly reduced likelihood of suffering from overweight/obesity (recessive model ORa = 0.20, $p = 0.025$). A marginally significant interaction between copper levels and *GCLM* variant was observed ($p$ interaction = 0.074).

### *3.3.2. Metal transport-related gene polymorphisms*

Concerning *ATP7B* SNPs (**Table 6**), in the recessive model for rs1061472 A/G (AA+AG vs GG), carriers of GG genotype who were exposed to higher levels of chromium had greater odds of being overweight/obese (ORa = 4.20, p = 0.035) than those with AA or AG genotype. For high molybdenum levels, despite the fact that the association of carrying the gene polymorphism with being overweight/obese was not relevant, interaction between molybdenum and rs1061472 proved statistically significant in both the recessive genetic model and the allele contribution model ($p$ interaction = 0.041 and $p$ interaction = 0.010, respectively). Interaction terms were also statistically relevant for high exposure to lead in the codominant ($p$ interaction = 0.019) and recessive models ($p$ interaction = 0.010).

In relation to *ATP7B* rs1801243 A/C polymorphism, borderline significances were found for chromium, copper and lead. In the recessive model (AA+AC vs CC), individuals with CC genotype and exposed to higher chromium levels had greater odds of overweight/obesity (ORa = 4.09, $p = 0.081$) compared to those with AA or AC genotype, with the interaction being meaningful ($p$ interaction = 0.043).

By contrast, children who carried one or two copies of C alleles (AC or CC genotypes) were 74% and 78% less likely to be overweight/obese when exposed to higher copper and lower lead concentrations, respectively (p = 0.079 for copper, and p = 0.092 for lead). Marginal interaction was detected between lead levels and *ATP7B* rs1801243 (*p* interaction = 0.089 and *p* interaction = 0.088 for the codominant and recessive models, respectively).

**Table 6.** Main effects between metal(loid) exposure and *ATP7B* polymorphisms on excess body weight.

| | **Low exposure (< median)** | | | **High exposure (≥ median)** | | | ***p*-int** |
|---|---|---|---|---|---|---|---|
| | ORa | *p* | SE | ORa | *p* | SE | |
| *ATP7B* rs1061472 | | | | | | | |
| **Chromium** (Ref. AA) | | | | | | | |
| AG | 1.51 | 0.625 | 1.28 | 0.63 | 0.614 | 0.57 | *0.072** |
| GG | 0.44 | 0.389 | 0.42 | 3.05 | 0.221 | 2.79 | |
| AA+AG vs GG (Rec) | 0.33 | 0.131 | 0.24 | 4.20 | **0.035** | 2.86 | **0.012** |
| **Molybdenum** (Ref. AA) | | | | | | | |
| AG | 1.23 | 0.801 | 1.01 | 0.79 | 0.809 | 0.79 | *0.089** |
| GG | 0.57 | 0.532 | 0.51 | 2.76 | 0.290 | 2.64 | |
| AA+AG vs GG (Rec) | 0.50 | 0.323 | 0.35 | 3.27 | 0.078 | 2.20 | **0.041** |
| A vs G | 0.73 | 0.462 | 0.31 | 2.01 | 0.138 | 0.95 | **0.010** |
| **Lead** (Ref. AA) | | | | | | | |
| AG | 0.97 | 0.967 | 0.84 | 0.97 | 0.972 | 0.96 | **0.019** |
| GG | 0.29 | 0.186 | 0.27 | 3.35 | 0.192 | 3.11 | |
| AA+AG vs GG (Rec) | 0.29 | *0.082** | 0.21 | 3.43 | *0.062** | 2.27 | **0.010** |
| A vs G | 0.52 | 0.126 | 0.22 | 2.36 | *0.077** | 1.14 | *0.057** |
| *ATP7B* rs1801243 | | | | | | | |
| **Chromium** | | | | | | | |
| GG+AG vs AA (Rec) | 0.42 | 0.220 | 0.30 | 4.09 | *0.081** | 3.30 | **0.043** |
| **Copper** (Ref. AA) | | | | | | | |
| AC | 1.02 | 0.979 | 0.88 | 0.26 | *0.079** | 0.20 | 0.798 |
| CC | 0.67 | 0.663 | 0.61 | 0.77 | 0.741 | 0.62 | |
| **Lead** (Ref. AA) | | | | | | | |
| AC | 0.39 | 0.232 | 0.31 | 0.54 | 0.457 | 0.45 | *0.089** |
| CC | 0.22 | *0.092** | 0.20 | 1.39 | 0.684 | 1.12 | |
| AA+AC vs CC (Rec) | 0.43 | 0.226 | 0.30 | 1.98 | 0.310 | 1.34 | *0.088** |
| A vs C | 0.49 | *0.096** | 0.21 | 1.26 | 0.602 | 0.57 | *0.093** |

ORa: Odds Ratio adjusted for age, sex, and creatinine; SE: standard error; *p*-int: *p* for interaction; Ref: reference category; Dom: dominant model; Rec: recessive model. Chromium (median: 0.260 µg/L); Molybdenum (median: 58.201 µg/L); Lead (median: 0.196 µg/L); Copper (median: 5.052 µg/L). The bold indicates significant *p* values lower than 0.05, and italics (*) means *p* values lower than 0.1.

## 4. Discussion

To the best of our knowledge, this case-control study of Spanish children is the first to assess the combined effect of urinary metal(loid) levels and detoxification system-related polymorphisms in childhood excess body weight (overweight and obesity). The most relevant results were obtained for *GSTP1* rs1695, *GCLM* rs3789453, *ATP7B* rs1061472 and rs1801243 genetic variants. Among them, *GSTP1* rs1695 and *ATP7B* rs1061472 showed significant effects in increased excess weight in those children carrying at least one copy of the risk G allele and highly exposed to chromium and lead. On the contrary, *GCLM* rs3789453 and *ATP7B* rs1801243 appeared to have a protective function against excess weight in those exposed to copper and lead.

The GST gene family (*GSTP1, GSTM1,* and *GSTT1*) plays an important role in protecting cells against oxidative stress, and considering the obesity-associated oxidative damage, genetic defects in the GST antioxidant system could be involved in the pathogenesis of metabolic diseases (Chielle et al., 2017; Pietrocola and Bravo-San Pedro, 2021; Rahbar et al., 2020). In our first phenotype-genotype association study without including metal exposure, children carrying one or two copies of high-risk G allele at *GSTP1* rs1695 had increased odds of presenting overweight/obesity at borderline significance. Very limited evidence exists on the influence of *GSTP1* polymorphism on excess weight. In line with our results, a cross-sectional study conducted on individuals aged 18-30 years old demonstrated, for first time, that young adults with at least one G allele were more vulnerable to obesity (OR = 2.43, 95% CI: 1.18 – 5.01) (Chielle et al., 2017).

*GSTP1* rs1695 variant (A to G transition) is one of the most common missense variants located within exon 5 of *GSTP1* gene, and leads to amino acid substitution of isoleucine (Ile) with valine (Val) at position 105 (Ile105Val) (Gong et al., 2021). This replacement causes a significant loss of affinity of GST enzymes to conjugate GSH to xenobiotic compounds, including metal(loid)s (Rahbar et al., 2020). In this present gene-environment association study, consistent interactions between rs1695 A/G and the exposure to lead and chromium were detected, indicating for the first

time that genetic alterations coupled with metal exposure could have an effect on overweight/obesity.

A recent study conducted on lead- and cadmium-exposed children by Yohannes et al. (2022) found that *GSTP1* rs1695 was significantly related to increased susceptibility to lead toxicity. Nevertheless, there are no reports assessing the effects of interactions between this variant and metals on overweight/obesity.

γ-Glutamyl-cysteine ligase (GCL) with its catalytic (GCLC) and modifier (GCLM) subunits is another important enzyme within the GSH system. As mentioned above, GSTs are responsible for GSH conjugation, while GCL is involved in GSH synthesis (Barcelos et al., 2015; Chan et al., 2020). Therefore, genetic variations in the GCL complex might also have an impact on the body burden of metals. Particularly, *GCLC* rs761142, *GCLC* rs17883901 (C129T) and *GCLM* rs41303970 (C588T) have been shown to modulate concentrations of mercury and lead (Barcelos et al., 2015; Chan et al., 2020; Parajuli et al., 2016; Wahlberg et al., 2018). Surprisingly, we found suggestive associations for *GCLM* rs3789453 and excess weight that have not been previously reported on. In this case, the most significant results were obtained for low exposure to copper, where reduced odds of overweight/obesity were identified in children inheriting the AA variant genotype.

To support this finding, it is necessary to focus on the role of copper in the GSH pathway and obesity. Copper possesses a high affinity for thiol groups (-SH) contained in GSH, being GSH a chelator of copper (da Silva Fonseca et al., 2021; Lu et al., 2015). In fact, copper deficiency induced upregulation of GCL mRNA resulting in an increased biosynthesis of GSH in male rats (Uthus et al., 2007). On the other hand, a meta-analysis by Gu et al. (2020) indicated that an excess of copper was prevalent among obese children and adults. So, considering that increased copper exposure contributes to obesity through oxidative stress disorder and antioxidant imbalance (Gu et al., 2020), we propose that *GCLM* rs3789453 could promote the protective role of GSH against an oxidative stress situation in the presence of lower copper concentrations.

Regarding common genetic variants in *ATP7B* affecting metal transport, only rs1801243 A/C showed a marginal inverse association with overweight/obesity. The following studies deal with *ATP7B* genetic variants in copper metabolism disorders, such as Alzheimer's and Wilson's diseases (Kumari et al., 2018; Squitti et al., 2017; Wang et al., 2021), but no studies exist addressing their roles in childhood obesity.

ATP7B encodes copper-transporting proteins that are in charge of maintaining cell copper homeostasis (Hilário-Souza et al., 2016). Genetic dysfunction of *ATP7B* results in intracellular copper accumulation, and consequently, the development of copper disorders (McCann et al., 2019; Muchenditsi et al., 2017). In our study, we obtained that subjects carrying rs1801243 AC genotype had decreased odds of overweight/obesity at borderline significance for higher concentrations of copper. For low exposure to lead, associations with decreased odds and interactions were near significance. This finding was in accordance with the aforementioned association between this SNP and overweight/obesity, suggesting that the *ATP7B* rs1801243 variant exerts a protective role against excess body weight when exposure to metals occurs.

More importantly, we identified statistically significant interactions between the *ATP7B* rs1061472 A/G variant and high exposure to chromium, molybdenum and lead, showing a marginal association with elevated overweight/obesity odds. Until now, ATP7B has been typically associated with copper export and, whether or not it could serve as a transporter of other metals in humans, requires further investigation (Harder et al., 2022). Parajuli et al. (2016) reported for first time a significant association of rs732774 and rs1061472 with lower hair and blood mercury levels, indicating that the ATP7B protein might have an affinity for other metals.

As far as we are concerned, this study is the first of its kind to investigate whether the associations between gene polymorphisms related to the detoxification system of metal(loid)s and overweight/obesity depend on urinary metal(loid) concentrations in Spanish children. Our findings suggest that genetic variants of the GSH system (*GSTP1* rs1695 and *GCLM* rs3789453) and metal transport (*ATP7B*

rs1061472 and rs1801243) are responsible for the interindividual susceptibility to the adverse effects of metal(loid)s on body weight regulation. Moreover, we found some evidence of the role of *GSTP1* rs1695 as a genetic predisposing factor of excess weight; while *ATP7B* rs1801243 appeared to display a protective role against overweight/obesity that has never been previously reported on.

In this regard, our results have implications for public health. We provide evidence of the importance of exploring genetic variations in the presence of metal(loid) exposure on excess body weight in Spanish children. The urinary metal(loid) levels of cadmium, mercury, lead, chromium, manganese and nickel found in our study population were in line with those obtained in other studies of Spanish male adolescents aged 15-17 years old, by the INMA-Granada cohort (Casteillo et al., 2020; Rodríguez-Carrillo et al., 2022). In Andalusian children and adolescents aged 5-17 years old, the levels of arsenic were much lower than in our population (Aguilera et al, 2010). With respect to studies carried out in other countries, the same trends were observed; our study sample shows similar urinary levels of the majority of metal(loid)s, except for arsenic and nickel whose concentrations were lower and higher than ours, respectively, in children aged 6-19 years old participating in the NHANES cohort (Shan, 2022) and Mexicans aged 8–14 years old (Lewis et al., 2018). After seeing these results, replication of our findings is warranted in future analyses with larger and different populations in order to provide insightful clues about the reduction of childhood excess weight in Spain and around the world.

We used urine sampling as a biomarker of exposure to metal(loid)s. Urine is a non-invasive biological sample and is especially useful among children due to its simple and rapid collection. This matrix is one of the biomarkers used to estimate the internal dose of chemicals through human biomonitoring (Astolfi et al., 2020).

One limitation of our study was the sample size. Our results may have been compromised by the small size and lack of sufficient statistical power to detect significant associations. Nevertheless, we have been able to detect trends that can be reproduced in large populations. An additional limitation pertains to the fact that we

assessed exposure to metal(loid)s through a single urine sample which, due to the short half-life of certain metals, is not representative of long-term exposure. Furthermore, metal(loid) speciation analysis was not performed, which would have allowed us to know the predominant chemical form more related to the outcome of interest. Finally, analyses were carried out taking into account exposure to individual metal(loid)s, but it should be taken into consideration that humans are often exposed to a mixture of them, as well as co-exposed to other pollutants.

In conclusion, the findings of the present case-control study evidenced that interaction effects could exist between genetic variants within GSH and metal transporting systems and exposure to metal(loid)s on excess body weight among Spanish children. The exploration of the genetic background of detoxifying pathways is a key target to study how genetic variation impacts metal body burden and body sensitivity. For this reason, and in view of the lack of studies assessing gene-environment interactions on the risk of obesity, we support considering genetic and environmental factors as a causal crosstalk rather than individual contributors to the risk of developing obesity.

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**CRediT authorship contribution statement**

**Viviana Ramírez:** Data curation, Investigation, Methodology, Visualization, Writing-original draft, and Writing-reviewing and editing. **Inmaculada Salcedo-Bellido:** Data curation, Formal analysis, Supervision, Writing-original draft, and Writing-reviewing and editing. **Lourdes Rodrigo:** Supervision, Writing-reviewing and editing. **Fernando Gil Hernández**: Methodology. **Pablo Olmedo Palma:** Methodology. **Luis Javier Martínez-González:** Conceptualization and

Supervision. **María Jesús Álvarez-Cubero**: Conceptualization, Supervision, Writing-reviewing and editing. **Ana Rivas**: Conceptualization, Funding acquisition, Project administration, Supervision, Writing-reviewing and editing.

**Data availability.** The data that has been used is confidential.

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**Appendix A.** Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.162333.

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# Exploring the role of genetic variability and exposure to bisphenols and parabens on excess body weight in Spanish children

**Viviana Ramírez[a,b,c,d,1], Yolanda Gálvez-Ontiveros[a,c,d], Vega Almazán Fernández de Bobadilla[e], Patricia González-Palacios[a,d], Inmaculada Salcedo-Bellido[c,f,g], Cristina Samaniego-Sánchez[a,c,d], María Jesús Álvarez-Cubero[b,c,h], Luis Javier Martínez-González[b,h,*], Alberto Zafra-Gómez[c,d,i,1], Ana Rivas[a,c,d,1]**

[a]Department of Nutrition and Food Science, Faculty of Pharmacy, University of Granada, Granada, Spain

[b]GENYO. Centre for Genomics and Oncological Research: Pfizer / University of Granada / Andalusian Regional Government PTS Granada - Avenida de la Ilustración, 114, 18016 Granada, Spain

[c]Instituto de Investigación Biosanitaria ibs.GRANADA, Granada, Spain

[d]Institute of Nutrition and Food Technology "Jose Mataix Verdú," Biomedical Research Center, University of Granada, Granada, Spain

[e]Health Center of Maracena, Granada, Spain.

[f]Department of Preventive Medicine and Public Health, University of Granada, Granada, Spain.

[g]Consortium for Biomedical Research in Epidemiology & Public Health (CIBER en Epidemiología y Salud

Pública-CIBERESP), Madrid, Spain.

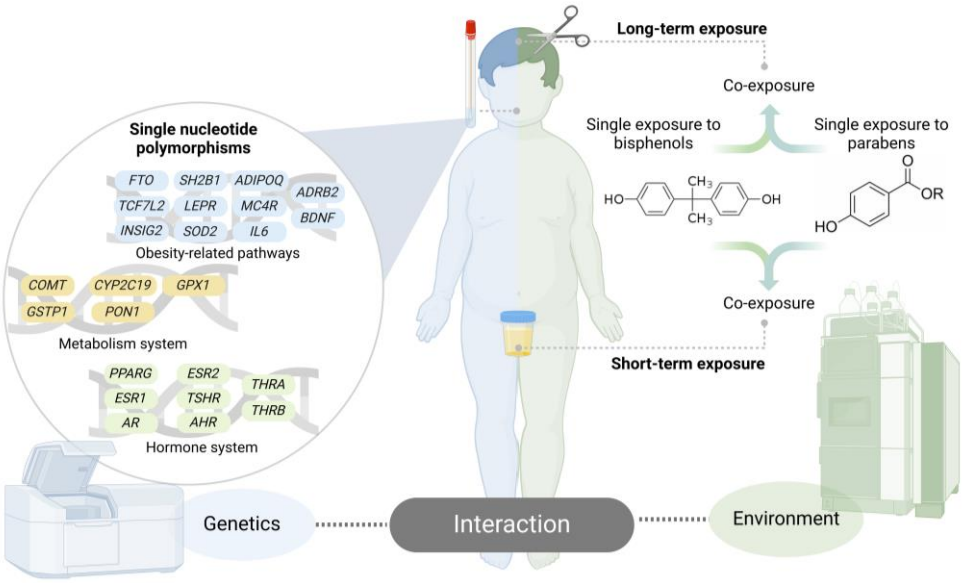
[h]Department of Biochemistry and Molecular Biology III and Immunology, Faculty of Medicine, University of Granada, Granada, Spain.

[i]Department of Analytical Chemistry, University of Granada, Granada, Spain.

[1] These authors contributed equally to the work.

*Corresponding author. Email: luisjavier.martinez@genyo.es (L.J. Martínez-González).

## Graphical abstract





## Abstract

Gene-environment interaction studies are emerging as a promising tool to shed light on the reasons for the rapid increase in excess body weight (overweight and obesity). We aimed to investigate the influence of several polymorphisms on excess weight in Spanish children according to a short- and long-term exposure to bisphenols and parabens, combining individual approach with the joint effect of them. This case-control study included 144 controls and 98 cases children aged 3-12 years. Thirty SNPs in genes involved in obesity-related pathways, xenobiotic metabolism and hormone systems were genotyped using the GSA microchip technology and qPCRs with Taqman® probes. Levels of bisphenols and parabens in urine and hair were used to assess short- and long-term exposure, respectively, via UHPLC-MS/MS system. *LEPR* rs9436303 was identified as a relevant risk variant for excess weight ($OR_{Dom:AAvsAG+GG}$=2.65, $p$<0.001), and this effect persisted across exposure-stratified models. For long-term exposure, *GPX1* rs1050450 was associated with increased excess weight at low single paraben exposure ($OR_{GvsA}$=2.00, $p$=0.028, $p$-interaction=0.016), whereas *LEPR* rs1137101 exhibited a protective function at high co-exposure ($OR_{Dom:AAvsAG+GG}$=0.17, $p$=0.007, $p$-interaction=0.043). *ESR2* rs3020450 ($OR_{Dom:GGvsAG+AA}$=5.17, $p$=0.020, $p$-

interaction=0.028) and *CYP2C19* rs4244285 ($OR_{Dom:GGvsAG+AA}$=3.54, *p*=0.039, *p*-interaction=0.285) were identified as predisposing variants at low and high co-exposure, respectively. In short-term exposure, higher odds were observed for *INSIG2* rs7566605 at high bisphenol exposure ($OR_{CvsG}$=2.97, *p*=0.035, *p*-interaction=0.017) and for *GSTP1* rs1695 at low levels ($OR_{Dom:AAvsAG+GG}$=5.38, *p*=0.016, *p*-interaction=0.016). At low and medium co-exposure, *SH2B1* rs7498665 ($OR_{AvsG}$=0.17, *p*=0.015, *p*-interaction=0.085) and *MC4R* rs17782313 ($OR_{AvsG}$=0.10, *p*=0.023, *p*-interaction=0.045) displayed a protective effect, whereas *ESR2* rs3020450 maintained its contributing role ($OR_{GvsA}$=3.12, *p*=0.030, *p*-interaction=0.010). Our findings demonstrate for the first time that understanding the genetic variation in excess weight and how the level of exposure to bisphenols and parabens might interact with it, is crucial for a more in-depth comprehension of the complex polygenic and multifactorial aetiology of overweight and obesity.

**Highlights**

- *LEPR* rs9436303 strongly contributed to excess weight.
- Several genetic variants were related to excess weight at short- and long-term exposure.
- SNP effect depended on exposure level, leading to significant interactions.
- More complete answer to the multifactorial and polygenetic aetiology of obesity.

**Keywords**: overweight; obesity; children; gene polymorphisms; bisphenol; paraben

## 1. Introduction

Childhood excess body weight (overweight and obesity) constitute one of the most serious health issues facing the developed world due to its epidemic growth since 1975 (Wickramasinghe et al., 2021). Data from the World Health Organization (WHO) in 2022 reveal that over 390 million children and adolescents aged between 5 and 19 years were affected by overweight, including obesity, and about 37 million children under the age of 5 were overweight (WHO, 2024). European countries have reported the highest prevalence rates of overweight and obesity, although they have

recently plateaued or have started to decrease in some countries, including Spain (Buoncristiano et al., 2021; López-Sobaler et al., 2019; Spinelli et al., 2021). According to the ALADINO studies conducted between 2011 and 2019 on Spanish schoolchildren aged 6 to 9 years, the prevalence of overweight and obesity has significantly decreased over the four-year period. However, it still remains high, with overweight and obesity rates of 23.3% and 17.3% respectively in 2019, compared to 26.2% and 18.3% in 2011 (AESAN, 2020).

Children suffering from obesity are likely to remain obese into adulthood and are more vulnerable to non-communicable diseases (Wickramasinghe et al., 2021). Principally, unhealthy eating habits and lack of physical activity are the major risk factors, but they are not enough to explain the unstoppable growth of obesity cases. Crucially, obesity possesses a strong heritable component in which multiple candidate genes are implicated, mainly those regulating feeding behaviour, energy balance and body mass (fat mass and obesity-associated gene (*FTO*), leptin (*LEP*), leptin receptor (*LEPR*), melanocortin 4 receptor (*MC4R*), Src-homology-2 domain-containing putative adapter (*SH2B1*), and brain-derived neurotrophic factor (*BDNF*), among others) (Martins et al., 2018; Littleton et al., 2020). As consequence, alterations to expression of these genes result in interindividual variation in body mass index (BMI) leading to obese phenotype. In these terms, Genome Wide Association Studies (GWAS) have uncovered hundreds of single nucleotide polymorphisms (SNPs) as the most common inherited genetic variations associated with BMI heritability, although they account for around 6% of BMI variability (Bradfield et al., 2019; Loos and Yeo, 2021; Seral-Cortes et al., 2022).

*FTO* rs9939609 (Danaher et al., 2019), *LEPR* rs1137101 (Raskiliene et al., 2021), *SH2B1* rs7498665 (Aerts et al., 2015), *MC4R* rs17782313 (Resende et al., 2021) and *BDNF* rs1695 (Mitchell et al., 2013) have been identified as common predisposing genetic risk factors for childhood obesity. Insulin-induced gene 2 (*INSIG2*), which regulates adipogenesis and lipid synthesis, is another strong candidate gene for obesity, and the rs7566605 has demonstrated to contribute to its development (Vourdoumpa et al., 2023).

Importantly, despite the majority of BMI loci have been identified in adult population, current evidence supports that children and adults share a similar genetic profile (Bradfield et al., 2019; Littleton et al., 2020; Seral-Cortes et al., 2022).

On the other hand, considering that an obesogenic environment might modulate genetic contribution to obesity risk, gene-environment interaction studies are emerging as a promising tool to shed light on the reasons for the rapid increase in obesity, mainly in paediatric population (Goodarzi, 2017; Loos and Yeo, 2021). Endocrine disrupting chemicals (EDCs) are environmental pollutants with well-established hormonal activity (Nadal et al., 2017). Additionally, they are called obesogenic chemicals due to their ability to induce metabolic disruptions through activation of nuclear transcription factors (such as peroxisome proliferator-activated receptor gamma (PPARγ)), and other nuclear hormone receptors (like estrogen (ERs), androgen (ARs), progesterone (PGR), and thyroid receptors (TRs)) (Egusquiza and Blumberg, 2020; Mohajer et al., 2021). These EDC-receptor interactions cause adipose tissue disfunction, lipid storage, and energy imbalance, thereby influencing body composition and conferring susceptibility to weight gain (Andújar et al., 2019; Mohajer et al., 2021).

Childhood exposure to EDCs occurs principally through breastfeeding and diet, followed by contaminated air and dermal absorption (Ramírez et al., 2022a). Parabens and bisphenols are well-known EDCs industrially used as food additives and plasticizers in food containers, respectively (Naomi et al., 2022; Wei et al., 2021). While a large number of epidemiological studies have reported relevant associations between urinary bisphenol levels and increasing risk of obesity in children (Bhandari et al., 2013; Mustieles et al., 2019; Liu, B. et al., 2019; Li et al., 2017; Vafeiadi et al., 2016), the scientific evidence on parabens is quite limited (Berger et al., 2021, Leppert et al., 2020; Moscoso-Ruiz et al., 2022). In human biomonitoring, urine and hair has been proven to be suitable matrices to assess recent and long-term exposure to EDCs, drugs, and pharmaceuticals, respectively (Katsikantami et al., 2019).

Concerning gene-environment interactions in obesity, only two prospective studies examined and demonstrated that children of mothers carrying *PON1* genetic variants and exposed to pesticides were more likely to have increased fat mass and BMI (Etzel et al., 2020; Tinggaard et al., 2016). *PON1* gene codes for paraoxonase-1, a phase II biotransformation enzyme involved in detoxification of xenobiotics (Etzel et al., 2020). Other phase II detoxifying enzymes include glutathione S-transferases (e.g. GSTP1), glutathione peroxidases (e.g. GPX1) and catechol O-methyltransferase (COMT) (Rahbar et al., 2020). Whereas microsomal cytochrome P450 (CYP450) enzymes are responsible for phase I xenobiotic metabolism (Martínez-González et al., 2020). Hence, genetic variants in these enzymatic systems could interfere with the proper metabolism and elimination of exogenous compounds. In fact, one study suggested that *GSTP1* rs1695 may be involved in detoxification of BPA metabolites (Lin et al., 2018).

Nonetheless, to our knowledge no human study has investigated the interaction between exposure to bisphenols and/or parabens and genetic alterations in association with childhood excess body weight. Our objective was therefore to examine whether polymorphisms of obesity-related genes, and those coding for metabolising enzymes and hormone receptors are predisposing factors for overweight and obesity in a sample of Spanish children according to a short- and long-term exposure to total bisphenols and parabens, combining individual approach with the joint effect of them.

