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Programa de Doctorado en Nutrición y Ciencia de los Alimentos

# EXPOSICIÓN A BISFENOL A Y ANÁLOGOS CON ACTIVIDAD DISRUPTORA ENDOCRINA EN NIÑOS/AS EN EDAD ESCOLAR Y SU RELACIÓN CON LA OBESIDAD

Memoria presentada por

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*A mi Familia*

*A todos los que me han acompañado en esta aventura*

*El coraje no siempre se aprecia a primera vista. Frecuentemente es ese susurro al final del día que dice: «No voy a tirar la toalla; voy a continuar».*

*Rafael Santandreu*

## RESUMEN

La presente Memoria de Tesis Doctoral se centra en la determinación del bisfenol A (BPA) y sus análogos en muestras de alimentos, de consumo habitual en la población española, y matrices biológicas, obtenidas de una población infantil, mediante cromatografía de líquidos de ultra alta resolución acoplada a espectrometría de masas en tándem triple cuadrupolo (UHPLC-MS/MS). En este trabajo de investigación se investiga principalmente el posible impacto de la exposición dietética al BPA y sus análogos con actividad disruptora endocrina y valora su posible asociación con el sobrepeso/obesidad en una población escolar española. Por otro lado, dado que en algunos de los trabajos presentados en esta Tesis Doctoral se han utilizado métodos multirresiduos, se ha aprovechado la ocasión para estudiar varios parabenos por su analogía en estructura con los bisfenoles.

En las últimas tres décadas, hemos sido testigos de un marcado aumento en la prevalencia y la incidencia global del sobrepeso y la obesidad, planteando un grave desafío de salud pública. Recientemente, entre otros factores, se está vinculando este aumento con la exposición a contaminantes químicos con actividad disruptora endocrina y obesogénica, como los bisfenoles presentes en los envases plásticos de alimentos u otras fuentes. Algunas teorías, como las de los obesógenos, postulan que ciertos compuestos químicos ambientales pueden desencadenar cambios en las vías metabólicas relacionadas con la acumulación de grasa y el equilibrio energético promoviendo la diferenciación de los adipocitos. Estudios previos han insinuado que la exposición al BPA y sus análogos pueden alterar la fisiología del tejido adiposo mediante los mecanismos mencionados anteriormente. Investigar estos efectos, especialmente durante las primeras etapas de la vida debido a una mayor vulnerabilidad, es crucial, ya que la exposición a estas sustancias químicas durante la infancia puede tener efectos más intensos y pronunciados incluso a dosis más bajas. A fin de estudiar los posibles efectos de los bisfenoles sobre el sobrepeso/obesidad en etapas de

la vida más vulnerables, como la infancia, se han fijado un total de cuatro objetivos.

Con el propósito de alcanzar los objetivos fijados para la presente Tesis, se han reclutado un total de 128 casos y 194 controles, con un tamaño total de muestra de 322 niños/as de 3 a 12 años. De los cuales se obtuvieron datos dietéticos, antropométricos, sociodemográficos y estilos de vida, además de muestras de orina, saliva, uña y pelo.

El primer objetivo se basa en determinar cuantitativamente la presencia de bisfenoles y parabenos en alimentos de consumo habitual en España. Con la ayuda de encuestas nutricionales y análisis estadístico (regresión lineal stepwise) se pudo obtener los principales grupos de alimentos que aportan el 95% de la ingesta energética y de nutrientes. Posteriormente, se compraron un total de 98 muestras de alimentos en diferentes supermercados nacionales. Para detectar la presencia de los disruptores endocrinos (DEs) en alimentos se utilizó un metodo multirresiduos optimizado previamente por el grupo de investigación con algunas modificaciones, utilizando como técnica de análisis la UHPLC-MS/MS. De los 98 alimentos analizados, se obtuvo que el 52% de las muestras presentaban concentraciones detectables de bisfenoles. El BPA fue el bisfenol detectado con mayor frecuencia en las muestras de alimentos analizadas, seguido del bisfenol S (BPS) y el bisfenol E (BPE). El bisfenol AF (BPAF), el bisfenol B (BPB) y el bisfenol P (BPP) no se encontraron en ninguna de las muestras analizadas. En relación a los parabenos, se detectaron en un 57% de los 98 alimentos analizados, siendo metilparabeno (MetPB) el parabeno detectado con mayor frecuencia (49%), seguido de etilparabeno (EthPB, 16%) y propilparabeno (PropPB, 10%), mientras que butilparabeno (ButPB) presento una frecuencia de detección menor que la del resto (8%).

El segundo objetivo se centró en identificar otras posibles fuentes de exposición alimentaria a los bisfenoles. La determinación del BPA y sus análogos en los alimentos permitió evaluar la exposición dietética a los mismos, centrándose en los alimentos previamente analizados. Para

evaluar la exposición dietética al BPA y al BPS se utilizó un cuestionario de frecuencia de consumo de alimentos (FFQ). Entre los varones se obtuvieron los siguientes resultados, los casos tuvieron una mayor exposición al BPA procedente de alimentos procesados y legumbres, mientras que los controles tuvieron una mayor exposición al BPS procedente de frutas. En las niñas, los casos tuvieron una mayor exposición al BPA procedente de la carne y huevos y las legumbres, mientras que los controles tuvieron una mayor exposición al BPS y a los bisfenoles totales procedentes de los lácteos. Para el resto de los alimentos no se hayaron diferencias estadísticamente significativas.

El tercer objetivo, se focalizó en analizar el BPA y análogos en muestras de saliva, orina, uña y pelo obtenidas de la población de estudio. Al igual que con los alimentos los métodos empleados fueron optimizados previamente por el grupo de investigación. Se detectaron al BPA y el BPAF en las cuatro matrices, seguidos del BPF que se detectó en uña, orina y pelo, el BPS en orina, saliva y pelo, y el BPAP en uña y saliva. Uno de los principales hallazgos fue que el pelo y la uña presentaron las concentraciones más elevadas del BPA y sus análogos en la población de estudio. En saliva y uña, los niveles más altos del BPA y los bisfenoles totales se dieron en los casos. Mientras que en la orina el pelo, el grupo de control presentó la concentración más alta al BPA y los bisfenoles totales, sin diferencias estadísticamente significativas. Por otro lado, en el cabello se analizaron los niveles de parabenos presentando éstos concentraciones mucho más elevadas que los bisfenoles, siendo el MetPB seguido del EthPB los principales parabenos detectados. Asimismo, se hallaron las concentraciones más altas el grupo de los casos.

El último y cuarto objetivo se centró en establecer la posible relación entre la exposición al BPA y análogos y el sobrepeso/obesidad infantil, así como entre los parabenos presentes en el cabello y la ganacia de peso. Una vez se determinó la exposición dietética a los bisfenoles y su presencia en las muestras biológica, se utilizaron modelos de regresión logística binaria para analizar la influencia de la exposición dietética y la presencia en muestras biológicas de los DEs sobre el sobrepeso/obesidad

de la población de estudio. Con relación a la exposición dietética, los hallazgos revelaron una mayor probabilidad de sobrepeso u obesidad en las niñas expuestas a niveles elevados del BPA proveniente de carne y huevos. Sin embargo, no se observaron asociaciones consistentes en los chicos. En el caso de las muestras biológicas, los resultados sugieren que la presencia del BPF en uñas está asociado con sobrepeso/obesidad en los escolares (OR:4,87; $p$=0,020). Asimismo, se mostró que las concentraciones más altas del PropPB presentaron una probabilidad 4,67 veces mayor de tener sobrepeso u obesidad ($p$= 0,039) en los varones, pero no en las chicas. Con respecto a los demás DEs los hallazgos no fueron relevantes.

Para concluir, los resultados obtenidos durante la realización de la presente Tesis Doctoral indican una asociación estadísticamente significativa entre la exposición dietética al BPA presente en carne y huevos y la presencia de sobrepeso y obesidad en niñas. Por otro lado, los resultados sugieren una asociación positiva entre la presencia del BPF en las uñas de los escolares y el ProPB en el pelo de los chicos con el sobrepeso u obesidad. Con respecto a los demás DEs los hallazgos no fueron estadísticamente significativos.

Aunque el BPA sigue siendo el principal bisfenol hallado en muestras de alimentos, el presente trabajo de investigación observa que la ingesta diaria total del BPS en la dieta de los escolares supera a la del BPA. La ausencia de límites toxicológicos para los análogos está contribuyendo al aumento de la detección de estos, tanto en alimentos, como en muestras biológicas. Por otro lado, pese a que los parabenos no han sido los DEs objeto de estudio los hallazgos encontrados en dos de los trabajos de la Tesis Doctoral subrayan la necesidad de revisar la regulación y uso de parabenos (MetPB y EthPB junto con sus sales) en productos de consumo como conservantes alimentarios, así como de promover investigaciones adicionales para comprender mejor sus efectos en la salud infantil.

Los hallazgos obtenidos durante la realización de la presente tesis han permitido la publicación 3 artículos en revistas científicas de alto

impacto, y otros 2 que se encuentran actualmente en fase de publicación. Además, a través de la colaboración en 5 proyectos de investigación con el grupo EXPODIET, se ha trabajado en otras investigaciones que han derivado en la publicación de otros 14 artículos científicos. En resumen, durante el periodo de formación predoctoral se han publicado un total de 19 artículos científicos, todos ellos en revistas Q1, en su mayoría, y Q2 indexadas en el *Journal Citation Reports* (JCR). A continuación, se muestran las referencias bibliográficas de estos trabajos.

**Artículos de la Memoria de la Tesis**

**Gálvez-Ontiveros, Y**., Moscoso-Ruiz, I., Rodrigo, L., Aguilera, M., Rivas, A., & Zafra-Gómez, A. (2021). Presence of Parabens and Bisphenols in Food Commonly Consumed in Spain. *Foods*, 10(1), 92. https://doi.org/10.3390/foods10010092

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**Gálvez-Ontiveros, Y**., Páez, S., Monteagudo, C., & Rivas, A. (2020). Endocrine Disruptors in Food: Impact on Gut Microbiota and

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**Artículos colaboraciones durante la Tesis Doctoral**

Andújar, N., **Gálvez-Ontiveros, Y**., Zafra-Gómez, A., Rodrigo, L., Álvarez-Cubero, M. J., Aguilera, M., Monteagudo, C., & Rivas, A. A. (2019). Bisphenol A analogues in food and their hormonal and obesogenic effects: A review. *Nutrients*, 11(9), 2136. https://doi.org/10.3390/nu11092136

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## ABSTRACT

This Doctoral Thesis is based on the determination of bisphenol A (BPA) and analogues in food samples, commonly consumed in the Spanish population, and biological matrices, from a child population, by ultra high-performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry (UHPLC-MS/MS). This research work has mainly focused on investigating the possible impact of dietary exposure to BPA and analogues with endocrine disrupting activity and assessing its possible association with overweight/obesity in a Spanish school population. On the other hand, since in some of the works presented in this Thesis Report multi-residue methods have been used, the opportunity has been taken to study several parabens due to their analogy with bisphenols. On the other hand, since in some of the works presented in this Doctoral Thesis multi-residue methods have been used, the opportunity has been taken to study several parabens due to their analogy with bisphenols.

Over the past three decades, we have witnessed a marked increase in the global prevalence and incidence of overweight and obesity, posing a serious public health challenge. Recently, among other factors, this increase is being linked to exposure to chemical contaminants with endocrine disrupting and obesogenic activity, such as bisphenols in plastic food packaging or other sources. Some theories, such as those of obesogens, postulate that certain environmental chemical compounds may trigger changes in metabolic pathways related to fat accumulation and energy balance and promote adipocyte differentiation. Previous studies have suggested that exposure to BPA and its analogues may alter adipose tissue physiology through the mechanisms mentioned above. Investigating these effects, especially during the early stages of life due to increased vulnerability, is crucial, as exposure to these chemicals during childhood can have stronger and more pronounced effects even at lower doses. In order to study the possible effects of bisphenols on overweight/obesity at more vulnerable life stages, such as infancy, a total of four targets have been set.

In purpose to achieve the objectives set for this Thesis, a total of 128 cases and 194 controls were recruited, with a total sample size of 322 children aged 3 to 12 years. Dietary, anthropometric, sociodemographic and lifestyle data were obtained, as well as urine, saliva, nail and hair samples.

The first objective is based on quantitatively determining the presence of bisphenols and parabens in commonly consumed foods in Spain. With the help of nutritional surveys and statistical analysis (stepwise linear regression), the main food groups providing 95% of the energy and nutrient intake could be obtained. Subsequently, a total of 98 food samples were purchased in different national supermarkets. To detect the presence of endocrine disruptors (DEs) in food, a multi-residue method previously optimised by the research group was used with some modifications, using UHPLC-MS/MS as the analysis technique. Of the 98 foods analysed, 52% of the samples showed detectable concentrations of bisphenols. BPA was the most frequently detected bisphenol in the analysed food samples, followed by bisphenol S (BPS) and bisphenol E (BPE). Bisphenol AF (BPAF), bisphenol B (BPB) and bisphenol P (BPP) were not found in any of the samples analysed. Parabens were detected in 57% of the 98 foods analyzed, with methylparaben (MetPB) being the most frequently detected paraben (49%), followed by ethylparaben (EthPB, 16%) and propylparaben (PropPB, 10%), while butylparaben (ButPB) had a lower frequency of detection than the rest (8%).

The second objective focused on identifying other possible sources of dietary exposure to bisphenols. The determination of BPA and analogues in food allowed the assessment of dietary exposure to BPA and analogues, focusing on the foods previously analysed. A food frequency questionnaire (FFQ) was used to assess dietary exposure to BPA and BPS. Among males, the following results were obtained: cases had a higher exposure to BPA from processed foods and legumes, while controls had a higher exposure to BPS from fruits. In girls, cases had higher exposure to BPA from meat and eggs and legumes, while controls had higher exposure to

BPS and total bisphenols from dairy. For all other foods no statistically significant differences were found.

The third objective focused on analysing BPA and analogues in saliva, urine, nail and hair samples obtained from the study population. As with food, the methods used were previously optimised by the research group. BPA and BPAF were detected in all four matrices, followed by BPF which was detected in nail, urine and hair, BPS in urine, saliva and hair, and BPAP in nail and saliva. One of the main findings was that hair and nail had the highest concentrations of BPA and analogues in the study population. In saliva and nail, the highest levels of BPA and total bisphenols were found in the cases. While in urine and hair, the control group had the highest concentration of BPA and total bisphenols, with no statistically significant differences. On the other hand, the levels of parabens in the hair were analyzed, presenting much higher concentrations than bisphenols, with MetPB followed by EthPB as the main parabens detected. Likewise, the highest concentrations were found in the group of cases.

The last and fourth objective focused on establishing the possible relationship between exposure to BPA and analogues and childhood overweight/obesity, as well as between parabens present in hair and weight gain. Once dietary exposure to bisphenols and their presence in biological samples were determined, binary logistic regression models were used to analyse the influence of dietary exposure and the presence of DEs in biological samples on overweight/obesity in the study population. In relation to dietary exposure, the findings revealed an increased likelihood of overweight or obesity in girls exposed to high levels of BPA from meat and eggs. However, no consistent associations were observed in boys. In the case of biological samples, the results suggest that the presence of BPF in nails is associated with overweight/obesity in schoolchildren (OR:4.87; $p$=0.020). Likewise, it was shown that the highest concentrations of PropPB presented a 4.67 times greater probability of being overweight or obese ($p$= 0.039) in boys, but not in girls. With respect to the other DEs the findings were not relevant.

To conclude, the results obtained during this Doctoral Thesis indicate a statistically significant association between dietary exposure to BPA present in meat and eggs and the presence of overweight and obesity in girls. On the other hand, the results suggest a positive association between the presence of BPF in the nail of schoolchildren and ProPB in the hair of boys with overweight or obesity. With respect to the other DEs the findings were not statistically significant.

Although BPA remains the main bisphenol found in food samples, the present research work shows that the total daily intake of BPS in the diet of schoolchildren exceeds that of BPA. The absence of toxicological limits for analogues is contributing to the increased detection of analogues in both food and biological samples. On the other hand, despite the fact that parabens have not been the DEs under study, the findings found in two of the works of the Doctoral Thesis underline the need to review the regulation and use of parabens (MetPB and EthPB together with their salts) in consumer products as food preservatives, as well as to promote additional research to better understand their effects on children's health.

The findings obtained during this thesis have led to the publication of 3 articles in high-impact scientific journals, and another 2 are currently in the publication phase. In addition, through the collaboration in 5 research projects with the EXPODIET group, we have collaborated in other research that has led to the publication of another 14 scientific articles. In summary, during the pre-doctoral training period, a total of 19 scientific articles have been published, all of them in Q1 journals, mostly Q1, and Q2 journals indexed in the Journal Citation Reports (JCR). The bibliographical references of these works are shown below.

**Thesis Memory Articles**

**Gálvez-Ontiveros, Y.**, Moscoso-Ruiz, I., Rodrigo, L., Aguilera, M., Rivas, A., & Zafra-Gómez, A. (2021). Presence of Parabens and Bisphenols in Food Commonly Consumed in Spain. *Foods*, 10(1), 92. https://doi.org/10.3390/foods10010092

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**Gálvez-Ontiveros, Y**., Moscoso-Ruiz, I., Almazán Fernández de Bobadilla, V., Monteagudo, C., Giménez-Martínez, R., Rodrigo, L., Zafra-Gómez, A., & Rivas, A. (2023). Levels of Bisphenol A and its analogs in nails, saliva, and urine of children: a case control study. *Frontiers in nutrition*, 10, 1226820. https://doi.org/10.3389/fnut.2023.1226820

**Gálvez-Ontiveros, Y**., González-Palacios, P., Ramírez, V., Monteagudo, C., Samaniego-Sánchez, C., Rivas, A., Zafra-Gómez, A. Hair as an Indicator of Prolonged Exposure to Endocrine Disruptors and its Relation to Weight Gain in a Child Population. **Enviado a Obesity Research Journal (Under review).**

**Gálvez-Ontiveros, Y**., Páez, S., Monteagudo, C., & Rivas, A. (2020). Endocrine Disruptors in Food: Impact on Gut Microbiota and Metabolic Diseases. *Nutrients*, 12(4), 1158. https://doi.org/10.3390/nu12041158

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# ÍNDICE DE ABREVIATURAS

Relación de abreviaturas que aparecen en la presente Memoria de Tesis Doctoral. En algunos casos se ha mantenido la correspondiente abreviatura en inglés debido a su frecuente utilización en el lenguaje científico.

| | |
|---|---|
| **AESAN** | Agencia Española de Seguridad Alimentaria y Nutrición |
| **AR** | Receptor de andrógenos |
| **BAT** | Tejido adiposo pardo o marrón |
| **BPA** | Bisfenol A |
| **BPA-$d_{16}$** | Bisfenol A deuterado-16 |
| **BPAF** | Bisfenol AF |
| **BPAP** | Bisfenol AP |
| **BPB** | Bisfenol B |
| **BPC** | Bisfenol C |
| **BPE** | Bisfenol E |
| **BPF** | Bisfenol F |
| **BPFL** | Bisfenol FL |
| **BPM** | Bisfenol M |
| **BPP** | Bisfenol P |
| **BPS** | Bisfenol S |
| **ButPB** | Butilparabeno |
| **BPZ** | Bisfenol Z |
| **C** | Carbono |
| **C18** | Octadecilo |
| **DEs** | Disruptores endocrinos |
| **DS** | Desviación estándar |
| **dSPE** | Extracción en fase sólida dispersiva |
| **DSR** | Desviación estándar relativa |
| **EC** | Energía de colisión |
| **ECNT** | Enfermedades crónicas no transmisibles |
| **EFSA** | Autoridad Europea de Seguridad Alimentaria |
| **ER** | Receptor de estrógenos |
| **ES** | Error estándar |
| **ESI** | Ionización por electrospray |
| **EthPB** | Etilparabeno |
| **EthPB-$d_5$** | Etilparabeno deuterado-5 |
| **GR** | Receptor de glucocorticoides |
| **HCl** | Ácido clorhídrico |

| | |
|---|---|
| **IDT** | Ingesta diaria tolerable |
| **IL-6** | Interleucina-6 |
| **IMC** | Índice de masa corporal |
| **IQR** | Rango intercuartílico |
| **isoButPB** | Isobutilparabeno |
| **isoPropPB** | Isopropilparabeno |
| **JCR** | Journal Citation Report |
| **LC-MS** | Cromatografía de líquidos acoplada a espectrometría de masas simple |
| **LOD** | Límite de detección |
| **LOQ** | Límite de cuantificación |
| **MeOH** | Metanol |
| **MetPB** | Metilparabeno |
| **$MgSO_4$** | Sulfato de magnesio |
| **MRM** | Monitoreo de reacciones múltiples |
| **MSC** | Células madre mesenquimales |
| **$N_2$** | Nitrógeno |
| **NaCl** | Cloruro de sodio |
| **NaOH** | Hidróxido de sodio |
| **NIH** | Instituto Nacional del Cáncer |
| **NR** | Receptores hormonales nucleares |
| **OMS** | Organización Mundial de la Salud |
| **OR** | Odds ratio |
| **PPAR-γ** | Receptor activado por el proliferador de peroxisomas-γ |
| **PropPB** | Propilparabeno |
| **PSA** | Amina primaria-secundaria |
| **QuEChERS** | Quick, Easy, Cheap, Effective, Rugged, and Safe |
| **ROS** | Especies reactivas de oxigeno |
| **SPE** | Extracción en fase sólida |
| **T3** | Triyodotironina |
| **$t_R$** | Tiempo de retención |
| **UHPLC-MS/MS** | Cromatografía de líquidos de ultra alta resolución acoplada a espectrometría de masas en tándem triple cuadrupolo |
| **VC** | Voltaje del cono |
| **WAT** | Tejido adiposo blanco |

## ÍNDICE DE TABLAS



## ÍNDICE DE IMÁGENES



## ÍNDICE DE FIGURAS

# 1. INTRODUCCIÓN

## 1.1. Sobrepeso y Obesidad

El Sobrepeso y la obesidad se definen según la Organización Mundial de la Salud (OMS) como, «*una acumulación anormal o excesiva de grasa que puede ser perjudicial para la salud*» (Organización Muldial de la Salud [OMS], 2021). La obesidad tiene componentes inflamatorios, directa e indirectamente, relacionados con enfermedades crónicas importantes no transmisibles (ECNT) como la diabetes, la aterosclerosis, la hipertensión y varios tipos de cáncer (Lindberg et al., 2020; Yong et al., 2023; Khanna et al., 2022).