## 2. Material and Methods

### *2.1. Study design, setting and participants*

Participants enrolled in this case-control study were recruited between 2020 and 2023 from different elementary schools and primary care centres in Granada, Spain. Eligible cases had to meet the following inclusion criteria: (1) diagnosis of overweight or obesity, (2) children aged between 3 and 12 years, and (3) residence at least 6 months continuously in the study area. Children suffering from obesity secondarily to other pathology or pharmacological treatment were excluded from the study. The same inclusion criteria were applied to the control group, except for the

diagnosis of overweight and obesity. The study aims and procedures were fully explained to all parents or legal tutors of the children before they signed the written informed consent. The study protocol was approved by the Biomedical Research Ethics Committees of the Province of Granada (references: 0922-N-19; 1939-M1-22; 1742-N-23).

A total of 144 controls and 98 cases having available measured levels of bisphenols and parabens in urine or hair, and genomic DNA adequate concentration were included in the present research. We attempted to address the sources of bias due to unavailability of biological samples by comparing baseline characteristics between subjects with and without these data (**Table S1**). Non-significant differences were found.

### *2.2. Data collection and variables*

Face-to-face interviews were conducted to all participants' parents or guardians by trained interviewers. In this way, information on sociodemographic aspects, lifestyles, dietary patterns, and anthropometric data by qualified personnel (weight and height) was collected. For this study, gender and age were selected for covariate adjustment in regression models.

Body weight (kg) and height (cm) were determined with children barefooted and in their underwear using a portable Tanita floor scale (model MC 780-S MA) and a stadiometer (model SECA 214 (20-207 cm)), respectively. BMI was calculated as the weight (kg) divided by height ($m^2$). Children were classified as underweight (BMI<18.5 kg/$m^2$), normal weight (18.5≥BMI<25 kg/$m^2$), overweight (BMI≥25 kg/$m^2$) or obese (BMI≥30 kg/$m^2$) following the sex- and age-specific cut-off points described by Cole et al. (2000, 2007). These BMI cut-off values cover the age range from 2 to 18 years old at 6-month intervals.

### *2.3. DNA isolation and genotyping assays*

Two buccal swab samples were taken from each participant and preserved at -20 ºC until DNA extraction. Genomic DNA extraction was based on proteinase K and salt/ethanol purification (Ramírez et al., 2022b). DNA concentration was

quantified with the Qubit™ 4.0 fluorometer using the Qubit dsDNA BR Assay Kit (Invitrogen™). DNA samples were stored at – 20ºC until the genotyping step.

Firstly, more than 100 genetic variants reported in the 1000 Genome Project were considered, and then, 30 of them with a minor allele frequency (MAF) higher than 10% were selected from Ensembl (https://www.ensembl.org/index.html) and The National Centre for Biotechnology Information SNP website (https://www.ncbi.nlm.nih.gov/) as possible genetic biomarkers. Gene panel consisted of: 13 polymorphisms of 11 candidates genes involved in signalling **pathways related to obesity** (*FTO* rs9939609 and rs8050136; *TCF7L2* rs7903146, *INSIG2* rs7566605, *SH2B1* rs7498665, *LEPR* rs1137101 and rs9436303; *SOD2* rs4880, *ADIPOQ* rs1501299, *MC4R* rs17782313, *IL6* rs1800795, *ADRB2* rs1042714, and *BDNF* rs6265), 6 genetic variants of 5 **metabolising enzyme encoding genes** (*COMT* rs4680, *GSTP1* rs1695, *CYP2C19* rs4244285, *PON1* rs662 and rs854560; and *GPX1* rs1050450) and 11 SNPs within genes encoding **hormone receptors and nuclear transcription factors** (*PPARG* rs1801282 and rs3856806; *ESR1* rs2234693 and rs9340799; *AR* rs6152, *ESR2* rs3020450, *TSHR* rs179247, *AHR* rs4410790 and rs6968865; *THRA* rs939348, and *THRB* rs3752874). Information regarding the gene, chromosome location, and allele frequencies of the selected SNPs are shown in Supplementary **Table 2**.

For SNP genotyping assays, 24 SNPs were genotyped using the llumina® Infinium® Global Screening Array (GSA)-24 BeadChips according to manufacturer's recommendations as described by Ramírez et al. (2023). DNA samples were scanned on the iScan platform and GSA data were analysed with the software llumina® GenomeStudio V2010.3.

In Taqman assays, 6 SNPs were genotyped using the following commercially available Taqman® probes (Applied Biosystems™ Taqman SNP Genotyping Assays): C_29715216_10 for *LEPR* rs9436303, C_175686987_10 for *GPX1* rs1050450, C_2259750_20 for *PON1* rs854560, C_11608716_10 for *AR* rs6152, C_26928532_10 for *TSHR* rs179247, and C_27495838_10 for *THRB* rs3752874. Quantitative PCRs (qPCRs) for SNP Genotyping were conducted in the

QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems™) and data output were processed and analysed with the software QuantStudio™ Real-Time PCR v1.3.1 (Ramírez et al., 2022b).

Those SNPs presenting a call rate less than 95% and deviated from Hardy-Weinberg equilibrium (HWE, $p < 0.05$) were excluded from the final analysis. Samples with overall call rates less than 95% were also excluded.

### *2.4. Sample collection and determination of bisphenols and parabens*

For this study, a total of 12 bisphenols (BPA, BPS, BPE, BPB, BPF, BPAF, BPC, BPZ, BPAP, BPM, BPP and BPFL) and 6 parabens (MetPB, EthPB, PropPB, iPropPB, ButPB and iButPB) were measured in hair and urine samples. Validation parameters, LOD, LOQ, recovery, calibration ranges, etc. can be checked in previous studies published by our research group (Moscoso-Ruiz et al., 2022; Rodriguez-Gomez et al., 2017).

**Urine treatment**. A spot urine sample from each participant's first morning void was collected in a sterile polyethylene container and stored at -80ºC until analysis (n=122). Parabens and bisphenols were extracted according to the methodology previously developed by Moscoso-Ruiz et al. (2022). Briefly, after the dispersive liquid-liquid microextraction, samples were analysed in the ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) system.

The determination of creatinine levels in urine was performed by the Ángel Méndez Soto Clinical Analysis Laboratory (Granada, Spain). Urine concentrations of bisphenols and parabens were standardised by creatinine content (ng/g creatinine).

**Hair treatment**. Hair samples (3-5 cm) were obtained by cutting from the posterior region of the vertex, as close as possible to the scalp (n=232). They were stored in aluminium foil at room temperature until processing and analysis (Rodriguez-Gomez et al., 2017). After successive steps of washing to remove contaminants and surface residues, samples were lyophilised and pulverised. Then, aliquots of 0.05 g of hair powder were digested with 0.5 ml of acetic acid/methanol

mixture at 38 ºC for 12 h. Extraction of analytes was performed by adding 1 ml of acetonitrile. After 15 min of centrifugation, the organic phase was evaporated until dryness, reconstituted in 250 µl water/methanol, centrifuged, and analysed by UHPLC-MS/MS system.

### *2.5. Statistical analysis*

Descriptive analysis was performed using mean, standard deviation (SD), median, 25$^{th}$ and 75$^{th}$ percentiles (interquartile range) for quantitative variables, and frequency and percentages for categorical variables. Student's *t*-test, U Mann-Whitney test and chi-square test were used to examine the differences between controls and cases for parametric, non-parametric, and qualitative variables, respectively. Kolmogorov-Smirnov test with Lilliefors correction was applied to check the normality of continuous data.

Additionally, chi-square tests were also applied to verify the Hardy-Weinberg equilibrium (HWE, $p > 0.05$). Linkage disequilibrium (LD) analyses were conducted using the SNPStats software (https://snpstats.net/start.htm). SNPs in the same gene with a $r^2$ parameter greater than 0.5 were considered to be in LD. The analyses were undertaken under the dominant (Dom) or recessive model (Rec), also the allelic contribution was considered. An *in silico* analysis was performed using the Ensembl VEP (Variant Effect Predictor) tool to predict the functional impact and malignancy of the genetic variants (https://www.ensembl.org/info/docs/tools/vep/index.html).

Firstly, multivariable logistic regression models were used to estimate odds ratio (OR) and 95% confidence intervals (95% CI) to evaluate the association of each genetic variant with overweight and obesity. BMI dichotomised as normal weight and overweight/obesity was the dependent variable. Models were adjusted for the covariates of gender and age according to the three statistical criteria described by Jager et al. (2008), changes in OR of more than 10%, and information from previous studies (Chen et al., 2022; Marcos-Pasero et al., 2020; Olza et al., 2017). The bisphenol and paraben levels were categorised based on the median built from the cut-off point according to the distribution of the control group, using the low concentration (≤ median values) as the reference group. To explore gene-

environment interactions in excess weight, the product term "polymorphism x bisphenol and paraben levels" was entered in each model. When assessing the co-exposure to both contaminants, three categories were obtained based on the level of exposure: low exposure to both bisphenols and parabens, medium exposure (high exposure to at least one of them) and high exposure to both. Hence, two *p* values for interaction were obtained. Statistical significance was set at $p$ value $\leq 0.05$. In addition, a Bonferroni-corrected *p* value was applied to the multifactorial logistic regressions to account for the multiple SNP testing ($p \leq 0.002$). All statistical analyses were performed with IBM SPSS Statistics 25 (Armonk, NY, USA).

## 3. Results

### *3.1. General characteristics of participants*

**Table 1** displays the study population's baseline characteristics. The mean age was significantly higher in the case group than in the control group (8.9 (2.6) vs 7.3 (2.5), $p < 0.001$). The percentage of boys and girls was the same in both study groups (50% boys and 50% girls). No significant differences were observed in creatinine levels.

**Table 1**: General characteristics of the study population.

| | **Controls (N=144)** | **Cases (N=98)** | ***p*** |
|---|---|---|---|
| Age (years), mean (SD) | 7.3 (2.5) | 8.9 (2.6) | **<0.001**[a] |
| Gender, n (%) | | | |
| Boys | 72 (50.0) | 49 (50.0) | 1.000[b] |
| Girls | 72 (50.0) | 49 (50.0) | |
| Weight (kg), median (IQR) | 24.10 (19.47-30.70) | 48.55 (37.37-60.25) | **<0.001**[a] |
| Height (cm), mean (SD) | 124.4 (18.5) | 139.6 (16.6) | **<0.001**[c] |
| BMI (kg/m$^2$), median (IQR) | 15.79 (14.71-17.00) | 23.83 (22.07-26.82) | **<0.001**[a] |
| Creatinine (g/L), median (IQR) | 0.86 (0.58-1.18) | 1.11 (0.57-1.35) | 0.111[a] |

[a]U Mann-Whitney test; [b]Chi-square test; [c]Student's *t*-test. The bold indicates significant *p* values < 0.05.

**Table 2** shows the concentrations of bisphenols and parabens determined in hair and urine and the distribution of exposure frequencies divided into low and high exposure for single exposure to bisphenol and paraben levels; and low, medium and high exposure for co-exposure. No significant differences existed between controls

and cases. Detection rate and concentration of the specific bisphenols and parabens are listed in Supplementary **Tables 3 and 4**.

**Table 2**: Concentration of bisphenols and parabens in hair (ng/g) and urine (ng/g creatinine) and distribution of exposure frequencies for controls and cases.

| | **Hair bisphenols and parabens** | | | **Urine bisphenols and parabens** | | |
|---|---|---|---|---|---|---|
| | Controls (N=138) | Cases (N=94) | *p* | Controls (N=79) | Cases (N=43) | *p* |
| **Bisphenols, median (IQR)** | 410.01 (238.86-892.37) | 355.08 (197.73-1051.37) | 0.725[a] | 2138.58 (1398.14-4318.95) | 2033.09 (1364.41-3417.68) | 0.374[a] |
| **Total bisphenols, n (%)** | | | | | | |
| Low exposure (<= median) | 69 (50.0) | 53 (56.4) | 0.339[b] | 40 (50.6) | 22 (51.2) | 0.995[b] |
| High exposure (> median) | 69 (50.0) | 41 (43.6) | | 39 (49.4) | 21 (48.8) | |
| **Parabens, median (IQR)** | 1865.90 (1044.83-4157.42) | 2295.46 (1008.82-6255.11) | 0.364[a] | 4529.47 (2673.89-15671.42) | 7070.72 (2872.92-22198.30) | 0.233[a] |
| **Total parabens, n (%)** | | | | | | |
| Low exposure (<= median) | 69 (50.0) | 42 (44.7) | 0.426[b] | 40 (50.6) | 18 (41.9) | 0.354[b] |
| High exposure (> median) | 69 (50.0) | 52 (55.3) | | 39 (49.4) | 25 (58.1) | |
| **Total exposure, n (%)** | | | | | | |
| Low exposure to both EDCs | 37 (26.8) | 26 (27.7) | 0.990[b] | 24 (30.4) | 13 (30.2) | 0.595[b] |
| Medium exposure (high exposure to at least one EDC) | 64 (46.4) | 43 (45.7) | | 32 (40.5) | 14 (32.6) | |
| High exposure to both EDCs | 37 (26.8) | 25 (26.6) | | 23 (29.1) | 16 (37.2) | |

[a]U Mann-Whitney test; [b] Chi-square test.

### *3.2. Genetic association analysis*

All SNPs were in HWE ($p > 0.05$, **Table S2**), except for *AR* rs6152, which was excluded from the analysis. Likewise, *PPARG* rs1801282 C/G and rs3856806 G/A variants were discarded from the statistical analysis because their MAF was below 10%. Thus, 27 SNPs were included for the statistical analysis, leading to a rigorous significance level ($p \leq 0.002$). A strong linkage was observed between *FTO* rs9939609 and rs8050136 ($r^2 = 0.99$), followed by *AHR* rs4410790 and rs6968865 ($r^2 = 0.98$), and *ESR1* rs2234693 and rs9340799 ($r^2 = 0.70$). From the battery of selected SNPs, 9 genetic variants showed significant results along the association analyses ($p \leq 0.05$), and one SNP (*LEPR* rs9436303 A/G) remained statistically

significant after Bonferroni correction (**Table 3** and **Figures 1** and **2**). The full results are available in the supplementary material (**Tables S5-S12**).

**Table 3** details the distribution of genotype and allele frequencies among controls and cases, and the association of each genetic variant with overweight and obesity. The frequencies were statistically different for *LEPR* rs9436303 carriers ($p < 0.001$). Here, most of the children with AG or GG genotypes were classified within the case group (57.1%), whereas 66% of the children carrying the wild-type AA genotype belonged to the control group. Likewise, carrying one or two copies of the rs9436303 minor G allele significantly contributed to an increased likelihood of developing overweight and obesity (OR = 2.65, $p < 0.001$). For *GSTP1* rs1695 A/G, the allele frequencies were distributed differently between controls and cases ($p = 0.049$), but the association did not reach significance. No significant differences were found between the associations of genotype and allele frequencies of the other SNPs with susceptibility to overweight and obesity.

**Table 3**: Distribution of genotypes and alleles between controls and cases and associations of each genetic variant with overweight and obesity.

| SNP | Model | Genotype/ allele | Controls | Cases | *p*[a] | ORa | 95% CI | *p* |
|---|---|---|---|---|---|---|---|---|
| | | | N (%) | N (%) | | | | |
| *INSIG2* rs7566605 | Rec | CC + CG | 75 (52.1) | 50 (51.0) | 0.871 | 1.00 | | |
| | | GG | 69 (47.9) | 48 (49.0) | | 1.07 | 0.62-1.84 | 0.810 |
| | | C | 95 (33.0) | 61 (31.1) | 0.667 | 1.00 | | |
| | | G | 193 (67.0) | 135 (68.9) | | 1.09 | 0.73-1.65 | 0.674 |
| *SH2B1* rs7498665 | Dom | AA | 59 (41.8) | 39 (40.6) | 0.852 | 1.00 | | |
| | | AG + GG | 82 (58.2) | 57 (59.4) | | 1.04 | 0.60-1.81 | 0.891 |
| | | A | 183 (64.9) | 123 (64.1) | 0.853 | 1.00 | | |
| | | G | 99 (35.1) | 69 (35.9) | | 1.04 | 0.69-1.55 | 0.865 |
| *LEPR* rs1137101 | Dom | AA | 44 (30.8) | 41 (42.3) | 0.068 | 1.00 | | |
| | | AG + GG | 99 (69.2) | 56 (57.7) | | 0.59 | 0.34-1.04 | 0.070 |
| | | A | 157 (54.9) | 122 (62.9) | 0.082 | 1.00 | | |
| | | G | 129 (45.1) | 72 (37.1) | | 0.73 | 0.50-1.09 | 0.121 |
| *LEPR* rs9436303 | Dom | AA | 95 (66.0) | 42 (42.9) | **<0.001** | 1.00 | | |
| | | AG + GG | 49 (34.0) | 56 (57.1) | | 2.65 | 1.51-4.65 | **<0.001**** |
| | | A | 229 (79.5) | 130 (66.3) | **0.001** | 1.00 | | |
| | | G | 59 (20.5) | 66 (33.7) | | 1.95 | 1.26-3.01 | **0.003*** |
| *MC4R* rs17782313 | Dom | AA | 89 (61.8) | 64 (65.3) | 0.579 | 1.00 | | |
| | | AG + GG | 55 (38.2) | 34 (34.7) | | 0.81 | 0.46-1.42 | 0.452 |
| | | A | 226 (78.5) | 160 (81.6) | 0.396 | 1.00 | | |
| | | G | 62 (21.5) | 36 (18.4) | | 0.78 | 0.48-1.26 | 0.308 |

**Table 3** (*continued*)

| SNP | Model | Genotype/ allele | Controls | Cases | $p^a$ | ORa | 95% CI | *p* |
|---|---|---|---|---|---|---|---|---|
| | | | N (%) | N (%) | | | | |
| *GSTP1* rs1695 | Dom | AA | 69 (47.9) | 38 (38.8) | 0.160 | 1.00 | | |
| | | AG + GG | 75 (52.1) | 60 (61.2) | | 1.36 | 0.79-2.36 | 0.269 |
| | | A | 197 (68.4) | 117 (59.7) | **0.049** | 1.00 | | |
| | | G | 91 (31.6) | 79 (40.3) | | 1.36 | 0.92-2.03 | 0.128 |
| *CYP2C19* rs4244285 | Dom | GG | 104 (72.7) | 62 (63.9) | 0.147 | 1.00 | | |
| | | AG + AA | 39 (27.3) | 35 (36.1) | | 1.36 | 0.75-2.43 | 0.310 |
| | | G | 239 (83.6) | 155 (79.9) | 0.304 | 1.00 | | |
| | | A | 47 (16.4) | 39 (20.1) | | 1.20 | 0.73-1.97 | 0.481 |
| *GPX1* rs1050450 | Dom | GG | 72 (51.1) | 47 (48.0) | 0.637 | 1.00 | | |
| | | AG + AA | 69 (48.9) | 51 (52.0) | | 1.01 | 0.58-1.74 | 0.982 |
| | | G | 203 (72.0) | 133 (67.9) | 0.331 | 1.00 | | |
| | | A | 79 (28.0) | 63 (32.1) | | 1.12 | 0.74-1.70 | 0.600 |
| *ESR2* rs3020450 | Dom | GG | 53 (36.8) | 31 (31.6) | 0.407 | 1.00 | | |
| | | AG + AA | 91 (63.2) | 67 (68.4) | | 1.28 | 0.72-2.27 | 0.396 |
| | | G | 170 (59.0) | 114 (58.2) | 0.850 | 1.00 | | |
| | | A | 118 (41.0) | 82 (41.8) | | 1.04 | 0.70-1.53 | 0.861 |

[a]Chi-square test. Dom: dominant model; Rec: recessive model; ORa: Odds ratio adjusted for age and gender.
* Significant *p* values ≤ 0.05. ** Significant *p* values after Bonferroni correction ($p \leq 0.002$).

### *3.3. Influence of genetic variants on overweight and obesity according to short- and long-term exposure to bisphenols and parabens*

Herein, the contribution of each genetic variant to overweight and obesity occurrence was assessed by stratifying the study population into low and high exposure to total bisphenols and parabens individually (Figure 1A-B, Figure 2A-B, Tables S7, S8, S10, and S11). Then, associations were evaluated according to combined exposure to both contaminants (Figure 1C, Figure 2C, Tables S9 and S12). Firstly, the distribution of genotypes and allele frequencies according to low, medium, and high exposure was investigated, but non-significant differences were found for the 9 SNPs selected from the association analyses (**Table 4**). The results for the remaining SNPs are shown in Supplementary **Table 6**.

When the exposure factor was entered into logistic regression models, several genetic polymorphisms gained importance, which was not observed in the previous genetic association analysis. Consequently, SNP-exposure interaction was explored to verify if the variant effect depended on the level of exposure.

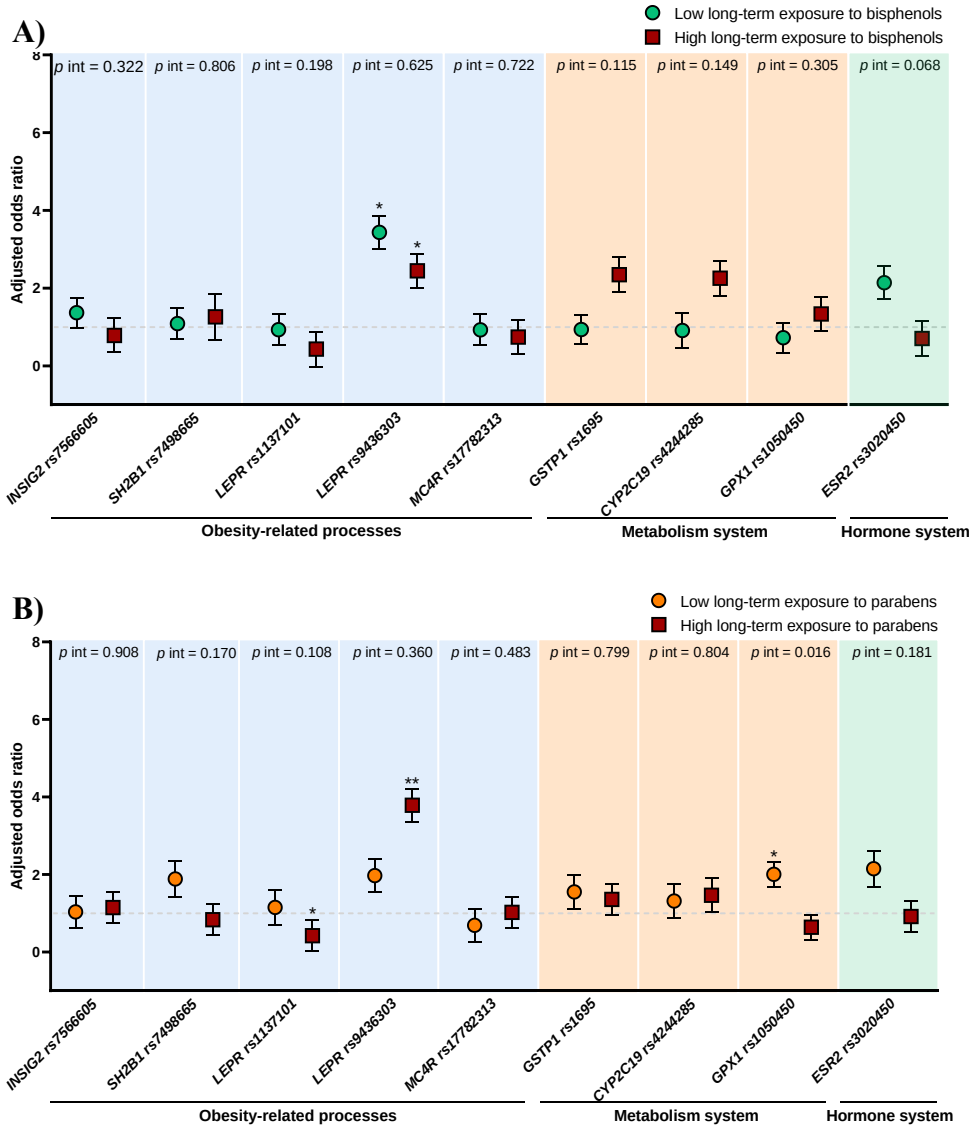
**Table 4**: Distribution of genotypic and allelic frequencies of genetic variants according to co-exposure to bisphenols and parabens.

| SNP | Bisphenols and parabens in hair | | | | Bisphenols and parabens in urine | | | |
|---|---|---|---|---|---|---|---|---|
| | Low exposure (N=63) | Medium exposure (N=107) | High exposure (N=62) | $p^a$ | Low exposure (N=37) | Medium exposure (N=46) | High exposure (N=39) | $p^a$ |
| *INSIG2* rs7566605 (Rec) | | | | | | | | |
| CC + CG | 30 | 52 | 39 | 0.140 | 18 | 23 | 18 | 0.939 |
| GG | 33 | 55 | 23 | | 19 | 23 | 21 | |
| C | 35 | 69 | 47 | 0.230 | 23 | 29 | 21 | 0.781 |
| G | 91 | 145 | 77 | | 51 | 63 | 57 | |
| *SH2B1* rs7498665 (Dom) | | | | | | | | |
| AA | 24 | 42 | 30 | 0.435 | 15 | 17 | 14 | 0.909 |
| AG + GG | 38 | 62 | 31 | | 22 | 29 | 25 | |
| A | 75 | 136 | 85 | 0.318 | 46 | 57 | 48 | 0.997 |
| G | 49 | 72 | 37 | | 28 | 35 | 30 | |
| *LEPR* rs1137101 (Dom) | | | | | | | | |
| AA | 21 | 42 | 20 | 0.577 | 13 | 20 | 13 | 0.584 |
| AG + GG | 41 | 64 | 42 | | 24 | 26 | 26 | |
| A | 70 | 129 | 71 | 0.681 | 45 | 58 | 48 | 0.955 |
| G | 54 | 83 | 53 | | 29 | 34 | 30 | |
| *LEPR* rs9436303 (Dom) | | | | | | | | |
| AA | 41 | 56 | 34 | 0.258 | 22 | 23 | 22 | 0.673 |
| AG + GG | 22 | 51 | 28 | | 15 | 23 | 17 | |
| A | 102 | 153 | 88 | 0.108 | 56 | 65 | 56 | 0.759 |
| G | 24 | 61 | 36 | | 18 | 27 | 22 | |
| *MC4R* rs17782313 (Dom) | | | | | | | | |
| AA | 38 | 68 | 41 | 0.795 | 27 | 33 | 26 | 0.811 |
| AG + GG | 25 | 39 | 21 | | 10 | 13 | 13 | |
| A | 97 | 175 | 99 | 0.566 | 63 | 77 | 62 | 0.626 |
| G | 29 | 39 | 25 | | 11 | 15 | 16 | |
| *GSTP1* rs1695 (Dom) | | | | | | | | |
| AA | 26 | 47 | 30 | 0.719 | 17 | 21 | 17 | 0.974 |
| AG + GG | 37 | 60 | 32 | | 20 | 25 | 22 | |
| A | 79 | 135 | 86 | 0.440 | 47 | 59 | 53 | 0.819 |
| G | 47 | 79 | 38 | | 27 | 33 | 25 | |
| *CYP2C19* rs4244285(Dom) | | | | | | | | |
| GG | 45 | 75 | 40 | 0.582 | 27 | 33 | 26 | 0.761 |
| AG + AA | 17 | 31 | 22 | | 10 | 12 | 13 | |
| G | 103 | 177 | 99 | 0.680 | 63 | 75 | 63 | 0.770 |
| A | 21 | 35 | 25 | | 11 | 15 | 15 | |
| *GPX1* rs1050450 (Dom) | | | | | | | | |
| GG | 24 | 57 | 35 | 0.085 | 20 | 17 | 19 | 0.148 |
| AG + AA | 38 | 48 | 27 | | 14 | 29 | 20 | |
| G | 80 | 154 | 91 | 0.180 | 50 | 55 | 55 | 0.141 |
| A | 44 | 56 | 33 | | 18 | 37 | 23 | |
| *ESR2* rs3020450 (Dom) | | | | | | | | |
| GG | 19 | 39 | 22 | 0.694 | 16 | 14 | 8 | 0.101 |
| AG + AA | 44 | 68 | 40 | | 21 | 32 | 31 | |
| G | 72 | 129 | 71 | 0.798 | 42 | 51 | 39 | 0.669 |
| A | 54 | 85 | 53 | | 32 | 41 | 39 | |

Dom: dominant model; Rec: recessive model. [a]Chi-square test.

### *3.3.1. Genetic association analysis according to long-term exposure to bisphenols and parabens*

The total content of bisphenols and parabens in hair samples was used as an indicator of long-term exposure. Focusing on obesity-related genetic variants (**Fig. 1A** and **Table S7**), under the dominant model, the *LEPR* rs9436303 A/G variant remained a risk variant at low and high exposure to bisphenols (OR = 3.43, $p = 0.003$, and OR = 2.45, $p = 0.038$, respectively), so the interaction was not significant ($p$ interaction = 0.625).



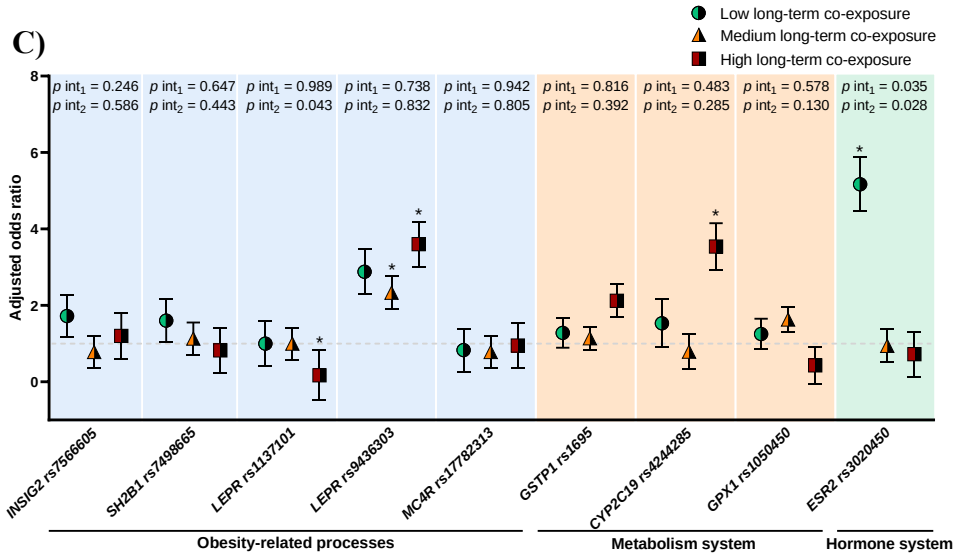



**Fig. 1**. Influence of genetic polymorphisms on overweight and obesity according to long-term exposure to A) bisphenols, B) parabens, and C) combined exposure to both. Odds ratio adjusted for age and gender; *p* $int_1$: *p* for interaction SNP x low exposure (reference category) vs medium exposure; *p* $int_2$: *p* for interaction SNP x low exposure vs high exposure. *$p \leq 0.05$; ** Bonferroni corrected *p* value ≤ 0.002.

With high paraben exposure **(Fig. 1B** and **Table S8**), the risk association was strengthened (OR = 3.78, *p* = 0.002, *p* interaction = 0.360). In the same scenario, another *LEPR* rs1137101 A/G variant conferred protection against excess weight (OR = 0.42, *p* = 0.035, *p* interaction = 0.108).

With regard to genetic variability within metabolising enzymes, two genetic variants in the CYP2C19 (rs4244285 G/A) and GSTP1 (rs1695 A/G) phase I and II enzymes appeared to increase the odds of overweight and obesity at borderline significance at high bisphenol exposure (OR = 2.25, *p* = 0.067, *p* interaction = 0.149 for rs4244285, and OR = 2.35, *p* = 0.056, *p* interaction = 0.115 for rs1695). In the stratification analysis of single exposure to parabens, each high-risk A allele of *GPX1* rs1050450 G/A was significantly associated with greater odds of overweight and obesity (OR = 2.00, *p* = 0.028, *p* interaction = 0.016) at low exposure compared with the low-risk G allele. The nominally significant interaction showed that the effect of rs1050450 was exposure level dependent.

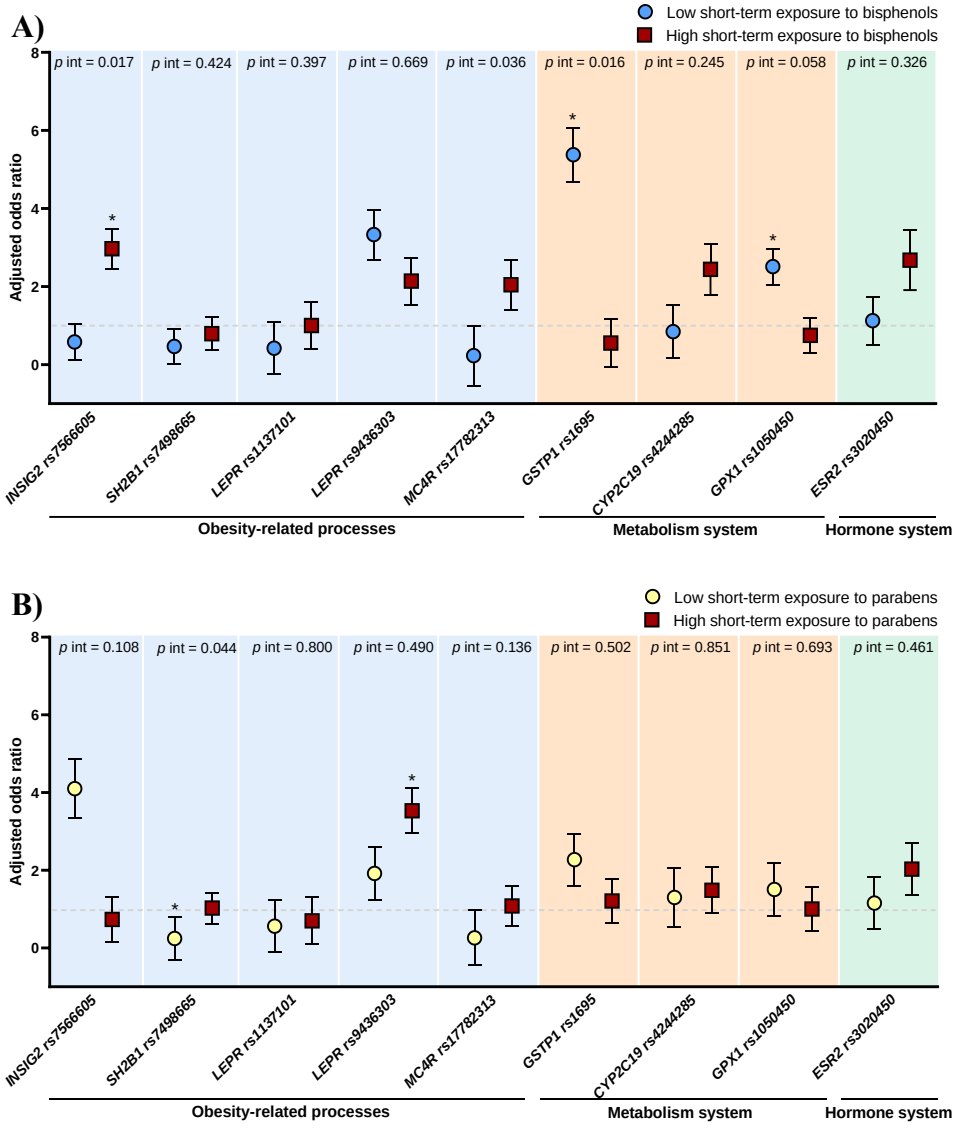
In the situation of co-exposure to bisphenols and parabens (**Fig. 1C** and **Table S9**), the differential effect of *LEPR* SNPs was observed at high exposure to both pollutants. *LEPR* rs1137101 retained its protective effect (OR = 0.17, $p$ = 0.007, $p$ interaction = 0.043), whereas the increased odds of developing overweight and obesity associated with *LEPR* rs9436303 was appreciated in the three exposure groups, with the effect being greater at the highest exposure dose (OR = 2.88, $p$ = 0.074 for low exposure, OR = 2.33, p = 0.047 for medium exposure, and OR = 3.60, $p$ = 0.030 for high exposure). For *CYP2C19* rs4244285, carriers of AG or AA genotypes and highly exposed to both chemical compounds were more likely to suffer from overweight and obesity than those with GG genotype, although the interaction was not significant (OR = 3.54, $p$ = 0.039, $p$ interaction = 0.285). The other two variants in metabolising enzymes showed associations close to significance above high co-exposure (OR = 2.12, $p$ = 0.082, $p$ interaction = 0.392 for *GSTP1* rs1695, and OR = 0.43, $p$ = 0.079, $p$ interaction = 0.130 for *GPX1* rs1050450).

On the other hand, there was a significant and positive association between rs3020450 G/A in the estrogen receptor 2 gene (*ESR2*) and overweight and obesity in those children who inherited one or two copies of the high-risk A allele (OR = 5.17, $p$ = 0.020, $p$ interaction = 0.028). This effect was seen at lower co-exposure doses, resulting in an important interaction.

### *3.3.2. Genetic association analysis according to short-term exposure bisphenols and parabens*

Creatinine-adjusted urinary bisphenols and parabens were used as an indicator of short-term exposure. In the stratification analysis of bisphenol exposure (**Fig. 2A** and **Table S10**), a new association was found between the *INSIG2* rs7566605 C/G polymorphism and increased odds of exhibiting excess weight at high levels of bisphenols (OR = 2.97, $p$ = 0.035, $p$ interaction = 0.017). In contrast, *MC4R* rs17782313 A/G seemed to have a protective effect against increasing odds at low exposure, leading to a significant SNP-bisphenol interaction (OR = 0.23, $p$ = 0.056, $p$ interaction= 0.036). For its part, *GSTP1* rs1695 gained importance in its linkage

with overweight and obesity depending on the level of exposure (OR = 5.38, $p$ = 0.016, $p$ interaction = 0.016 for low exposure). Additionally, *GPX1* rs1050450 showed to be a predisposing factor at low exposure dose (OR = 2.51, $p$ = 0.047, $p$ interaction = 0.058).



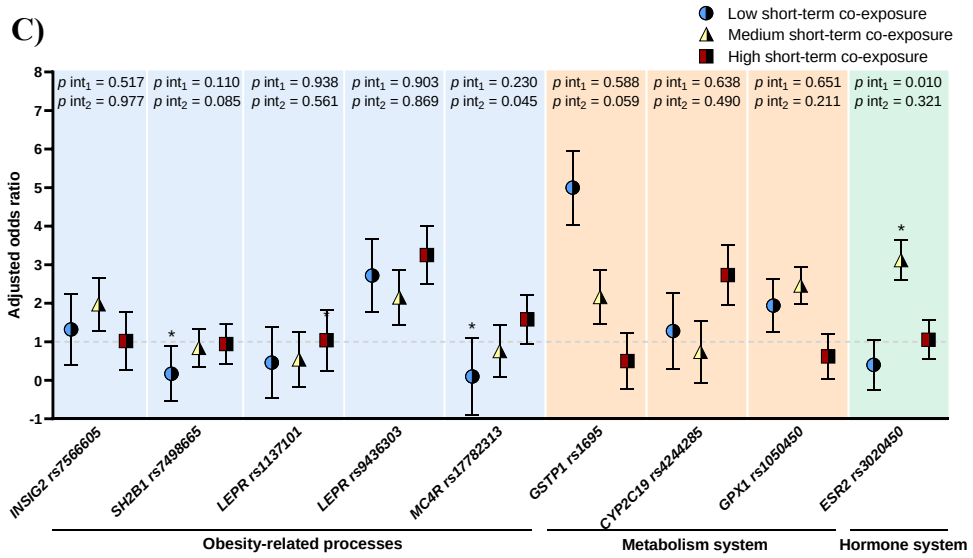



**Fig. 2**. Influence of genetic polymorphisms on overweight and obesity according to short-term exposure to A) bisphenols, B) parabens, and C) combined exposure to both. Odds ratio adjusted for age and gender; *p* $int_1$: *p* for interaction SNP x low exposure (reference category) vs medium exposure; *p* $int_2$: *p* for interaction SNP x low exposure vs high exposure. *$p \leq 0.05$.

In the case of individual exposure to parabens (**Fig. 2B** and **Table S11**), associations were particularly observed with obesity-related genetic variations. The minor G allele of two variants showed a protective trend in terms of low exposure (OR = 0.24, $p = 0.010$, *p* interaction = 0.044 for *SH2B1* rs7498665, and OR = 0.26, $p = 0.057$, *p* interaction = 0.136 for *MC4R* rs17782313). While the marked effect of *LEPR* rs9436303 in favour of excess body weight persisted independently of the exposure (OR = 3.53, $p = 0.029$, *p* interaction = 0.490 for high exposure).

When co-exposure to both bisphenols and parabens was explored (**Fig. 2C** and **Table S12**), the protective function of the *SH2B1* and *MC4R* variants remained in the low exposure group with signs of interaction (OR = 0.17, $p = 0.015$, *p* interaction = 0.085 for rs7498665, and OR = 0.10, $p = 0.023$, *p* interaction = 0.045 for rs17782313). Conversely, *GSTP1* and *GPX1* variants slightly contributed to excess weight development in the context of low and medium exposure, respectively (OR = 5.00, $p = 0.092$, *p* interaction = 0.059 for rs1695, and OR = 2.46, $p = 0.065$, *p*

interaction = 0.651 for rs1050450). More importantly, the high-risk A allele of *ESR2* rs3020450 significantly predisposed to overweight and obesity when the subject was highly exposed to at least one pollutant (OR = 3.12, $p = 0.030$, $p$ interaction = 0.010). A sensitivity analysis including creatinine levels as a confounding factor was performed (Table S13), and the direction of association was maintained.

### *3.3.3. In silico analysis: functional impact and clinical effects*

Intron variants and missense variants were the most common predicted functional consequences (see Supplementary **Table 14**). In terms of ClinVar clinical significance, *TCF7L2* rs7903146, *SOD2* rs4880, *IL6* rs1800795, *BDNF* rs6265, *PON1* rs662, and *ESR1* rs2234693 are considered as genetic risk or likely risk factors. Although the effect of the *LEPR* rs1137101 missense variant was predicted to be benign, the Polyphen score on the ENST00000344610.12 and ENST00000371058.1 transcripts predicted a possibly damaging effect. Similarly, SIFT and PolyPhen values of *GPX1* rs1050450 in the ENST00000703796.1 and ENST00000704374.1 transcripts predicted a deleterious and possibly damaging impact, respectively. On the other hand, the clinical significance of other variants including *SH2B1* rs7498665, *GSTP1* rs1695, and *CYP2C19* rs4244285 has been described as benign.

## 4. Discussion

The present study aimed to assess the complex interplay between several genetic variants and exposure to bisphenols and parabens, alone and in combination, on odds for developing excess body weight in Spanish children. Herein, the *LEPR* rs9436303 emerged as a relevant risk variant for excess weight, and its effect persisted after stratification by bisphenol and paraben exposure levels. For long-term exposure, *GPX1* rs1050450 was associated with increased excess weight at low single exposure to parabens, whereas *LEPR* rs1137101 exhibited a protective function in those highly co-exposed to bisphenols and parabens. *ESR2* rs3020450 and CYP*2C19* rs4244285 were identified as predisposing variants at low and high co-exposure, respectively. In short-term exposure, a higher likelihood of overweight and obesity was observed for *INSIG2* rs7566605 at high bisphenol exposure and for

*GSTP1* rs1695 and *GPX1* rs1050450 at low levels. Under situation of low and medium co-exposure, *SH2B1* rs7498665 and *MC4R* rs17782313 displayed a protective effect, whereas *ESR2* rs3020450 maintained its role in favour of excess weight.

The analysis of bisphenol and paraben exposure levels in the two study groups revealed that controls exhibited higher bisphenol levels than cases, though this difference was not statistically significant. A study by Melough et al. (2022) reported that adherence to healthy dietary patterns does not appear to be associated with a low exposure to EDCs such as bisphenols. This lack of association may be attributable to the widespread contamination across the food chain, including fresh products (Melough et al., 2022; González et al., 2020). This may explain the higher levels of contaminants in biological matrices from healthy lifestyle individuals. Thus, it may be that not all individuals exposed to a specific environmental factor will develop a disease. Likewise, not all individuals who inherit certain genetic variants will develop a disease (Virolainen et al., 2023). For this reason, we believe that studying the combined effect of genetic and environmental factors could help to fill the knowledge gaps in the aetiology of obesity.

SNPs are the largest source of sequence variation in the human genome, and the *FTO* rs9939609 was the first variant to be associated with overweight and obesity in the childhood and adulthood (Frayling et al., 2007). Since then, genetic variability in predisposition to obesity has become increasingly important, and numerous SNPs associated with BMI variability have been identified to date (Loos and Yeo, 2021). In our study, *FTO* rs9939609 and rs8050136 variants did not show significant associations in the phenotype-genotype analysis. Nevertheless, genetic variation in genes involved in the leptin pathway is of importance in the present study.

Leptin is one of the body energy sensors that act in the hypothalamus through its receptor (LEPR) regulating food intake, energy expenditure, and body weight status (Olza et al., 2017). Thus, genetic alterations in the leptin gene or its receptor can impair leptin-mediated signalling, leading to leptin resistance. It results in higher production of leptin by adipose tissue as a compensatory mechanism (Cissé et al.,

2022). The *LEPR* rs1137101, an arginine (A) to glutamine (G) transition at position 223 (Q223R), is one of the most common polymorphisms and it is believed to be related to increased body weight (Raskiliene et al., 2021). In Spanish children aged 6 to 8 years, this association was proved (Marcos-Pasero et al., 2020), whereas this SNP did not show effect in Lithuanian children aged 12 to 13 years (Raskiliene et al., 2021).

In our present cohort, we found that rs1137101 variant conferred protection against excess weight in children co-exposed to higher levels of bisphenols and parabens in data from hair. However, the other *LEPR* variant, rs9436303, showed to be a relevant risk variant: (1) in the genetic association analysis, (2) at low and high exposure to bisphenols, (3) at high paraben exposure, and (4) mainly at high exposure to both bisphenols and parabens. This revealed that the SNP-obesity association was independent of the level of exposure. In line with the first point, several studies have reported that carrying the rs9436303 G allele is associated with obesity-related traits in children and adolescents (Alves et al., 2019; Cissé et al., 2022; Olza et al., 2017; Ramírez et al., 2022b). Regarding the impact of EDCs on leptin signalling, there is evidence pointing to an increase in leptin levels following exposure to BPA and parabens in animals (Haq et al., 2020; Marraudino et al., 2019) and humans (Rönn et al., 2014). In this way, we assumed that the modulations in body weight resulted from the additive effect between *LEPR* gene polymorphisms and exposure to bisphenols and parabens, as previously shown in adolescents and young adults (Ramírez et al., 2022b).

Importantly, when leptin binds to LEPR, it stimulates neurons expressing proopiomelanocortin (POMC), which produces melanocortin peptides (α-MSH, β-MSH and γ-MSH) that bind to the melanocortin receptor (MC4R). This binding triggers signalling pathways that lead to decreased appetite and increased energy expenditure (Flores-Dorantes et al., 2020; Mera-Charria et al., 2023). In turn, the *SH2B1* gene acts as a positive regulator of leptin-melanocortin pathway (Aerts et al., 2015). Therefore, genetic variants in these genes, such as *SH2B1* rs7498665 and *MC4R* rs17782313, have proven to be predisposing risk factors for childhood obesity

(Aerts et al., 2015; Dastgheib et al., 2021; Krishnan et al., 2017; López-Rodríguez et al., 2020; Resende et al., 2021). In the present child population, these polymorphisms displayed a protective role in the case of low urinary exposure to bisphenols and parabens. Although the opposite effect was not significant at high exposure, the SNP-exposure interaction was significant, suggesting that the effect of the genetic variants varies with the degree of exposure. As far as we know, there are no studies linking bisphenols or parabens to *SH2B1* alterations, while only one study noticed a downregulation of *Mc3r, Mc4r* in Wistar rats exposed to BPA via drinking water (Patisaul et al., 2012).

Another strong candidate gene for obesity is *INSIG2*, which regulates adipogenesis and lipid synthesis (Kaulfers et al., 2015). In paediatric population, the rs7566605 C/G polymorphism has demonstrated to be involved in obesity development (Liu, F. et al., 2015; Vourdoumpa et al., 2023). Consistent with this, we found a positive association with overweight and obesity at high urinary levels of bisphenols, indicating a significant interaction. In human adipose-derived mesenchymal stem cells, the treatment with vitamin D plus BPA affected adipose function by promoting up-regulation of *INSIG2* expression (Salehpour et al., 2021).

On the other hand, changes in adipose tissue function lead to increased secretion of pro-inflammatory cytokines, creating a phenomenon of low-grade chronic inflammation and oxidative stress (Hernández-Guerrero et al., 2018; Pietrocola and Bravo-San Pedro, 2021). Under this scenario, the organism has detoxifying enzymatic systems that protect against oxidative stress, such as GSTs (e.g. GSTP1) and GPX1 (Rahbar et al., 2020). In fact, common variants like *GSTP1* rs1695 and *GPX1* rs1050450 may play a role in the susceptibility of obesity among children (Chielle et al., 2016; Hernández-Guerrero et al., 2018; Mera-Charria et al., 2023; Ramírez et al., 2023). In addition, *in vitro* studies have evidenced that BPA, BPS and MetPB could downregulate the mRNA expression of *GSTP1* and *GPX1*, resulting in increased intracellular oxidative stress (Cha et al., 2014; Nguyen, M. et al., 2022; Shan et al., 2023). Likewise, another study investigated the interaction between BPA and *GSTP1* rs1695 in children with asthma and found that those homozygous for the

variant G allele had lower urinary concentrations of BPA glucuronide (Lin et al., 2018). Herein, the authors suggested that *GSTP1* gene may be involved in detoxification of BPA metabolites. In agreement with this finding, we reported that carrying the variant alleles of *GSTP1* rs1695 (G) and *GPX1* rs1050450 (A) was corresponded with a higher likelihood of overweight and obesity at low levels of urinary bisphenols with evidence of interaction. Role of *GPX1* variant in bisphenol detoxification has not yet been assessed. Thus, we support that genetic variability within detoxifying enzymes could interrupt the proper degradation and excretion of toxic substances, thereby favouring the development of inflammatory diseases like obesity.

Similarly, the xenobiotic metabolism takes place in two phases: phase I by CYP450 enzymes, and phase II (e.g. GSTP1 and GPX1). CYP450-dependent oxidation is a minor pathway for bisphenol metabolism and is catalysed mainly by CYP1A2, CYP2C9, CYP3A4 isoforms (Ramírez et al., 2021). In the present child sample, individuals with *CYP2C19* rs4244285 AG or AA genotypes and higher accumulation of bisphenols and parabens in hair tended to be overweight or obese. To date, it is known that CYP2C19 is involved in drug metabolism, but there is not enough data on bisphenol or paraben metabolism (Kvitne et al., 2022). Further, patients with obesity had lower CYP2C19 activity, possibly attributable to frequent variants that produce non-functional proteins (Chaudhry et al., 2015; Kvitne et al., 2022). We therefore hypothesise that *CYP2C19* gene variations may impair the metabolism and excretion of bisphenols and parabens in urine, leading to their accumulation in the body.

Lastly, obesogenic chemicals are multitarget compounds that can act through multiple hormone sensitive elements (Marraudino et al., 2019). In adipocytes, estrogens via both membrane and nuclear receptors (ESR1 and ESR2) inhibit lipogenesis and help modulate food consumption and energy expenditure (Heindel et al., 2022; Lustig et al., 2022). Indeed, interactions between estrogens and adipokines have been described, leading to estrogen-influenced leptin sensitivity in the brain (Rönn et al., 2014). In our SNP-exposure interaction analysis, carrying the

high-risk A allele of *ESR2* rs3020450 significantly predicted overweight and obesity at low and medium co-exposure assessed in hair and urine, respectively. Although, this SNP has not yet been associated with obesity, the aforementioned role of ESR2 on leptin pathway supports our finding. Furthermore, bisphenols have been shown to negatively affect brain estrogen receptor expression patterns, even at low doses (Patisaul et al., 2012; Rebuli et al., 2014).

One limitation of our study was the sample size; we have been able to detect nominal significance ($p \leq 0.05$) in the gene-environment interaction study, which did not reach the rigorous Bonferroni correction due to the high quantity of analysed genetic markers. Even so, we did highlight new SNP-obesity associations that had not been explored previously. As this study was designed as a proof-of-concept investigation, the preliminary findings, particularly in a vulnerable population within the context of the current global prevalence of excess weight, represent a significant contribution to the field that merits consideration for future research involving larger populations. Apart from the sample size, the genotype and allele frequencies vary across the different populations, and this may explain the discrepancies found between studies.

The main strength of the current research is that it sheds light on the complex interplay between genetic and environmental factors in childhood overweight and obesity. From the literature available to date, it is important to note that 1) the SNP-obesity association has been addressed and 2) the impairment of some signalling pathways or gene expression by exposure to bisphenols and parabens has been investigated. However, there is a lack of research assessing the synergistic effect when genetic variation and exposure to disrupting substances coexist. Exposure to these compounds is continuous and ubiquitous, contributing to the environment dynamic (Heindel et al., 2022). Furthermore, polygenic obesity is the most common form of obesity, caused by the cumulative effect of genetic variants in multiple genes (Littleton et al., 2020). All this make difficult to develop a simple, biological plausible model that accurately reflects the direct relationship between environmental exposure and genetic polymorphisms.

We have demonstrated that some polymorphisms, such as *LEPR* rs9436303, maintain their risk effect independently of the level of exposure. Meanwhile, other variants have no consequences per se, but in the presence of exposure, their effect (protective or risk) varies with the degree of exposure (*INSIG2* rs7566605, *SH2B1* rs7498665, *LEPR* rs1137101, *MC4R* rs17782313, *GSTP1* rs1695, *CYP2C19* rs4244285, *GPX1* rs1050450, and *ESR2* rs3020450). It indicates that genetics interacts with an ever-changing environment, and therefore studying gene-environment interactions gives us a more holistic approach to dealing with human disease aetiology (Arango et al., 2021; Virolainen et al., 2023). Specially, studying the contribution of synergistic interplay between genetic and environmental factors could provide a more comprehensive understanding of the mechanisms driving human disease risk (Virolainen et al., 2023).