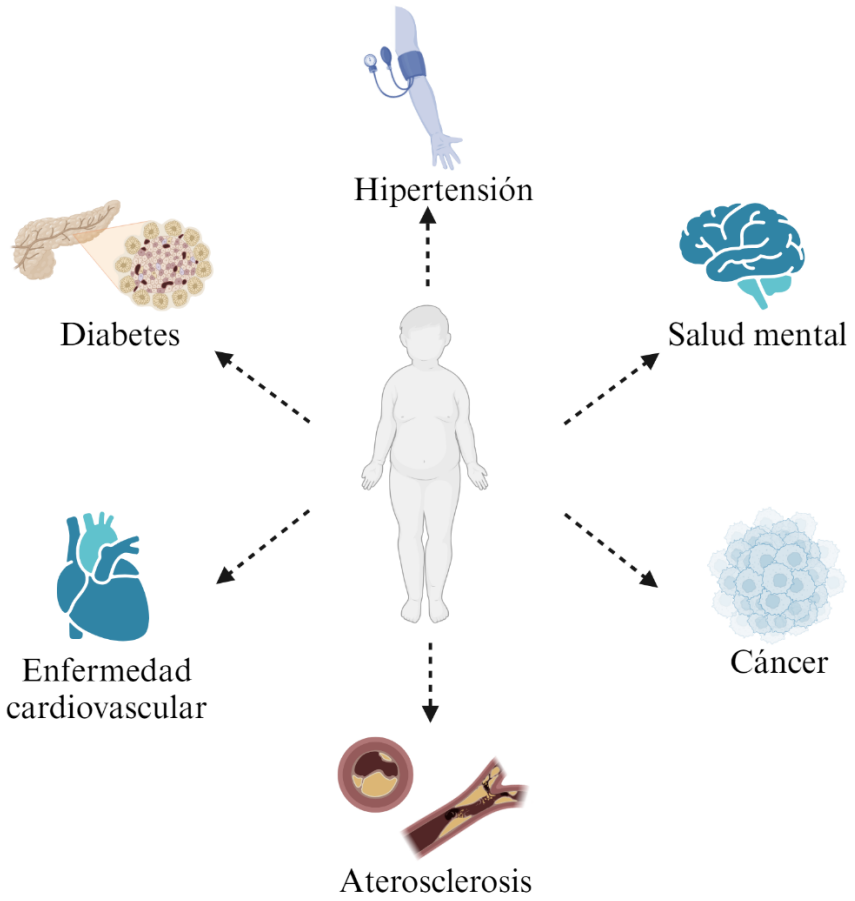



**Figura 1.** Interconexión entre la obesidad y otras ECNT. Elaborado con Biorender.com

El sobrepeso y la obesidad se caracterizan por una inflamación sistémica de bajo grado, en la que parece estar implicado el tejido adiposo. Las personas con sobrepeso/obesidad tienen niveles circulatorios alterados de citoquinas inflamatorias, como interleucina-6 (IL-6) secretada por los adipocitos (Khanna et al., 2022). La IL-6 es una de las citoquinas proinflamatorias sintetizada en un 40% en el tejido adiposo. Se ha observado que niveles elevados de IL-6 se asocian con un índice de masa corporal (IMC) elevado, mayor circunferencia de la cintura y grasa visceral (Valero et al., 2018; Khanna et al., 2022).

Tanto el sobrepeso como la obesidad son considerados ECNT y actualmente son un importante problema de salud pública que afecta negativamente tanto a la salud física como mental de quienes lo padecen (Muscogiuri et al., 2022). En 2022, 2.500 millones de adultos presentaron sobrepeso, de los cuales 890 millones eran obesos a nivel mundial. En un informe publicado el 14 de abril de 2023, prevé que para 2035 el 51% de la población global presentará sobrepeso/obesidad (OMS, 2023; Elika, 2023). Con relación a el sobrepeso/obesidad infantil, la OMS lo considera uno de los desafíos más graves del siglo XXI, puesto que, las cifras de sobrepeso/obesidad infantil están creciendo exponencialmente. Para 2035, se espera alrededor de 400 millones de niños/as presenten sobrepeso/obesidad en el mundo (OMS, 2017; OMS, 2023; ELIKA, 2023). La obesidad infantil se asocia a una mayor probabilidad de sufrir muerte prematura, pudiendo anular los beneficios conseguidos a nivel sanitario que han contribuido al aumento de la esperanza de vida (Lindberg et al., 2020).

Los países de ingresos bajos y medios presentan en cifras absolutas mayor número de niños/as con sobrepeso y obesidad en comparación con los países de altos ingresos. Asimismo, en los países de altos ingresos se ha observado que la probabilidad de obesidad infantil es más alta en los grupos socioeconómicos más bajos. Esto es debido al acceso deficiente a información de salud pública. Además, los estatus socioeconómicos más bajos se relacionan con un mayor nivel de angustia psicológica, asociado a su vez con una mayor alimentación emocional. Por consiguiente, la

alimentación emocional muestra una asociación positiva con la ganancia de peso de quienes la padecen (OMS, 2016).

Una preocupación adicional con relación a la obesidad infantil son los efectos negativos que puede tener en las interacciones sociales. El acoso es común y las actitudes negativas hacia los niños/as obesos pueden aumentar el riesgo de sufrir trastornos alimentarios, aislamiento social y reducción de la actividad física (Marcus et al., 2022). En consecuencia, actualmente existe un poderoso movimiento denominado «activismo corporal». Este movimiento lucha en contra de los estereotipos de belleza fijados en las últimas décadas, y defiende la diversidad corporal (Ellison, 2013). Pero es importante evaluar cuidadosamente los riesgos que conlleva un determinado peso corporal sobre la salud, y comparar estos riesgos con los posibles beneficios para la salud manteniendo un peso saludable. Se considera peso saludable aquel que nos permite mantener un buen estado de salud y calidad de vida, sin que conlleve la existencia de un riesgo para la salud (International Agency for Research on Cancer [IARC], 2016). En otras palabras, estar demasiado delgado o mantener un peso excesivamente alto puede no ser un peso saludable, por lo que debemos luchar por mantener un peso corporal dentro de los límites de salud.

### *1.1.1. Entorno obesogénico*

Actualmente, muchos niños/as crecen en un entorno obesogénico que favorece la ganancia de peso e incluso es desencadenante de la obesidad. Se denomina entorno obesogénico, según la OMS, a un «*entorno que fomenta la ingesta calórica elevada y el sedentarismo. Se tienen en cuenta los alimentos disponibles, asequibles, accesibles y promocionados; las oportunidades para practicar una actividad física; y las normas sociales en relación con la alimentación y la actividad física*» (OMS, 2016). A simple vista, la causa de esta pandemia de obesidad parece simple: demasiada ingesta calórica y poco ejercicio físico.

Debido a los cambios en la dieta causados por la globalización y el estilo de vida sedentario, se ha producido un desequilibrio en el balance energético, lo que ha llevado a un aumento de peso en la sociedad. El tejido adiposo que sirve como principal sistema de almacenamiento y transporte de energía del cuerpo, es crucial para controlar el equilibrio energético. Cuando el cuerpo almacena demasiada energía en forma de grasa, se desarrolla el sobrepeso/obesidad (Yong et al., 2023). Sin embargo, el aumento del aporte energético de las comidas y el sedentarismo no son factores exclusivos del sobrepeso/obesidad. El sobrepeso y la obesidad tienen una etiología multifactorial que incluye factores genéticos, ambientales, socioeconómicos y/o conductuales o psicológicos, siendo resultado de la interacción de estos diferentes factores (Goodarzi, 2018; Khanna et al., 2022). En la Figura 2 se muestran los diferentes factores influyentes en el desarrollo del sobrepeso/obesidad.

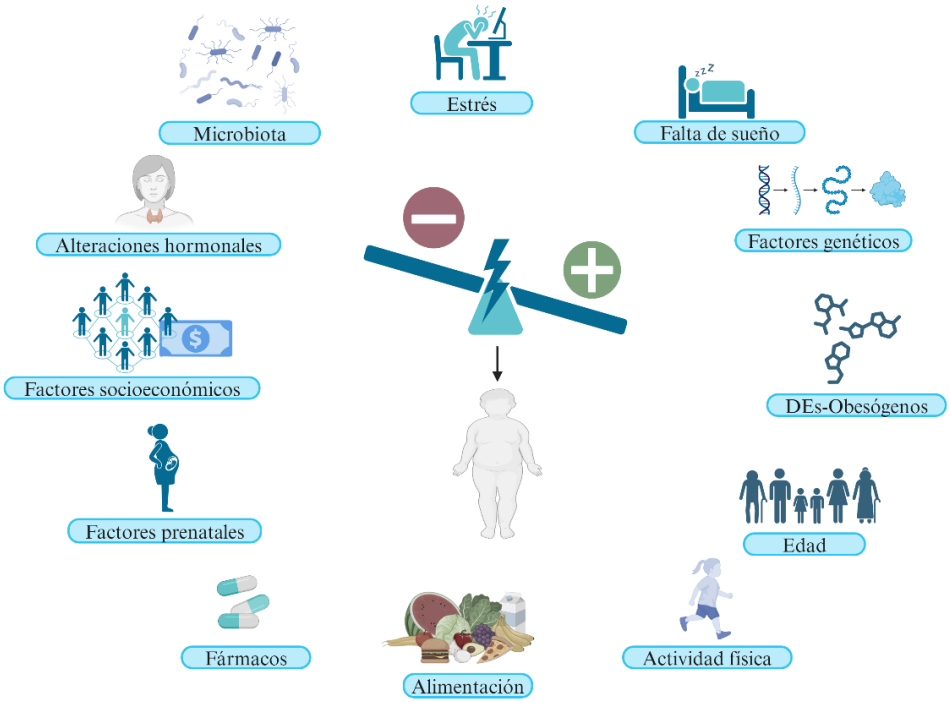



**Figura 2**. Factores asociados al sobrepeso/obesidad. Elaborado con BioRender.com

El sobrepeso y la obesidad como otras ECNT (diabetes mellitus, hipertensión, dislipemias, enfermedades cerebrovasculares, entre otras) pueden prevenirse o disminuir el riesgo de padecerlas con hábitos dietéticos saludables o un estilo de vida activo (OMS, 2024). Por ello, la OMS ha elaborado un total de 6 recomendaciones para combatir la obesidad infantil (OMS, 2016). Estas recomendaciones vienen detalladas en la Tabla 1.

**Tabla 1.** Recomendaciones para combatir la obesidad infantil elaboradas por la OMS.

| | |
|---|---|
| ***Recomendación 1*** | Aplicar programas integrales que promuevan la ingesta de alimentos sanos y reduzcan la ingesta de alimentos malsanos y bebidas azucaradas entre niños/as y adolescentes. |
| ***Recomendación 2*** | Aplicar programas integrales que promuevan la actividad física y reduzcan los comportamientos sedentarios en niños/as y adolescentes. |
| ***Recomendación 3*** | Integrar y fortalecer las orientaciones para la prevención de las enfermedades no transmisibles con las pautas actuales para la atención pregestacional y prenatal a fin de reducir el riesgo de obesidad infantil. |
| ***Recomendación 4*** | Ofrecer orientaciones y apoyo al establecimiento de una dieta sana y de pautas de sueño y de actividad física durante la primera infancia a fin de que los niños/as crezcan de forma adecuada y adquieran hábitos saludables. |
| ***Recomendación 5*** | Aplicar programas integrales que promuevan entornos escolares saludables, conocimientos básicos en materia de salud y nutrición y actividad física en niños/as y adolescentes en edad escolar. |
| ***Recomendación 6*** | Ofrecer a niños/as y jóvenes con obesidad servicios para el control del peso corporal que reúnan diversos componentes y se centren en la familia y en la modificación del tipo de vida. |

### *1.1.2. Diagnóstico de sobrepeso y obesidad*

En la actualidad, el sobrepeso y la obesidad se suelen clasificar mediante el IMC, el cual corresponde a la relación entre el peso expresado en kg y la altura en m² (OMS, 2024). Esta medida fue descrita por primera vez por Adolphus Quetelet a mediados del siglo XIX (Bray, 2023).

$$IMC = \frac{kg}{\mathrm{m}^2}$$

El IMC ayuda a clasificar a la población en las diferentes categorías existentes. Las categorías que engloban al IMC son, bajo peso, normopeso, sobrepeso u obesidad. A su vez la obesidad viene dividida en tres categorías, obesidad tipo I o moderada, obesidad tipo II o severa y obesidad mórbida o extrema (OMS, 2024). En la Tabla 2 se muestran las diferentes categorías del IMC.

**Tabla 2.** Clasificación IMC

| | |
|---|---|
| ***Bajo peso*** | < 18,5 kg/m² |
| ***Normopeso*** | 18,5 – 24,9 kg/m² |
| ***Sobrepeso*** | 25,0 – 29,9 kg/m² |
| ***Obesidad tipo I o moderada*** | 30,0 – 34,9 kg/m² |
| ***Obesidad tipo II o severa*** | 35,0 – 39,9 kg/m² |
| ***Obesidad Mórbida o extrema*** | ≥ 40,0 kg/m² |

Por ello, se considera que un adulto tiene sobrepeso cuando presenta un IMC entre 25-29,9 kg/m² u obesidad cuando el IMC es ≥ 30 kg/m², independientemente del sexo y la edad.

En el caso de los niños/as y adolescentes, el IMC es específico con respecto al sexo y la edad. El sobrepeso viene definido por la OMS para menores de 5 años como «*el peso para la estatura con más de dos desviaciones típicas por encima de la mediana establecida en los patrones de crecimiento infantil de la OMS*», y la obesidad es «*el peso para la estatura con más de tres desviaciones típicas por encima de la mediana establecida en los patrones de crecimiento infantil de la OMS*». En el

rango de edad de 5 a 19 años, según la OMS, el sobrepeso es «*el IMC para la edad con más de una desviación típica por encima de la mediana establecida en los patrones de crecimiento infantil de la OMS*», y la obesidad es «*el IMC para la edad con más de dos desviaciones típicas por encima de la mediana establecida en los patrones de crecimiento infantil de la OMS*» (OMS, 2024). En las Tablas 3 y 4 se muestra las diferentes categorías del IMC para niños/as y adolescentes en función del sexo y la edad.

**Tabla 3.** IMC en función de la edad, de niñas de 5 a 18 años (OMS 2007).

| *Edad (años:meses)* | Desnutrición severa < -3 DS (IMC) | Desnutrición moderada < -3 a < -2 DS (IMC) | Normopeso ≤ -2 a ≤ +1 DS (IMC) | Sobrepeso >+1 a ≤ +2 DS (IMC) | Obesidad >+2 DS (IMC) |
|---|---|---|---|---|---|
| *5:1* | <11,8 | 11,8 – 12,6 | 12,7 – 16,9 | 17,0 – 18,9 | ≥ 19,0 |
| *5:6* | <11,7 | 11,7 – 12,6 | 12,7 – 16,9 | 17,0 – 19,0 | ≥ 19,1 |
| *6:0* | <11,7 | 11,7 – 12,6 | 12,7 – 17,0 | 17,1 – 19,2 | ≥ 19,3 |
| *6:6* | <11,7 | 11,7 – 12,6 | 12,7 – 17,1 | 17,2 – 19,5 | ≥ 19,6 |
| *7:0* | <11,8 | 11,8 – 12,6 | 12,7 – 17,3 | 17,4 – 19,8 | ≥ 19,9 |
| *7:6* | <11,8 | 11,8 – 12,7 | 12,8 – 17,5 | 17,6 – 20,1 | ≥ 20,2 |
| *8:0* | <11,9 | 11,9 – 12,8 | 12,9 – 17,7 | 17,8 – 20,6 | ≥ 20,7 |
| *8:6* | <12,0 | 12,0 – 12,9 | 13,0 – 18,0 | 18,1 – 21,0 | ≥ 21,1 |
| *9:0* | <12,1 | 12,1 – 13,0 | 13,1 – 18,3 | 18,4 – 21,5 | ≥ 21,6 |
| *9:6* | <12,2 | 12,2 – 13,2 | 13,3 – 18,7 | 18,8 – 22,0 | ≥ 22,1 |
| *10:0* | <12,4 | 12,4 – 13,4 | 13,5 – 19,0 | 19,1 – 22,6 | ≥ 23,8 |
| *10:6* | <12,5 | 12,5 – 13,6 | 13,7 – 19,4 | 19,5 – 23,1 | ≥ 24,4 |
| *11:0* | <12,7 | 12,7 – 13,8 | 13,9 – 19,9 | 20,0 – 23,7 | ≥ 23,8 |
| *11:6* | <12,9 | 12,9 – 14,0 | 14,1 – 20,3 | 20,4 – 24,3 | ≥ 24,4 |
| *12:0* | <13,2 | 13,2 – 14,3 | 14,4 – 20,8 | 20,9 – 25,0 | ≥ 25,1 |
| *12:6* | <13,4 | 13,4 – 14,6 | 14,7 – 21,3 | 21,4 – 25,6 | ≥ 25,7 |
| *13:0* | <13,6 | 13,6 – 14,8 | 14,9 – 21,8 | 21,9 – 26,2 | ≥ 26,3 |
| *13:6* | <13,8 | 13,8 – 15,1 | 15,2 – 22,3 | 22,4 – 26,8 | ≥ 26,9 |
| *14:0* | <14,0 | 14,0 – 15,3 | 15,4 – 22,7 | 22,8 – 27,3 | ≥ 27,4 |
| *14:6* | <14,2 | 14,2 – 15,6 | 15,7 – 23,1 | 23,2 – 27,8 | ≥ 27,9 |
| *15:0* | <14,4 | 14,4 – 15,8 | 15,9 – 23,5 | 23,6 – 28,2 | ≥ 28,3 |
| *15:6* | <14,5 | 14,5 – 15,9 | 16,0 – 23,8 | 23,9 – 28,6 | ≥ 28,7 |
| *16:0* | <14,6 | 14,6 – 16,1 | 16,2 – 24,1 | 24,2 – 28,9 | ≥ 29,0 |
| *16:6* | <14,7 | 14,7 – 16,2 | 16,3 – 24,3 | 24,4 – 29,1 | ≥ 29,2 |
| *17:0* | <14,7 | 14,7 – 16,3 | 16,4 – 24,5 | 24,6 – 29,3 | ≥ 29,4 |
| *17:6* | <14,7 | 14,7 – 16,3 | 16,4 – 24,6 | 24,7 – 29,4 | ≥ 29,5 |
| *18:0* | <14,7 | 14,7 – 16,3 | 16,4 – 24,8 | 24,9 – 29,5 | ≥ 29,6 |

*DS: desviación estándar; IMC: índice de masa corporal*

**Tabla 4.** IMC en función de la edad, de niños de 5 a 18 años (OMS 2007).

| *Edad (años:meses)* | Desnutrición severa < -3 DS (IMC) | Desnutrición moderada < -3 a < -2 DS (IMC) | Normopeso ≤ -2 a ≤ +1 DS (IMC) | Sobrepeso >+1 a ≤ +2 DS (IMC) | Obesidad >+2 DS (IMC) |
|---|---|---|---|---|---|
| *5:1* | <12,1 | 12,1 – 12,9 | 13,0 – 16,6 | 16,7 – 18,3 | ≥ 18,4 |
| *5:6* | <12,1 | 12,1 – 12,9 | 13,0 – 16,7 | 16,8 – 18,4 | ≥ 18,5 |
| *6:0* | <12,1 | 12,1 – 12,9 | 13,0 – 16,8 | 16,9 – 18,5 | ≥ 18,6 |
| *6:6* | <12,2 | 12,2 – 13,0 | 13,1 – 16,9 | 17,0 – 18,7 | ≥ 18,8 |
| *7:0* | <12,3 | 12,3 – 13,0 | 13,1 – 17,0 | 17,1 – 19,0 | ≥ 19,1 |
| *7:6* | <12,3 | 12,3 – 13,1 | 13,2 – 17,2 | 17,3 – 19,3 | ≥ 19,4 |
| *8:0* | <12,4 | 12,4 – 13,2 | 13,3 – 17,4 | 17,5 – 19,7 | ≥ 19,8 |
| *8:6* | <12,5 | 12,5 – 13,3 | 13,4 – 17,7 | 17,8 – 20,1 | ≥ 20,2 |
| *9:0* | <12,6 | 12,6 – 13,4 | 13,5 – 17,9 | 18,0 – 20,5 | ≥ 20,6 |
| *9:6* | <12,7 | 12,7 – 13,5 | 13,6 – 18,2 | 18,3 – 20,9 | ≥ 21,0 |
| *10:0* | <12,8 | 12,8 – 13,6 | 13,7 – 18,5 | 18,6 – 21,4 | ≥ 21,5 |
| *10:6* | <12,9 | 12,9 – 13,8 | 13,9 – 18,8 | 18,9 – 21,9 | ≥ 22,0 |
| *11:0* | <13,1 | 13,1 – 14,0 | 14,1 – 19,2 | 19,3 – 22,5 | ≥ 22,6 |
| *11:6* | <13,2 | 13,2 – 14,1 | 14,2 – 19,5 | 19,6 – 23,0 | ≥ 23,1 |
| *12:0* | <13,4 | 13,4 – 14,4 | 14,5 – 19,9 | 20,0 – 23,6 | ≥ 23,7 |
| *12:6* | <13,6 | 13,6 – 14,6 | 14,7 – 20,4 | 20,5 – 24,2 | ≥ 24,3 |
| *13:0* | <13,8 | 13,8 – 14,8 | 14,9 – 20,8 | 20,9 – 24,8 | ≥ 24,9 |
| *13:6* | <14,0 | 14,0 – 15,1 | 15,2 – 21,3 | 21,4 – 25,3 | ≥ 25,4 |
| *14:0* | <14,3 | 14,3 – 15,4 | 15,5 – 21,8 | 21,9 – 25,9 | ≥ 26,0 |
| *14:6* | <14,5 | 14,5 – 15,6 | 15,7 – 22,2 | 22,3 – 26,5 | ≥ 26,6 |
| *15:0* | <14,7 | 14,7 – 15,9 | 16,0 – 22,7 | 22,8 – 27,0 | ≥ 27,1 |
| *15:6* | <14,9 | 14,9 – 16,2 | 16,3 – 23,1 | 23,2 – 27,4 | ≥ 27,5 |
| *16:0* | <15,1 | 15,1 – 16,4 | 16,5 – 23,5 | 23,6 – 27,9 | ≥ 28,0 |
| *16:6* | <15,3 | 15,3 – 16,6 | 16,7 – 23,9 | 24,0 – 28,3 | ≥ 28,4 |
| *17:0* | <15,4 | 15,4 – 16,8 | 16,9 – 24,3 | 24,4 – 28,6 | ≥ 28,7 |
| *17:6* | <15,6 | 15,6 – 17,0 | 17,1 – 24,6 | 24,7 – 29,0 | ≥ 29,1 |
| *18:0* | <15,7 | 15,7 – 17,2 | 17,3 – 24,9 | 25,0 – 29,2 | ≥ 29,3 |

*DS: desviación estándar; IMC: índice de masa corporal*

El IMC se ha utilizado consistentemente en una gran cantidad de estudios epidemiológicos y se ha recomendado para uso individual en la práctica clínica con el objetivo de guiar las recomendaciones para la pérdida y el control del peso (Winter et al., 2014; Khanna et al., 2022). Sin embargo, se han planteado limitaciones en el uso de IMC como

indicador de sobrepeso u obesidad, ya que el IMC no es un buen indicador de la composición corporal, debido a que no distingue entre masa grasa y masa magra en relación con el peso corporal. Se ha demostrado que hay individuos que presentan el mismo IMC, la misma estatura y peso, pero con respecto a su composición corporal no presentan semejanzas (Izquierdo-Aguilar & Aguilar-Rodríguez, 2020; Khanna et al., 2022). Es decir, un individuo puede presentar un peso elevado por presentar un mayor porcentaje de masa magra, mientras otro posee el mismo peso por exceso del porcentaje masa grasa (Figura 3).

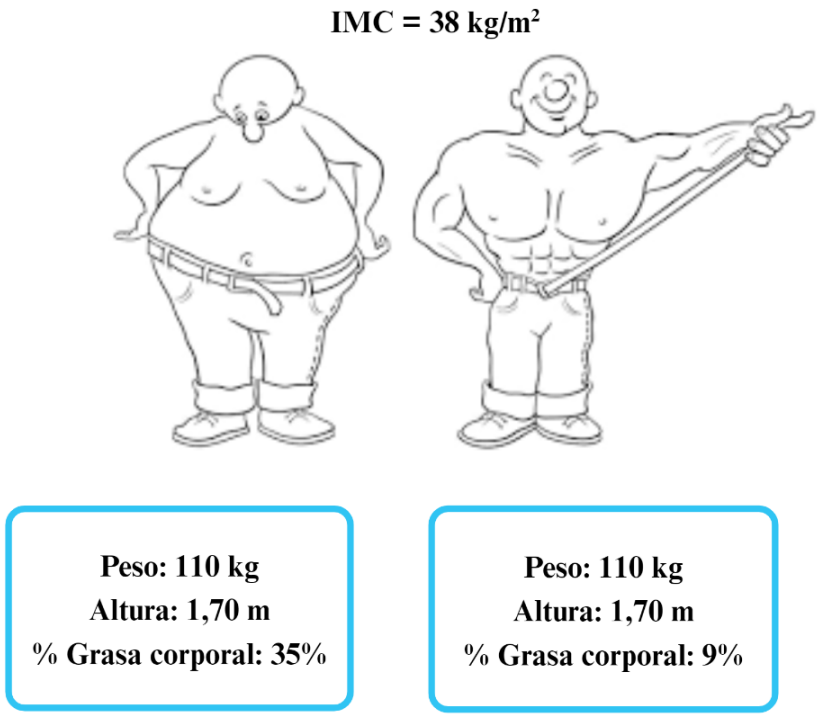



**Figura 3.** IMC obeso vs IMC culturista. Elaborado con BioRender.com

En 2008, se publicó un estudio realizado por Romero-Corral et al., en el que se demuestra que el IMC tiene un rendimiento diagnóstico limitado para identificar correctamente a los individuos con exceso de grasa corporal, particularmente para aquellos con un IMC entre 25 y 30 kg/m$^2$. El IMC tiene una buena correlación general con el porcentaje de grasa corporal, pero no logra discriminar entre el porcentaje de grasa corporal

y el porcentaje de masa magra. Por ello, en los últimos años ha aumentado la reflexión sobre cual es el mejor parámetro antropométrico para el diagnóstico del sobrepeso/obesidad. Para un correcto diagnóstico del grado de sobrepeso/obesidad se puede recurrir a la toma de IMC junto con otras medidas antropometrícas (pliegues y dimensiones corporales como, la circunferencia de la cintura y el índice cintura-cadera) o el uso de la bioimpedancia eléctrica, tanto desde un punto de vista clínico como epidemiológico (Valero et al., 2018).

### *1.1.3. La obesidad comienza en el útero*

Como se señaló anteriormente, la pandemia de obesidad también ha alcanzado a la población infantil. Es preocupante que la obesidad esté aumentando incluso en niños/as menores de 2 años. Esto es particularmente alarmante. Si bien se puede argumentar que los escolares y adultos pueden comer más y ser más sedentarios, resulta complicado afirmar que los bebés de hoy en día consumen más calorías y se ejercitan menos que las generaciones anteriores. Por lo tanto, es más probable que los bebés nazcan con un mayor porcentaje de grasa y/o que ocurran cambios en el entorno prenatal y posnatal en comparación con el pasado. La alimentación durante el embarazo, el estrés y la regulación de la insulina han sido vinculados con el riesgo de obesidad en la descendencia, lo que sugiere que factores del entorno prenatal y la infancia juegan un papel crucial en determinar la predisposición a la obesidad y otras enfermedades en etapas posteriores (Heindel & Blumberg, 2019).

Con respecto al medio ambiente, el ejemplo más claro se observa en el reino animal. En 2011, se publicó un estudio sobre 12 especies animales que vivían cerca del entorno humano en las sociedades industrializadas. En el estudio se observó un aumento de peso en esas especies entorno al 33,2% - 37,2% en el periodo comprendido entre 1985 y 2005. Entre las especies animales estudiadas incluían animales domésticos (perros y gatos), animales salvajes y modelos animales de laboratorio. Con relación a los animales domésticos se podría suponer que comen más y son más

sedentarios. En cambio, los animales de laboratorio viven en ambientes donde sus dietas están estrictamente controladas (Klimentidis et al., 2011). Así que, en vez de señalar al equilibrio energético como la causa del aumento en la obesidad, sería más lógico concluir que algo ha cambiado en el entorno de estos animales, lo cual los está volviendo obesos al mismo tiempo que a los humanos.

Como se mencionó anteriormente, la causa del sobrepeso y la obesidad es multifactorial. Sin embargo, los hallazgos arrojan serias dudas sobre que estos factores sean suficientes para explicar el aumento de sobrepeso/obesidad en la población mundial, e implican fuertemente la importancia de otros factores de riesgo (Heindel & Blumberg, 2019).

En las últimas décadas, se ha demostrado que una amplia variedad de compuestos químicos, presentes en el ambiente, son capaces de actuar alterando el sistema endocrino de los individuos influyendo en la adipogénesis, y pudiendo contribuir al aumento de la prevalencia del sobrepeso/obesidad (Heindel et al., 2022). Por ello, se empiezan a tener en cuenta otros factores de riesgo predisponentes como, la presencia de contaminantes químicos ambientales con actividad disruptora endocrina y obesogénica (Heindel & Blumberg, 2019; Heindel et al., 2022). La evidencia recogida hasta hoy sobre la actividad disruptora endocrina y obesógena de estos compuestos se ha observado principalmente en estudios *in vitro* e *in vivo*, siendo los estudios epidemilógicos escasos (Heindel et al., 2022).

## 1.2. Disruptores Endocrinos

La capacidad que tienen algunos compuestos químicos de síntesis de interferir en el sistema hormonal humano se conoce desde los años 40, cuando se utilizó el fármaco dietilestilbestrol con la esperanza de prevenir abortos espontáneos y partos prematuros (Rogers et al., 2023). Unos veinte años después en 1962 se publicó el libro «Primavera Silenciosa» de Rachel Carson, en el que se advertía de los efectos perjudiciales sobre el medio ambiente de los plaguicidas. Desde mediados del siglo XX, se ha

ido tomando conciencia de los efectos nocivos que presenta determinados compuestos químicos sobre la salud humana, animal y el medio ambiente. A finales del siglo XX se publicó el libro titulado «Nuestro futuro robado», escrito por Theo Colborn, John Peterson Myers y Dianne Dumanosk, donde, por primera vez se reúnen pruebas alarmantes de diferentes estudios científicos para presentar la base de este nuevo peligro de una manera científica y fácil de entender (Pombo-Arias et al., 2020).

El término «Disruptor Endocrino» apareció por primera vez en una conferencia organizada por la Dra. Theo Colborn, del World Wildlife Fund, en Wingspread, Wisconsin, en el año 1991, en el cual, un grupo de expertos se reunió para evaluar las causas de los efectos adversos observados en estudios *in vivo* y epidemilógicos, en el que se observaron daños en el sistema reproductor e inmunitario y cánceres hormono dependientes entre otros efectos. Los expertos propusieron la hipótesis de que la exposición a contaminantes químicos ambientales producía efectos perjudiciales sobre los animales y los seres humanos, a estos compuestos químicos los denominaron disruptores endocrinos (DEs) (Marconetto et al., 2022).

Desde que se utilizó por primera vez el término DEs, varios grupos y agencias han propuesto numerosas definiciones (Di Pietro et al., 2023). Una de las definiciones establecidas para los DEs según la Comisión Europea es «*sustancias, tanto naturales como químicas, que pueden alterar las funciones del sistema hormonal y, en consecuencia, causar efectos adversos en las personas o los animales*» (Comisión Europea, 2016a). De acuerdo con la Comisión Europea, los DEs deben considerarse como tales cuando presentan tres efectos (Lauretta et al., 2019):

1) Actividad endocrina.
2) Actividad mediada endocrina nociva y/o patológica.
3) Relación causa-efecto entre sustancias, exposición y actividad endocrina.

El conocimiento científico en este área sigue creciendo y, por tanto, la comprensión de lo que es un DEs sigue siendo objeto de debate científico.

### *1.2.1. Clasificación de los Disruptores Endocrinos*

La lista de DEs es muy amplia y crece cada día, comprendiendo desde compuestos químicos sintetizados por el hombre hasta compuestos que se encuentran de manera natural en el medio ambiente. Debido al amplio catálogo de DEs actualmente existen diversas clasificaciones, en función de diferentes criterios: (I) según el origen; (II) en función de la naturaleza; (III) en función del lugar de aparición (Kabir et al., 2015).

(I) Según el origen:

- Hormonas artificiales o naturales (fitoestrógenos, anticonceptivos y fármacos para el tratamiento de enfermedades tiroideas).
- Fármacos con efectos hormonales secundarios (naproxeno y metoprolol).
- Sustancias químicas industriales y de uso doméstico (ftalatos, retardantes de la llama y bisfenoles).
- Subproductos de procesos industriales o domésticos (hidrocarburos aromáticos policíclicos y dioxinas).

(II) En función de la naturaleza:

- Nuturales. Compuestos químicos que se encuentran en los alimentos de forma natural (fitoestrógenos).
- Sintéticos. Este grupo esta constituido por compuestos químicos sintetizados por el hombre como, por ejemplo, disolventes o lubricantes industriales (dioxinas, bifenilos policlorados y polibromados), plásticos (BPA), plastificantes, pesticidas, fungicidas y algunos fármacos (dietilestilbestrol).

(III) En función del lugar de aparición:

- Pesticidas.
- Sustancias químicas en productos de uso cotidiano: productos de cuidado e higiene personal (parabenos), textil, electrodomésticos (retardadores de la llama) entre otros.
- Materiales en contacto con los alimentos (BPA).

### *1.2.2. Mecanismos de acción de los Disruptores Endocrinos*

Los DEs son compuestos exógenos que se encuentran en el medio ambiente y que pueden interactuar con las vías endocrinas del cuerpo y contribuir a efectos adversos sobre la salud. El sistema endocrino es importante para la salud humana y animal, ya que regula y controla la liberación de hormonas (Darbre, 2017; Monneret, 2017; Varghese & Hall, 2023). Las hormonas actúan en cantidades muy pequeñas y en momentos precisos para regular multitud de funciones del organismo como el desarrollo, el crecimiento, la reproducción, el metabolismo y la inmunidad entre otras. Los efectos de los DEs sobre la salud pueden sentirse mucho después de que haya cesado la exposición. La exposición a DEs puede tener efectos para toda la vida e incluso consecuencias para la próxima generación, es decir, efectos multigeneracionales (Monneret, 2017).

Los DEs pueden interactuar con el sistema endocrino mediante el bloqueo de la acción hormonal por competición con el receptor hormonal, suplantación o mimetización de las hormonas endógenas y/o, a través de un aumento o disminución de los niveles de actividad hormonal (Darbre, 2017; Monneret, 2017; Varghese & Hall, 2023). En la Figura 4 se muestra de forma gráfica el bloqueo de la acción hormonal por competención con el receptor.

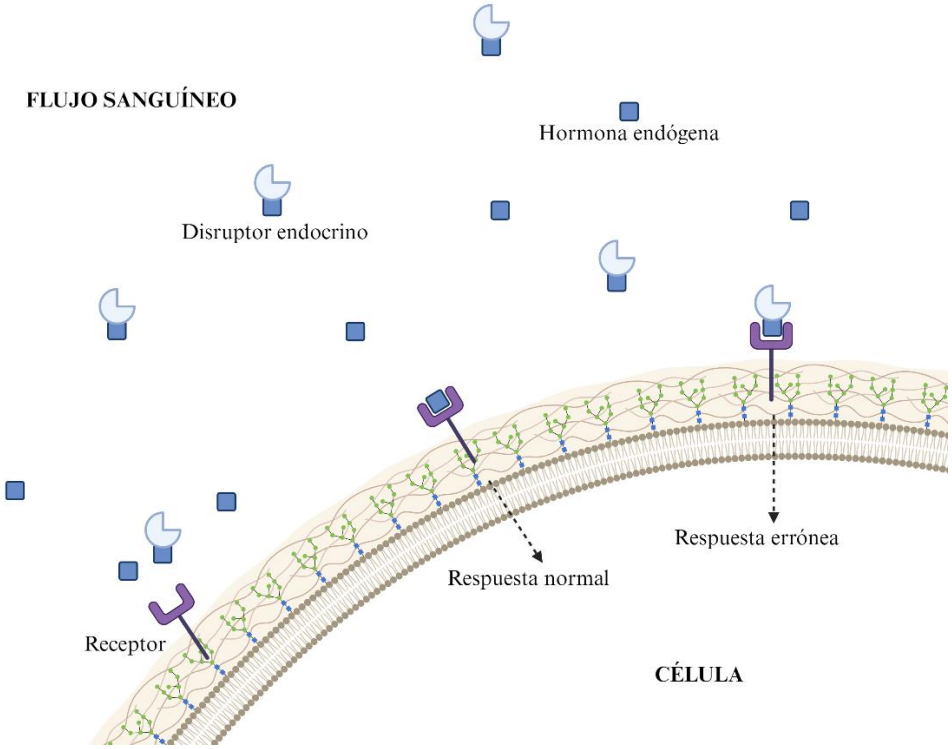



**Figura 4.** Bloqueo de la acción hormonal por competición con el receptor hormonal. Elaborado con Biorender.com

Esta interacción puede presentar efectos adversos sobre las funciones del organismo, siendo estos efectos potenciados durante la fase prenatal y en las fases iniciales de la vida. La etapa de la infancia es más sensible a los efectos de los DEs que la adulta, debido principalmente a diferencias a nivel fisiológico y anatómico, pero también a nivel farmacocinético, dietético y conductual (Di Pietro et al., 2023).