Additionally, we used urine and hair as reliable indicators of short- and long-term exposure, respectively. Both biological matrices have the advantage of being easy and non-invasive to collect, which is particularly important in the child population. However, urine concentrations of bisphenols and parabens vary throughout the day between individuals, even within the same person due to their short half-life (Gálvez-Ontiveros et al., 2023; Nguyen, H. T. et al., 2023). For its part, hair has been proven to be a suitable matrix to assess long-term exposure to contaminants and drugs given their accumulation during hair growth (Katsikantami et al., 2019; Robin et al., 2022). That is why some genetic association studies have used hair as a useful target organ for the deposition of xenobiotics, and urine as a window of their metabolism and excretion (Parajuli et al., 2015; Wang et al., 2012). At the same time, differential findings of the effects of the SNPs on the response are not surprising given the different biological nature of the matrices. For each matrix, the significance of the associations and interactions differed, but the direction of the effect was the same for both matrices. Another aspect to highlight is that humans are often exposed to multiple pollutants simultaneously; therefore, the analyses were conducted to account for co-exposure to both phenols, in addition to examining their effects separately. As that EDCs follow a particular dose-response curve with

optimal effects at lower doses, it was important to evaluate the effects by stratifying based on the level of exposure (Vandenberg et al., 2012).

## 5. Conclusions

In conclusion, our findings demonstrate for the first time that understanding the genetic variation along obesity-related biological pathways, antioxidant defence system, metabolising enzymes, and hormonal processes, and how the level of exposure to bisphenols and parabens might interact with it, is crucial for a more in-depth comprehension of the complex polygenic and multifactorial aetiology of obesity. In this way, by determining the extent of the genetic impact in the presence of an obesogenic environment, effective intervention strategies could be developed to prevent or reduce the incidence of overweight and obesity. This raises the need for further research into the complex relationships between genetic polymorphisms and environmental exposures in large and diverse populations.

**Ethics Statement**. The present study has been approved by the Biomedical Research Ethics Committees of the Province of Granada (references: 0922-N-19; 1939-M1-22; 1742-N-23) and the study has been performed in accordance with the ethical standards. All subjects gave written informed consent and had parental permission to participate in this study.

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**CRediT authorship contribution statement**

**Viviana Ramírez:** Methodology, Formal analysis, Investigation, Writing- Original draft preparation, Visualization. **Yolanda Gálvez-Ontiveros:** Methodology, Investigation. **Vega Almazán Fernández de Bobadilla:** Resources. **Patricia González-Palacios:** Methodology, Investigation. **Inmaculada Salcedo-Bellido:**

Conceptualization, Formal analysis, Supervision and Writing-reviewing and editing, Visualization. **Cristina Samaniego-Sánchez:** Supervision and Writing-reviewing and editing. **María Jesús Álvarez-Cubero:** Conceptualization, Writing – Review and Editing, Visualization, Supervision. **Luis Javier Martínez-González:** Conceptualization, Writing – Review and Editing, Visualization, Supervision. **Alberto Zafra-Gómez:** Methodology, Resources, Supervision. **Ana Rivas:** Conceptualization, Writing-reviewing and editing, Supervision, Funding acquisition, Project administration.

**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.

**Appendix A. Supporting information**. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.117206.

**Data availability**. The data that has been used is confidential.

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# **Chapter II.** Effects of cognition-related genetic polymorphisms and dietary exposure to EDCs during childhood

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# Influence of genetic polymorphisms on cognitive function according to dietary exposure to bisphenols in a sample of Spanish schoolchildren

**Viviana Ramírez[1,2,3,4,†], Patricia González-Palacios[1,3], Pablo José González-Domenech[5], Sonia Jaimez-Pérez[6], Miguel A. Baca[7], Lourdes Rodrigo[8], María Jesús Álvarez-Cubero[2,3,9,*,†], Celia Monteagudo[1,3,*,†], Luis Javier Martínez-González[2,9,†] and Ana Rivas[1,3,4,†]**

[1] Department of Nutrition and Food Science, Faculty of Pharmacy, University of Granada, 18071 Granada, Spain; vivianarl@ugr.es (V.R.); pgonzapa@ugr.es (P.G.P.); amrivas@ugr.es (A.R.)

[2] GENYO. Centre for Genomics and Oncological Research: Pfizer / University of Granada / Andalusian Regional Government PTS Granada—Avenida de la Ilustración, 114 - 18016 GRANADA, Spain; luisjavier.martinez@genyo.es (L.J.M.G.)

[3] Instituto de Investigación Biosanitaria ibs.GRANADA, 18012 Granada, Spain

[4] Institute of Nutrition and Food Technology "Jose Mataix Verdú", Biomedical Research Center, Health Sciences Technological Park, University of Granada, 18016 Granada, Spain

[5] Department of Psychiatry, Faculty of Medicine, University of Granada, 18012 Granada, Spain; pgdomenech@ugr.es (P.J.G.D.)

[6] Virgen de las Nieves University Hospital, 18014 Granada, Spain; soniajaimezperez@gmail.com (S.J.P.)

[7] Clinica MenSana, 18009 Granada, Spain; mabaca@cop.es (M.A.B.)

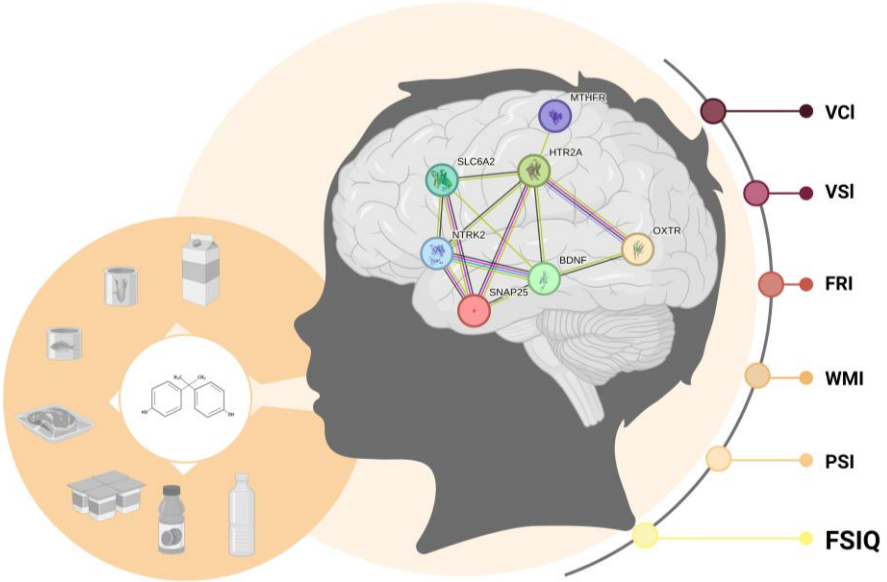
[8] Department of Legal Medicine, Toxicology and Physical Anthropology, Faculty of Medicine, University of Granada, 18012 Granada, Spain; lourdesr@ugr.es (L.R.)

[9] Department of Biochemistry and Molecular Biology III, Faculty of Medicine, University of Granada, 18012 Granada, Spain

* Correspondence: mjesusac@ugr.es (M.J.Á.C.); celiams@ugr.es (C.M.)

† These authors contributed equally to this work.

**Graphical abstract**




**Abstract**

Background: Neurodevelopmental disorders (NDDs) like intellectual disability (ID) are highly heritable, but the environment plays an important role. For example, endocrine disrupting chemicals (EDCs), including bisphenol A (BPA) and its analogues, have been termed neuroendocrine disruptors. This study aimed to evaluate the influence of different genetic polymorphisms (SNPs) on cognitive function in Spanish schoolchildren according to dietary bisphenol exposure. Methods: A total of 102 children aged 6–12 years old were included. Ten SNPs in genes involved in brain development, synaptic plasticity, and neurotransmission (*BDNF*, *NTRK2*, *HTR2A*, *MTHFR*, *OXTR*, *SLC6A2*, and *SNAP25*) were genotyped. Then, dietary exposure to bisphenols (BPA plus BPS) was estimated and cognitive functions were assessed using the WISC-V Spanish form. Results: *BDNF* rs11030101-T and *SNAP25* rs363039-A allele carriers scored better on the fluid reasoning domain, except for those inheriting the *BDNF* rs6265-A allele, who had lower scores. Secondly, relevant SNP–bisphenol interactions existed in verbal comprehension (*NTRK2* rs10868235 ($p$-int = 0.043)), working memory (*HTR2A* rs7997012 ($p$-int = 0.002), *MTHFR* rs1801133 ($p$-int = 0.026), and *OXTR* rs53576 ($p$-int = 0.030)) and fluid reasoning (*SLC6A2* rs998424 ($p$-int = 0.004)).

Conclusions: Our findings provide the first proof that exploring the synergistic or additive effects between genetic variability and bisphenol exposure on cognitive function could lead to a better understanding of the multifactorial and polygenic aetiology of NDDs.

**Keywords:** cognitive function; neurodevelopmental disorders; genetic polymorphism; dietary exposure; bisphenols

## 1. Introduction

DSM-V (Diagnostic and Statistical Manual of Mental Disorders, fifth edition) defines neurodevelopmental disorders (NDDs) as a heterogenous group of mental health conditions that occur during the developmental period and negatively affect brain functioning [1]. NDDs include attention-deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), and intellectual disability (ID), which lead to behavioural problems, poorer learning, memory dysfunction, and delayed motor development [2,3]. Among these, cognitive impairments in general and ID in particular constitute major conditions of NDDs with diverse aetiologies, affecting about 1% of children in the world [4,5]. They are characterised by both impaired cognitive functioning (intellectual quotient (IQ) < 70) and adaptive behaviour [6].

Non-genetic causes such as infections, autoimmunity, and environmental factors are described in NDD pathogenesis, but advances in biomolecular knowledge (e.g., genotyping/sequencing approaches) have identified hundreds of candidate genes to be involved in neurodevelopment, revealing the importance of a genetic contribution [7,8]. In fact, ID has emerged as the most common manifestation under genetic abnormalities [5,9]. Structural variants such as copy number variations (CNVs) and point mutations like single nucleotide variants (SNVs) have been found in patients suffering from neurodevelopmental alterations [10]. In certain cases, one single de novo mutation could be the causative factor, while in other scenarios, the risk of developing NDDs could be influenced by a complex interplay between rare and common genetic variants [7]. Specifically, single nucleotide polymorphisms (SNPs), which are common SNVs occurring with a frequency of at least 1%, have shown to contribute to mild intellectual impairment [11]. Brain-derived neurotrophic

factor (*BDNF*) rs6265 (Val66Met) is one of the most extensively studied missense variants within the prodomain region of BDNF, with functional consequences on memory, cognition, and behaviour [12].

Although the identification of NDD-causing genes is essential for understanding the underlying biological mechanisms responsible for the onset of these disorders, the molecular diagnosis is quite challenging and still unknown in many patients [2]. This highlights the complex and multifactorial nature of NDDs and the need to examine other risk factors at the same time. Endocrine disrupting chemicals (EDCs) such as bisphenol A (BPA) and its analogues are able to cross the blood–brain barrier and, as the developing brain is particularly sensitive to these compounds, EDCs have been termed neuroendocrine disruptors [13]. BPA migration from food packaging into foodstuffs is a significant contamination source by which BPA enters the food chain, and for this reason, dietary consumption has been considered the primary contributor to BPA exposure, followed by contaminated air and dermal absorption [14]. To date, BPA exposure during childhood has been more frequently related to adverse behavioural outcomes, whereas evidence for effects on cognitive functioning is still weak [15,16]. For this reason, the exploration of the synergistic or additive effect between the environmental factor and genetic vulnerability could lead to a better understanding of the multifactorial and polygenic aetiology of NDDs [17,18]. To the best of our knowledge, there is growing evidence of interactions between gene polymorphisms and pesticides/heavy metals in cognitive development and the etiopathogenesis of disorders such as ASD and ADHD [10]; nonetheless, no human studies examining NDD-associated genetic variants in the presence of bisphenol exposure are available.

Therefore, the purpose of the current study was to evaluate the influence of different genetic polymorphisms on cognitive function in Spanish schoolchildren aged between 6 and 12 years according to dietary exposure to bisphenols.

## 2. Materials and Methods

### *2.1. Study subjects and data collection*

Participants enrolled in this study were recruited from different elementary schools and health centres in Granada, Spain, between 2020 and 2023 as part of a

larger research project. Inclusion criteria for the selection of the study population were (1) schoolchildren aged between 6 and 12 years, and (2) having lived in the study area for at least 6 months continuously. Children whose parents or legal tutors agreed to participate and signed the written informed consent form were contacted by the paediatric clinical centre specialised in neurodevelopmental disorders. The study protocol was approved by the Ethics Committee of Provincial Biomedical Research of Granada (1742-N-23).

A total of 102 children with available estimates of dietary exposure to bisphenols, good quality DNA samples, and neurodevelopmental tests assessing cognitive function were finally selected for the current study.

Face-to-face interviews were conducted with all participants' parents or guardians by trained interviewers. The structured questionnaire was based on a sociodemographic section (gender and age of children and educational level, occupational rank, and marital status of parents or legal guardians), lifestyles (physical and dietary patterns) and anthropometric data collected by qualified personnel (weight and height).

*2.2. DNA isolation and genotyping assays*

For genotyping, DNA was extracted from buccal swabs using a procedure based on proteinase K digestion and saline purification. DNA quantification was performed using the Qubit™ 4.0 fluorometer (Invitrogen™ by ThermoFisher Scientific, MA, USA) with the Qubit dsDNA BR Assay Kit (Invitrogen™ by ThermoFisher Scientific, Oregon, USA). DNA samples were frozen at −20 °C until the genotyping step.

Ten SNPs were selected based on two selection criteria: (1) a minor allele frequency (MAF) higher than 10% within the Iberian population and (2) a greater number of studies on the association with neurodevelopmental functions in healthy and clinical populations. These SNPs are in genes involved in brain development and synaptic plasticity (*BDNF* rs6265 and rs11030101; neurotrophic receptor tyrosine kinase 2 (*NTRK2*) rs2289656 and rs10868235; methylenetetrahydrofolate reductase (*MTHFR*) rs1801133; and synaptosome associated protein 25 (*SNAP25*)

rs363039) and neurotransmitter systems (5-hydroxytryptamine receptor 2A (*HTR2A*) rs6314 and rs7997012; oxytocin receptor (*OXTR*) rs53576; and solute carrier family 6 member 2 (*SLC6A2*) rs998424).

Information on the gene, chromosomal location, variant effect, genotype, and allele frequencies were obtained from Ensembl "https://www.ensembl.org/index.html (accessed on 22 January 2024)" and The National Centre for Biotechnology Information SNP website "https://www.ncbi.nlm.nih.gov/ (accessed on 22 January 2024)" and are listed in **Table 1**.

**Table 1.** Information on the selected SNPs in the Spanish reference population (N = 107) and in our cohort (N=102).

| Gene name | Gene function | rs ID | Chr position (GRCh38/hg38) | Reference/ variant allele | Variant effect | MAF (N) | | HWE $p^b$ |
|---|---|---|---|---|---|---|---|---|
| | | | | | | IBS[a] | Our Cohort | |
| *BDNF* | Neuronal development, synaptogenesis, plasticity | rs6265 (Val66Met) | chr11:27658369 | C/T or G/A | Missense variant | T: 0.210 (45) | A: 0.211 (43) | 0.132 |
| | | rs11030101 | chr11: 27659197 | A/T | 5 prime UTR variant | T: 0.435 (93) | T: 0.392 (80) | 0.264 |
| *HTR2A* | Learning, cognitive abilities | rs6314 (His452Tyr) | chr13: 46834899 | G/A | Missense variant | A: 0.107 (23) | A: 0.103 (21) | 0.324 |
| | | rs7997012 | chr13: 46837850 | A/G | Intron variant | A: 0.388 (83) | A: 0.333 (68) | 0.766 |
| *MTHFR* | Brain development, synaptic plasticity | rs1801133 (C677T) | chr1: 11796321 | G/A | Missense variant | A: 0.444 (95) | A: 0.377 (77) | 0.823 |
| *OXTR* | Social, working, spatial, episodic memory formation | rs53576 | chr3: 8762685 | A/G | Intron variant | A: 0.308 (66) | A: 0.294 (60) | 0.384 |
| *SLC6A2* | Mood, attention, stress response regulation | rs998424 | chr16: 55698034 | G/A | Intron variant | A: 0.308 (66) | A: 0.377 (77) | 0.536 |
| *SNAP25* | Brain development, synaptic plasticity | rs363039 | chr20: 10239848 | G/A | Intron variant | A: 0.383 (82) | A: 0.328 (67) | 0.653 |
| *NTRK2* | Neuronal development, synaptogenesis, plasticity | rs2289656 | chr9: 84948647 | G/A | Intron variant | A: 0.206 (44) | A: 0.181 (37) | 0.273 |
| | | rs10868235 | chr9: 84878840 | C/T or G/A | Intron variant | C: 0.486 (104) | A: 0.480 (98) | 0.831 |

MAF: minor allele frequency. [a]IBS: Iberian population MAF values from the Ensembl database "https://www.ensembl.org/index.html (accessed on 22 January 2024)". [b]HWE: Hardy–Weinberg equilibrium by the chi-square test.

Two types of genotyping technologies were performed: (1) Infinium Global Screening Array (GSA)-24 BeadChip and (2) Taqman SNP Genotyping Assays. In the first place, 7 SNPs were genotyped using the microarray technology on the iScan system by Ilumina® Infinium® HTS Assay (Illumina, Inc., CA, USA) according to the method previously described by Ramírez et al. (2023) [19]. GSA data were read and analysed with the software llumina® GenomeStudio v2010.3.

In Taqman assays, 3 SNPs were genotyped by the following commercially available Taqman® probes (Applied Biosystems™ Taqman SNP Genotyping Assays): C___1751785_10 for *BDNF* rs11030101, C___3020067_10 for *SLC6A2* rs998424, and C____327976_10 for *SNAP25* rs363039. Quantitative PCRs (qPCRs) were performed on the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems™, USA) and data outputs were read and processed with the software QuantStudio™ Real-Time PCR v1.3.1 [20].

Those SNPs presenting a call rate of less than 95% that deviated from Hardy–Weinberg equilibrium (HWE, $p < 0.05$) and samples with an overall call rate of less than 95% were excluded from the final statistical analysis.

### *2.3. Bisphenol exposure assessment*

Daily dietary exposure to total bisphenols (BPA plus BPS) was estimated on an individual basis by multiplying the daily intake of different foods (g/day) by the corresponding bisphenol content in each food item (ng/g of food). The dietary information was recorded for the last 12 months through a semi-quantitative food frequency questionnaire (FFQ). This food survey was designed to ask about the food frequency (g of food per day) of 112 food items categorised into 13 groups, e.g., dairy products, meat and meat products, vegetables, legumes, and cereals, among others [21]. After that, the bisphenol content was chemically determined via an ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) system following the methodology described by Galvez-Ontiveros et al. (2021) [22]. Finally, BPA intake from all food sources analysed was summed for all individuals to estimate the total exposure dose (ng/day).

### *2.4. Neurodevelopmental assessment*

Cognitive functions in children aged 6–12 years were assessed using the Spanish form of the Weschler Intelligence Scale for Children—Fifth Edition (WISC-V), administrated by licensed and trained psychologists in childhood neurodevelopment. The WISC-V assesses various cognitive domains, providing a comprehensive profile of a child's cognitive abilities. The test is composed of 10 primary subtests, which can be combined into composite quotients, yielding five age-standardised primary indices: Verbal Comprehension Index (VCI), Visual Spatial Index (VSI), Fluid Reasoning Index (FRI), Working Memory Index (WMI), and Processing Spead Index (PSI). The Full-Scale Intelligence Quotient (FSIQ) is derived from seven primary subtests, typically Similarities, Vocabulary, Block Design, Matrix Reasoning, Figure Weights, Digit Span, and Coding.

For this study, the five primary indices and FSIQ scores (mean = 100, standard deviation (SD) = 15) were selected to address the cognitive profiles and IQ.

### *2.5. Data analysis*

Descriptive analyses of quantitative variables were carried out using the means and SDs for parametric variables, and medians and interquartile ranges (IQRs) in the case of non-parametric variables. The qualitative variables are presented in terms of frequencies and percentages. The Kolmogorov–Smirnov test with Lilliefors correction was performed to check the normality of continuous data.

To assess Hardy–Weinberg equilibrium (HWE), chi-square tests were applied ($p > 0.05$) in the codominant model. Linkage disequilibrium (LD) analyses were performed using SNPStats software "https://snpstats.net/start.htm (accessed on 10 February 2024)". SNPs were in LD when they had an $r^2$ value higher than 0.5. After verifying HWE and LD, the analyses were undertaken within the dominant or recessive model, and the contribution per allele was tested.

Student's *t*-test and the Mann–Whitney test were conducted for parametric and non-parametric variables, respectively. They were used to compare WISC-V index scores for each different genetic variant.

Crude odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated using binary logistic regression models to evaluate the influence of the genetic variants on WISC-V index scores. The WISC-V index was entered as the dependent variable, each genetic polymorphism as the independent variable, and dietary exposure to bisphenols (stratified by low and high exposure according to median values expressed as ng/day) was input as the selecting variable. Multivariable logistic regression models were then fitted that included sex, age, body mass index (BMI), and/or parental education level as potential confounders of neurological testing [23,24]. Sex and age were used as confounding factors in all analyses, and BMI and parental education level were included in the model if they produced changes in the OR of more than 10%. To explore gene–environment interactions in cognitive functions, the interaction term "polymorphism x exposure level" was added to the logistic regressions. Statistical significance was based on a $p$ value $\leq 0.05$. In addition, Bonferroni's correction was applied to the multifactorial logistic regression $p$ values to account for the multiple testing of 10 different SNPs ($p \leq 0.005$). All statistical analyses were performed with IBM SPSS Statistics 25 (Armonk, NY, USA) and RStudio 2023.12.0.

## 3. Results

### *3.1. Characteristics of participants*

Baseline characteristics of the study population are shown in **Table 2**. Of the 102 children included, 53 (52%) were boys and the mean age was 8.7 ± 2.1 years. The estimated daily dietary exposure dose for total bisphenols was 17306.3 ng/day. The educational level of the parents was classified into primary, secondary, and university education, with most of parents belonging to university category (50%). Regarding overall cognitive performance, the mean of the WISC-V FSIQ was 101.1 (12.7).

**Table 2.** General characteristics of the study population (N=102).

| | |
|---|---|
| Age in years, mean (SD) | 8.7 (2.1) |
| Gender, n (%) | |
| Boys | 53 (52.0) |
| Girls | 49 (48.0) |
| Weight in kg, mean (SD) | 36.9 (15.0) |

| | |
|---|---|
| Height in cm, mean (SD) | 134.8 (18.8) |
| BMI in kg/m$^2$, mean (SD) | 19.3 (4.9) |
| Bisphenols in ng/day, median (IQR) | 17306.3 (9674.2–27067.7) |
| Bisphenol A | 6823.7 (3575.9–12305.9) |
| Bisphenol S | 6976.4 (3459.9–17472.7) |
| Parental education level, n (%) | |
| Up to primary | 12 (11.8) |
| Secondary | 38 (37.3) |
| University | 51 (50.0) |
| Missing data | 1 (0.9) |
| WISC-V indices | |
| Verbal Comprehension Index (VCI), median (IQR) | 106.0 (95.0–113.0) |
| Visual Spatial Index (VSI), mean (SD) | 102.5 (15.3) |
| Fluid Reasoning Index (FRI), median (IQR) | 106.0 (94.0–115.0) |
| Working Memory Index (WMI), mean (SD) | 101.9 (14.2) |
| Processing Spead Index (PSI), median (IQR) | 86.0 (77.0–92.0) |
| Full-Scale Intelligence Quotient (FSIQ), mean (SD) | 101.1 (12.7) |

SD: standard deviation; BMI: body mass index; IQR: interquartile range; bw: body weight.

### *3.2. Genetic Variants and WISC-V Scores*

All SNPs achieved HWE ($p > 0.05$, Table 1). The MAFs of each locus were in agreement with those established for the Iberian population; only for *NTRK2* rs10868235 G/A was the variant A allele the minor allele in our cohort instead of the previously reported reference G allele. Those SNPs within the same gene were not in LD (*BDNF* rs6265/rs11030101 $r^2 = 0.17$; *HTR2A* rs6314/rs7997012 $r^2 = 0.06$; and *NTRK2* rs2289656/rs10868235 $r^2 = 0.04$).

**Table 3** shows in detail the mean and median values of the WISC-V index scores obtained for each genetic variant. For the first *BDNF* rs6265/rs11030101 variant pair, opposite effects were found. Children with *BDNF* rs6265 AG/AA genotypes had significantly lower FRI scores than those homozygous for the reference G allele ($p = 0.030$). On the contrary, children who carried one or two copies of the rs11030101 minor T allele displayed significantly higher FRI ($p = 0.009$) scores than children who showed the wild AA genotype, suggesting a protective effect.