### *1.2.3. Efecto Dosis-Respuesta*

En la actualidad, hay controversias acerca de la relación entre la dosis-respuesta de los DEs. Se ha observado que los DEs presentan la particularidad de ocasionar un determinado efecto incluso a bajas concentraciones, y estas concentraciones corresponden a los niveles de exposición a los que la población general está diariamente expuesta (Marconetto et al., 2022). Actualmente no existe un umbral de

concentración exacto para prevenir los efectos tóxicos de los DEs. Por ello, no se pueden establecer niveles de exposición seguros para la población, ya que la sensibilidad a los DEs varía entre individuo.

Con respecto a las curvas dosis-respuesta, los DEs no presentan curvas dosis-respuesta tradicionales, no siguen un patrón lineal, sino que presentan curvas dosis-respuesta no monótonas, en forma de U o U invertida (Marconetto et al., 2022; Yue et al., 2023).

Hay que tener en cuenta que los individuos de una población suelen exponerse a múltiples DEs al mismo tiempo, pudiendo ser el efecto negativo causa de la mezcla de diferentes compuestos químicos. De manera individual, algunos DEs pueden no desencadenar un efecto, pero cuando se combinan entre ellos, pueden generar una respuesta sinérgica, antagónica o aditiva. Los desafíos en la investigación y determinación de los niveles de toxicidad de los DEs se han visto dificultados debido a este efecto cóctel (Marconetto et al., 2022; Dutta et al., 2023).

### *1.2.4. Vías de absorción y fuentes de exposición a los Disruptores Endocrinos*

Los DEs son un grupo muy heterogéneo de compuestos químicos, de origen y estructura muy diferente. Como se mencionó anteriormente, algunos de ellos están presentes en la naturaleza de forma natural, pero la mayoría son compuestos químicos de síntesis que han sido liberados al medio ambiente por actividades humanas sin ningún conocimiento previo de sus efectos adversos. Estos compuestos originalmente fueron diseñados y sintetizados para llevar a cabo funciones concretas como, control de plagas en los cultivos, mejora de la conservación de los productos de higiene personal o formando parte de la estructura de algunos plásticos otorgándole determinadas propiedades como, termorresistencia y flexibilidad, entre otras (Darbre, 2017, Marconetto et al., 2022).

En la segunda mitad del siglo XX, los DEs experimentaron su primer auge de desarrollo. En 2019, la producción total de productos químicos

peligrosos se estimó en 211 millones de toneladas en toda la Unión Europea. Casi 1.000 compuestos químicos se han clasificado como DEs. Siendo esto solo una pequeña fracción del total de sustancias químicas conocidas en el medio ambiente (Macedo et al., 2023).

Las fuentes de exposición a los DEs son diversas y varían ampliamente en todo el mundo. Se observa en diferentes estudios que los DEs están presentes en el aire que respiramos, el agua que bebemos e incluso en el suelo en el que se cultivan nuestros alimentos. Esto es debido principalmente a la actividad industrial, por ello los DEs son liberados al medio que nos rodea, distribuyéndose fácilmente por toda la cadena trófica e ingresando en el organismo a través de la vía alimentaria mayoritariamente. Debido a su naturaleza lipófila y su resistencia a la degradación, algunos compuestos pueden acumularse en el cuerpo, persistiendo en los tejidos grasos. Mientras que otros compuestos entran al cuerpo de forma regular y son rápidamente excretados, lo que los hace pseudo-persistentes (Kabir et al., 2015; Macedo et al., 2023).

Una de las características más distintivas de los DEs es su ubicuidad. Este hecho hace que hoy en día estemos expuestos a ellos de forma constante por diversas vías de exposición, como la ingestión (alimentos), la inhalación y la absorción dérmica (Marconetto et al., 2022). La ingestión o vía oral es la principal vía de exposición a los DEs, estos van a llegar al interior del organismo tras el consumo de alimentos tanto de origen vegetal como animal, siendo la via principal de exposición. La inhalación o vía respiratoria y la absorción o vía dérmica son las otras dos vías de entrada de los DEs al organismo, a las que contribuyen mucho la contaminación ambiental y el uso de cosméticos (Olea, 2022; Dutta et al., 2023). En la Figura 5 se muestran las principales fuentes de exposición a los DEs en función de las tres vías de entrada en el organismo humano.

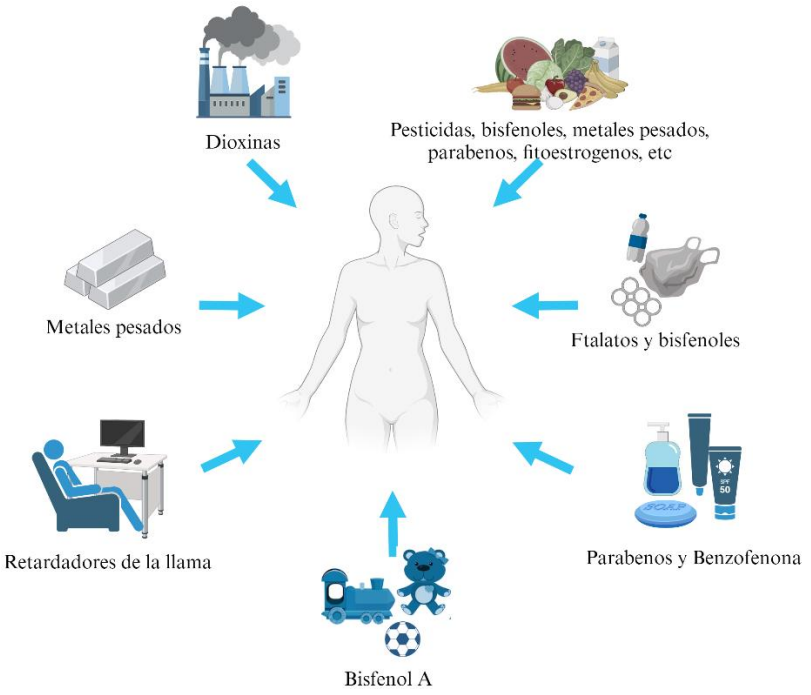



**Figura 5.** Fuentes de exposición a los DEs. Elaborado con Biorender.com

### *1.2.5. Efectos adversos sobre la salud de los DEs*

La exposición a los DEs tiene impactos negativos sobre algunos sistemas fisiológicos del organismo, especialmente al alterar el equilibrio hormonal. Se ha demostrado que los DEs afectan a el sistema reproductivo, tiroideo, neurológico y adiposo, e incluso estimulan el crecimiento tumoral. Además, los efectos sobre la salud dependen del momento de exposición, ya sea la etapa prenatal, durante la infancia, adolescencia o edad adulta (Zlatnik, 2016; Yilmaz et al., 2020; Ahn & Jeung, 2023; Macedo et al., 2023).

Algunos compuestos químicos con actividad disruptora endocrina, como los bisfenoles, los parabenos, los compuestos organoclorados, los retardantes de llama polibromados, los alquilfenoles y los ftalatos, se han ido evidenciando gradualmente como posibles factores de riesgo de enfermedades reproductivas, tumorales, neuronales y metabólicas (Darbre, 2018; Ahn & Jeung, 2023).

A continuación, se mencionan algunos de los efectos adversos más profundamente estudiados de los DEs sobre la salud humana.

### a) Efectos sobre salud reproductiva

Los DEs ejercen sus efectos tóxicos alterando la fisiología de las hormonas que promueven el crecimiento y desarrollo de los tejidos reproductivos. Estos compuestos químicos exógenos impiden la unión de las hormonas a sus respectivos receptores, como el receptor de estrógenos (ER) y el receptor de andrógenos (AR), lo que puede provocar efectos agonistas o antagonistas (Yilmaz et al., 2020).

Se ha observado que los DEs afectan tanto a la salud reproductiva femenina como masculina. Con respecto al sistema reproductor femenino, se ha observado asociaciones con la infertilidad, fecundidad y abortos espontáneos. En relación con los hombres, la exposición a los DEs se asocia con una peor calidad del esperma, difusión eyaculatoria, distancia anogenital más corta y desarrollo anormal de los genitales (Zlatnik, 2016).

Adicionalmente, la preocupación por los DEs y la reproducción se ve aumentada por la evidencia emergente que sugiere que algunos efectos pueden tener impactos que se extienden más allá de la primera generación. Los efectos multigeneracionales de los DEs se observaron por primera vez con el fármaco dietilestilbestrol. Las hijas cuyas madres tomaron dietilestilbestrol durante el embarazo presentaron una mayor probabilidad de sufrir efectos adversos en el sistema reproductor, incluidos cánceres vaginales e incompetencia cervical. También se observaron efecto en la tercera generación (nietos), quienes presentaron una mayor probabilidad de hipospadias, lo que sugiere que los DEs pueden causar efectos transgeneracionales a través de mecanismos epigenéticos (Zlatnik, 2016).

### b) Desarrollo de cáncer estrógeno dependiente

De acuerdo con la literatura encontrada, se ha observado un incremento en los casos de cáncer de mama, ovario y endometrio, y se

plantea la posibilidad de que los DEs y otros factores ambientales estén contribuyendo a estas enfermedades relacionadas con las hormonas, ya que alrededor del 80% de los DEs son potencialmente cancerígenos (Yilmaz et al., 2020; Macedo et al., 2023).

El cáncer de mama, endometrio, ovárico, próstata, testícular y tiroides son cánceres hormono dependientes, todos ellos comparten un mecanismo único. Las hormonas endógenas y exógenas favorecen la proliferación celular. Los DEs funcionan como hormonas exógenas, promoviendo la proliferación celular y favoreciendo la progresión tumoral (Yilmaz et al., 2020; Ahn & Jeung, 2023).

Hoy en día, los mecanismos cancerígenos que tienen lugar tras la exposición a los DEs no se comprenden completamente. Pueden ocurrir cambios genéticos directos (genotoxicidad), pero también pueden influir otros posibles mecanismos. Incluso la susceptibilidad genética individual a algunos DEs también puede estar en juego (Macedo et al., 2023).

### c) Alteraciones en el neurodesarrollo

Dado que la etapa prenatal y la primera infancia son las etapas más importantes para el correcto desarrollo y funcionamiento del sistema neuroendocrino, la exposición a los DEs durante estos periodos del desarrollo es de gran preocupación (Ramírez et al., 2022). Datos recientes sugieren que la exposición a DEs se asocia con una mayor incidencia de la ansiedad, la depresión, las alteraciones neuroconductuales, el déficit de atención, la peor coordinación motora fina y un menor coeficiente intelectual (Yilmaz et al., 2020).

A pesar de la falta de claridad sobre cómo estas sustancias afectan al neurodesarrollo, hay indicios de que la alteración endocrina puede estar involucrada. Se cree que algunos DEs interfieren con la actividad normal de la hormona tiroidea (triyodotironina (T3)), lo que a su vez causa deterioros cognitivos y conductuales. Puesto que la actividad hormonal tiroidea puede desempeñar un papel crucial en el desarrollo neurológico

normal del cerebro, estas interacciones pueden causalmente desempañar un papel en el desarrollo neurológico anormal (Yilmaz et al., 2020).

### d) Enfermedades Metabólicas

Cada vez hay más evidencia de la asociación entre las enfermedades metabólicas y la exposición a los DEs. Éstos tienen la capacidad de inducir alteraciones en el metabolismo, lo que puede resultar en trastornos metabólicos y contribuir significativamente a la propagación mundial de enfermedades metabólicas. Varios estudios han señalado una asociación significativa entre la exposición a diferentes DEs y diversos factores que influyen en las enfermedades metabólicas, como la obesidad, la diabetes tipo II, el hígado graso no alcohólico y el síndrome metabólico (Heindel et al., 2017; Ahn & Jeung, 2023).

Con relación a los mecanismos de acción, se han sugerido varios mediante los cuales los DEs pueden contribuir al desarrollo de enfermedades metabólicas (Romano-Mozo, 2012; Ahn & Jeung, 2023). En el caso de la obesidad, como posibles mecanismos se plantean mediante la alteración de los puntos de ajuste metabólicos (set-points), alteración del control del apetito y perturbación de la homeostasis lipídica durante las etapas del desarrollo. A pesar de que el periodo fetal es clave debido a la reprogramación de la expresión génica a través de cambios epigenéticos que pueden influir en el desarrollo posterior de la obesidad, así mismo, la exposición de los adultos a determinados DEs también puede desencadenar la aparición de obesidad. Por otro lado, el mecanismo de alteración endocrina asociado con la diabetes tipo II es la estimulación de los ER-α en las células β del páncreas, lo que resulta en una señalización excesiva de la insulina que conduce a la resistencia a la insulina en el hígado y los músculos y al agotamiento de las células β (Romano-Mozo, 2012). Con respecto al hígado graso no alcohólico, los DEs pueden promover la apareción de esteatosis hepática al incrementar la absorción de ácidos grasos libres, incrementar la lipogénesis, disminuir la exportación de triglicéridos a través de lipoproteínas de muy baja densidad y/o disminuir la β-oxidación de los ácidos grasos. Esto es debido

a que los DEs actúan como agonistas o antagonistas de los receptores hormonales nucleares (NR) alterando su función. Los NR presentan funciones cruciales sobre el metabolismo de los lípidos hepáticos (Ahn & Jeung, 2023).

En la Figura 6 se muestran de manera esquematizada los principales efectos sobre la salud de los DEs sobre algunos sistemas del organismo humano.

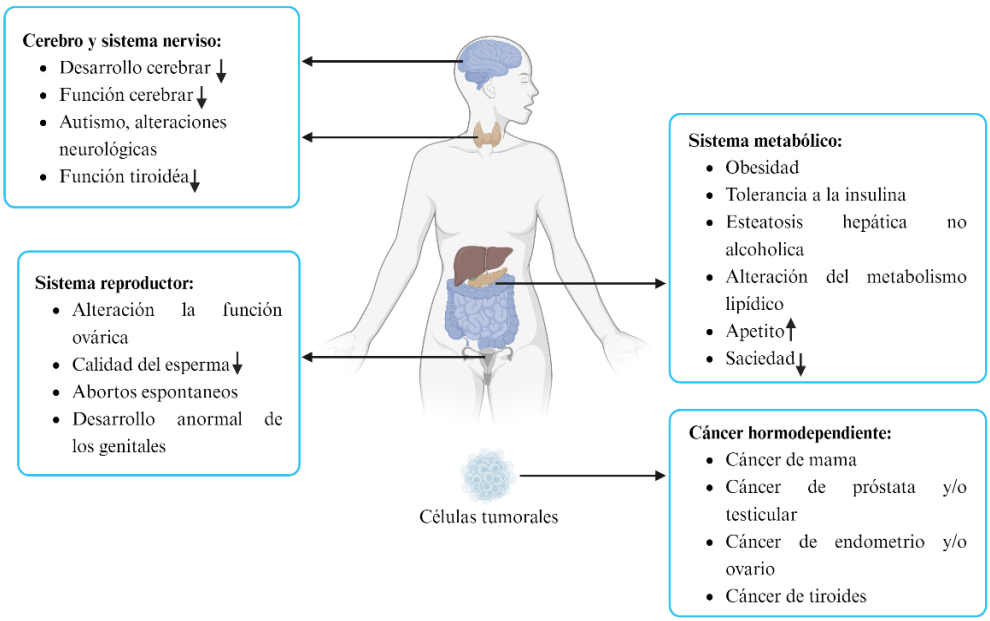



**Figura 6.** Efectos adversos sobre la salud humana de los DEs. Elaborado con Biorender.com

Aunque gran parte de la investigación sobre los DEs se ha centrado en la alteración de la homeostasis hormanal del organismo, cada vez hay más evidencias de que algunos DEs también pueden conducir a la alteración del metabolismo lipídico. En las últimas dos décadas se ha asociado la exposición a determinados DEs con el desarrollo de la obesidad, sugiriendo que el aumento de la prevalencia a nivel mundial está relacionado con el aumento de la producción de compuestos químicos ambientales con actividad obesogénica (Darbre, 2017).

## 1.3. Hipótesis sobre los obesógenos

En 2002, Baille-Hamilton publicó el primer artículo que establecía una relación entre la exposición a DEs y la obesidad. En su artículo titulado *«Toxinas químicas: una hipótesis para explicar la epidemia global de obesidad»,* se planteó la hipotesis de que la producción creciente de estos compuestos estaba vinculada a la epidemia actual de obesidad. Baille-Hamilton revisó diversos estudios donde se relacionaba la exposición a varios DEs (pesticidas, retardantes de la llama, metales pesados, plásticos y disolventes) con una mayor ganancia de peso. En 2006, apareció por primeravez el termino «obesógeno» en el artículo publicado por Grün & Blumberg (Grün & Blumberg, 2006). Tres años después estos dos autores publicaron una revisión titulada «*Los disruptores endocrinos como obesógenos*», donde observaron que diversos DEs alteraban el equilibrio energético favoreciendo la ganancia de peso. En base a estos hallazgos propusieron que los obesógenos tenían la capacidad de alterar los mecanismos homeostáticos para el control del peso, de este modo los individuos expuestos presentan una probabilidad mayor de aumento de peso a pesar de mantener una correcta alimentación y un estilo de vida activo (Heindel et al., 2017, 2022).

La hipótesis del obesógeno plantea dos puntos importantes (Heindel et al., 2017):

1) La predisposición a la obesidad comienza durante las primeras etapas del desarrollo (prenatal, postnatal y la infancia).
2) La predisposición a la obesidad se debe en parte a la influencia de un conjunto específico de DEs que modifican la programación del desarrollo y, en consecuencia, alteran el punto de ajuste para el aumento de peso en etapas posteriores.

### *1.3.1. Concepto Obesógeno*

El termino «obesógenos» se definió por primera vez como «*compuestos químicos xenobióticos que regulan y promueven de forma inapropiada la acumulación de lípidos y la adipogénesis*» (Grün & Blumberg, 2006).

Los obesógenos pueden afectar directamente y/o indirectamente sobre el tejido adiposo. Por un lado, pueden favorecer directamente promoviendo la diferenciación de los preadipocitos a adipocitos a partir de células madre. También pueden alterar el número de adipocitos, aumentar el almacenamiento de triglicéridos y cambiar la tasa de creación o destrucción de adipocitos. Por otro lado, pueden influir indirectamente al modificar el equilibrio energético a favor del almacenamiento de energía, afectar a la tasa metabólica basal, alterar la microbiota intestinal para promover el almacenamiento de energía y perturbar el control hormonal del apetito y la saciedad (Heindel & Blumberg, 2019).

Según esta perspectiva, pueden tener un papel en el aumento de peso al influir sobre el tejido adiposo, el consumo de alimentos y el metabolismo. Así, los compuestos químicos con actividad disruptora endocrina y obesogénica afectan a factores nucleares y otras vías endocrinas durante las etapas del desarrollo, lo que resulta en etapas posteriores de la vida en la obesidad (Heindel & Blumberg, 2019).

### *1.3.2. Tejido adiposo como órgano endocrino*

El sistema endocrino desempeña un papel crucial en la regulación del metabolismo de los macronutrientes (grasas, hidratos de carbono y proteínas), asegurando que estos nutrientes proporcionen la energía necesaria para el organismo en todo momento. Las hormonas son las encargadas del almacenamiento del exceso de energía en tiempos de abundancia y de su movilización en tiempos de necesidad y, sobre todo, de mantener la glucemia constante. Se puede esperar que cualquier cambio en estos procesos hormonales puede causar un desequilibrio en el

metabolismo. La principal reserva de energía del cuerpo la proporciona la grasa contenida en los adipocitos del tejido adiposo. Hoy en día, se reconoce al tejido adiposo como un órgano con función endocrina capaz de secretar hormonas (leptina, adiponectina y citoquinas proinflamatorias como IL-6 entre otras) (Darbre, 2017). Con respecto a la actividad hormonal del tejido adiposo, la leptina está involucrada en la regulación de la ingesta y el equilibrio energético, la adiponectina se encargada de la homeostasis de los hidratos de carbono, y la IL-6 se vincula con la inflamación y la obesidad (Esteve Ràfols, 2014). De este modo, cualquier interferencia en el control hormonal de las funciones del tejido adiposo podría ocasionar la acumulación inapropiada de grasa y, en consecuencia, obesidad (Darbre, 2017).

Actualmente, se distiguen dos tipos de tejido adiposo, el blanco y el pardo o marrón. El tejido adiposo blanco (WAT) constituye más del 95% del peso de la grasa corporal. Es el responsable de la función endocrina del tejido adiposo y se encuentra ampliamente distribuido en el cuerpo, dispuesto en dos compartimientos subcutáneo y visceral. También, es el responsable del almacenamiento de energía en los triglicéridos. El tejido adiposo pardo o marrón (BAT) representa alrededor del 1% al 2% en los adultos, siendo más abundante en las primeras etapas de la vida, disminuyendo marcadamente después de las 8 semanas de vida. Principalmente, El BAT se localiza en áreas céntricas e internas, distribuido de tal manera que genera calor para calentar la sangre que va hacia los órganos vitales. Se encuentra en la región axilar, subescapular, interescapular, intercostal, cervical e inguinal. Como se ha mencionado, la característica más destacada de BAT es su capacidad para generar calor a través de la termogénesis, sin necesidad de temblores, para mantener la temperatura corporal (Esteve Ràfols, 2014; Frigolet & Gutiérrez-Aguilar, 2020; Kowalczyk et al., 2023).

El tejido adiposo representa entre 20% y 28% de la masa corporal de los individuos sanos, este porcentaje varía dependiendo del sexo y del estado energético, lo que significa que el % de grasa puede representar hasta el 80% del peso corporal en individuos obesos (Frigolet & Gutiérrez-

Aguilar, 2020). El volumen de la masa de tejido adiposo está determinado por el agrandamiento de los adipocitos existentes (hipertrofia) y por un aumento en el número de adipocitos (hiperplasia). El número de adipocitos en adultos permanece bastante constante sin importar si la persona es obesa o delgada, ya que, durante la niñez y la adolescencia se establece y mantiene ese número. Durante la edad adulta, la masa de WAT aumenta mediante hipertrofia. La hipertrofia puede ser desencadenada por la sobrenutrición, la cual provoca una mayor acumulación de grasa en los adipocitos, por consiguiente, los adipocitos sufren una hipertrofia. En contraposición, la lipólisis ocurre en los adipocitos cuando se reduce la ingesta de calorías (Esteve Ràfols, 2014; Kowalczyk et al., 2023).

Los obesógenos pueden actuar alterando la biología del tejido adiposo por diversos mecanismos tales como regular el desarrollo de las células madre y preadipocitos hacia su diferenciación en adipocitos maduros y/o el tamaño de los adipocitos, así como el contenido de triglicéridos de los adipocitos, entre otros (García-Mayor et al., 2012; Heindel et al., 2022).

### *1.3.3. Mecanismo de acción de los obesógenos*

Para establecer una relación de causa-efecto entre la exposición a compuestos químicos y la obesidad, es necesario identificar los mecanismos de acción de dichos compuestos. Los obesógenos tienen la capacidad de afectar directamente la fisiología del tejido adiposo por diversos mecanismos (Desai et al., 2018; Heindel & Blumberg, 2019; Shahnazaryan et al., 2019; Guerrero-Meza et al., 2022)

Un posible mecanismo es la alteración de la biología del adipocito, a través de la inducción de hiperplasia y/o hipertrofia. En relación a la hiperplasia, puede ser mediante la activación de la adipogénesis por medio de dianas moleculares presentes en el tejido adiposo tales como el receptor activado por el proliferador de peroxisomas-γ (PPAR-γ), ER y receptor de glucocorticoides (GR) (Shahnazaryan et al., 2019). La adipogénesis «*es el proceso por el cual las células progenitoras similares*

*a los fibroblastos restringen su destino hacia células adipogénicas, acumulan nutrientes y se convierten en adipocitos maduros que almacenan grandes cantidades de triglicéridos*» (Guerrero-Meza et al., 2022). Los obesógenos pueden unirse al PPAR-γ, al ER y al GR activándolos y estimulando la diferenciación de las células madre mesenquimales (MSC) hacia los adipocitos. Con respecto a la hipertrofia, los obesógenos pueden aumentar directamente la expresión de genes implicados en el almacenamiento de lípidos en los adipocitos, como la lipoproteína lipasa, o mediante la activación del GR (Shahnazaryan et al., 2019; Heindel et al., 2019).

Otro posible mecanismo que aumenta la adipogénesis es por medio de un estado inflamatorio o un aumento del estrés oxidativo causado por la exposición a obesógenos. Los niveles elevados de especies reactivas de oxígeno (ROS) se asocian con una diferenciación de las MSC hacia los adipocitos. Por otro lado, algunos obesógenos aumentan los niveles de citoquinas proinflamatorias, como la IL-6, promoviendo la adipogénesis (Shahnazaryan et al., 2019).

Los obesógenos también pueden influir en el control hormonal del hambre y la saciedad mediante la alteración de la función del hipotálamo, que es la región del cerebro responsable de controlar la conducta alimentaria. Se ha observado en estudios en animales que la exposición a determinados obesógenos en las primeras etapas de la vida altera las vías de señalización tanto presinápticas como postsinápticas, promoviendo comportamientos adictivos y compulsivos y dando como resultado un mayor consumo de alimentos y la posterior ganancia de peso (Desai et al., 2018; Shahnazaryan et al., 2019; Heindel et al., 2022).

En los últimos años, los mecanismos epigenéticos han generado un gran interés como posibles reguladores de la expresión genética en el tejido adiposo (Shahnazaryan et al., 2019). La epigenética se define como «*estudio de los cambios que activan o inactivan los genes sin cambiar la secuencia del ADN, a causa de la edad y la exposición a factores ambientales (alimentación, ejercicio, medicamentos y sustancias*

*químicas). Estos cambios modifican el riesgo de enfermedades y a veces pasan de padres a hijos*» (National Cancer Institute (NIH), https://www.cancer.gov/espanol/publicaciones/diccionarios/diccionario-cancer/def/epigenetica). Se ha reunido evidencia que indica que la exposición a obesógenos durante el desarrollo puede provocar cambios duraderos en la actividad genética en tejidos importantes para regular el metabolismo. Se ha demostrado que la exposición a determinados obesógenos causa cambios de las histonas que estimula la diferenciación de las MSC hacia los adipocitos (Shahnazaryan et al., 2019).

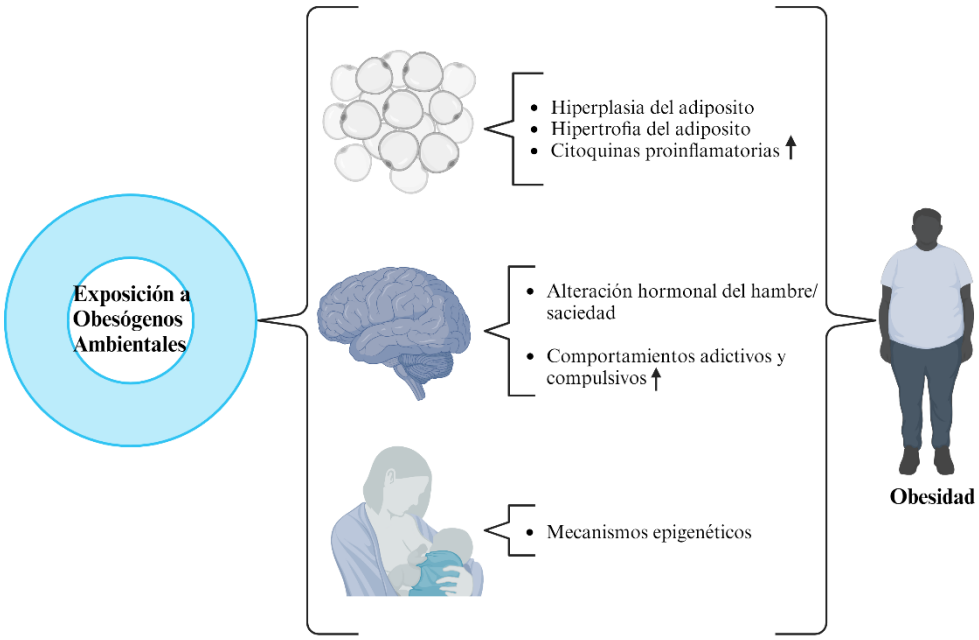



**Figura 7.** Resumen de los efectos de los obesógenos y su relación con la obesidad. Elaborado con Biorender.com

### *1.3.4. Susceptibilidad del desarrollo a los obesógenos*

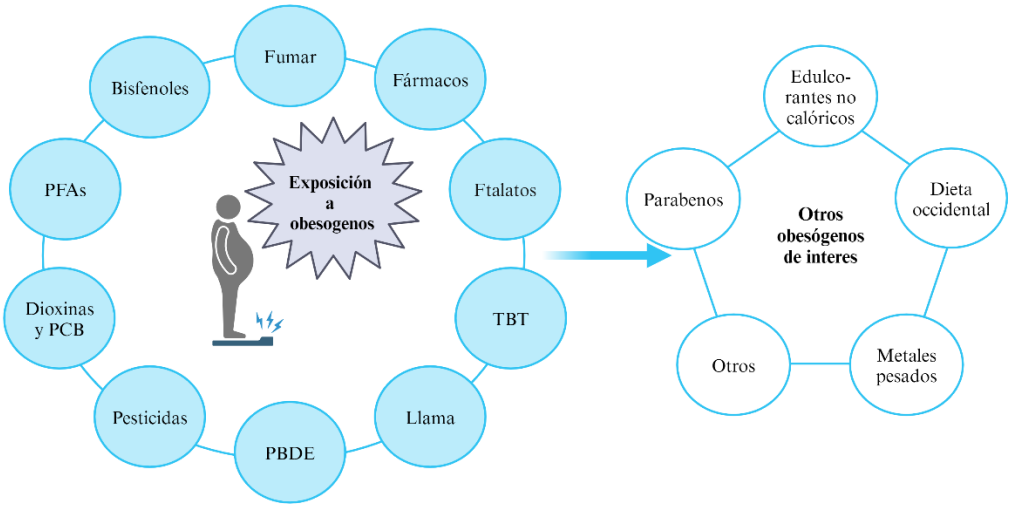
La preconcepción, el embarazo y la primera infancia son los momentos más sensibles a la exposición a obesógenos, ya que, en estas etapas los organismos son extremadamente vulnerables a sustancias químicas con efecto similar a las hormonas (García-Mayor et al., 2012; Darbre, 2017; Heindel et al., 2022). Se ha demostrado que los efectos de los obesógenos son más fuertes, más pronunciados y en concentraciones

más bajas en las primeras etapas del desarrollo de los individuos. Esto se debe a que después del nacimiento, los efectos protectores que ocurren en la edad adulta, como la reparación del ADN, un sistema inmunológico fuerte, las enzimas desintoxicantes del hígado y la barrera hematoencefálica, no están completamente funcionales en esta etapa. Además, el metabolismo aumenta durante el período fetal e intrauterino en comparación con etapas posteriores del desarrollo, lo que puede exacerbar los efectos de los obesógenos (García-Mayor et al., 2012). Asimismo, la exposición a estos compuestos químicos durante las primeras etapas del desarrollo puede causar cambios que perduran durante toda la vida y a través de generaciones (Heindel et al., 2022).

A esto hay que añadir que los niños/as por naturaleza son curiosos y confiados. Esta actitud de la infancia es causante de que los mas pequeños esten desprotegidos ante estos compuestos químicos. Nacemos sin experiencia y para adquirirla tocamos, chupamos e incluso ingieremos objetos que encuentra a nuestro alcance (tierra, juguetes, objetos domésticos entre otros). A su vez, los niños/as mientras no saben caminar se mueven por casa gateando o reptando estando expuestos a pontenciales contaminantes presentes en el suelo, lo que puede aumentar su dosis de ingesta de contaminantes específicos que se acumulan en el polvo y otras matrices (Ferguson & Solo-Gabriele, 2016).

### *1.3.5. Obesógenos*

Entre los DEs que se que han demostrado poseer propiedades obesogénicas, existen alrededor de 50 compuestos químicos y clases de compuestos químicos se clasifican como obesógenos (Darbre, 2017; Heindel et al., 2022). Entre los obesógenos más relevantes encontramos los siguientes compuestos (Figura 8).




TBT: tributilo de estaño
PBDE: éteres de polibromodifenilos
PCB: bifenilos policlorados
PFAs: sustancias per- y polifluoroalquiladas

**Figura 8.** Obesógenos ambientales más relevantes. Elaborado con Biorender.com

En la presente Tesis Doctoral nos vamos a centrar en el BPA y sus análogos como posibles potenciales obesógenos ambientales en la población escolar.

## 1.4. Bisfenol A y análogos

La estructura básica de una molécula de bisfenol contiene dos anillos de benceno unidos a través de un átomo de carbono (C) central (Figura 9). Algunos bisfenoles pueden tener un átomo de azufre en lugar de un átomo de C central, como es el caso del bisfenol S (BPS) (İyİgÜndoĞdu et al., 2020).

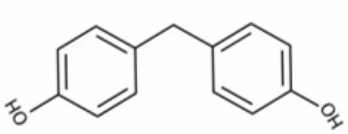



**Figura 9.** Estructura química general de los bisfenoles. Elaborado con Scifinder

El bisfenol más utilizado es el BPA, fue descubierto por primera vez por Dianin en 1891, pero no se utilizó comercialmente hasta la década de 1940, cuando se convirtió en un componente de las resinas epoxi (Warner & Flaws, 2018; İyİgÜndoĞdu et al., 2020). En la década de 1950, el BPA resurgió como base del plástico policarbonato, un nuevo plástico con alta resistencia y transparencia. En la década de 1970, la producción del BPA alcanzó más de 500 millones de libras por año (226.796,19 toneladas por año) (Warner & Flaws, 2018). En las ultimas décadas, el BPA ha sido uno de los plastificantes más producidos y utilizados en todo el mundo, con una producción estimada de 5 a 6,8 millones de toneladas por año (İyİgÜndoĞdu et al., 2020). Hoy en día, debido a las regulaciones y la prohibición del uso del BPA en numerosas aplicaciones se está reduciendo su producción en muchos países. Además, se está explorando, por parte de la industria, la búsqueda de nuevas alternativas como los análogos del BPA, para producir productos «libres del BPA» (Ni et al., 2023).

### *1.4.1. Fuentes de exposición y metabolización del bisfenol A y análogos*

El BPA es una sustancia química ampliamente utilizada como monómero base para la producción de plásticos policarbonato y resinas-fenólicas, altamente resistentes al calor y que proporciona elasticidad a los materiales plásticos (González-Casanova et al., 2023). Las fuentes de exposición comunes incluyen plásticos policarbonato (botellas de agua y dispositivos eléctricos), resinas epoxi (recubrimiento de latas metálicas para envasado de alimentos y tuberías de suministro de agua), papel térmico (tickets de compra), juguetes de plástico, materiales dentales (selladores dentales y empastes) y polvo doméstico entre otras (González-Casanova et al., 2023; Li et al., 2023; Numsriskulrat et al., 2023). La exposición humana al bisfenol es por tanto ubícua.

La entrada de los bisfenoles al organismo se produce por vía oral, dérmica e inhalatoria. Estimándose que el 90% de la exposición es a través de los alimentos (vía oral) y, sólo el 5% corresponde a las otras dos vías (González-Casanova et al., 2023; Ni et al., 2023). Otra vía de

exposición a destacar es la materno-fetal, ya que el BPA puede atravesar la barrera placentaria (González-Casanova et al., 2023).

Tras la exposición, los bisfenoles se metabolizan en el hígado gracias a la uridina 5′-difosfato-glucuronosiltransferasas a los glucurónidos correspondientes y posteriormente se eliminan en la orina en 24 h en el caso del BPA o 48 h para el BPS (Numsriskulrat et al., 2023). La vida media del BPA en el organismo humano se estima aproximadamente 5 - 6 horas (Grettchen-Flores, 2019; González-Casanova et al., 2023). Aunque la excreción del BPA se produce rápidamente por orina, la exposición en los humanos es continúa debido a su ubicuidad (González-Casanova et al., 2023).

Sin embargo, se ha demostrado que una parte del compuesto que ingresa en el organismo tiende a bioacumularse en los diversos tejidos y matrices biológicas cómo, el tejido graso, el pelo, la uña o la placenta (Rodríguez-Gómez et al., 2017; Martín-Pozo et al., 2020; Fernández et al., 2021; González-Casanova et al., 2023). Existe una gran preocupación en la comunidad científica en base a los posibles efectos acumulativos y la exposición continua a los bisfenoles. Estos hechos hacen muy necesario seguir investigando para poder adoptar medidas que permitan mitigar los pontenciales riesgo.

### *1.4.2. Efectos adversos demostrados de los bisfenoles*

Hace más de 50 años, el BPA fue identificado como una sustancia química inofensiva mediante los métodos toxicológicos tradicionales. Históricamente, la toxicidad se evaluaba mediante pruebas de dosis altas bajo el supuesto de una curva dosis-respuesta lineal. Sin embargo, actualmente se conoce que los bisfenoles como otros DEs no siguen curvas dosis-respuesta lineales (Warner & Flaws, 2018).

La actividad estrogénica del BPA fue descubierta accidentalmente en 1993, cuando el grupo de Krishan et al. (1993) observaron que el BPA constituyente en los matraces de policarbonato se filtraba y provocaba un aumento en la tasa de proliferación de células de cáncer de mama

(Vasiljevic & Harner, 2021). A pesar de las dudas existentes desde hace mucho tiempo sobre su seguridad, el BPA se ha seguido empleando en productos de consumo en todo el mundo debido a la dificultad de desarrollar alternativas asequibles económicamente y seguras (Warner & Flaws, 2018). Actualmente, existe evidencia acerca del vínculo entre la exposición al BPA y la incidencia de diversos problemas de salud, como hipertensión, obesidad, diabetes tipo II, enfermedades cardiovasculares y cáncer (Algonaiman et al., 2023). Debido a la preocupación en los últimos años sobre la toxicidad del BPA ha llevado consigo la búsqueda de nuevas alternativas. Sin embargo, los análogos del BPA presentan similitudes estructurales y funcionales. Dado a estas semejanzas con el BPA, es probable que tengan impactos similares en la salud (Heindel et al., 2022; Algonaiman et al., 2023).

La Figura 10 muestra las principales fuentes de exposición y efectos adversos sobre la salud del BPA y sus análogos.