**Table 3.** Scoring of each WISC-V index by genetic variant.

| | | VCI [a] | | VSI [b] | | FRI [a] | | WMI [b] | | PSI [a] | | FSIQ [b] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | Median (IQR) | *p* | Mean (SD) | *p* | Median (IQR) | *p* | Mean (SD) | *p* | Median (IQR) | *p* | Mean (SD) | *p* |
| ***BDNF* rs6265 (Dom)** | | | | | | | | | | | | | |
| GG | 61 | 108.0 (95.0-116.0) | 0.444 | 102.6 (12.9) | 0.920 | 106.0 (95.5-115.0) | **0.030** | 101.3 (14.7) | 0.605 | 83.0 (76.0-92.0) | 0.251 | 102.1 (12.0) | 0.338 |
| AG + AA | 41 | 106.0 (95.0-111.0) | | 102.3 (18.5) | | 100.0 (88.0-112.0) | | 102.8 (13.6) | | 89.0 (80.0-95.0) | | 99.6 (13.6) | |
| G | 161 | 106.0 (95.0-114.5) | 0.520 | 102.5 (14.5) | 0.967 | 106.0 (94.0-115.0) | 0.069 | 101.6 (14.4) | 0.572 | 86.0 (77.0-92.0) | 0.278 | 101.4 (12.4) | 0.476 |
| A | 43 | 106.0 (95.0-111.0) | | 102.4 (18.3) | | 100.0 (88.0-112.0) | | 103.0 (13.5) | | 89.0 (80.0-95.0) | | 99.9 (13.6) | |
| ***BDNF* rs11030101 (Dom)** | | | | | | | | | | | | | |
| AA | 35 | 103.0 (92.0-113.0) | 0.119 | 100.5 (13.5) | 0.355 | 94.0 (91.0-109.0) | **0.009** | 101.4 (13.1) | 0.805 | 86.0 (77.0-95.0) | 0.753 | 97.8 (11.5) | 0.056 |
| AT + TT | 67 | 108.0 (98.0-118.0) | | 103.5 (16.2) | | 106.0 (97.0-118.0) | | 102.2 (14.8) | | 83.0 (77.0-92.0) | | 102.8 (13.0) | |
| A | 124 | 106.0 (95.0-113.0) | 0.261 | 101.5 (14.8) | 0.277 | 103.0 (91.0-112.0) | **0.014** | 101.7 (13.7) | 0.808 | 86.0 (77.8-95.0) | 0.404 | 99.9 (12.5) | 0.101 |
| T | 80 | 108.0 (95.8-118.0) | | 103.9 (16.1) | | 106.0 (97.0-117.3) | | 102.2 (15.0) | | 83.0 (77.0-92.0) | | 102.9 (12.6) | |
| ***HTR2A* rs6314 (Dom)** | | | | | | | | | | | | | |
| GG | 83 | 103.0 (95.0-113.0) | 0.117 | 102.4 (15.4) | 0.960 | 106.0 (91.0-115.0) | 0.433 | 102.4 (14.4) | 0.507 | 86.0 (77.0-92.0) | 0.812 | 100.6 (13.0) | 0.425 |
| AG + AA | 19 | 111.0 (100.0-118.0) | | 102.6 (15.4) | | 106.0 (97.0-115.0) | | 99.9 (13.6) | | 83.0 (77.0-95.0) | | 103.2 (11.1) | |
| G | 183 | 106.0 (95.0-113.0) | 0.109 | 102.5 (15.4) | 0.942 | 106.0 (94.0-115.0) | 0.443 | 102.1 (14.3) | 0.536 | 86.0 (77.0-92.0) | 0.799 | 100.8 (12.8) | 0.385 |
| A | 21 | 111.0 (103.0-115.5) | | 102.2 (15.2) | | 106.0 (98.5-113.5) | | 100.1 (13.3) | | 89.0 (77.0-95.0) | | 103.4 (10.7) | |
| ***HTR2A* rs7997012 (Rec)** | | | | | | | | | | | | | |
| AA + AG | 56 | 108.0 (95.0-116.0) | 0.718 | 103.9 (16.0) | 0.310 | 106.0 (94.0-115.0) | 0.167 | 102.3 (14.4) | 0.739 | 83.0 (77.8-92.0) | 0.741 | 102.0 (13.0) | 0.426 |
| GG | 46 | 104.5 (98.0-113.0) | | 100.8 (14.5) | | 106.0 (91.0-112.0) | | 101.4 (14.1) | | 86.0 (77.0-95.0) | | 100.0 (12.3) | |
| A | 68 | 108.0 (95.0-115.3) | 0.734 | 104.6 (16.2) | 0.160 | 106.0 (94.0-115.0) | 0.202 | 101.9 (14.1) | 0.992 | 83.0 (77.0-92.0) | 0.858 | 102.7 (12.8) | 0.215 |
| G | 136 | 106.0 (95.0-113.0) | | 101.4 (14.8) | | 106.0 (91.0-112.0) | | 101.9 (14.3) | | 86.0 (77.0-92.0) | | 100.3 (12.5) | |
| ***MTHFR* rs1801133 (Dom)** | | | | | | | | | | | | | |
| GG | 39 | 106.0 (95.0-111.0) | 0.218 | 98.5 (14.5) | **0.038** | 103.0 (91.0-115.0) | 0.177 | 100.4 (14.5) | 0.388 | 86.0 (80.0-92.0) | 0.354 | 98.4 (12.5) | 0.087 |
| AG + AA | 63 | 108.0 (95.0-116.0) | | 104.9 (15.4) | | 106.0 (97.0-115.0) | | 102.9 (14.0) | | 83.0 (77.0-92.0) | | 102.8 (12.6) | |
| G | 127 | 106.0 (95.0-113.0) | 0.462 | 100.9 (15.5) | 0.061 | 103.0 (91.0-115.0) | 0.214 | 101.0 (14.4) | 0.243 | 86.0 (77.0-92.0) | 0.634 | 100.0 (12.8) | 0.060 |
| A | 77 | 108.0 (95.0-116.0) | | 105.1 (14.7) | | 106.0 (97.0-113.5) | | 103.4 (13.7) | | 83.0 (77.0-93.5) | | 102.9 (12.3) | |

**Table 3** (*continued*)

| | | VCI [a] | | VSI [b] | | FRI [a] | | WMI [b] | | PSI [a] | | FSIQ [b] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | Median (IQR) | *p* | Mean (SD) | *p* | Median (IQR) | *p* | Mean (SD) | *p* | Median (IQR) | *p* | Mean (SD) | *p* |
| ***OXTR* rs53576 (Rec)** | | | | | | | | | | | | | |
| AA + AG | 53 | 103.0 (95.0-113.0) | 0.283 | 103.0 (15.0) | 0.709 | 103.0 (91.0-110.5) | 0.078 | 100.8 (13.8) | 0.435 | 83.0 (77.0-92.0) | 0.941 | 99.6 (13.4) | 0.202 |
| GG | 49 | 106.0 (98.0-118.0) | | 101.9 (15.8) | | 109.0 (94.0-115.0) | | 103.1 (14.7) | | 86.0 (77.0-92.0) | | 102.8 (11.7) | |
| A | 60 | 106.0 (95.0-113.0) | 0.806 | 104.5 (16.6) | 0.215 | 103.0 (91.0-114.3) | 0.318 | 101.1 (13.4) | 0.606 | 86.0 (77.8-92.0) | 0.863 | 100.6 (13.8) | 0.694 |
| G | 144 | 106.0 (95.0-113.0) | | 101.6 (14.7) | | 106.0 (94.0-115.0) | | 102.2 (14.5) | | 86.0 (77.0-92.0) | | 101.3 (12.1) | |
| ***SLC6A2* rs998424 (Dom)** | | | | | | | | | | | | | |
| GG | 41 | 100.0 (95.0-112.0) | 0.211 | 102.1 (14.5) | 0.862 | 103.0 (91.0-113.5) | 0.363 | 100.7 (15.3) | 0.494 | 83.0 (77.0-92.0) | 0.368 | 99.7 (12.8) | 0.362 |
| AG + AA | 61 | 108.0 (95.0-116.0) | | 102.7 (16.0) | | 106.0 (94.0-115.0) | | 102.7 (13.5) | | 86.0 (80.0-93.5) | | 102.0 (12.5) | |
| G | 127 | 103.0 (95.0-113.0) | 0.111 | 102.8 (15.2) | 0.733 | 106.0 (91.0-112.0) | 0.155 | 101.9 (14.7) | 0.969 | 86.0 (77.0-92.0) | 0.687 | 100.5 (12.8) | 0.396 |
| A | 77 | 106.0 (96.5-116.0) | | 102.0 (15.6) | | 106.0 (94.0-116.5) | | 102.0 (13.4) | | 86.0 (77.0-95.0) | | 102.1 (12.4) | |
| ***SNAP25* rs363039 (Dom)** | | | | | | | | | | | | | |
| GG | 45 | 103.0 (92.0-111.0) | **0.026** | 102.1 (14.1) | 0.825 | 100.0 (91.0-107.5) | **0.012** | 99.7 (12.8) | 0.166 | 86.0 (80.0-93.5) | 0.512 | 98.8 (11.8) | 0.096 |
| AG + AA | 57 | 108.0 (98.0-117.0) | | 102.8 (16.4) | | 109.0 (94.0-118.0) | | 103.6 (15.1) | | 83.0 (77.0-92.0) | | 103.0 (13.1) | |
| G | 137 | 106.0 (95.0-113.0) | 0.082 | 102.1 (14.5) | 0.597 | 103.0 (91.0-112.0) | **0.004** | 100.8 (13.9) | 0.113 | 86.0 (80.0-92.0) | 0.239 | 100.1 (12.2) | 0.097 |
| A | 67 | 108.0 (95.0-118.0) | | 103.3 (17.0) | | 109.0 (94.0-118.0) | | 104.2 (14.6) | | 83.0 (77.0-92.0) | | 103.2 (13.3) | |
| ***NTRK2* rs2289656 (Dom)** | | | | | | | | | | | | | |
| GG | 70 | 108.0 (97.3-116.0) | 0.260 | 103.3 (16.6) | 0.406 | 106.0 (93.3-112.8) | 0.651 | 102.7 (15.0) | 0.436 | 84.5 (77.0-92.0) | 0.560 | 101.7 (13.4) | 0.456 |
| AG + AA | 32 | 104.5 (92.0-112.5) | | 100.6 (12.2) | | 106.0 (94.0-115.0) | | 100.3 (12.4) | | 87.5 (77.0-95.0) | | 99.7 (10.9) | |
| G | 167 | 106.0 (95.0-116.0) | 0.181 | 102.9 (15.9) | 0.372 | 106.0 (94.0-112.0) | 0.428 | 102.3 (14.6) | 0.394 | 86.0 (77.0-92.0) | 0.695 | 101.4 (13.0) | 0.414 |
| A | 37 | 103.0 (92.0-112.0) | | 100.4 (12.1) | | 106.0 (94.0-115.0) | | 100.1 (12.0) | | 86.0 (77.0-95.0) | | 99.6 (10.8) | |
| ***NTRK2* rs10868235 (Dom)** | | | | | | | | | | | | | |
| GG | 27 | 103.0 (93.0-113.0) | 0.291 | 97.6 (11.5) | 0.052 | 103.0 (91.0-112.0) | 0.407 | 99.3 (11.7) | 0.260 | 86.0 (77.0-95.0) | 0.921 | 97.5 (11.3) | 0.083 |
| AG + AA | 75 | 108.0 (95.0-116.0) | | 104.2 (16.2) | | 106.0 (94.0-115.0) | | 102.9 (15.0) | | 86.0 (77.0-92.0) | | 102.4 (12.9) | |
| G | 106 | 106.0 (95.0-113.0) | 0.405 | 100.8 (13.9) | 0.096 | 106.0 (91.0-112.0) | 0.453 | 101.1 (13.8) | 0.409 | 86.0 (77.0-92.0) | 0.875 | 100.0 (12.5) | 0.201 |
| A | 98 | 108.0 (97.3-113.0) | | 104.3 (16.6) | | 106.0 (94.0-115.0) | | 102.8 (14.6) | | 86.0 (77.0-92.0) | | 102.3 (12.7) | |

Dom: dominant model; Rec: recessive model. The bold indicates significant $p$ values < 0.05. [a]Mann–Whitney test. [b]Student's *t*-test.

This protective trend was maintained for other genetic variants. For example, children inheriting at least one copy of the variant allele of *MTHFR* rs1801133 G/A and *SNAP25* rs363039 G/A obtained better scores on the visual spatial ($p = 0.038$ for rs1801133), verbal comprehension, and fluid reasoning domains ($p = 0.026$ and $p = 0.004$ for rs363039, respectively).

Looking at these results, more significant differences in fluid reasoning scores were observed under the dominant model of *BDNF* rs6265/rs11030101 and *SNAP25* rs363039 variants (**Figure 1**).

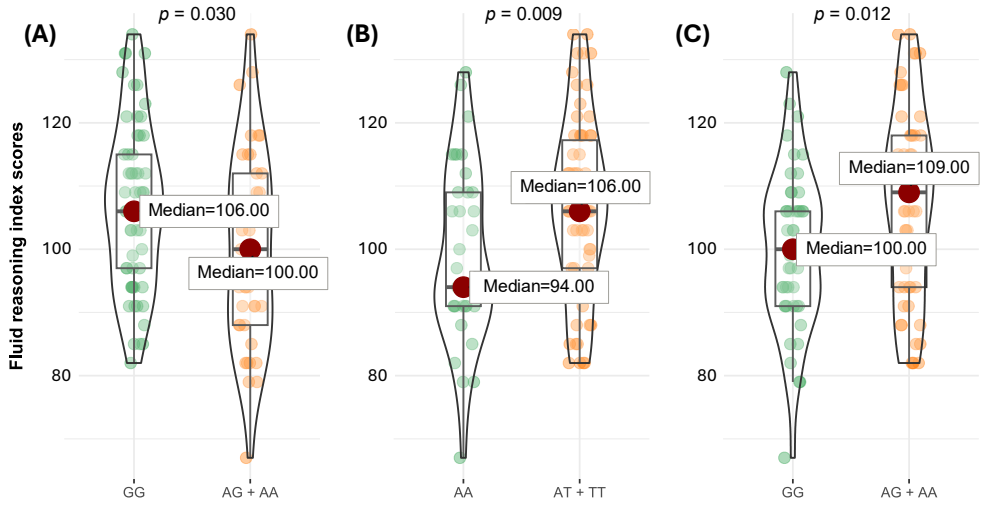



**Figure 1.** Fluid reasoning index scores obtained for (**A**) *BDNF* rs6265, (**B**) *BDNF* rs11030101, and (**C**) *SNAP25* rs363039.

### *3.3. Influence of genetic variants on the cognitive profile assessed by WISC-V according to dietary exposure to bisphenols*

Here, the contribution of each genetic variant to possible changes in cognitive function was addressed by dividing the population into groups with low and high exposure to bisphenols. When the dietary exposure factor was entered, highly significant associations between genetic polymorphisms and WISC-V indices were obtained, which were even stronger after adjustment for sex, age, BMI, and/or parental education levels as covariates. The SNP-by-bisphenol exposure interaction was also explored to verify if the effect of the variant depended on the magnitude of

exposure. Table 4 shows only the significant outcomes; the rest of the results are fully described in the Supplementary Material (**Table S1**).

**Table 4.** Influence of genetic polymorphisms on the cognitive profile assessed by WISC-V according to bisphenol exposure in children.

| | | **Unadjusted Logistic Regression Models** | | | | | | **Adjusted Logistic Regression Models** | | | | | | ***p*-int** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Low Exposure (≤Median) | | | High Exposure (>Median) | | | Low Exposure (≤Median) | | | High Exposure (>Median) | | | |
| SNP | Index | OR | 95% CI | *p* | OR | 95% CI | *p* | OR | 95% CI | *p* | OR | 95% CI | *p* | |
| ***BDNF* rs11030101 (Ref. AA)** | | | | | | | | | | | | | | |
| AA vs AT + TT (Dom) | VCI | 0.29 | 0.08-1.02 | 0.053 | 0.91 | 0.28-2.89 | 0.869 | 0.18[d] | 0.04-0.85 | **0.031** | 0.68[c] | 0.18-2.59 | 0.575 | 0.302 |
| Ref. A vs T | | 0.49 | 0.22-1.08 | 0.078 | 1.19 | 0.53-2.70 | 0.672 | 0.26[d] | 0.09-0.73 | **0.011** | 1.15[d] | 0.50-2.64 | 0.738 | 0.067 |
| ***HTR2A* rs6314 (Ref. GG)** | | | | | | | | | | | | | | |
| GG vs AG + AA (Dom) | VCI | 0.34 | 0.08-1.48 | 0.150 | 0.35 | 0.08-1.62 | 0.180 | 0.15[d] | 0.02-0.94 | **0.042** | 0.21[d] | 0.03-1.33 | 0.098 | 0.820 |
| Ref. G vs A | | 0.32 | 0.08-1.29 | 0.109 | 0.33 | 0.08-1.35 | 0.122 | 0.22[d] | 0.05-1.04 | 0.055 | 0.23[d] | 0.04-1.22 | 0.084 | 0.946 |
| ***HTR2A* rs7997012 (Ref. AA)** | | | | | | | | | | | | | | |
| AA + AG vs GG (Rec) | WMI | 3.96 | 1.23-12.73 | **0.021** | 0.46 | 0.14-1.49 | 0.193 | 6.30[d] | 1.38-28.73 | **0.017** | 0.27[d] | 0.06-1.26 | 0.096 | **0.002*** |
| Ref. A vs G | | 2.74 | 1.08-6.94 | **0.033** | 0.63 | 0.27-1.46 | 0.281 | 3.42[b] | 1.22-9.53 | **0.019** | 0.49[d] | 0.18-1.30 | 0.152 | **0.007** |
| ***MTHFR* rs1801133 (Ref. GG)** | | | | | | | | | | | | | | |
| GG vs AG + AA (Dom) | WMI | 0.28 | 0.09-0.91 | **0.034** | 0.75 | 0.21-2.67 | 0.657 | 0.24[c] | 0.06-0.92 | **0.038** | 0.55[d] | 0.11-2.78 | 0.467 | 0.272 |
| Ref. G vs A | | 0.31 | 0.13-0.73 | **0.007** | 1.18 | 0.52-2.69 | 0.689 | 0.28[c] | 0.10-0.73 | **0.010** | 1.20[a] | 0.49-2.93 | 0.686 | **0.026** |
| ***MTHFR* rs1801133 (Ref. GG)** | | | | | | | | | | | | | | |
| GG vs AG + AA (Dom) | FSIQ | 0.38 | 0.12-1.21 | 0.101 | 0.93 | 0.28-3.11 | 0.902 | 0.32[d] | 0.08-1.29 | 0.111 | 0.68[d] | 0.16-2.83 | 0.599 | 0.226 |
| Ref. G vs A | | 0.42 | 0.18-0.97 | **0.041** | 1.43 | 0.64-3.18 | 0.382 | 0.36[b] | 0.14-0.91 | **0.030** | 1.43[a] | 0.63-3.27 | 0.393 | **0.025** |
| ***OXTR* rs53576 (Ref. AA)** | | | | | | | | | | | | | | |
| AA + AG vs GG (Rec) | FRI | 0.49 | 0.15-1.61 | 0.238 | 0.26 | 0.08-0.86 | **0.028** | 0.69[d] | 0.17-2.80 | 0.600 | 0.20[d] | 0.05-0.78 | **0.020** | 0.315 |
| Ref. A vs G | | 0.74 | 0.29-1.91 | 0.531 | 0.53 | 0.23-1.26 | 0.152 | 0.99[d] | 0.34-2.89 | 0.981 | 0.51[a] | 0.21-1.21 | 0.126 | 0.370 |
| ***OXTR* rs53576 (Ref. AA)** | | | | | | | | | | | | | | |
| AA + AG vs GG (Rec) | WMI | 0.91 | 0.30-2.74 | 0.869 | 0.24 | 0.07-0.80 | **0.021** | 1.08[d] | 0.29-4.02 | 0.905 | 0.08[d] | 0.01-0.50 | **0.007** | **0.030** |
| Ref. A vs G | | 0.97 | 0.40-2.31 | 0.937 | 0.42 | 0.17-1.07 | 0.070 | 1.14[d] | 0.43-3.04 | 0.787 | 0.27[d] | 0.09-0.83 | **0.023** | 0.066 |

**Table 4** (*continued*)

| | | Unadjusted Logistic Regression Models | | | | | | Adjusted Logistic Regression Models | | | | | | *p*-int |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Low Exposure (≤Median) | | | High Exposure (>Median) | | | Low Exposure (≤Median) | | | High Exposure (>Median) | | | |
| SNP | Index | OR | 95% CI | *p* | OR | 95% CI | *p* | OR | 95% CI | *p* | OR | 95% CI | *p* | |
| ***SLC6A2* rs998424 (Ref. GG)** | | | | | | | | | | | | | | |
| GG vs AG + AA (Dom) | FRI | 1.68 | 0.50-5.66 | 0.403 | 0.18 | 0.05-0.60 | **0.006** | 2.14[d] | 0.53-8.64 | 0.285 | 0.16[c] | 0.04-0.57 | **0.005*** | **0.004*** |
| Ref. G vs A | | 1.36 | 0.58-3.20 | 0.476 | 0.30 | 0.13-0.71 | **0.006** | 1.35[a] | 0.56-3.26 | 0.500 | 0.26[c] | 0.11-0.65 | **0.004*** | **0.004*** |
| ***SNAP25* rs363039 (Ref. GG)** | | | | | | | | | | | | | | |
| GG vs AG + AA (Dom) | FRI | 0.62 | 0.19-2.02 | 0.430 | 0.19 | 0.06-0.68 | **0.010** | 0.55[b] | 0.16-1.94 | 0.353 | 0.17[b] | 0.04-0.63 | **0.008** | 0.124 |
| Ref. G vs A | | 0.58 | 0.24-1.40 | 0.226 | 0.27 | 0.11-0.64 | **0.003** | 0.45[d] | 0.16-1.26 | 0.128 | 0.28[a] | 0.12-0.68 | **0.005*** | 0.258 |
| ***SNAP25* rs363039 (Ref. GG)** | | | | | | | | | | | | | | |
| GG vs AG + AA (Dom) | WMI | 0.41 | 0.13-1.27 | 0.124 | 0.56 | 0.17-1.86 | 0.344 | 0.36[b] | 0.11-1.19 | 0.094 | 0.29[d] | 0.07-1.27 | 0.099 | 0.859 |
| Ref. G vs A | | 0.53 | 0.23-1.24 | 0.144 | 0.51 | 0.22-1.17 | 0.112 | 0.43[b] | 0.17-1.09 | 0.075 | 0.33[d] | 0.11-0.95 | **0.040** | 0.775 |
| ***SNAP25* rs363039 (Ref. GG)** | | | | | | | | | | | | | | |
| GG vs AG + AA (Dom) | FSIQ | 0.29 | 0.09-0.92 | **0.035** | 0.41 | 0.13-1.33 | 0.137 | 0.19[d] | 0.05-0.82 | **0.026** | 0.28[d] | 0.07-1.09 | 0.067 | 0.820 |
| Ref. G vs A | | 0.42 | 0.18-0.99 | **0.047** | 0.57 | 0.25-1.30 | 0.181 | 0.26[d] | 0.09-0.78 | **0.016** | 0.59[a] | 0.25-1.37 | 0.221 | 0.378 |
| ***NTRK2* rs2289656 (Ref. GG)** | | | | | | | | | | | | | | |
| GG vs AG + AA (Dom) | VCI | 3.43 | 0.99-11.93 | 0.053 | 0.96 | 0.29-3.24 | 0.951 | 9.06[c] | 1.51-54.39 | **0.016** | 0.96[d] | 0.23-3.91 | 0.951 | 0.088 |
| Ref. G vs A | | 2.97 | 1.05-8.44 | **0.041** | 1.11 | 0.38-3.27 | 0.844 | 6.72[d] | 1.82-24.83 | **0.004*** | 0.89[b] | 0.28-2.82 | 0.837 | 0.062 |
| ***NTRK2* rs10868235 (Ref. GG)** | | | | | | | | | | | | | | |
| GG vs AG + AA (Dom) | VCI | 0.26 | 0.07-0.98 | **0.046** | 0.79 | 0.21-2.95 | 0.730 | 0.22[d] | 0.04-1.08 | 0.062 | 2.09[d] | 0.41-10.72 | 0.377 | **0.043** |
| Ref. G vs A | | 0.53 | 0.24-1.17 | 0.117 | 0.93 | 0.42-2.04 | 0.854 | 0.46[b] | 0.19-1.13 | 0.090 | 1.40[d] | 0.58-3.37 | 0.458 | 0.094 |
| ***NTRK2* rs10868235 (Ref. GG)** | | | | | | | | | | | | | | |
| GG vs AG + AA (Dom) | VSI | 0.31 | 0.08-1.30 | 0.110 | 1.00 | 0.27-3.66 | 1.000 | 0.18[d] | 0.04-0.88 | **0.034** | 5.35[d] | 0.60-47.42 | 0.132 | **0.020** |
| Ref. G vs A | | 0.92 | 0.41-2.06 | 0.840 | 1.08 | 0.49-2.37 | 0.841 | 0.66[b] | 0.27-1.62 | 0.362 | 1.56[b] | 0.61-4.03 | 0.357 | 0.199 |

Ref: reference category; Dom: dominant model; Rec: recessive model; *p*-int: *p* for interaction.

Bold indicates significant *p* values < 0.05, and the asterisk (*) means significant *p* values after Bonferroni's correction ($p < 0.005$). [a]Adjusted for gender and age. [b]Adjusted for gender, age, and BMI. [c]Adjusted for gender, age, and parental education level. [d]Adjusted for gender, age, BMI, and parental education level.

Focusing on SNP pairs for *BDNF* and its receptor *NTRK2*, the *BDNF* rs11030101 variant T allele conferred protection against verbal comprehension dysfunction (adjusted OR = 0.26, $p = 0.011$, $p$ interaction = 0.067).

*NTRK2* SNPs showed a dual effect, where the rs2289656 G/A polymorphism proved to be a risk variant (adjusted OR = 6.72, $p = 0.004$, $p$ interaction = 0.062 for VCI), whereas rs10868235 developed a protective function in two cognitive aspects (adjusted OR = 0.22, $p = 0.062$, $p$ interaction = 0.043 for VCI; and adjusted OR = 0.18, $p = 0.034$, $p$ interaction = 0.020 for VSI), and the interaction was significant.

With regards to the serotonin signalling pathway, two variants within the *HTR2A* gene were explored and, once again, opposite associations were observed. The rs6314 G/A polymorphism seemed to confer a protective effect on poorer verbal comprehension at low exposure (adjusted OR = 0.15, $p = 0.042$, $p$ interaction = 0.820) In contrast, the rs7997012 A/G effect differed based on the exposure degree: a significant decline in working memory was appreciated at low exposure levels (adjusted OR = 6.30, $p = 0.017$), whereas a modest improvement was observed at high levels (adjusted OR = 0.27, $p = 0.096$). After Bonferroni's correction, strong interaction evidence ($p$ interaction = 0.002) resulted from this differential effect.

For the *MTHFR* rs1801133 G/A polymorphism in the per-allele contribution model, the presence of the variant A allele was associated with a reduced likelihood of cognitive dysfunctions than the presence of the reference G allele at a low exposure dose (adjusted OR = 0.28, $p = 0.010$, $p$ interaction = 0.026 for WMI; and adjusted OR = 0.36, $p = 0.030$, $p$ interaction = 0.025 for FSIQ).

Lastly, a protective role was observed for the genetic variants *OXTR* rs53576 A/G (adjusted OR = 0.08, $p = 0.007$, $p$ interaction = 0.030 for WMI) and *SLC6A2* rs998424 G/A (adjusted OR = 0.16, $p = 0.005$, $p$ interaction = 0.004 for FRI) in terms of high exposure. After Bonferroni's correction, the association and interaction persisted for *SLC6A2* rs998424. Other genetic variants, such as *SNAP25* rs363039 G/A, maintained their remarkable protective function independently of the exposure, resulting in a non-significant interaction ($p$ interaction of 0.258, 0.775, and 0.378 for FRI, WMI and FSIQ, respectively).

**Figure 2** highlights the associations and interactions obtained mainly for the verbal comprehension, working memory, and fluid reasoning domains.

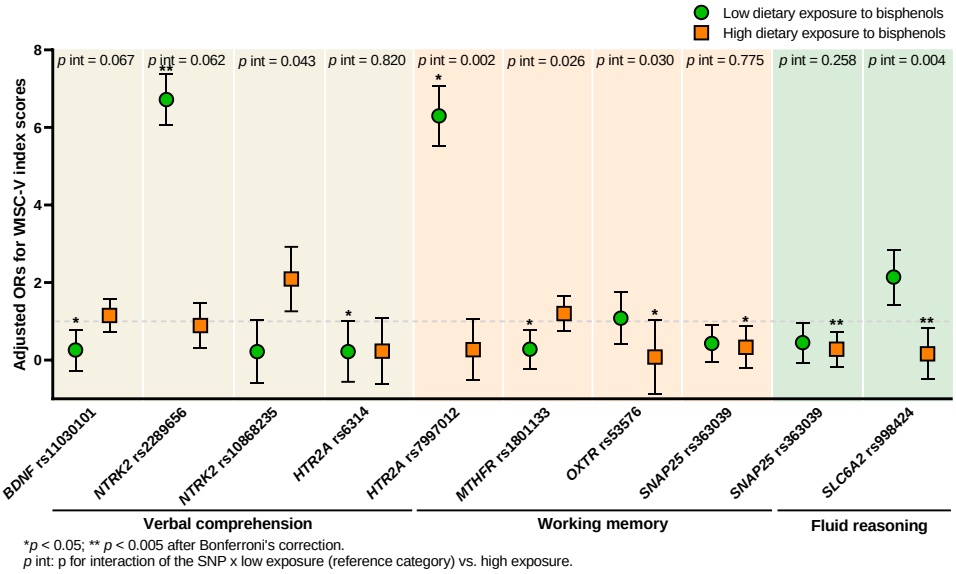



**Figure 2.** Influence of genetic polymorphisms on specific cognitive domains based on the level of bisphenol exposure.

## 4. Discussion

As far we know, our findings suggest for the first time that neurodevelopment-related gene polymorphisms play an important role in cognition measured through WISC-V in Spanish children exposed to dietary bisphenols. The main outcomes of the current research included the following aspects: (1) significant differences in fluid reasoning scores were observed mainly for *BDNF* rs6265/rs11030101 and *SNAP25* rs363039 variants, and (2) consistent associations of *BDNF* rs11030101, *NTRK2* rs2289656/rs10868235, *MTHFR* rs1801133, *HTR2A* rs7997012, *OXTR* rs53576, and *SLC6A2* rs998424 with certain cognitive domains and global intelligence index were obtained in the presence of bisphenol exposure, resulting in relevant SNP–bisphenol interactions.