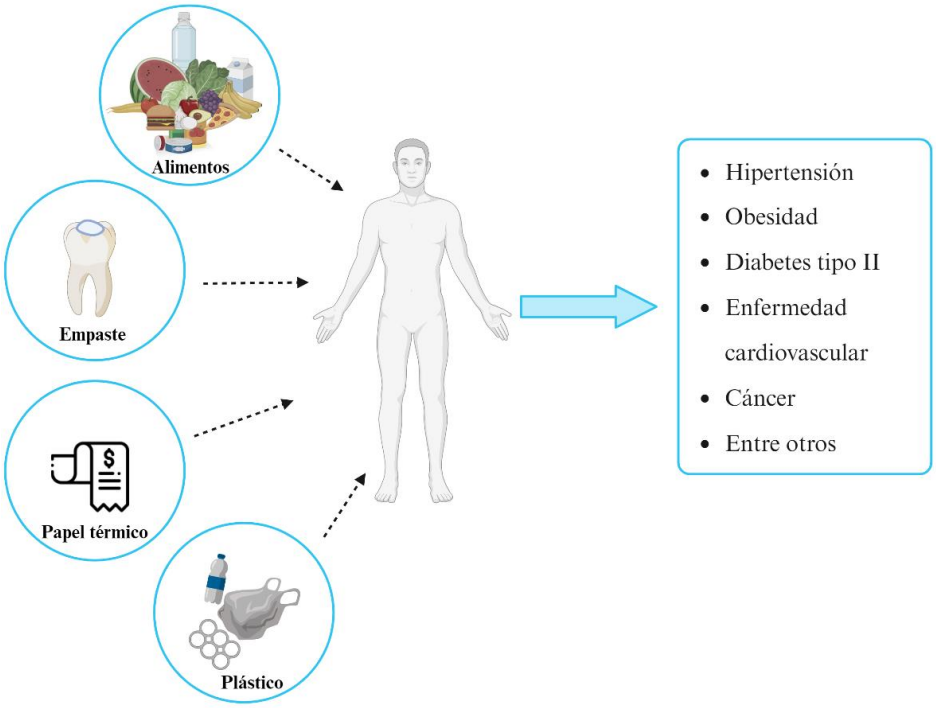



**Figura 10.** Fuentes de exposición al bisfenol A y sus análogos y sus efectos adversos sobre la salud. Elaborado con Biorender.com

En la presente Tesis Doctoral nos concierne la preocupación del aumento de la prevalencia del sobrepeso y la obesidad a nivel mundial. La evidencia actual sugiere que el BPA y sus análogos podrían ser un factor que contribuye a esta epidemia, formando parte del grupo de los llamados compuestos obesógenos (Heindel et al., 2022; González-Casanova et al., 2023). Por ello, nos vamos a enfocar en el posible efecto de los bisfenoles sobre el sobrepeso/obesidad.

### *1.4.3. Mecanismo de acción de los bisfenoles como obesógenos ambientales*

Diversos estudios *in vitro* han mostrado efecto proadipogénico asociado a la exposición al BPA, el BPS, el bisfenol F (BPF) y el bisfenol AF (BPAF) en líneas celulares 3T3-L1 de ratón y en preadipocitos humanos dado a su capacidad de unirse al NR. Los bisfenoles inducen la hiperplasia y/o hipertrofia por diversos mecanismos (Heindel et al., 2022).

Por un lado, estudios *in vitro* utilizando la línea celular murina 3T3-L1 han mostrado que la exposición al BPA produce efectos transitorios sobre la metilación del promotor PPARγ y la acumulación de lípidos (Longo et al., 2020). Asimismo, en estudios *in vivo* la exposición crónica al BPA y el BPS provoca la acumulación de lípidos en células hepáticas de pez zebra (*Danio rerio*), favoreciendo la aparición de la esteatosis hepática no alcohólica, mediante la desregulación en los genes relacionados con el metabolismo de los lípidos y/o a través del estrés del retículo endoplásmico (Qin et al., 2020; Sun et al., 2020). Además, se ha observado que la exposición de células HepG2 hepáticas humanas a bajas concentraciones del BPA indujo disfunción mitocondrial, alteración del metabolismo lipídico e inflamación (Huc et al., 2012).

El BPA también induce respuestas inflamatorias. La inducción de citoquinas proinflamatorias como la IL-6 en los adipocitos y la expresión reducida de la hormona antiinflamatoria (adiponectina) causaron inflamación, llevando consigo un adipocito disfuncional (Heindel et al., 2022).

Cabe señalar que, en ciertos modelos animales, la exposición al BPA durante el desarrollo disminuye la cantidad de neuronas de saciedad y aumenta la cantidad de neuronas del apetito en el cerebro, aumentando así la ingesta de alimentos y, por tanto, el aumento de peso (Desai et al., 2018).

A continuación, se muestras una figura resumen de los diferentes mecanismos de acción de los bisfenoles como obesógenos (Figura 11).

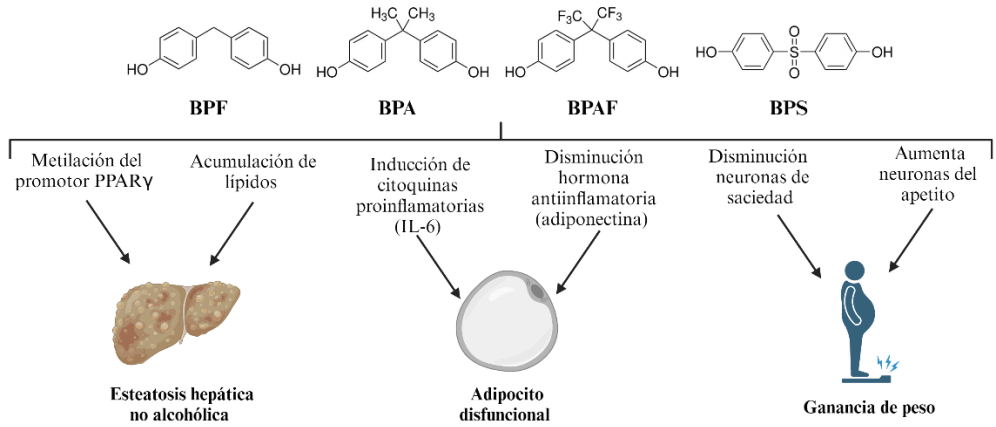



**Figura 11**. Resumen de los efectos de los bisfenoles y su relación con la obesidad. Elaborado con Biorender.com

### *1.4.4. Legislación con relación al bisfenol A*

Dada la incertidumbre que existía en 2015, la Autoridad Europea de Seguridad Alimentaria (EFSA) estableció una ingesta diaria tolerable (IDT) provisional, reduciendo la IDT establecia previamente de 50 a 4 µg/kg pc/día, enfatizando la necesidad de datos adicionales sobre los efectos toxicológicos del BPA para comprender el potencial riesgo para la salud de la presencia del BPA en productos alimentarios provenientes de materiales plásticos en contacto con ellos (Agencia Española de Seguridad Alimentaria y Nutrición [AECOSAN], 2023; González-Casanova et al., 2023). En abril de 2023, la nueva reevaluación de la EFSA decidió establecer una nueva IDT de 0,2 ng/kg pc/día reemplazando la IDT propuesta provisionalmente de 4 µg/kg pc/día (AECOSAN, 2023).

Con respecto al material en contacto con los alimentos que presenta el BPA en su composición, se aprobó en 2018 el Reglamento (UE) sobre el uso de BPA en los barnices y revestimientos destinados a entrar en contacto con los alimentos, en él se estableció el límite tolerable de 0,05 mg de BPA por kg de alimento para los materiales y objetos plásticos en contacto con los alimentos a fin de garantizar que la exposición al BPA siga siendo inferior a la IDT (Comisión Europea, 2018). En enero de 2020 en la Unión Europea entró vigor la prohibición del uso de BPA en el papel térmico (Comisión Europea, 2016b).

Para proteger a la población más vulnerable contra la exposición al BPA, la Comisión Europea tomó la medida de prohibir el uso del BPA en objetos y materiales destinados específicamente a entrar en contacto con alimentos para lactantes (Comisión Europea, 2018). En este sentido, la cantidad de BPA permitida en los juguetes de los niños/as también se ha limitado a 0,04 mg/L el límite de migración en los juguetes (Comisión Europea, 2017).

Dadas las crecientes regulaciones y prohibiciones sobre el uso del BPA la industria ha buscado alternativas, llevando consigo en los últimos años un aumento del uso de los análogos (İyİgÜndoĞdu et al., 2020).

### *1.4.5. Motivo de preocupación del uso de las alternativas al BPA*

Los compuestos químicos utilizados para reemplazar al BPA también presentan la estructura básica de una molécula de bisfenol y pueden tener efectos similares en los organismos. Según estudios científicos, los análogos del BPA pueden provocar efectos tóxicos similares e incluso mayores que los del BPA (İyİgÜndoĞdu et al., 2020).

Metanálisis realizados confirman la asociación entre la exposición al BPA y la obesidad en niños/as y adultos (Rancière et al., 2015; Kim et al., 2019). Aunque limitada, la evidencia preliminar sobre alternativas al BPA sugiere lo mismo. Se ha demostrado que el BPF presenta alguna evidencia sobre la relación entre la obesidad infantil y adolescente (Liu et al., 2019). En un estudio basado en la encuesta NHANES de 2013-2016,

el BPS y el BPF mostraron asociaciones significativas con la obesidad, al igual que el BPA (Choi et al., 2022). Actualmente, la evidencia preliminar sugiere que el BPS y el BPF no constituyen alternativas seguras.

Como se ha mencionado anteriormente, la exposición al BPA ha disminuido en los últimos años en todo el mundo debido a las regulaciones y prohibiciones de su uso. Sin embargo, a medida que aumentan las tasas de detección de los análogos, principalmente del BPF y el BPS, surge un patrón preocupante que sugiere su presencia generalizada y efectos similares sobre la salud (Numsriskulrat et al., 2023).

### *1.4.6. Bisfenoles objeto de estudio de la presente Tesis Doctoral*

Hoy en día, existen veinticuatro análogos del BPA (Heindel et al., 2022). Sin embargo, en la presente Tesis van a ser objeto de estudio el BPA y sus 11 análogos mas empleados y representativos (BPF; BPS; BPAF; Bisfenol AP, BPAP; Bisfenol B, BPB; Bisfenol E, BPE; Bisfenol C, BPC; Bisfenol FL, BPFL; Bisfenol M, BPM; Bisfenol P, BPP; Bisfenol Z, BPZ). En la Tabla 5 se muestran los 12 bisfenoles considerados en la presente Tesis.

**Tabla 5.** Masa molecular, estructura química, nombre científico y número CAS de los bisfenoles objeto de estudio de la presente Tesis Doctoral.

| Nombre científico (IUPAC) | Abreviatura | Número CAS | Masa molecular (g/mol) | Estructura química |
|---|---|---|---|---|
| *4,4'-(propane-2,2-diyl)diphenol* | BPA | 80-05-7 | 228,29 | $C_{15}H_{16}O_2$ |
| *4,4'-(1,1,1,3,3,3-Hexafluoropropane-2,2-diyl)diphenol* | BPAF | 1478-61-1 | 336,23 | $C_{15}H_{10}F_6O_2$ |
| *4-[1-(4-hydroxyphenyl)-1-phenylethyl]phenol* | BPAP | 1571-75-1 | 290,36 | $C_{20}H_{18}O_2$ |
| *4-[2-(4-hydroxyphenyl)butan-2-yl]phenol* | BPB | 77-40-7 | 242,31 | $C_{16}H_{18}O_2$ |
| *4-[2,2-dichloro-1-(4-hydroxyphenyl)ethenyl]phenol* | BPC | 79-97-0 | 256,34 | $C_{17}H_{20}O_2$ |
| *4-[1-(4-hydroxyphenyl)ethyl]phenol* | BPE | 2081-08-5 | 214,26 | $C_{14}H_{14}O_2$ |
| *4-[(4-hydroxyphenyl)methyl]phenol* | BPF | 620-92-8 | 200,23 | $C_{13}H_{12}O_2$ |
| *4-[9-(4-hydroxyphenyl)fluoren-9-yl]phenol* | BPFL | 3236-71-3 | 350,41 | $C_{25}H_{18}O_2$ |
| *4-[2-[3-[2-(4-hydroxyphenyl)propan-2-yl]phenyl]propan-2-yl]phenol* | BPM | 13595-25-0 | 346,46 | $C_{24}H_{26}O_2$ |
| *4,4'-(1,4-Phenylendi-2,2-propandiyl)diphenol* | BPP | 2167-51-3 | 346.46 | $C_{24}H_{26}O_2$ |
| *4-(4-hydroxyphenyl)sulfonylphenol* | BPS | 80-09-1 | 250.27 | $C_{12}H_{10}O_4S$ |
| *4-[1-(4-hydroxyphenyl)cyclohexyl]phenol* | BPZ | 843-55-0 | 268,35 | $C_{18}H_{20}O_2$ |

### *1.4.7. Otros compuestos analizados en la presente Tesis Doctoral*

Por otro lado, dado que en algunos de los trabajos presentados en esta Memoria de Tesis Doctoral se han utilizado métodos multirresiduo, se ha aprovechado la ocasión para estudiar varios parabenos por su analogía con los bisfenoles. Los parabenos y los bisfenoles comparten similitudes estructurales, puesto que ambas clases de compuestos contienen anillos fenólicos (Li et al., 2022), lo que hace que su estudio sea especialmente relevante. La tabla 6 recoge los parabenos que han sido analizados también en la presente Tesis Doctoral.

**Tabla 6.** Masa molecular, estructura química, nombre científico y número CAS de los parabenos estudiados en la Tesis Doctoral.

| Nombre científico (IUPAC) | Abreviatura | Número CAS | Masa molecular (g/mol) | Estructura química |
|---|---|---|---|---|
| ***Methyl 4-hydroxybenzoate*** | MetPB | 99-76-3 | 152,15 | $C_8H_8O_3$ |
| ***Ethyl 4-hydroxybenzoate*** | EthPB | 120-47-8 | 166,17 | $C_9H_{10}O_3$ |
| ***Propyl 4-hydroxybenzoate*** | PropPB | 94-13-3 | 180,20 | $C_{10}H_{12}O_3$ |
| ***isoPropyl 4-hydroxybenzoate*** | isoPropPB | 4191-73-5 | 180,20 | $C_{10}H_{12}O_3$ |
| ***Buthyl 4-hydroxybenzoate*** | ButPB | 94-26-8 | 194,23 | $C_{11}H_{14}O_3$ |
| ***isoButhyl 4-hydroxybenzoate*** | IsoButPB | 4247-02-3 | 194,23 | $C_{11}H_{14}O_3$ |

### *1.4.8. Técnica analítica empleada para la determinación de obesógenos disruptores endocrinos*

La presente Tesis Doctoral se llevó a cabo en colaboración entre los Departamentos de Nutrición y Bromatología, y Química Analítica de la Universidad de Granada. Todos los análisis de muestras de alimentos y matrices biológicas (saliva, orina, uña y pelo) se llevaron a cabo en las insatalaciones del Departamento de Química Analítica.

Las muestras biológicas recogidas de la población objeto de estudio fueron no invasivas en todos los casos. Estas muestras se obtienen sin

procedimientos invasivos ni técnicas que penetren en la piel o los tejidos corporales, lo que garantiza un proceso de recogida indoloro y seguro (NIH, https://www.cancer.gov/publications/dictionaries/cancer-terms/def/noninvasive). Utilizar métodos de muestreo no invasivos tiene numerosos beneficios, sobre todo cuando se trabaja con niños/as. Estos métodos ofrecen ventajas como la reducción de las molestias para la población pediátrica. Además, las muestras seleccionadas para la presente Tesis nos aportan información valiosa sobre los patrones de exposición a largo plazo (uña y pelo) y a corto plazo (saliva y orina) a BPA y análogos.

Dado que se prevé que las concentraciones identificadas en las muestras investigadas se sitúen en niveles traza, el análisis de los bisfenoles se realizó mediante técnicas de elevada sensibilidad como es la cromatografía de líquidos de ultra alta resolución acoplada a espectrometría de masas en tándem triple cuadrupolo (UHPLC-MS/MS). La técnica UHPLC-MS/MS ha demostrado ser muy ventajosa en el campo de la salud ambiental, especialmente en la determinación de compuestos químicos ambientales tales como los DEs (Martín-Pozo et al., 2021; Metcalfe et al., 2022). Una de las principales ventajas de la técnica seleccionada reside en su sensibilidad y selectividad, que permiten detectar y cuantificar con veracidad y precisión los compuestos diana incluso en matrices complejas (Martín-Pozo et al., 2021; Metcalfe et al., 2022). Esta capacidad es crucial para identificar estos compuestos, que pueden tener importantes implicaciones para la salud pública y la protección del medio ambiente.

**Imagen 1.** Equipo UHPLC-MS/MS empleado en la determinación del BPA y sus análogos en los alimentos y las matrices biológicas

Además, por su concepción básica, la técnica de UHPLC-MS/MS ofrece una mayor resolución a tiempos más cortos de análisis en comparación con las técnicas analíticas convencionales, permitiendo el análisis simultáneo de múltiples compuestos en tiempos cortos. Esto no sólo agiliza el proceso analítico, sino que también mejora la eficacia general de la detección y caracterización de los contaminantes ambientales (Martín-Pozo et al., 2021; Metcalfe et al., 2022).

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## 2. HIPÓTESIS Y JUSTIFICACIÓN DEL ESTUDIO

Puesto que en las últimas décadas se ha observado un incremento de la prevalencia de la obesidad infantil, se hace necesario el estudio de los factores contribuyentes a dicho incremento y su relación con el ambiente. Los hallazgos arrojan serias dudas sobre que los factores tradicionales sean suficientes para explicar el aumento del sobrepeso/obesidad a nivel mundial, y dejan ver la importancia de otros factores de riesgo como la exposición a sustancias químicas de síntesis como el BPA y sus análogos. Actualmente existe un desacuerdo entre los estudios científicos sobre la posible relación entre la exposición al BPA y sus análogos y su consiguiente actividad disruptora endocrina y obesógena con el desarrollo de sobrepeso/obesidad. Debido a esta situación se plantea la necesidad, en primer lugar, de determinar de forma cuantitativa la presencia de estos compuestos en muestras de alimentos consumidos de manera habitual por la población escolar y estimar su ingesta en la población.

Como base poblacional se ha utilizado la perteneciente a los proyectos de investigación en los que el grupo viene trabajando en los últimos años, es decir, EFSA GP/EFSA/ENCO/2018/03, ISCIII-FEDER PI17/01758, PI20/01278 y PI23/01359; Consejería de Salud y Familias PE-0250-2019; Fundación MAPFRE 30.BF.88.08.01; Consejería de Economía, Conocimiento, Empresas y Universidad P18-RT-4247; y Plan Nacional 2019 EIN2019-103431. Por otro lado, es necesario llevar a cabo un seguimiento de la exposición de dicha población a través de la determinación de la concentración del BPA y sus análogos en las muestras biológicas tomadas a los sujetos objeto de estudio (saliva, orina, uña y pelo). Estos compuestos se están utilizando masivamente en la industria del plástico sin que existan trabajos científicos previos que evalúen los niveles de exposición a los mismos en población infantil. Además, dado que los bisfenoles presentan actividad obesógena en diferentes trabajos experimentales se estudiará si existe una relación entre el grado de exposición a estos compuestos y el sobrepeso/obesidad infantil.