Gene polymorphisms selected for this study are located in genes responsible for key neurodevelopmental processes, and it is well known that NDDs such as ADHD, ASD, and ID are genetically linked through common genetic alterations [6].

*BDNF* and its receptor tropomyosin receptor kinase B (TrkB), encoded by the *NTRK2* gene, are an essential regulatory system for neuronal development, synaptogenesis, and plasticity [25]. The possible involvement of *BDNF* in cognitive dysfunction was observed in children with ID showing reduced BDNF protein levels [26]. Furthermore, it has been evidenced that *BDNF* and *NTRK2* variants are associated with changes in hippocampal volume and altered performance on learning and memory tasks [25,27]. *BDNF* rs6265 (Val66Met) is one of the most extensively studied missense variants within the prodomain region of *BDNF*, with functional consequences for memory, cognition, and behaviour [12].

In our study, rs6265 variant A allele carriers had lower scores on the fluid reasoning domain, whereas children with the rs11030101 T allele experienced a better scenario for this cognitive component. These polymorphisms have been reported to be associated with other psychiatric and neurological disorders like major depressive disorder (MDD) [28,29], schizophrenia, or epilepsy [30]. However, no associations were found with cognitive outcomes [31,32].

Our gene–environment association analysis revealed interactions between variants in the *BDNF-NTRK2* system, such as rs10868235, and exposure to bisphenols in the context of verbal comprehension and visual spatial skills. Although there are no studies assessing interactions between these SNPs and dietary contaminants in neurodevelopment, some evidence suggests that BPA may interfere with the *BDNF* signalling pathway, leading to behavioural and cognitive impairments [33,34].

Like the *BDNF-NTRK2* system, *MTHFR* and *SNAP25* are involved in brain development and synaptic plasticity, respectively [35,36]. Firstly, proper folate metabolism is required for normal brain development, and so disruptions in this process may contribute to neurological disorders [35]. MTHFR is a key folate metabolism enzyme, whose deficiency has been correlated with common variants like rs1801133 (C677T) and rs1801131 (A1298C) [37]. We found that the presence of the variant A allele of the rs1801133 G/A polymorphism was linked to higher scores for working memory and FSIQ at a low bisphenol exposure dose (Table 4).

This finding makes sense given the peculiar U-shaped dose–response curve followed by bisphenols, indicating the importance of investigating effects at both low and high exposure levels. In line with our result, the rs1801133 A allele was also found to attenuate the negative effect of *COMT* Val homozygosity on IQ in patients with schizophrenia [38]. A meta-analysis by Sun et al. (2021) did not find associations between this *MTHFR* SNP and mild cognitive impairment [39]. At the level of gene–environment interactions, possible connections of bisphenols with disrupted *MTHFR* metabolic functions have not yet been established.

For its part, the *SNAP25* gene is involved in synaptic plasticity, neuronal maturation, and neurotransmission [36]. In children with borderline intellectual functioning, *SNAP25* polymorphisms were associated with lower scores for the perceptual reasoning index and FSIQ [36]. In the present child population, the *SNAP25* rs363039 G/A variant maintained its protective function in fluid reasoning, working memory, and overall IQ, independent of the exposure. In agreement with this finding, the A allele of rs363039 was reported to be beneficial for working memory in individuals with ADHD [40].

On the other hand, we have also focused on genetic changes at the level of neurotransmitter systems (*HTR2A, OXTR,* and *SLC6A2*). The serotonin 2A receptor, encoded by the *HTR2A* gene, is located in brain regions essential for learning and cognition. In fact, polymorphisms within this gene, such the rs6314 (His452Tyr), have been associated with altered memory processes [41]. Consistent with this, we found that the *HTR2A* rs7997012 A/G variant was related to altered working memory at low bisphenol exposure, whereas the opposite effect was modestly observed at high levels, resulting in a strong interaction. This finding shed light that genetics interact with a dynamic environment, leading to differential effects depending on the exposure level. Conversely, the other variant, *HTR2A* rs6314, maintained its protective role against poorer verbal comprehension independent of the exposure level. Until now, evidence from animal studies has demonstrated that mixtures of EDCs, including BPA, could impair mouse behaviour by modifying the brain expression of *Htr1a* and *Htr2a* [42].

Another variant that showed a protective effect on working memory was the *OXTR* rs53576 A/G polymorphism in children with high bisphenol levels. This polymorphism is located in the gene encoding the receptor for oxytocin, a neuromodulator involved in forming social, working, spatial, and episodic memory [43]. *OXTR* rs53576 has been proven to be associated with poorer social cognition in children but also with protective social traits, such as prosocial and empathic behaviour [44–46]. Meanwhile, the *OXTR* rs53576–bisphenol interaction found in our study could make sense from in vivo studies. Here, perinatal exposure to BPA, alone or in a mixture, alters oxytocin and *OXTR* expression in a sex- and region-specific manner [42,47].

Finally, *OXTR* rs53576 also showed protection for fluid reasoning, but the interaction was not significant. However, a strong interaction was obtained for the *SLC6A2* rs998424 G/A variant. Polymorphic variants in this gene coding for the norepinephrine transporter have been implicated in ADHD-related impairments, such as altered intrinsic brain activity, visual memory, and attention in children [48–50]. As aforementioned, BPA exposure may affect the serotonergic and oxytocin systems in the brain, but the effects on the norepinephrine system remain unclear.

One limitation of our study was the sample size. Although this is a limitation of several genetic association studies [36,45,51], the insightful findings of our small-scale study highlight the value of further larger studies to replicate and validate the results. It is well established that adverse neurodevelopmental effects of bisphenols are more pronounced in early age [13]. To date, evidence of the effects of childhood BPA exposure on cognitive function remain inconclusive [15,16]. One study addressed associations of urinary BPA concentrations with WISC-IV scores at different ages [15], while another study used age as an adjusting variable [16]. Given the limited sample size, it was not possible to perform an age-stratified analysis, but the regression models were adjusted for age to minimise potential confounding effects.

An additional limitation is that the particular effect of each SNP varies depending on which allele is designated as the “risk” allele. This is the reason why

contradictory results can be obtained between different studies for the same genetic variant. Furthermore, the study design (neurodevelopment assessment tool, ethnic heterogeneity, and selected study population) could explain the inconsistencies between studies. There are several non-dietary sources of human exposure to bisphenols, which were not considered for the purpose of this study; however, the largest contribution to total exposure comes from food intake, accounting for more than 90%, confirming that a dietary exposure assessment is the first step in addressing the bisphenol-associated health problems [52].

The main strength of the current study lies in providing insightful evidence on the influence of genetic polymorphisms on childhood cognitive function in the presence of exposure to bisphenols. Firstly, carriers of the *BDNF* rs11030101 T and *SNAP25* rs363039 A alleles obtained better scores on the fluid reasoning domain, except for those inheriting the *BDNF* rs6265 A allele, who had lower scores. In comparison with previous WISC versions, in WISC-V, the perceptual reasoning domain is divided into FRI and VSI, and the fluid reasoning domain could be a good indicator of intellectual functioning, as we have shown [53].

Secondly, we reported relevant SNP–bisphenol interactions in certain cognitive domains. Genetic variants in genes responsible for vital neurodevelopmental processes, such as brain development and synaptic plasticity (*BDNF* rs11030101, *NTRK2* rs2289656 and rs10868235, and *MTHFR* rs1801133) and neurotransmission (*HTR2A* rs7997012, *OXTR* rs53576, and *SLC6A2* rs998424) presented consistent associations with verbal comprehension, working memory, and fluid reasoning. The effects on these cognitive abilities depended on the level of exposure to bisphenol. Two aspects need to be highlighted here. (1) Genetics interact with an environment that is constantly changing, and for this reason the study of gene–environment interaction gives us a more complete answer to disease aetiology [54]; (2) EDCs, including bisphenols, follow a particular dose–response curve, with optimal effects at low doses, and so it is important to assess effects at low concentrations [55]. Additionally, (3) working memory is a cognitive domain involved in many aspects of neurodevelopment, and given the significance found in this area, we support considering the selected SNPs as genetic markers of cognitive alterations in

individuals with NDDs. Similarly, the Weschler Intelligence Scales are the most widely used instruments for measuring cognitive function, and the latest version, the WISC-V, has undergone changes that may make it more reliable for assessing cognitive dysfunction in the etiopathogenesis of NDDs [53,56].

## 5. Conclusions

In conclusion, our findings demonstrate that SNPs related to brain development, synaptic plasticity, and neurotransmission are associated with differences in cognitive domains assessed by WISC-V, specifically fluid reasoning, verbal comprehension and working memory, in children exposed to bisphenols, revealing important SNP–bisphenol interactions. The exploration of gene–environment interactions could lead to a better understanding of the multifactorial and polygenetic aetiology of NDDs. For this reason, and in view of the lack of studies assessing the combined effects of genetic variability and exposure to bisphenols on cognitive function, we support considering them as interactive factors rather than individual contributors to NDDs.

**Supplementary Materials**. The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/nu16162639/s1, Table S1. Influence of genetic polymorphisms on the cognitive profile of children assessed by WISC-V according to bisphenol exposure.

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# Health risk assessment of exposure to bisphenol A on a Spanish population sample

**Viviana Ramírez[a,b,c,1], Virginia Robles-Aguilera[a], Yolanda Gálvez-Ontiveros[a,b,c], Patricia González-Palacios[a,c], Ulrike Bernauer[d], Caterina Curato[d], Andrea Gall[d], Matthias Herzler[d], Katherina Siewert[d], Patrick Tarnow[d], Achim Trubiroha[d], Sebastian Zellmer[d], María Jesús Alvarez-Cubero[e], Claudia Lorenz[d,*,1], Celia Monteagudo[a,b,*,1], Alberto Zafra-Gómez[b,c,f,1], Thomas Tietz[d,1], Ana Rivas[a,b,c,1]**

[a] Department of Nutrition and Food Science, Faculty of Pharmacy, University of Granada, Granada, Spain

[b] Instituto de Investigación Biosanitaria ibs.GRANADA, Granada, Spain

[c] Institute of Nutrition and Food Technology "Jose Mataix Verdú," Biomedical Research Center, University of Granada, Granada, Spain

[d] German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany

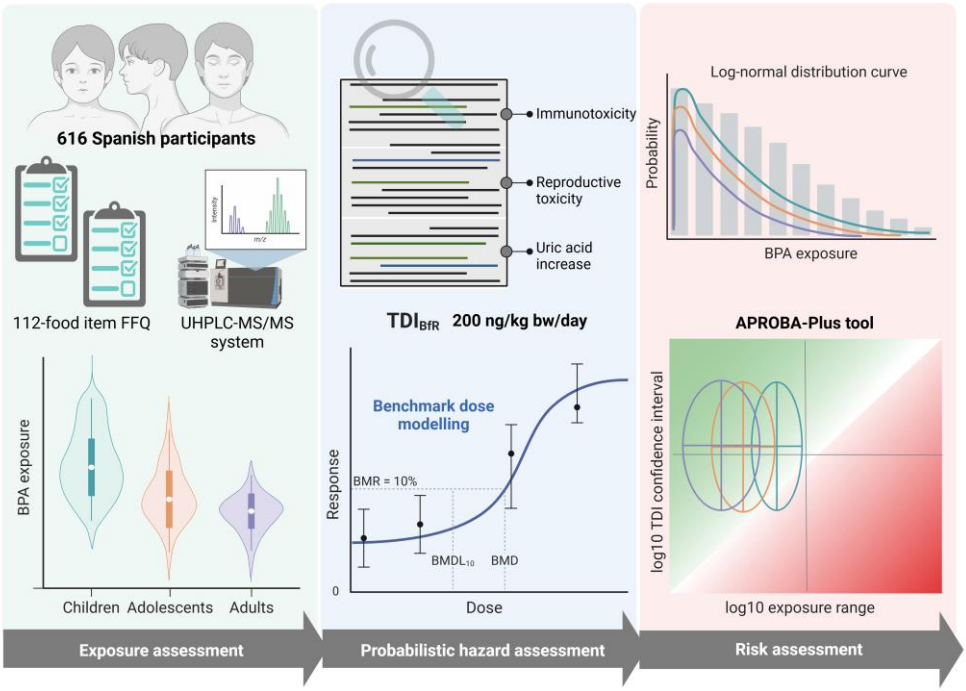
[e] Department of Biochemistry and Molecular Biology III and Immunology, Faculty of Medicine, University of Granada, Granada, Spain.

[f] Department of Analytical Chemistry, University of Granada, Granada, Spain.

[1] These authors contributed equally to the work.

*Corresponding author. E-mail address: Claudia. Lorenz@bfr.bund.de (C.Lorenz); celiams@ugr.es (C.Monteagudo).

## Graphical abstract




## Abstract

Bisphenol A (BPA) is a chemical compound used as a raw material in the manufacture of plastic food contact materials (FCM) made of epoxy resins and polycarbonate plastics. This study aimed to contribute to a reliable estimate of dietary BPA exposure using age-specific daily consumption data representative of the Spanish population and to compare it to the BfR-derived provisional tolerable daily intake (TDI) to perform a comprehensive risk assessment. A total of 213 children (3-9 years), 281 adolescents (10-17 years), and 122 adults (18-39 years) were included for the current risk assessment. In a probabilistic approach, exposure data were transformed into a log-normal distribution and combined with hazard characterisation data using the APROBA-Plus tool. Based on reproductive toxicity as a critical endpoint, the BfR derived a TDI of 200 ng/kg bw/day. Significant differences in total BPA exposure were observed between the age groups ($p$=1.53e-41 for lower bound (LB); $p$=1.49e-42 for upper bound (UB)). Median exposure in children (201.7 (LB) to 211.3 (UB) ng/kg bw/day) was slightly higher than the BfR's TDI. Canned tuna turned out to be the major contributor to dietary BPA exposure in

the three age groups, with 44.7-46.1% in children, 46.9-49% in adolescents, and 54.7-57.7% in adults. The probabilistic risk characterisation showed that a health risk is not negligible for at least parts of children. These findings provide evidence that BPA exposure close to the BfR's TDI may be of particular concern for the child population and may serve as a basis for risk assessment.

**Keywords**: Bisphenol A; Dietary exposure; Food contact materials; Children; Adolescents; Adults

## 1. Introduction

Bisphenol A (BPA) is a synthetic chemical compound commonly used as a basic component for the industrial production of containers for storing food and drinks made of epoxy resins and polycarbonate plastics (Dualde et al. 2019). The growing demand of BPA by the plastic manufacturing industries since 1950 and its extensive food- and non-food related applications have led to the ubiquitous and continuous presence of BPA in environmental matrices and human body fluids (Akash et al. 2020; Lee et al. 2018; Wang et al. 2022).

BPA intake from food has been described as the main contributor to overall human exposure, followed by dermal absorption and air inhalation (Rubin et al., 2019). BPA is included in the Union list of Commission Regulation (EU) No 10/2011 as a monomer in the production of plastic food contact materials (FCM) (European Commission 2018). The European Food Safety Authority (EFSA) identified FCM as the main source for BPA entering the food chain (EFSA 2015a). In particular, can linings made from epoxy resins contribute to dietary exposure (Wang et al. 2023). However, also food products that are not canned or packed in plastic can contain BPA, showing that contamination could occur at different stages across the farm-to-fork production chain, beyond the packaging (González et al. 2020).

Based on animal and human evidence, BPA exposure has been associated with a wide spectrum of adverse effects on human health. Since 2016, it is classified under CLP as toxic to reproduction (European Commission 2008). In consequence, it was identified as substance of very high concern (SVHC) under the REACH Regulation

in 2017 (European Commission 2006). The endocrine disrupting properties of BPA on human health and the environment have been the reasons for its reidentifications as SVHC in 2017 and 2018, respectively. Moreover, multiple effects have been reported including changes in the kidney and liver, immunological and metabolic changes, developmental toxicity, as well as neoplastic effects (EFSA 2023). In general, the available body of literature on BPA is extensive. However, this situation, which is actually desirable in regulatory terms, also means a great variety and variability of the reported (non-) effects.

In April 2023, EFSA published a re-evaluation of the risks of BPA to public health (EFSA 2023). In it, a new health-based guidance value (HBGV) was derived based on effects on the immune system, which were identified as the most sensitive endpoint. Compared to the 2015 scientific opinion by EFSA, the tolerable daily intake (TDI) was significantly reduced from 4.000 ng per kg body weight (bw) per day to 0.2 ng/kg bw per day (EFSA 2015a; EFSA 2023). The CEP Panel concluded that there is a health concern from BPA exposure for all age groups. The German Federal Institute for Risk Assessment (BfR) and other regulatory agencies do not support this new TDI due to several scientific and methodological divergences which is amongst others reflected in several critical comments during the public consultation on the EFSA opinion and joint publications of EFSA with European Medicines Agency (EMA) and BfR, respectively (BfR 2022; BfR 2023; EFSA 2023; EMA 2023). The BfR has derived another TDI of 200 ng/kg bw per day, which is 20 times lower than the previous temporary TDI derived by EFSA in 2015 (BfR 2023). However, since current dietary exposure data were not available at this time, BfR did not conclude on the risk of BPA exposure.

In hazard assessment, TDI derivation is usually done in a deterministic approach by dividing a suitable point of departure (PoD), such as a No Observed Adverse Effect level (NOAEL) or a Benchmark Dose Lower Confidence Limit (BMDL), by respective assessment and uncertainty factors (e.g. for intra- and inter-species extrapolation, study time correction etc.). However, a single standard assessment factor cannot be adequate for a huge number of substances. In order to be protective in most cases, i.e. for most chemicals, standard assessment factors are

chosen very conservatively and reflect a worst-case assumption in each case. If – like for BPA – a vast amount of studies exist, a single point assessment factor will most likely not be representative for the whole database (e.g. on toxicokinetics in different species). In such a case, one is tempted to use a conservative approach for each and every assessment factor, thereby omitting a significant percentage of study results. Consequently, the deterministically derived TDI which results from the multiplication of several conservative individual values, is more of an overestimating worst-worst-case estimate and thus more conservative than desired or necessary (WHO IPCS 2018). In contrast, the probabilistic approach calculates for each aspect of hazard assessment uncertainty distributions which are finally combined to an overall probability distribution of the position of the true TDI value. In doing so, the probabilistic approach transparently shows the uncertainty of the assessment in the form of an exposure range in which the real TDI lies, whereas the deterministic approach derives an exact TDI that suggests a grade of precision, which is most likely not justified. When deriving a TDI for BPA, BfR applied a probabilistic approach (WHO IPCS 2018), thereby combining log-normal distributions for the individual uncertainty and assessment factors.

A reliable risk assessment of a substance requires current exposure data. EFSA did not update the dietary exposure estimates from 2015 but compared its newly derived TDI with data mainly collected before 2012 (EFSA 2015a). However, due to several regulatory measures since 2012 as well as technical improvements it is very likely that the current BPA exposure of consumers has declined significantly (Boon et al. 2017; Sirot et al. 2018). The present study aims to contribute to a reliable estimate of BPA exposure from the diet using age-specific data on the daily dietary intake of the Spanish population as representatives. Afterwards, the exposure estimates were compared to the BfR-derived TDI to perform a comprehensive risk assessment. In a probabilistic approach, the exposure data were transferred to a log-normal distribution and combined with the data on hazard characterisation. The results are visualised and discussed with respect to possible health risks.

## 2. Materials and methods

### *2.1. Study population*

Participants enrolled in this study were part of previous larger research projects funded by the Instituto de Salud Carlos III (Ministry of Health, Spain) and EFSA. Adolescents and adults were recruited from high schools located in Talavera de la Reina (Toledo, Spain) between 2017 and 2018 (Monteagudo et al. 2021; Robles-Aguilera et al. 2021). Children were recruited from different health and educational centres in Granada (Spain) between 2020 and 2023 (Gálvez-Ontiveros et al. 2023; Moscoso-Ruiz et al. 2023). All subjects participating in the research projects provided a written informed consent. The study protocol was approved by the Ethics Committees of the University of Granada.

For the exposure assessment, anthropometric (height and weight) and dietary BPA data were considered. The age groups for children, adolescents and adults were defined according to the Spanish Dietary Datasets ENALIA 1 (National Dietary Survey on Children and Adolescents) and ENALIA 2 (National Food Survey on Adults, the Elderly and Pregnant Women) which are included in the EFSA Comprehensive European Consumption Database. A total of 213 children aged 3 to 9 years, 281 adolescents aged 10 to 17 years, and 122 adults aged 18 to 39 years were included for the current risk assessment.

### *2.2. Exposure assessment*

Assessment of dietary exposure to BPA is needed to provide accurate estimations and to identify potential food sources contributing most to overall exposure. Herein, total dietary exposure to BPA was estimated on an individual basis by multiplying the daily food consumption (g/day) of each food item by its corresponding BPA content (ng/g of food) and dividing this value by the body weight in kg for each participant (ng/kg bw per day).

Data on food consumption (g/day) for the last 12 months were obtained from a semi-quantitative food frequency questionnaire (FFQ) completed by each respondent in a face-to-face interview by trained nutritionists. In case of children under 18 years of age, the questionnaire was answered by parents or legal tutors. According to the method previously described by (Robles-Aguilera et al. 2021), FFQ was designed to ask about the consumption frequency of 96 food items grouped as

dairy products, meat and meat products, vegetables, legumes, and cereals, among others. The consumption frequency was categorised as never or hardly ever, 1-3 times per month, once a week, 2-4 times per week, 5-6 times per week, once a day, 2-3 times per day, 4-6 times per day, and more than 6 times per day. It was also specified the portion size (g/serving) based on the recommended amounts of each food group for the Spanish population (Monteagudo et al. 2021). Methodology pertaining to sample analysis and determination of BPA concentrations in the selected foods was previously published (Galvez-Ontiveros et al. 2021).

BPA content (ng/g) was quantified using the ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) system (Galvez-Ontiveros et al. 2021). For left-censored data, lower bound (LB) and upper bound (UB) substitution methods were applied to samples with concentrations below the limit of detection (LOD) or quantification (LOQ). In the LB approach, concentrations of BPA below the LOD (not detected) were assigned a value of zero and those below the LOQ (detected but not quantified) were replaced by the LOD. For UB method, these left-censored data were handled through the substitution of LOD and LOQ, respectively. In this way, BPA exposure was estimated. BPA occurrence in foods was combined with the corresponding consumption levels obtaining the daily BPA intake (ng/day). For all individuals, BPA intake from all food items was summed to estimate the total BPA exposure adjusted for body weight (ng/kg per day). From this deterministic exposure assessment, results are displayed for both the average (mean and standard deviation (SD); median and interquartile range (IQR)) and the high exposure scenarios ($90^{th}$ (P90) and $95^{th}$ (P95) percentiles). The Kruskal-Wallis test was applied to compare the estimated daily BPA intake between the three age groups. Holm-Bonferroni method was used as adjustment method for *p*-values for multiple comparisons.

Additionally, food items were grouped in accordance with the EFSA Food Classification to identify the main contributors to the total BPA exposure (EFSA 2015b). All statistical analyses were performed with RStudio 2023.12.0.

### *2.3. Hazard identification and characterisation*

A detailed description of the methodological approach is published in the BfR opinion on BPA (BfR 2023). Briefly, a literature screening was performed to identify new data that could call into question the provisional TDI derived by EFSA in 2015 (EFSA 2015a). Thereby, the focus was set on the three most relevant, i.e. sensitive toxicological endpoints identified by EFSA in its 2023 opinion (EFSA 2023). 139 and 1905 studies were recorded focussing on immunotoxicity and reproductive toxicity of BPA, respectively (**Figure 1**). There were reports of only four studies on the third most sensitive endpoint according to EFSA, "increased uric acid levels". All study reports revealed by systematic literature screening were verified with reference to relevance and methodology.

As a result, 26 and 529 articles on immunotoxicity and reproductive toxicity, respectively, were considered in the assessment. The studies on the relationship between BPA and changes of uric acid levels did not meet the quality requirements and were therefore not considered. The remaining studies were further sorted into three Tiers reflecting the respective weight of evidence. In doing so, value was placed on exposure characterisation, study design and traceability (for details see (BfR 2023)). Study reports with assigned Tier 3 were considered only qualitatively, whereas studies assigned as Tier 1 and 2 were used for quantitative hazard characterisation. Data were extracted directly (where given) or from images using WebPlotDigitizer (https://automeris.io/). Each data set was analysed for a dose-response relation by benchmark dose (BMD) modelling to determine the PoD to derive a TDI.

For the endpoints in reproductive toxicity, a benchmark response (BMR) of 10% was applied. For the immunological effects a BMR of 100% was used. Calculation of BMDs and upper (P95) and lower (P05) confidence/credible limits (BMDU/Ls), respectively, was performed in accordance with the latest EFSA guidance on BMD modelling (EFSA 2022) by applying Bayesian or standard model averaging and using BMD-tools provided by EFSA (for details see (BfR 2023)). The results were checked for criteria recommended by EFSA for the use of BMDLs as a

PoD (EFSA, 2022). If no suitable BMDL could be identified, the NOAEL or LOAEL was used as PoD for TDI derivation. After reviewing, 112 PoDs were derived from all Tier 1 and 2 studies and respective data sets suitable for dose-response analysis (BfR 2023). Of the 112 values, 61 were related to male reproductive endpoints, 34 to female reproductive endpoints and 17 to immunological aspects.

The derived PoDs were submitted to a probabilistic hazard assessment according to the approach proposed by WHO IPCS (2018) to calculate individual TDI values from every PoD (WHO IPCS 2018). In contrast to the deterministic hazard assessment approach typically applied, for a probabilistic assessment uncertainty and assessment factors are interpreted as log-normal distributions. For example, the credible or confidence interval around the BMD calculated via BMD modelling is interpreted as a log-normal distribution characterised by the BMDL as $5^{th}$ percentile, the BMDU as $95^{th}$ percentile, and the BMD as median. Afterwards, the derived log-normal distributions for a specific PoD were combined to yield a log-normal distribution for the individual TDI, including 90 percent confidence interval. Thus, TDI derivation and uncertainty assessment were transparently combined, all available study data was included in the derivation of the distributions for the assessment factors, and over-conservatism was avoided. Assessment factor distributions were used to correct for different PoDs (BMDL, NOAEL, LOAEL), interspecies toxicokinetics and remaining uncertainties, inter-human toxicokinetic and toxicodynamic differences, and study duration. Conservative assumptions and a goal to protect at least 99% of the population with at least 95% certainty were used as suggested by WHO IPCS (2018).

Finally, the lowest lower confidence limit from all studies was selected as an overall TDI. For more details, please refer to BfR opinion (BfR 2023).

As discussed extensively elsewhere, effects on immunological biomarker were assessed as not suitable for quantitative hazard characterisation (BfR 2023). Nevertheless, every PoD, including those related to the immune system, was processed through the probabilistic hazard assessment to estimate conservatism of the finally selected TDI.

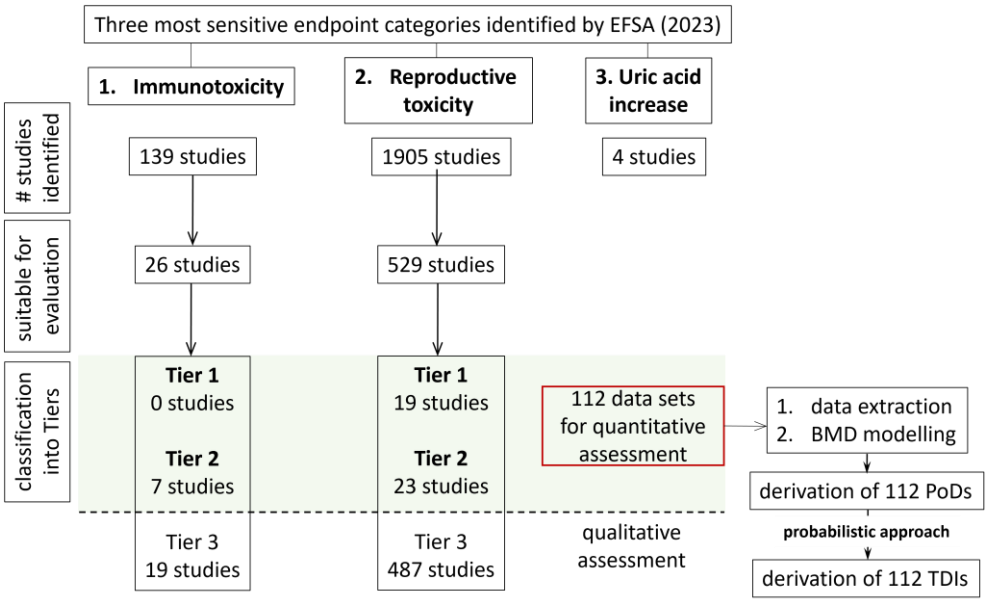



**Figure 1**. Flow-chart illustrating the approach of hazard identification and characterisation. A detailed description is given in BfR opinion on BPA (BfR 2023).

### *2.4. Risk characterisation*

The APROBA-Plus tool, which combines the output from the probabilistic hazard characterisation with the probabilistic exposure estimation, was applied to perform a comprehensive probabilistic risk assessment (Bokkers et al. 2017). Since lognormal distributions are used for the probabilistic risk assessment, overall exposure to BPA was fitted into lognormal distributions for each age group. Then, the 5$^{th}$ and 95$^{th}$ percentiles of the log-normal distribution were calculated as lower (LCL) and upper (UCL) confidence limits, representing the range of the exposure in the respective age group. In the last step, risk was characterised graphically by plotting the uncertainty range for the TDI against the range for the exposure.

## 3. Results

### *3.1. Hazard identification and characterisation*

Literature screening revealed some effects of BPA exposure on immunological biomarker in mice including changed TH17 frequency and related alterations, such as changed IL17 serum levels, or increased level of specific immunoglobulin E (IgE) in allergic lung inflammation. However, the reported effects were classified as

intermediate endpoints without a causal link to an apical endpoint. So far, there is no adverse outcome pathway (AOP) established and the transferability of effects in mice to humans is unclear. In BfR opinion on BPA (BfR 2023), multiple points of criticism are discussed extensively including effect size, study quality and adversity.

In summary, BPA effects on immunological biomarker were assessed as not suitable for the TDI derivation. Nevertheless, to assess to what extent immunological changes – should they occur in humans – would be covered by the final TDI, all available data sets of immunological studies were analysed for dose-response relation and processed through the probabilistic hazard assessment. Regarding reproductive toxicity, various effects on the male and female reproductive system were recorded, including for example impacts on sperm count, motility and morphology or ovary/ uterus histology and oestrous cycle, respectively. Overall, a wide range of effective exposure levels, spanning several orders of magnitude, was noted which was also reflected in the 95 individual TDI values that were calculated based on the respective endpoint. Data assessment identified a reduction of sperm count following oral BPA exposure as the most sensitive endpoint. The effect was consistently seen in rats, mice and rabbits, though at highly differing doses (compare **Figure 2**). Although studies in other strains or species showed relatively high effect levels, the only two studies available in Wistar rats reported LOAELs as low as 200 and 500 µg/kg bw/day after 60 and 90 days of BPA exposure, respectively (Liu et al. 2013; Srivastava and Gupta 2018).

It is not known whether or not possible inter-species and inter-strain differences may account for the highly differing results. However, for conservatism the BfR considers the effect seen at relatively low doses in Wistar rats relevant for humans. Based on the available data from Liu et al. (2013) and Srivastava and Gupta (2018), two respective PoDs were derived – a $BMDL_{10}$ of 26 µg/kg bw/day and a NOAEL of 50 µg/kg bw/day, respectively (BfR 2023). The results were submitted to the probabilistic hazard assessment described above. From the two studies, a 90% confidence interval for the final TDI ranging from 0.14 µg/kg bw/day (LCL from (Liu et al., 2013)) to 77.8 µg/kg bw/day (UCL from (Srivastava and Gupta 2018)) was calculated.

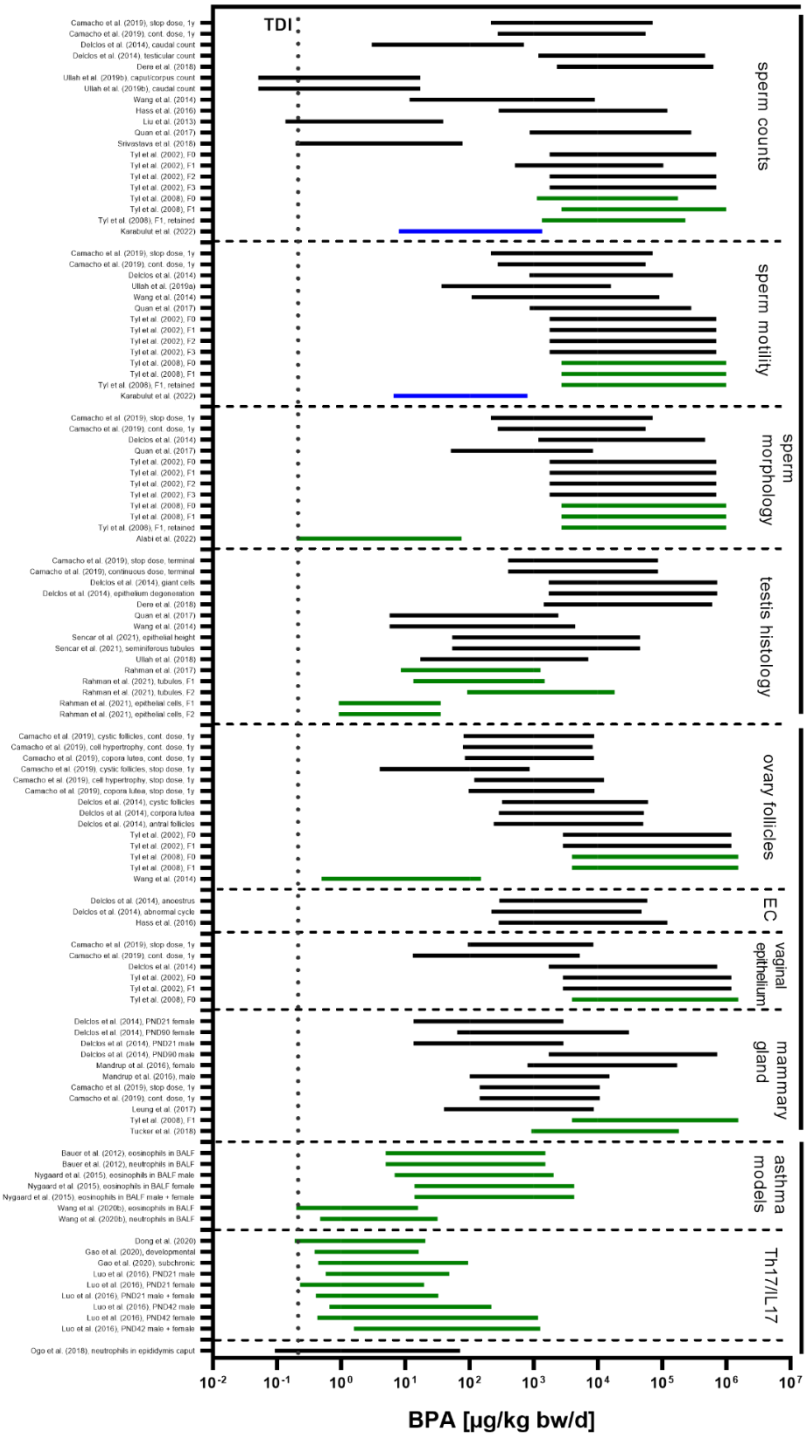



**Figure 2**. 90% Confidence intervals of the TDI values derived from all studies submitted to dose-response analysis in comparison to the TDI of 0.2 µg/kg bw/day. Image taken from BfR opinion (BfR 2023), study references as given there. Note: logarithmic scale. Note: the 2

studies by Ullah et al. were no effect studies (NOAEL = highest dose). Black = rat, green = mouse, blue = rabbit.

The final TDI was calculated to 200 ng/kg bw/day as rounded mean of the lower confidence limits from the two studies. It should be noted that this point estimation is a conservative value regarding the uncertainties in the assessment as represented by the above mentioned confidence interval for the true TDI value. However, as can be seen in Figure 1 the TDI is protective for all other endpoints related to reproductive toxicity. In addition, based on evaluations from other authorities (ECHA 2014; EFSA 2015a; EFSA 2023), **the TDI of 0.2 µg/kg bw/day as derived by the BfR** is also protective with respect to other toxicological endpoints (general toxicity, carcinogenicity, effects on brain and behaviour). Furthermore, although intermediate immunological effects were evaluated as not suitable for TDI derivation, the BfR TDI of 0.2 µg/kg bw/day would still be protective for a 100% increase of the respective markers. Thus, adverse immunological effects in humans – if at all – are unlikely to result from BPA exposure in the range of the TDI of 0.2 µg/kg bw/day.

### *3.2. BPA exposure assessment*

Overall external exposure estimates were derived by adding up the BPA exposure from all foods analysed. **Table 1** lists the mean and high external exposures ($90^{th}$ and $95^{th}$ percentiles) for children (3 to 9 years), adolescents (10 to 17 years), and adults (18 to 39 years).

The mean BPA intake for the children population ranged between 287.0 ng/kg bw (LB) and 296.2 ng/kg bw (UB) per day. For the highest estimated exposure in children ($95^{th}$ percentile), the total BPA exposure ranged from 794.6 ng/kg bw (LB) to 806.0 ng/kg bw (UB) per day.

In adolescents, total BPA exposure varied from 116.0 (LB) to 121.3 (UB) ng/kg bw/day for the mean exposure and from 391.4 (LB) to 399.7 (UB) ng/kg bw/day for the highest exposure scenario, respectively. In the case of adults, these dietary exposure estimates were 62.5 to 66.0 ng/kg bw/day and 228.5 to 231.2 ng/kg bw/day, respectively.

**Table 1**. Estimated total exposure to BPA for each age group (ng/kg bw/day) and percentage of subjects exceeding the TDI of 200 ng/kg bw/day (BfR 2023).

| | | LB (lower bound) | | | | | UB (upper bound) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | Mean (SD) | Median (IQR) | P90 | P95 | %TDI >200 | Mean (SD) | Median (IQR) | P90 | P95 | %TDI >200 |
| Children (3-9 yrs) | 213 | 287.0 (249.7) | 201.7 (121.9-380.9) | 634.2 | 794.6 | 51.6 | 296.2 (251.3) | 211.3 (127.1-389.5) | 656.1 | 806.0 | 53.5 |
| Adolescent (10-17 yrs) | 281 | 116.0 (136.2) | 71.9 (31.5-149.6) | 280.8 | 391.4 | 17.8 | 121.3 (137.5) | 76.7 (34.7-154.4) | 284.5 | 399.7 | 19.2 |
| Adults (18-39 yrs) | 122 | 62.5 (84.5) | 34.9 (12.9-74.0) | 140.8 | 228.5 | 4.9 | 66.0 (86.2) | 38.8 (15.9-79.3) | 145.5 | 231.2 | 5.7 |

SD: standard deviation; IQR: interquartile range; P90: 90$^{th}$ percentile; P95: 95$^{th}$ percentile.

The differences of total BPA exposure between the age groups were statistically significant (**Figure 3**). Median exposure in Spanish children (201.7 (LB) to 211.3 (UB) ng/kg bw/day) was slightly higher than the new TDI recommended by the BfR (200 ng/kg bw/day). The estimated daily dietary BPA intake was higher than BfR´s HBGV for 51.6% (LB) to 53.5% (UB) of the children included in this study (Table 1).

In adolescents and adults, the comparison of the dietary exposure estimates with the BfR-derived TDI showed that the mean exposure did not exceed this TDI. However, the high exposure scenarios (90$^{th}$ and 95$^{th}$ percentiles, except for the 90$^{th}$ percentile in adults) revealed values higher than 200 ng/kg bw/day.

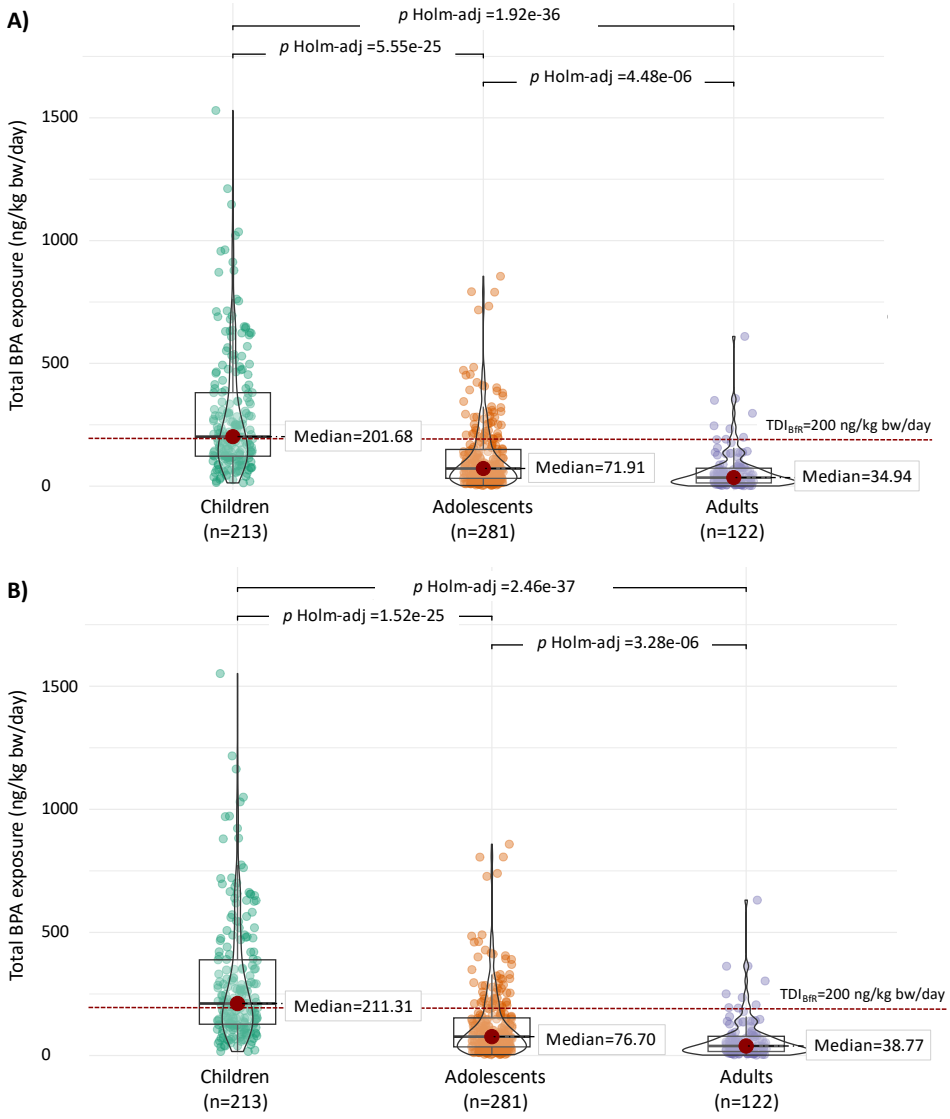



**Figure 3**. Total BPA exposure for each age group. A) Lower bound. B) Upper bound. Kruskal-Wallis test was applied to compare the estimated daily BPA intake between the different age groups ($p$ = 1.53e-41 for lower bound; $p$ = 1.49e-42 for upper bound). Holm-Bonferroni $p$ values for multiple comparisons are indicated as $p$ Holm-adj.

**Table 2** and **Figure 4** detail ten food pools that were covered by the EFSA FoodEx2 classification (EFSA 2015b) and their contribution to the total BPA exposure. Canned tuna turned out to be the major contributor to dietary BPA exposure in the three age groups, with 44.7-46.1% in children, 46.9-49% in adolescents, and 54.7-57.7% in adults. Milk and dairy products, in our assessment

mainly defined by sweetened and flavoured yoghurts, accounted for the second most percentage of total BPA exposure. With increasing age, the contribution of this food category decreased. Vegetables, meat, fruit, and grain-based products contributed modestly to overall exposure, while exposure from composite dishes, legumes, eggs, and sauces/condiments was minimal or absent in all age groups.

**Table 2**. Estimation of exposure to BPA from different food sources according to FoodEx2 level 1 main groups (ng/kg bw/day) (Galvez-Ontiveros et al. 2021).

| | | Children | | Adolescents | | Adults | |
|---|---|---|---|---|---|---|---|
| FoodEx2 level 1 Top level food groups | N | Mean LB | Mean UB | Mean LB | Mean UB | Mean LB | Mean UB |
| Grains and grain-based products | 19 | 7.1 | 7.8 | 7.0 | 7.7 | 2.9 | 3.4 |
| Vegetables and vegetable products | 11 | 27.6 | 29.7 | 10.9 | 11.9 | 6.6 | 7.3 |
| Legumes, nuts, oilseeds, and spices | 4 | 0.1 | 0.3 | 0.1 | 0.3 | 0.1 | 0.3 |
| Fruit and fruit products | 10 | 5.5 | 6.8 | 6.2 | 6.8 | 3.8 | 4.1 |
| Meat and meat products | 10 | 10.6 | 11.0 | 7.1 | 7.3 | 4.6 | 4.8 |
| Fish, seafood, amphibians, reptiles, and invertebrates | 3 | 132.5 | 132.9 | 57.1 | 57.5 | 36.3 | 36.6 |
| Milk and dairy products | 16 | 102.7 | 106.2 | 26.7 | 28.3 | 7.5 | 8.3 |
| Eggs and egg products | 1 | 0.0 | 0.3 | 0.0 | 0.1 | 0.0 | 0.1 |
| Composite dishes | 5 | 0.9 | 1.1 | 0.9 | 1.1 | 1.0 | 1.2 |
| Seasoning, sauces, and condiments | 3 | 0.0 | 0.1 | 0.0 | 0.1 | 0.0 | 0.0 |

LB: lower bound; UB: upper bound.

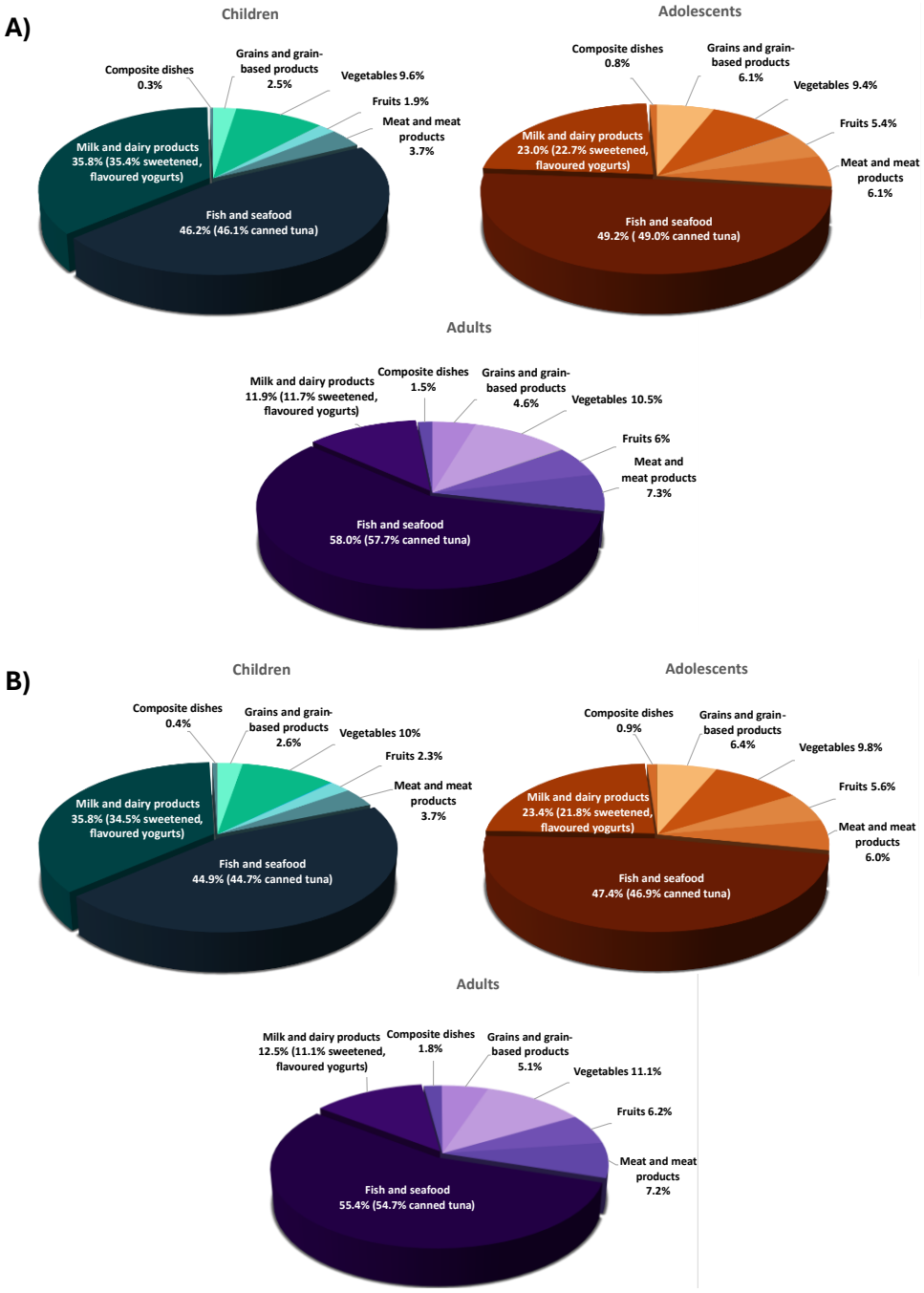



**Figure 4.** Main contributors of dietary BPA exposure in children, adolescents, and adults. A) Lower bound. B) Upper bound. Food items were grouped in accordance with the EFSA FoodEx2 Classification to identify the main contributors to the total BPA exposure (EFSA 2015b).

### *3.3. Probabilistic risk assessment*

For the probabilistic risk assessment, the results of the hazard characterisation and exposure assessment were combined. Therefore, log-normal probability distributions of dietary BPA exposure for the three age groups were fitted to the exposure data (**Figure 5**).

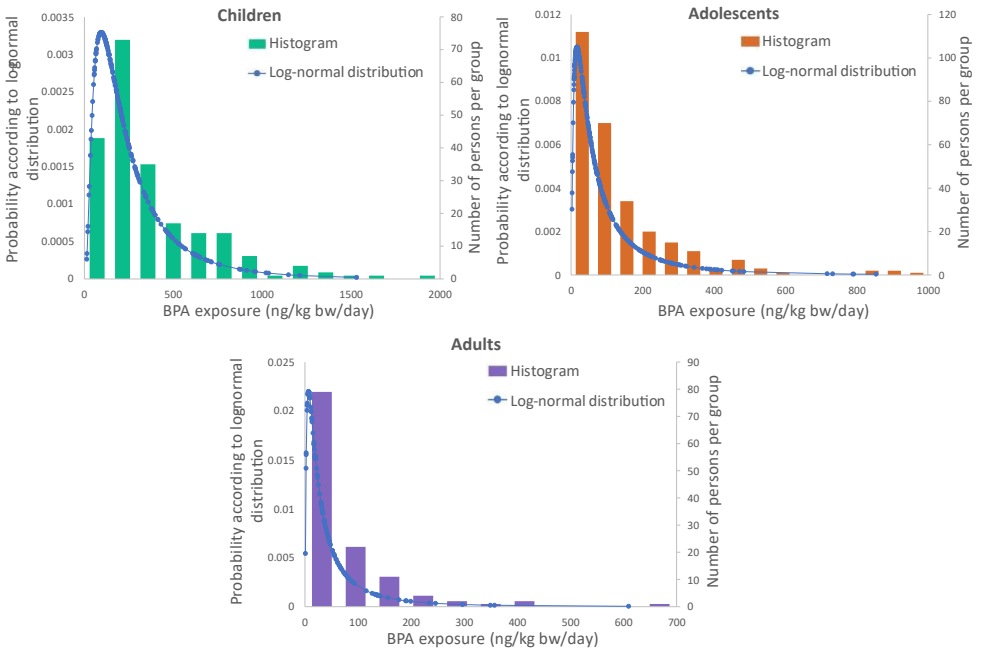



**Figure 5**. Log-normal distribution shape of dietary BPA exposure. Histogram and density plot of the total BPA exposure are shown. X-axis: total BPA exposure (ng/kg bw/day). Left Y-axis: log-normal transformed values using the probability density function (PDF). Right Y-axis: total number of individuals per group.

From these log-normal distributions, the 5$^{th}$ and 95$^{th}$ percentiles were calculated as a representation of the exposure range for every age group. Since UB and LB exposure did not differ significantly, only the values from the LB exposure estimation were used. As a representation of a possible health risk, this exposure range is plotted against the 90% confidence interval for the TDI (**Figure 6**). The 5$^{th}$ and 95$^{th}$ percentile points next to each other are connected to form an ellipsoid as a representation for the overall uncertainty and variation.