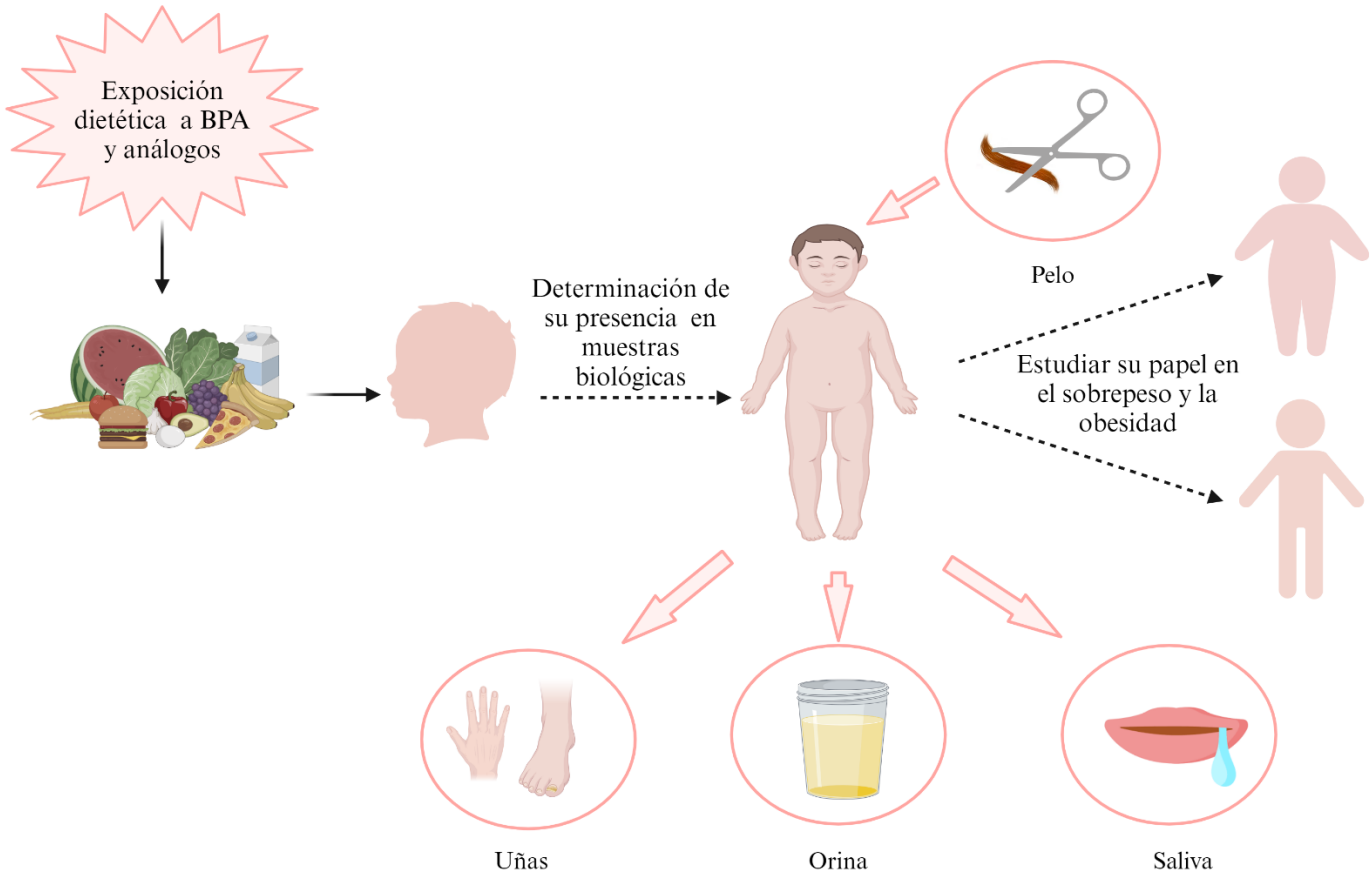


**Figura 12.** Resumen gráfico del enfoque de estudio de la presente Tesis Doctoral. Elaborado con Biorender.com

# 3. OBJETIVOS / OBJECTIVES

## 3.1. Objetivos

Dada la ubiquidad de los bisfenoles en el mundo actual y la falta de un consenso científico del posible papel del BPA y sus análogos sobre el sobrepeso y obesidad infantil, se requiere de más estudios epidemiológicos profundos que evalúen la actividad obesogénica de estos compuestos químicos.

El objetivo principal de este trabajo científico es estudiar la exposición alimentaria al BPA y sus análogos con actividad disruptora endocrina y valorar su posible asociación con el sobrepeso y la obesidad infantil en una población en edad escolar.

Los objetivos específicos son:

1. Determinar cuantitativamente la presencia del BPA y sus análogos en alimentos consumidos por la población escolar en estudio teniendo como fuente de información las encuestas nutricionales recogidas.
2. Identificar otras posibles fuentes de exposición a análogos del BPA en la población escolar.
3. Analizar los niveles de estos DEs en muestras de saliva, orina, uña y pelo recogidas en la población objeto de estudio.
4. Establecer la posible relación estadísticamente significativa entre la exposición a BPA y análogos, los hábitos de vida y nutricionales y el grado de obesidad/sobrepeso en los niños/as estudiados.

## 3.2. Objectives

Given the ubiquity of bisphenols worldwide and the lack of scientific consensus on the possible role of BPA and its analogues in childhood overweight and obesity, more deep epidemiological studies are needed to evaluate the obesogenic activity of these chemical compounds.

Therefore, the main objective of this work is to study dietary exposure to BPA and its analogues with endocrine disrupting activity and to assess its possible association with childhood overweight or obesity in a school population.

The specific objectives are:

1. To quantitatively determine the presence of BPA and analogues in the foods most consumed by the school population under study, using the nutritional surveys collected as a source of information.
2. Identify other possible sources of exposure to BPA analogues in the school population.
3. To analyse the levels of these DEs in saliva, urine, nails and hair samples collected from the project population.
4. To establish the possible statistically significant relationship between exposure to BPA and analogues, lifestyle and nutritional habits and the degree of obesity/overweight in the children studied.

# 4. MATERIALES Y MÉTODOS

## 4.1. Determinación de disruptores endocrinos en los alimentos más consumidos por la población escolar

### *4.1.1. Reactivos químicos*

Todos los reactivos empleados fueron de grado analítico. Se preparó agua ultrapura (18,2 MΩ-cm) con el sistema interno Milli-Q Plus® de Merck Millipore. Los compuestos metilparabeno (MetPB), etilparabeno (EthPB), propilparabeno (PropPB) y butilparabenos (ButPB) (pureza ≥ 99%) fueron suministrados por Alfa Aesar (Thermo Fisher Scientific, Kandel, Alemania). Del mismo modo los bisfenoles, BPA, BPF, BPS, BPAF, BPP (pureza ≥99%), BPE, BPB (pureza ≥ 98%) y BPA deuterado (BPA-d16, pureza ≥99%) fueron suministrados por Sigma-Aldrich (Madrid, España). El EthPB deuterado (EthPB-d5) se adquirió a Toronto Research Chemicals (Toronto Research Chemicals, NY, Canadá).

Se prepararon soluciones madre de bisfenoles y parabenos (100 mg $L^{-1}$) y los patrones internos BPA-$d_{16}$ y EthPB-$d_5$ (10 mg $L^{-1}$) en metanol (MeOH). La disolución patrón de trabajo se preparó diluyendo las soluciones madre de los siete bisfenoles y los cuatro parabenos (10 mg $L^{-1}$) con MeOH y se almacenó en botes de vidrio de color ámbar a −20°C hasta el análisis. Los estándares de calibración se prepararon añadiendo a la matriz alimentaria una disolución estándar de trabajo. Los disolventes empleados en la cromatografía de líquidos acoplada a espectrometría de masas simple (LC-MS), MeOH y acetonitrilo, fueron suministrados por VWR Chemicals (VWR International, Barcelona, España). La disolución de amoniaco al 25% para LC-MS utilizada como aditivo de la fase móvil y el hidróxido de sodio granulado (NaOH) de grado analítico (con una pureza ≥98%), procedían de Sigma-Aldrich (Madrid, España). El cloruro de sodio (NaCl) y el sulfato de magnesio ($MgSO_4$), se obtuvieron de Panreac (Barcelona, España). Los absorbentes, sílice

funcionalizada con grupos octadecilo (C18) y amina primaria-secundaria (PSA), se adquirieron a Scharlab (Barcelona, España).

### *4.1.2. Intrumentación y software*

El análisis de los once DEs se llevó a cabo utilizando un sistema Waters Acquity UHPLC™ I-Class (Waters Corporation, Milford, CT, EE.UU.). La detección espectrométrica se realizó con un equipo de Waters Cromatografía Xevo® TQ-XS (Waters Corporation, Milford, CT, EE.UU.) equipado con una fuente de ionización por electrospray (ESI) ortogonal Z-Spray™ (Waters Corporation, Milford, CT, EE.UU.). La columna cromatográfica empleada fue una Waters UPLC® BEH C18 (2,1 mm x 50 mm, tamaño de partícula de 1,7 µm). Las muestras de alimentos se liofilizaron utilizando un liofilizador ScanVac CoolSafe™ (Lynge, Dinamarca).

Se utilizaron diversos instrumentos de laboratorio, como un mezclador vórtex (IKA, Staufen, Alemania), una balanza de precisión GX400 (Mettler-Toledo, Columbus, OH, EE.UU.), una centrifugadora Universal 32 (Hettich, Tuttlingen, Alemania), una centrifugadora Spectrafuge™ 24D (Labnet International, Inc., Edison, NJ, EE.UU.), un evaporador-concentrador de muestras SBHC0NC (Stuart, Staffordshire, Reino Unido) y un baño de ultrasónico de la serie Ultrasons-HD (Selecta, Barcelona, España).

Para el tratamiento de datos, el análisis y el control del equipo se utilizó el software MassLynx 4.1 (Waters Corporation, Milford, CT, EE.UU.) de Waters.

### *4.1.3. Muestreo de alimentos*

Los alimentos incluidos en el presente estudio fueron seleccionados entre los más consumidos por la población infantil española. La mayoría de estos alimentos estaban envasados utilizando materiales como plástico, latas recubiertas internamente, bandejas de papel, cartón y papel de aluminio (Tabla 7). Los alimentos incluidos en este estudio se

obtuvieron de diferentes supermercados de la provincia de Granada. Se clasificaron según el sistema de clasificación de alimentos NOVA, que clasifica los alimentos en función de su naturaleza, extensión y propósito de los procesos industriales a los que se someten. Las categorías incluyen alimentos sin procesar o mínimamente procesados, alimentos procesados y alimentos ultraprocesados (Monteiro et al., 2018).

**Tabla 7.** Alimentos analizados.

| Grupo de alimentos | Alimentos (n=98) | Tipos de envases |
|---|---|---|
| Lácteos y derivados (n=21) | Leche entera (n=4)<br>Leche semifermentada (n=3)<br>Batido de chocolate (n=4)<br>Yogurt natural azucarado (n=2)<br>Yogurt sabores (n=1)<br>Yogurt líquido sabores (n=2)<br>Queso semicurado (n=2)<br>Queso de untar (n=1)<br>Queso para fundir (n=2) | Tetra brik / Plástico / Aluminio |
| Carne y derivados (n=12) | Pollo (n=1)<br>Jamón cocido (n=1)<br>Salchichón y chorizo (n=3)<br>Carne de hamburguesa (n=2)<br>Salchichas (n=1)<br>Jamón (n=1)<br>Pechuga de pavo en lonchas y tripa (n=2)<br>Mortadella (n=1) | Plástico |
| Huevos (n=1) | Huevos (n=1) | Plástico |
| Pescado y derivados (n=3) | Merluza congelada (n=1)<br>Atún de lata (n=2) | Cartón / Plástico / metal |
| Verduras y hortalizas (n=13) | Ajo congelado (n=1)<br>Cebolla congelada (n=1)<br>Perejil congelado (n=1)<br>Espinacas congeladas (n=1)<br>Tomate (n=2)<br>Zanahoria (n=2)<br>Lechuga (n=1)<br>Calabaza (n=1)<br>Champiñones y setas (n=1)<br>Pimiento verde (n=1)<br>Guacamole (n=1) | Envasados plástico / sin envasar |

**Tabla 7 (cont).** Alimentos analizados.

| Grupo de alimentos | Alimentos (n=98) | Tipos de envases |
|---|---|---|
| Verduras y hortalizas (n=13) | Ajo congelado (n=1)<br>Cebolla congelada (n=1)<br>Perejil congelado (n=1)<br>Espinacas congeladas (n=1)<br>Tomate (n=2)<br>Zanahoria (n=2)<br>Lechuga (n=1)<br>Calabaza (n=1)<br>Champiñones y setas (n=1)<br>Pimiento verde (n=1)<br>Guacamole (n=1) | Envasados plástico / sin envasar |
| Frutas (n=13) | Uvas (n=1)<br>Manzana y pera (n=3)<br>Mango congelado (n=1)<br>Arandanos y frambuesas (n=2)<br>Frutos rojos (congelados) (n=1)<br>Piña (n=1)<br>Melón (n=1)<br>Aceitunas con y sin relleno (n=3) | Envasados plástico / sin envasar |
| Legumbres (n=1) | Lentejas (n=1) | Plástico |
| Cereales (n=7) | Pan de hamburguesa (n=2)<br>Pan de sándwich (n=1)<br>Pasta (n=1)<br>Arroz (n=1)<br>Tortitas de arroz (n=1)<br>Maiz dulce (n=1) | Plástico / cartón papel |
| Dulces (n=9) | Bizcocho (n=1)<br>Pan de leche (n=1)<br>Bollito de leche y chocolate (n=3)<br>Croissant (n=2)<br>Magdalenas (n=2) | Plástico / papel |
| Ultraprocesados y snacks salados (n=18) | Picos con queso (n=1)<br>Empanadillas de atún congeladas (n=1)<br>Pizza (n=3)<br>Palitos de merluza congelados (n=1)<br>Ketchup (n=2)<br>Salsa de tomate (n=1)<br>Gusanitos de maíz (n=2)<br>Patatas de bolsa (n=5)<br>Gominolas (n=2) | Cartón / plástico / aluminio |

### *4.1.4. Métodos analíticos*

En la figura 13 se muestra esquemáticamente el pretratamiento seguido previo al análisis mediante la técnica UHPLC-MS/MS. El tratamiento de muestra se llevó a cabo siguiendo el método desarrollado por García-Córcoles et al. (2018), con algunas modificaciones.

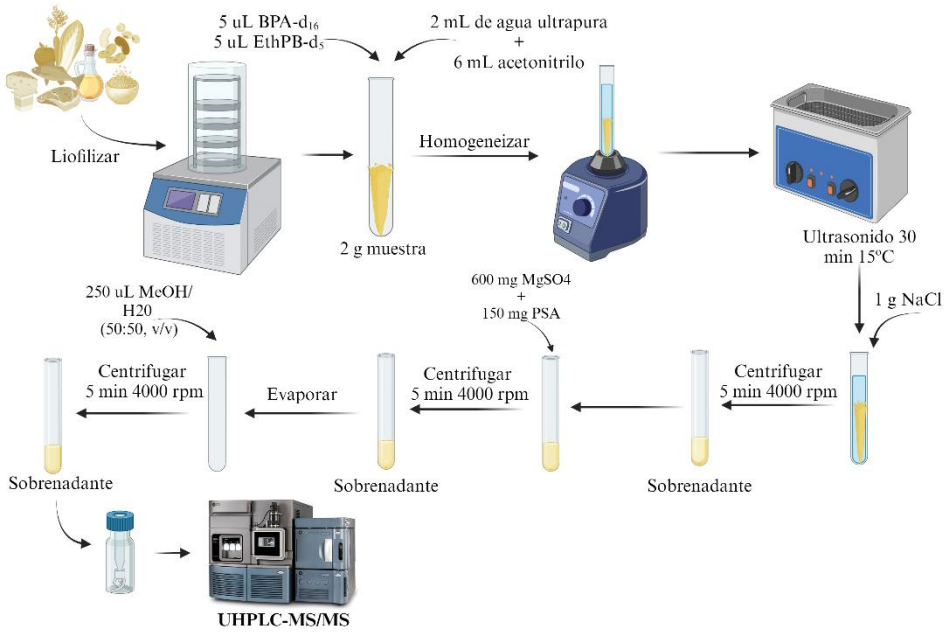


**Figura 13.** Esquema del pretratamiento, extracción y análisis de los DEs en alimentos. Elaborado con Biorender.com

Para el análisis cromatográfico se optimizó un nuevo método basado en la técnica de UHPLC-MS/MS.

### *4.1.5. Cromatografía de líquidos de ultra alta resolución acoplada a espectrometría de masas en tándem*

A continuación, se describen las condiciones cromatográficas optimizadas empleadas para el análisis. Como fase estacionaria, se empleó una columna Acquity UPLC® BEH C18 (2,1 mm x 50 mm, tamaño de partícula de 1,7 µm). La temperatura de la columna se fijó en 40ºC.

Esta temperatura además de disminuir la presión de trabajo mejoró la separación de los analitos y la forma de los picos cromatográficos. Basándonos en investigaciones previas del propio grupo, y teniendo en cuenta que el modo de ionización en el espectrómetro de masas fue electrospray negativo, se utilizó como fase móvil 0,025% (v/v) de amoníaco en agua (disolvente A) y MeOH (disolvente B) (Martín-Pozo et al., 2020). El caudal se fijó en 0,35 mL $min^{-1}$, con un volumen de inyección de 10 µL. El gradiente de la fase móvil B fue el siguiente: 0,0 a 1,0 minuto, 10% de disolvente B; 1,0 a 6,0 minutos, 10 a 90% B; 6,0 a 6,1 minutos, 90 a 100% B; 6,1 a 6,6 minutos de regreso al 10% B, tiempo de ejecución 10 minutos.

Para llevar a cabo una cuantificación de los compuestos de interés en las muestras de alimentos con la máxima especificidad y sensibilidad, se utilizó ionización por electrospray en modo de ionización negativo usando la modalidad de análisis de reacciones múltiples (MRM). La cuantificación se basó en la selección de la transición más abundante, mientras que la segunda transición sirvió para confirmar la identidad del analito, tal y como establecen las principales guías de validación seguidas.

### *4.1.6. Validación del método*

Con objeto de validar el método, se determinaron los parámetros de calidad de este. Según las guías de validación seguidas, se debe determinar la selectividad, la linealidad, la sensibilidad y la exactitud del método.

La selectividad del método se confirmó comparando un patrón con una muestra blanco no contaminada. Se analiza la señal de dicho blanco y se verifica la ausencia de picos interferentes que compartan las mismas transiciones seleccionadas a los tiempos de retención de los analitos de interés.

La sensibilidad se determinó a partir de la recta de calibrado. La sensibilidad analítica se define como la pendiente de dicha función. Se

determinaron también los límites de detección (LOD) y cuantificación (LOQ) a partir de la relación señal/ruido obtenida en el cromatograma. Los LODs y LOQs se definieron como la concentración de analito que produce una señal analítica de tres veces (LOD) y diez veces (LOQ) la relación señal-ruido.

Por último, la exactitud se evaluó mediante el empleo de muestras blanco dopadas. La precisión (inter e intradía) se determinó en términos de desviación estándar relativa (DSR) y la veracidad mediante el porcentaje de recuperación. El estudio se realizó a tres niveles de concentración: bajo, próximo al LOQ; medio, en la zona media de la recta de calibrado; y alto, a la mayor concentración del rango lineal de trabajo. Los tres niveles de concentración en este caso fueron 1, 100 y 250 ng $g^{-1}$. Se analizaron tres réplicas de cada muestra durante seis días diferentes.

Para minimizar el efecto matriz, se realizaron análisis cuantitativos basados en curvas de calibración en matriz. Estas curvas de calibración se generaron analizando cada analito en muestras en blanco enriquecidas con concentraciones crecientes: 0 ng $g^{-1}$, 1 ng $g^{-1}$, 5 ng $g^{-1}$, 10 ng $g^{-1}$, 25 ng $g^{-1}$, 50 ng $g^{-1}$, 100 ng $g^{-1}$ y 250 ng $g^{-1}$.

### *4.1.7. Estimación de la exposición dietética en niños/as españoles*

La ingesta alimentaria de parabenos y bisfenoles se calculó para niños/as en edad escolar. Se han recogido datos exhaustivos sobre su consumo de alimentos, hábitos dietéticos y medidas antropométricas a partir de diversas encuestas realizadas por la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) en colaboración con agencias europeas como la EFSA.