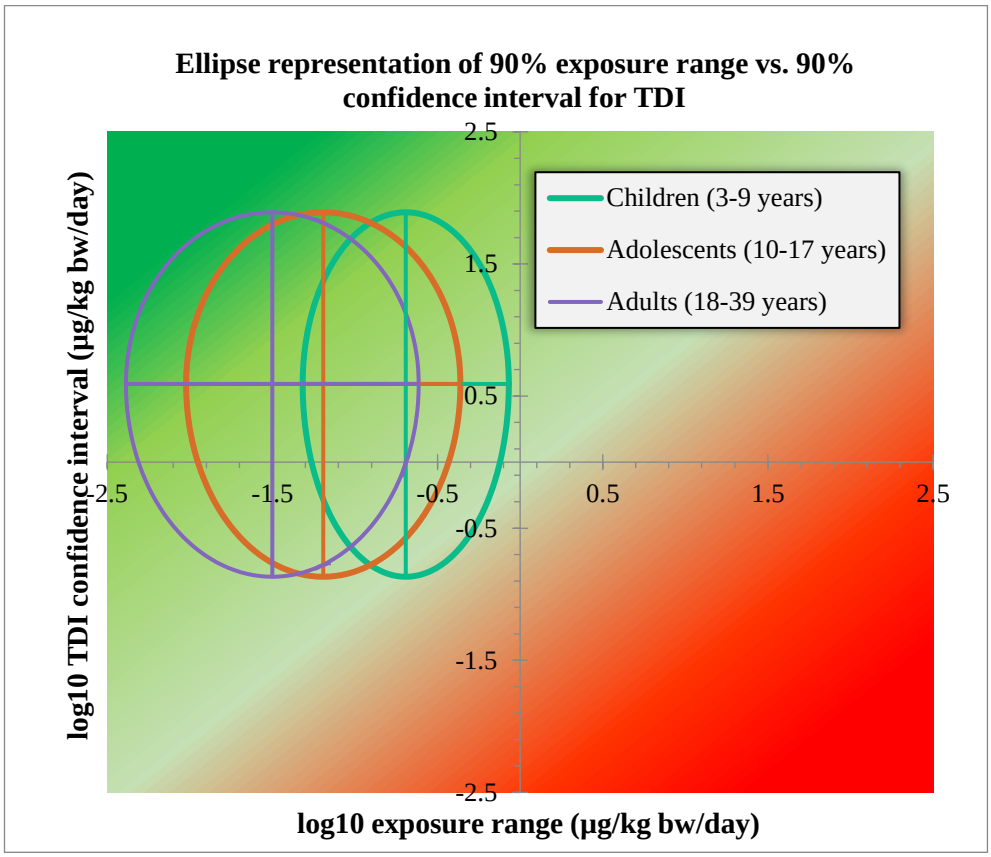



**Figure 6**. Risk visualisation for children, adolescents and adults as ellipse plot. X-axis: exposure range from 5$^{th}$ to 95$^{th}$ percentile. Y-axis: 90% confidence interval for the final TDI based on reduced sperm count in Wistar rats (Liu et al. (2013); Srivastava et al. (2018)). Note: logarithmic scale.

The more the ellipse is located to the lower right corner, the higher the probability for a possible health risk. When the ellipse is completely located to the left of the diagonal between lower left and upper right corner – as for adults and adolescents – a health risk is very unlikely. However, for children the ellipse is at least partly below this diagonal (or close to the red area), indicating that a health risk at least for parts of the age group is not negligible.

## 4. Discussion

BPA is present in a wide field of application with a variety of uses. It is, however, well-established that the main source of human BPA exposure is the diet

and food contact materials, respectively (EFSA, 2023). Recently, EFSA has dramatically lowered its provisional TDI to 0.2 ng/kg bw/day and concluded that the general population is at risk from (dietary) exposure to BPA (EFSA 2023). At the same time, BfR proposed its own HBGV of 200 ng/kg bw/day and concluded that "current exposure data are needed for a full risk assessment" (BfR 2023). The significant difference between both TDIs is the result of different approaches applied and divergencies within hazard assessment (BfR 2022). In contrast to EFSA, who identified the relative increase of TH17 cell frequency as the most sensitive and relevant endpoint, BfR derived its TDI based on two studies showing reduced sperm count after subchronic BPA exposure of adult Wistar rats (Liu et al. 2013; Srivastava and Gupta 2018). Dose-response analysis was performed by means of BMD modelling and results were submitted to a probabilistic uncertainty assessment according to the approach proposed by (WHO IPCS 2018). Therein, the distribution of possible human equivalent dose factors was combined with typical distributions for other uncertainties (e.g. interhuman variability, study duration), aiming to protect at least 99% of the population. In contrast, EFSA applied a deterministic approach considering uncertainties as default assessment factors, each representing a worst-case point estimate. As the approach used by BfR is scientifically sound and state of the art, the resulting TDI value is used here as HBGV for risk assessment. Overall, this TDI should be used for risk assessment of BPA.

Since the assessment of dietary BPA exposure by EFSA in 2015, no current data have been available that are suitable for an updated exposure estimation. Human biomonitoring (HBM) of urinary BPA concentrations as part of the recent European Initiative HBM4EU revealed a significant decline of the internal BPA exposure for the period from 2014 to 2020. Using a physiology-based pharmacokinetic (PBTK) model, the corresponding BPA intake, i.e. an external dietary exposure, was derived from the HBM data. However, since many of the samples were spot urine, the significance for the actual external BPA exposure is limited.

The concentration of urinary BPA varies from person to person throughout the day, and even within the same person due to its short half-life. Therefore, the ability to reflect long-term exposure levels from spot urine samples is limited. Urine

samples are commonly used as an indicator of short-term exposure in biomonitoring studies because of their easy and non-invasive collection, which is particularly important in the child population (Galvez-Ontiveros et al. 2021; Moscoso-Ruiz et al. 2023). This work presents reasonably up-to-date data of BPA exposure in the Spanish population, broken down by age. For a risk characterisation, the estimated daily BPA exposure for three different age groups was compared to the BfR-derived TDI of 200 ng/kg bw/day (BfR 2023). Children aged 3 to 9 years were the highest exposed group, with a mean dietary BPA exposure estimate in the range of TDI proposed by BfR. However, the estimated dietary exposure of more than half of the children exceeded the TDI. In a study assessing the dietary exposure to BPA in the French population, the measured values were lower than ours, ranging from 50 to 60 ng/kg bw/day for children and adolescents (3-17 years) and from 38 to 40 ng/kg bw/day for adults older than 18 years (Bemrah et al. 2014). However, in another study performed in Chinese participants, higher exposures to BPA were observed among 2- to 12-year-old children (331.3-403.7 ng/kg bw/day), 13- to 19-year-old adolescents (269.7-321.1 ng/kg bw/day), and 20- to 50-year-old adults (199.5-218.3 ng/kg bw/day) (Yao et al. 2020).

It is important to note that the results presented here are not directly comparable to the total dietary exposure obtained in these studies. There are differences in the food samples analysed, methods of exposure estimation and chemical analysis, and dietary habits between countries. In our study, we observed that exposure through canned tuna consumption was the major contributor to total BPA exposure in the three age groups investigated (44.7-57.7%). This is on the one hand due to the high BPA levels found in canned tuna samples (409 ng/g), previously reported (Galvez-Ontiveros et al. 2021). On the other hand, canned and raw tuna are one of the most consumed fish products by the Spanish population (Russo et al. 2019). Our finding was in line with the study by (Bemrah et al. 2014), in which canned food was assumed to account for 50% of the total exposure. It has been well-demonstrated that canned foods have higher BPA concentrations than non-canned foods (e.g. fresh, frozen, packed in plastic). However, it should be noted that it is not the can itself that is the source of BPA, but its coating inside. High BPA migration levels are mainly

found in canned foods stored in cans with epoxy-based protective linings. (González et al. 2020; Marchiandi et al. 2024; Wang et al. 2022; Wang et al. 2023). In contrast, samples from cans with non-epoxy or so-called "BPA-non-intent" coatings, respectively, have non-detectable or significantly lower BPA levels. In this context, it is also important to consider whether the food in the coated can is heated/sterilised for preservation. It has been shown that the BPA content increases with increasing duration and temperature during preservation. In contrast, the storage time of the food in a coated can or container does not play a decisive role in the amount of BPA transferred (Bayerisches et al. 2018; Munguia-Lopez et al. 2002). Accordingly, BPA levels of samples of canned food can vary significantly.

Besides sea food, milk and dairy products represented the group contributing second most to the dietary BPA exposure of the people examined in this study. Processed and ultra-processed yogurts made up the main part of this group. BPA levels in sweetened and flavoured yoghurt samples ranged from 12.3 to 60.85 ng/g and are published elsewhere (Galvez-Ontiveros et al. 2021). Breast milk, commercial milk and dairy products are an essential part of the diet, especially for infants and young children (Mercogliano and Santonicola 2018). The declining importance of dairy products in the diet with increasing age is reflected in both the decreasing BPA exposure estimates and the decreasing share of milk/dairy products in the overall diet. Dairy production starts with animal feed, followed by milk production on farms, raw milk collection, preservation, and processing. BPA can enter the milk chain at any of these stages, specially at the milk processing where some equipments, such as milking machines, storage tanks and transport pipes, can be made of polycarbonate or epoxy-based materials (Ghahremani et al. 2024; Mercogliano and Santonicola 2018). In addition, BPA is a fat-soluble chemical, which favours its bioaccessibility and accumulation in fatty dairy products (Mercogliano et al. 2021).

Other food groups including non-packed and packed vegetables, meat, fruits, and grain-based products also contributed, albeit only modestly, to overall BPA exposure. This demonstrates the ubiquitous nature of BPA and the vulnerability of food products to contamination that can occur along their entire production chain,

from the farm-to-fork, beyond to the packaging (González et al. 2020). Additionally, BPA has been found in groundwater and soil. The presence of BPA in irrigation water and agricultural soils could have an impact on crops and agricultural products (Li et al. 2021). However, a resulting significant impact on human exposure is unlikely.

One of the strengths of this study is that a large food consumption database was used that collects up-to-date data on exposure. Measuring dietary intake is considered one of the major methodological challenges in nutritional epidemiology (Sierra-Ruelas et al. 2021). Food frequency methods like FFQ have shown to be the most convenient tool for assessing long-term habitual intake patterns in large-scale prospective studies, mainly because of the ease of administration, rapid and unexpensive processing (Conrad and Nöthlings 2017; Notario-Barandiaran et al. 2020). However, the selection of foods, the clarity of the questions and the format and coding of the frequency of consumption responses need to be given particular attention in the design of the questionnaire. Additionally, hazard characterisation was combined with exposure assessment in a probabilistic way to perform for the first time a comprehensive risk characterisation using the APROBA-Plus tool. APROBA-Plus may be very useful as a quick approach of quantitatively determining uncertainties and characterising the risk. By visualising the uncertainties, APROBA tool provides useful information about the current situation of substance-derived risk. Nonetheless, a limitation of this tool is that the risk is illustrated graphically but not quantified.

## 5. Conclusion

Based on the updated TDI value for BPA recommended by the BfR and the estimated daily dietary exposure dose in the present study, the total exposure to BPA was exceeded by approximately 50% of the children aged 3 to 9 years, who are particularly vulnerable to food contaminants. For all age groups, canned fish was the predominant food source of BPA exposure. Dairy products and vegetables (fresh/plastic packaged) also contributed to total exposure, demonstrating that BPA food contamination could occur at any stage of the farm-to-fork production chain,

beyond packaging. The probabilistic risk characterisation showed that a health risk is not negligible for at least parts of the child population. Therefore, the results presented in this study provide evidence that BPA exposure close to the BfR-derived TDI may be of particular concern for the child population and may serve as a basis for designing future studies that include other food items in the child diet to obtain a more accurate dietary exposure assessment and subsequent risk characterisation.

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# 5. GLOBAL DISCUSSION. LIMITATIONS AND FUTURE PERSPECTIVES

Excess weight and neurodevelopmental disorders are bidirectionally related through different biological mechanisms and common risk factors (Braun, 2017). From a holistic perspective, the identification of gene-environment interactions and their contribution to disease aetiology provides a more comprehensive understanding of the mechanisms driving human diseases risk. With this in mind, and in line with the four models of gene-environment interactions described by Virolainen et al. (2023), this Doctoral Thesis focuses on the synergistic effect between genetic variability and exposure to EDCs with obesogenic and neurodisruptive activities (metal(loid)s, bisphenols and parabens), meaning that the risk modulation is greater when they are explored together than when they occur separately (Virolainen et al., 2023).

Importantly, pregnancy, childhood and adolescence are characterised by a high degree of physiological change, particularly in organs and endocrine-dependent systems. Consequently, these periods represent critical windows of vulnerability to the effects of EDCs, which can lead to irreversible damage in adulthood (Kahn et al., 2020; Lucaccioni et al., 2020). The human body possesses efficient metabolic systems to properly eliminate xenobiotic substances, including EDCs. Nonetheless, it has been demonstrated that genetic variations in this biological machinery could regulate the physiological response to external exposure to these chemical compounds, thereby modulating individual susceptibility to their adverse health effects (Hanioka et al., 2011; Joneidi et al., 2019; Tkalec et al., 2021).

The initial study of this Doctoral Thesis was designed as a point of departure to examine the influence of genetic polymorphisms within the detoxification system of one of the best-known EDCs, the group of metal(loid)s, on childhood excess weight. It was shown that *GSTP1* rs1695 and *ATP7B* rs1061472 contributed to excess weight in the presence of higher urinary chromium and lead levels. Whereas *GCLM* rs3789453 and *ATP7B* rs1801243 showed the opposite effect for copper and lead exposures. This was reflected in significant p-values for interaction. In accordance

with the most recent evidence, *GSTP1* rs1695 has been identified as a genetic predisposing variant for excess weight (Chielle et al., 2017) and has been found to be related to increased lead toxicity (Yohannes et al., 2022). It is notable that there is a lack of research assessing the combined effect of this variant with metal(loid) exposure on the prevalence of overweight/obesity. For its part, while *ATP7B* has been typically linked to copper export, further investigation is required to ascertain its potential role as a transporter of other metals and to elucidate its relationship with obesity (Harder et al., 2022).

In light of these findings, it becomes evident that an in-depth investigation into the genetic basis of detoxification pathways is crucial for understanding how genetic variation influences the body's response to external metal(loid) exposure.

To drive forward research in this field, a broader gene panel was designed, incorporating polymorphisms in genes involved in obesity-related metabolic pathways, xenobiotic metabolism and hormone systems. The aim was to investigate their role in excess weight according to a short- and long-term exposure to total bisphenols and parabens, integrating an individual-based approach with the joint effect of them. Here, we evidenced that *LEPR* rs9436303 was identified as a relevant risk variant for excess weight, and this effect persisted across exposure-stratified models. The variant rs9436303 G allele has been reported to be associated with obesity-related traits in children and adolescents (Alves et al., 2019; Cissé et al., 2022; Olza et al., 2017) and, in turn, there is evidence pointing to an increase in leptin levels following exposure to BPA and parabens in animals and humans (Rönn et al., 2014; ul Haq et al., 2020). Meanwhile, we demonstrated for first time that other variants have no consequences per se, but in the presence of exposure, their effect (protective or risk) varies with the degree of exposure to bisphenols and/or parabens (e.g., *GSTP1* rs1695, *GPX1* rs1050450, and *ESR2* rs3020450). It indicates that genetics interacts with an ever-changing environment, and therefore studying gene-environment interactions gives us a more holistic approach to dealing with human disease aetiology (Virolainen et al., 2023).

In both studies included in Chapter I, urine has been used as a reliable indicator of short-term exposure to certain metals and bisphenols/parabens (Gálvez-Ontiveros et al., 2023; Nguyen et al., 2024; Salcedo-Bellido et al., 2024). Additionally, given the rapid detoxification and elimination of bisphenols and parabens from the body, hair was used as an indicator of long-term exposure. That is why some genetic association studies have used hair as a useful target organ for the deposition of xenobiotics, and urine as a means of monitoring their metabolism and excretion (Parajuli et al., 2016; Wang, Y. et al., 2012).

The exposure to EDCs is ubiquitous and continuous, with dietary intake representing the primary source of overall human exposure (Ghassabian and Trasande, 2018; Mathiesen et al., 2021). Particularly, more than 90% of total BPA exposure comes from food intake, confirming that the dietary exposure assessment is the first step in addressing bisphenol-associated health issues (Martínez et al., 2018). For this reason, the impact of neurodevelopment-related genetic polymorphisms on specific cognitive domains and general cognitive function was addressed in Chapter II for both the low- and high-exposed dietary bisphenol children. To the best of our knowledge, there is growing evidence of interactions between gene variants and pesticides/heavy metals in cognitive development (Ramírez et al., 2022). However, no research has yet been conducted on bisphenol exposure. Our genetic study revealed a significant dual effect of *BDNF* variants on fluid reasoning, while the gene-environment association study identified relevant SNP-bisphenol interactions in verbal comprehension, working memory, and fluid reasoning.

*BDNF* rs6265 is one of the most studied missense variants in neurodevelopment (Szarowicz et al., 2022). Other genes, such as *SNAP25* and *OXTR*, play a significant role in synaptic plasticity, which is crucial for working memory (Abramova et al., 2020; Gao et al., 2015). On the other hand, BPA has shown to interfere with *BDNF* and neurotransmitter system signalling (Mustieles et al., 2022; Repouskou et al., 2020; Witchey et al., 2019). Brain development, synaptic plasticity and neurotransmission are essential processes for the proper development of the specific cognitive domains studied here, so studying the interconnection between the genetic

and environmental factors in each cognitive area may provide insightful clues to general cognitive dysfunction.

Returning to dietary intake as an important route of exposure, regulatory agencies have established reference values for BPA exposure. In 2023, EFSA drastically reduced its provisional TDI to 0.2 ng/kg bw/day and concluded that the general population is at risk from (dietary) exposure to BPA (EFSA, 2023). At the same time, BfR proposed its own TDI of 200 ng/kg bw/day, concluding that "current exposure data are needed for a full risk assessment" (BfR, 2023). Consequently, Chapter III focused on performing a comprehensive risk assessment of BPA by combining the data on dietary exposure of Spanish children, adolescents and adults to BfR's hazard characterisation. Approximately 50% of the children aged 3 to 9 years exceeded the BfR derived TDI. For at least a portion of this population, the health risk was not negligible. Food groups contributing to overall BPA exposure included canned fish, dairy products and fresh products. This is consistent with previous studies reporting that adherence to healthy dietary patterns does not appear to be associated with a low exposure to EDCs such as bisphenols. This lack of association may be due to the widespread contamination throughout the food chain, including fresh products (González et al., 2020; Melough et al., 2022).

One limitation of the studies included in this Doctoral Thesis is the relatively small sample size. Our results may have been compromised by the small size and lack of sufficient statistical power to detect significant associations. Even so, we did highlight novel SNP-disease associations and SNP-exposure interactions that had not been previously explored. As the studies were designed as a proof-of-concept investigation, the preliminary findings, especially in a vulnerable population within the context of the current global prevalence of excess weight and its bidirectional link with neurodevelopment, represent a significant contribution to the field that merits consideration for future research involving larger and diverse populations.

Due to the sample size, an experimental study of the common genetic profile in excess weight and neurodevelopment was not carried out. Through a review of the literature, a number of genetic variants were identified in patients suffering from

neurodevelopmental disorders and excess weight. The genetic changes are in genes implicated both in brain development and metabolic processes (*MYT1L, SH2B1, BDNF* and its receptor *TrkB, HTR2C, FTO,* and *TCF7L2*) (Ramírez et al., 2022). Genetic variants of some of these genes have been the subject of interest in this thesis. The subsequent step would be to perform an association analysis of variants of the above-mentioned genes with cognitive and behavioural/emotional aspects in children with excess weight.

As another future perspective, apart from replicating our findings in larger populations, a functional analysis selecting the most significant genetic variants would be interesting to validate our results.

# 6. CONCLUSIONS & CONCLUSIONES

## 6.1. Conclusions

Our findings demonstrate for the first time that exploration of the genetic variation along key disease-related biological systems (e.g., xenobiotic detoxification, hormone-dependent systems, metabolic processes, brain development and connectivity) and how the level of exposure to EDCs might interact with them, is crucial for a more in-depth understanding of the complex polygenic and multifactorial aetiology of excess weight and neurodevelopmental disorders. Importantly, given the significant genetic factor underlying both pathological scenarios, effective intervention strategies could be developed at the level of environmental exposure (e.g., reducing contact with EDCs) to prevent or decrease the incidence of obesogenic and neurodevelopmental outcomes. This raises the need for further research into the complex gene-environment interactions in large cohorts, especially in vulnerable populations.

1. The first case-control study suggests that genetic variants in the GSH system (*GSTP1* rs1695 and *GCLM* rs3789453) and metal transporting systems (*ATP7B* rs1061472 and rs1801243) are responsible for the interindividual susceptibility to the adverse effects of metal(loid)s on body weight regulation. Moreover, we found some evidence of the role of *GSTP1* rs1695 as a genetic predisposing factor of excess weight; while *ATP7B* rs1801243 appeared to display a protective role against overweight/obesity that has never been previously reported on.

2. Based on the subsequent genetic association analysis, the *LEPR* rs9436303 variant was proposed as a potential genetic marker for excess weight, independently of the level of exposure. Conversely, the magnitude of the effect of other genetic variants in obesity-related biological pathways, antioxidant defence systems, metabolising enzymes, and hormonal systems, differed between low and high exposure to total bisphenols and parabens, indicating evidence of gene-environment interactions.

3. Urine and hair proved to be reliable indicators of exposure in the short and long term, respectively. In this context, gene polymorphisms in phase II detoxifying enzymes (*GSTP1* rs1695 and *GPX1* rs1050450) were significantly associated with an increased likelihood of overweight and obesity at low urinary bisphenol levels. Whereas individuals with phase I CYP450 gene variations (*CYP2C19* rs4244285) and high long-term co-exposure to bisphenols and parabens exhibited a tendency towards excess weight. These findings provide insight into how genetic variability within detoxifying enzymes could interrupt the proper degradation and excretion of toxic substances, leading to their accumulation in the body.

4. In neurodevelopment, significant differences in fluid reasoning scores in individuals carrying *BDNF* (rs6265 and rs11030101) and *SNAP25* (rs363039) variants demonstrated that this domain is influenced by a substantial genetic component. In models stratified by dietary bisphenol exposure, SNPs related to brain development, synaptic plasticity, and neurotransmission were associated with differences in several WISC-V cognitive domains, specifically fluid reasoning, verbal comprehension and working memory, revealing important SNP-by-bisphenol interactions on childhood cognitive function.

5. Finally, the probabilistic risk characterisation of BPA indicated that at least some portion of the child population faces a non-negligible health risk. Therefore, BPA exposure close to the BfR-derived TDI (200 ng/kg bw/day) may be of particular concern for children and may serve as a basis for designing future studies that include more food items in the child diet to obtain a more accurate dietary exposure assessment and subsequent risk characterisation.

## 6.2. Conclusiones

Nuestros hallazgos demuestran, por primera vez, que la exploración de la variación genética en sistemas biológicos claves relacionados con la etiopatogenia – como la detoxificación de xenobióticos, el sistema hormonal, los procesos metabólicos, y el desarrollo y conectividad cerebral – y la forma en que el nivel de exposición a EDCs podría interactuar con ellos, es crucial para una mejor comprensión de la etiología compleja, poligénica y multifactorial del exceso de peso y de los trastornos del neurodesarrollo. Además, dada la importancia del factor genético subyacente en ambos escenarios patológicos, podrían desarrollarse estrategias de intervención efectivas a nivel de la exposición ambiental (por ejemplo, reduciendo el contacto con EDCs) para prevenir o disminuir la incidencia de eventos obesogénicos y neurodisruptivos. Esto subraya la necesidad de seguir investigando sobre las complejas interacciones gen-ambiente en grandes cohortes, especialmente en poblaciones vulnerables.

1. El primer estudio de caso-control sugiere que las variantes genéticas del sistema GSH (*GSTP1* rs1695 y *GCLM* rs3789453) y de los sistemas de transporte de metales (*ATP7B* rs1061472 y rs1801243) son responsables de la susceptibilidad interindividual a los efectos adversos de los metal(oide)s en la regulación del peso corporal. Además, encontramos indicios del papel de *GSTP1* rs1695 como factor genético de predisposición al exceso de peso, mientras que *ATP7B* rs1801243 parece desempeñar un papel protector frente al sobrepeso/obesidad, hallazgo que no se había reportado anteriormente.

2. En base a los subsecuentes análisis de asociación genética, se propuso a la variante *LEPR* rs9436303 como posible marcador genético del exceso de peso, independientemente del nivel de exposición. Por el contrario, la magnitud del efecto de otras variantes genéticas en rutas biológicas relacionadas con la obesidad, sistemas de defensa antioxidante, de detoxificación, y hormonales, difirió entre una exposición baja y alta a bisfenoles y parabenos totales, lo que indica la existencia de interacciones gen-ambiente.

3. La orina y el pelo resultaron ser indicadores fiables de la exposición a corto y largo plazo, respectivamente. En este contexto, los polimorfismos de genes de enzimas detoxificantes de fase II (*GSTP1* rs1695 y *GPX1* rs1050450) se asociaron significativamente con una mayor probabilidad de sobrepeso y obesidad en el grupo con bajos niveles urinarios de bisfenoles. Por su parte, los individuos con variaciones genéticas en las enzimas CYP450 de fase I (*CYP2C19* rs4244285) y con altos niveles de coexposición a bisfenoles y parabenos mostraron una tendencia al exceso de peso. Estos hallazgos demuestran cómo la variabilidad genética en el sistema enzimático de detoxificación podría interferir con la adecuada degradación y excreción de sustancias tóxicas, favoreciendo su acumulación en el organismo.

4. En el neurodesarrollo, las diferencias significativas en las puntuaciones de razonamiento fluido en individuos portadores de las variantes *BDNF* (rs6265 y rs11030101) y *SNAP25* (rs363039) demostraron que este dominio presenta un componente genético sustancial. En aquellos modelos estratificados según la exposición dietética a bisfenoles, los SNPs relacionados con el desarrollo cerebral, la plasticidad sináptica y la neurotransmisión se asociaron con una modulación de la puntuación de distintos dominios cognitivos, como el razonamiento fluido, la comprensión verbal y la memoria de trabajo, revelando importantes interacciones *SNP-bisfenoles* en el funcionamiento cognitivo infantil.

5. Por último, la evaluación probabilística de riesgo del BPA indicó que al menos una parte de la población infantil se enfrenta a un riesgo para la salud no despreciable. Por lo tanto, la exposición al BPA cercana a la dosis de ingesta derivada por el BfR (200 ng/kg pc/día) puede ser especialmente preocupante para los niños/as y puede servir de base para diseñar futuros estudios que cubran más alimentos de la dieta infantil, con el fin de obtener una evaluación de la exposición dietética más precisa y, por ende, una caracterización del riesgo más completa.

# 7. REFERENCES OF INTRODUCTION, JUSTIFICACTION AND GLOBAL DISCUSSION

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# ANNEXES

## Thesis memory articles:

- Ramírez, V., Salcedo-Bellido, I., Rodrigo, L., Hernández, F.G., Olmedo, P., Martínez-González, L.J., Álvarez-Cubero, M.J., Rivas, A., 2023. Association of genetic polymorphisms in detoxifying systems and urinary metal(loid) levels with excess body weight among Spanish children: A proof-of-concept study. Science of the Total Environment, 873:162333. doi: 10.1016/j.scitotenv.2023.162333.
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- Ramírez, V., Robles-Aguilera, V., Gálvez-Ontiveros, Y., González-Palacios, P., Bernauer, U., Curato, C., Gall, A., Herzler, M., Siewert, K., Tarnow, P., Trubiroha, A., Zellmer, S., Álvarez-Cubero, MJ., Lorenz, C., Monteagudo, C., Zafra-Gómez, A., Tietz, T., Rivas, A. 2024. Health risk assessment of exposure to bisphenol A on a Spanish population sample. *In process of submission to Journal of Hazardous Materials*.

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- Ramírez, V., Robles-Aguilera, V., Salcedo-Bellido, I., Galvez-Ontiveros, Y., Rodrigo, L., Martinez-Gonzalez, LJ., Monteagudo, C., Álvarez-Cubero, MJ., Rivas, A. 2021. Effects of genetic polymorphisms in body mass index according to dietary exposure to bisphenols and parabens. Chemosphere, 293:133421. doi: 10.1016/j.chemosphere.2021.
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