La exposición diaria total se determinó multiplicando la ingesta diaria de los grupos de alimentos (g $día^{-1}$) por las concentraciones medias de bisfenoles o parabenos (µg $kg^{-1}$) y dividiendo este resultado por el peso corporal en kg. La información sobre la ingesta diaria de grupos de alimentos se extrajo del estudio ENALIA, una encuesta transversal

llevada a cabo en colaboración de la AESAN y la EFSA. Este estudio recogió datos representativos a escala nacional sobre el consumo de alimentos en niños/as y adolescentes (López-Sobaler et al., 2019). Las concentraciones medias de los DEs se obtuvieron clasificando los alimentos analizados en grupos definidos por ENALIA, que incluían carne y derivados, pescado y derivados, cereales y derivados, verduras y derivados, frutas y derivados, lácteos y derivados, huevos, aperitivos salados y alimentos precocinados (https://www.aesan.gob.es/AECOSAN/web/seguridadalimentaria/subdetalle/enalia.htm#4).

El peso corporal utilizado para los cálculos de la exposición diaria total se estimó en 29,8 kg basándose en los datos proporcionados por una encuesta sobre peso y talla realizada en una muestra nacionalmente representativa de niños/as españoles de entre 6 y 9 años (Pérez-Farinós et al., 2013).

### *4.1.8. Estadística*

El análisis estadístico de los datos analíticos se realizó con SPSS v.23 (IBM® SPSS® Statistics, Armonk, NY, EE.UU.) y Statgraphics Plus 5.0 (versión 5, Statpoint Technologies Inc., Warrenton, VA, EE.UU.). Los valores/compuestos no detectados y no cuantificados se excluyeron del tratamiento de datos durante los cálculos. La fuerza de asociación entre las concentraciones de bisfenoles en las muestras de alimentos se evaluó mediante la correlación de Spearman. Se consideró significativo un valor de $p < 0,05$.

## 4.2. Determinación de bisfenoles en muestras biológicas, exposición dietética en la población escolar y su asociación con la obesidad

### *4.2.1. Diseño y población del estudio*

La presente Tesis Doctoral es un estudio de casos y controles realizado en diferentes centros de atención primaria y educativos de la provincia de Granada. Los participantes fueron reclutados desde enero de 2020 hasta enero de 2022.

Los casos y controles deben cumplir los siguientes criterios de inclusión:

(i) Diagnóstico de sobrepeso u obesidad (sólo casos).

(ii) Niños/as prepuberales con edades comprendidas entre 3 - 12 años.

(iii) Haber residido continuadamente en la zona de estudio durante al menos 6 meses.

El criterio de exclusión fue:

(i) Obesidad como síntoma de otras patologías, o como efecto secundario de un tratamiento farmacológico.

En este estudio se reclutaron un total de 128 casos y 194 controles, con un tamaño total de muestra de 322 participantes.

De los 322 sujetos que aceptaron participar, se trabajó con aquellos que recogieron y presentaron correctamente alguna de las muestras biológicas (saliva, orina, uña y pelo) y cumplimentaron el cuestionario de frecuencia de consumo de alimentos (FFQ). En la figura 14 se muestra el diagrama de flujo de selección de la población de estudio.

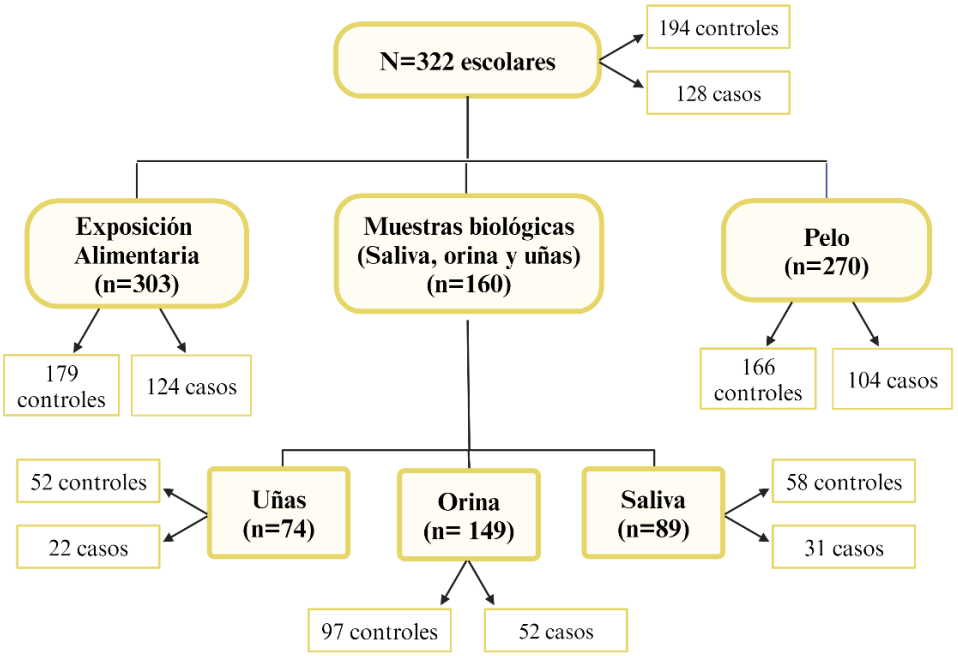


**Figura 14.** Diagrama de flujos de selección de la población de estudio. Elaborado con Biorender.com

El Comité de Ética de la Universidad de Granada aprobó el protocolo del estudio (referencia 1939-M1-22, Portal Ético de Investigación Biomédica de Andalucía). Todos los padres/madres o tutores legales de los participantes en el estudio fueron informados sobre el presente estudio y firmaron el consentimiento informado.

### *4.2.2. Recopilación de datos*

En la línea de base, entrevistadores formados realizaron entrevistas cara a cara a los padres/madres o tutores legales de los participantes. De este modo, se recogió información sociodemográfica (sexo y edad), y datos sobre el estilo de vida (hábito tabáquico de los miembros de la unidad familiar, actividad física extraescolar y dieta). Además, personal cualificado obtuvo medidas antropométricas como la altura (en cm) y el peso (en Kg). Concretamente, se pesó a los participantes con ropa ligera y sin zapatos utilizando una báscula portátil Tanita (modelo MC 780-S

MA). Se utilizó un estadiómetro (modelo SECA 214, con rango de 20-207 cm) para medir la altura. Durante la medición de la altura, la espalda, las nalgas y los talones de los participantes debían estar en contacto con la pared. El peso y la estatura se utilizaron para obtener el IMC, que se calculó como el peso dividido por la estatura al cuadrado. Así, los sujetos se clasificaron en bajo peso, normopeso, sobrepeso y obesidad (Cole et al., 2000, 2007).

La información dietética de los últimos 12 meses anteriores a la entrevista se obtuvo a través de los padres/madres o tutores legales de los participantes mediante un FFQ estando supervisado por nutricionistas formados. Esta información incluía datos sobre 112 alimentos clasificados en 13 grupos: productos lácteos (n=11), huevos, carne y derivados cárnicos (n=9), pescado y derivados del pescado (n=7), verduras y hortalizas (n=17), tubérculos (n=2), frutas y frutos secos (n=18), legumbres (n=4), cereales (n=12), alimentos precocinados o ultraprocesados (n=2), productos de panadería, bollería y dulces (n=13), grasas y aceites (n=5), bebidas no alcohólicas (n=5) y varios (n=7). Se especificó el tamaño de las raciones de cada alimento y 8 opciones de frecuencia de consumo: nunca, 1-3 veces al mes, 2-4 veces a la semana, 5-6 veces a la semana, una vez al día, 2-3 veces al día, 4-6 veces al día y más de 6 veces al día. Además, se registró información sobre el tipo de envase de los alimentos (plástico, vidrio, metal, cartón).

La versión española del KIDMED utilizada en el estudio procede de una investigación realizada previamente por López-Gajardo et al. (2022). Se trata de un formulario autocompletado dirigido a estimar la adherencia a la dieta mediterránea. Este cuestionario consta de 16 preguntas, de las cuales 4 de ellas reflejan connotaciones negativas asociadas a una dieta mediterránea adecuada, puntuadas negativamente (-1 punto). Además, hay 12 preguntas afirmativas que destacan aspectos positivos relacionados con dicho patrón dietético, puntuadas positivamente (+1 punto). Los sujetos encuestados se clasifican en tres grupos en fusión de su puntuación: baja adherencia o baja calidad de la

dieta (puntuación menor o igual a 3), adherencia moderada o calidad moderada de la dieta (puntuación de 4 a 7) y alta adherencia o alta calidad de la dieta (puntuación mayor o igual a 8).

### *4.2.3. Estimación de la exposición alimentaria a los bisfenoles*

El procedimiento de selección y análisis de alimentos ha sido descrito en un estudio previo por Monteagudo et al. (2021). Para determinar la exposición dietética diaria al BPA, el BPS y los bisfenoles totales (ng día$^{-1}$) de cada participante, se calculó multiplicando su consumo diario de alimentos (g día$^{-1}$) por el contenido de bisfenol correspondiente en cada alimento (ng g$^{-1}$ alimento). La ingesta media diaria de alimentos (g día$^{-1}$) se calculó multiplicando la frecuencia de consumo (raciones día$^{-1}$) por el tamaño estandar de ración (g ración$^{-1}$) establecido para la población española. Los niveles de cada bisfenol se cuantificaron mediante UHPLC-MS/MS, tal y como se ha descrito previamente en el apartado 4.1 de Material y Métodos. Del total de muestras de alimentos analizadas, un 52% de ellas presentaban concentraciones de bisfenoles superiores al nivel de cuantificación. La exposición dietética estimada se obtuvo dividiendo la ingesta dietética estimada de cada participante por su peso.

### *4.2.4. Determinación de los bisfenoles en muestras biológicas*

Las muestras biológicas recogidas para la presente Tesis Doctoral fueron saliva, orina, uña y pelo. Para la recolección de saliva, cada participante recibió un frasco de vidrio de boca ancha y se le indicó que durante una semana aproximadamente acumulara saliva pasivamente en ayunas, hasta que el frasco alcanzara aproximadamente la mitad de su capacidad. La orina se recogió en un bote de polipropileno, tomándose una única muestra, preferiblemente la primera orina del día debido a su mayor concentración. Tanto la saliva como la orina se almacenaron en los domicilios de los participantes en condiciones de congelación hasta su recogida y transporte al laboratorio. Para las muestras de uña, se entregó un tubo de polipropileno para recolectar uña tanto de las manos como de

los pies durante un período mínimo de 3 meses. La muestra de uña debe ser tomada en ausencia de cualquier tipo de esmalte. Las muestras de pelo se extrajeron el día de la entrevista de la zona occipital del cuero cabelludo, cortado lo más cerca posible de la raíz. Tras la recogida, todas las muestras se almacenaron a -80°C hasta su análisis en laboratorio, a excepción de la uña y el pelo, que se almacenaron a temperatura ambiente.

Se analizaron un total de 12 bisfenoles: BPA, BPF, BPS, BPAP, BPAF, BPB, BPE, BPC, BPFL, BPM, BPP y BPZ. Además, se determinaron seis parabenos: MetPB, EthPB, PropPB, isopropilparabeno (isoPropPB), ButPB e isobutilparabeno (isoButPB) en la muestra de cabello.

Los parámetros de calidad y para la validación del método han sido previamente publicados en diferentes trabajos científicos publicados por nuestro grupo de investigación (Rodríguez-Gómez et al., 2017; Martín-Pozo et al., 2020; Moscoso-Ruiz et al., 2022a,b). A continuación, se describen brevemente estas metodologías.

### a) Determinación de los bisfenoles en saliva humana

El método empleado para la determinación de los bisfenoles en saliva humana (n=89) fue desarrollado anteriormente por miembros del grupo de investigación (Moscoso-Ruiz et al., 2022a). En la figura 15 se muestra un esquema del procedimiento analítico que se llevó a cabo.

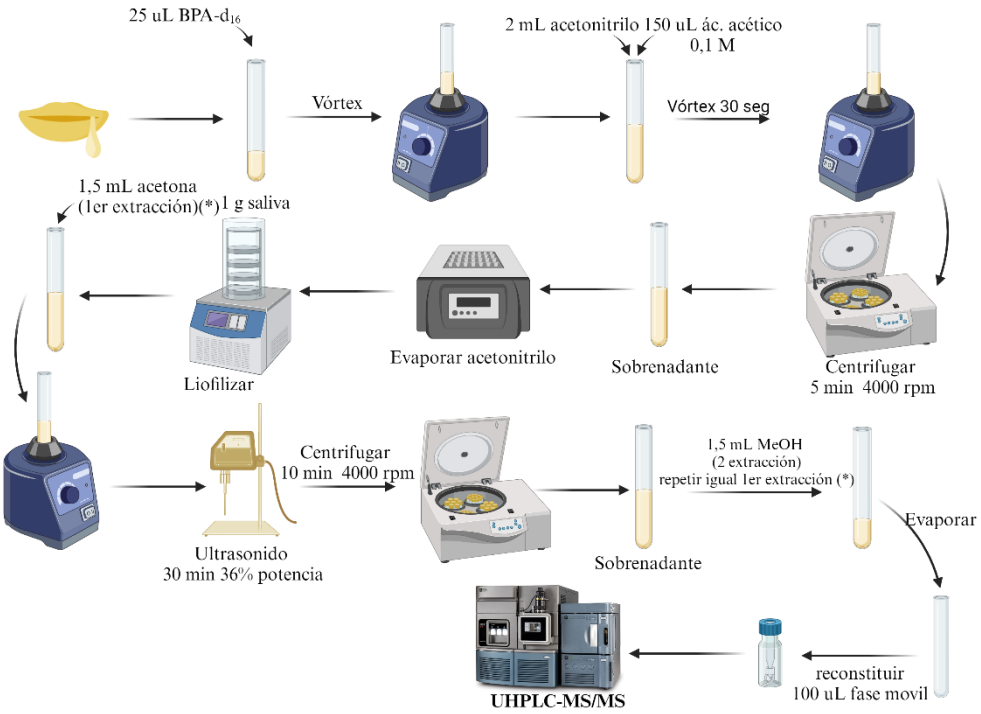


**Figura 15.** Protocolo pretratamiento, extracción y análisis de los bisfenoles en muestras de saliva. Elaborado con Biorender.com

### b) Determinación de los bisfenoles en orina humana

Las muestras de orina (n=149) fueron sometidas previamente a un tratamiento enzimático para desconjugar a los analitos. Las enzimas usadas fueron ß-glucuronidasa y ß-glucuronidasa/arilsulfatasa. El tratamiento enzimático seguido se describe en el trabajo de Moscoso-Ruiz et al. (2022b) y Vela-Soria et al. (2014). La figura 16 muestra el procedimiento analítico que se realizó.

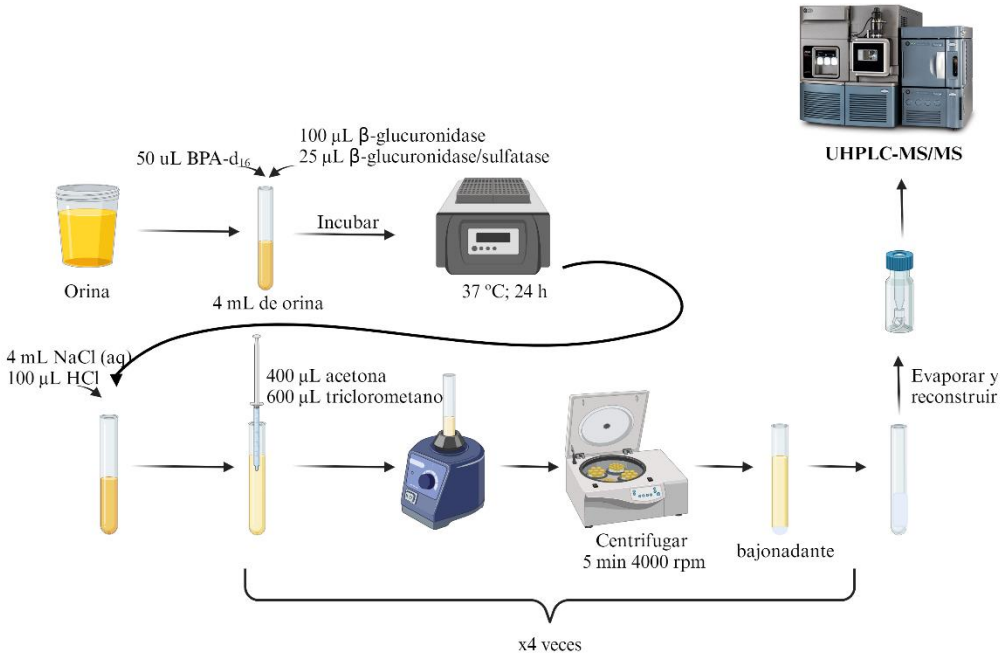


**Figura 16.** Protocolo pretratamiento, extracción y análisis de los bisfenoles en muestras de orina. Elaborado con Biorender.com

Además, se determinó los niveles de creatinina de las muestras de orina a través del Laboratorio de Análisis Clínicos Ángel Méndez Soto. El método utilizado fue el método clásico de Jaffé, basado en la medición fotométrica de la cinética de reacción de la creatinina con ácido pícrico a 37°C (Peake & Whiting, 2006; Weber & van Zanten, 1991). El kit de reactivos se adquirido a Biosystems (Barcelona, España).

### c) Determinación de los bisfenoles en muestra de uña humana

El método para la determinación de los bisfenoles en uña (n=74) también fue desarrollado por miembros de este grupo de investigación tal y como se describe en el artículo Martín-Pozo et al. (2020). A continuación, se muestra un esquema del procedimiento analítico seguido (Figura 17).

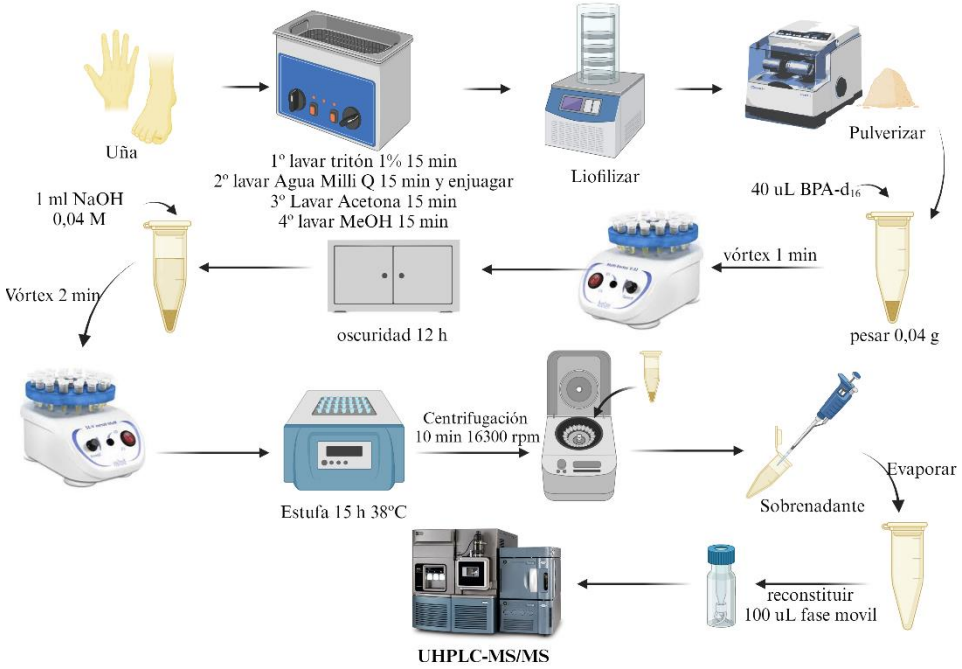


**Figura 17.** Protocolo pretratamiento, extracción y análisis de los bisfenoles en muestras de uña. Elaborado con Biorender.com

### d) Determinación de los bisfenoles y los parabenos en muestras de pelo humano

Para la determinación de los DEs en el pelo (n=318), se siguió el protocolo discrito por Rodríguez-Gómez et al. (2017). En la figura 18 se puede observar el esquema del procedimiento analítico.

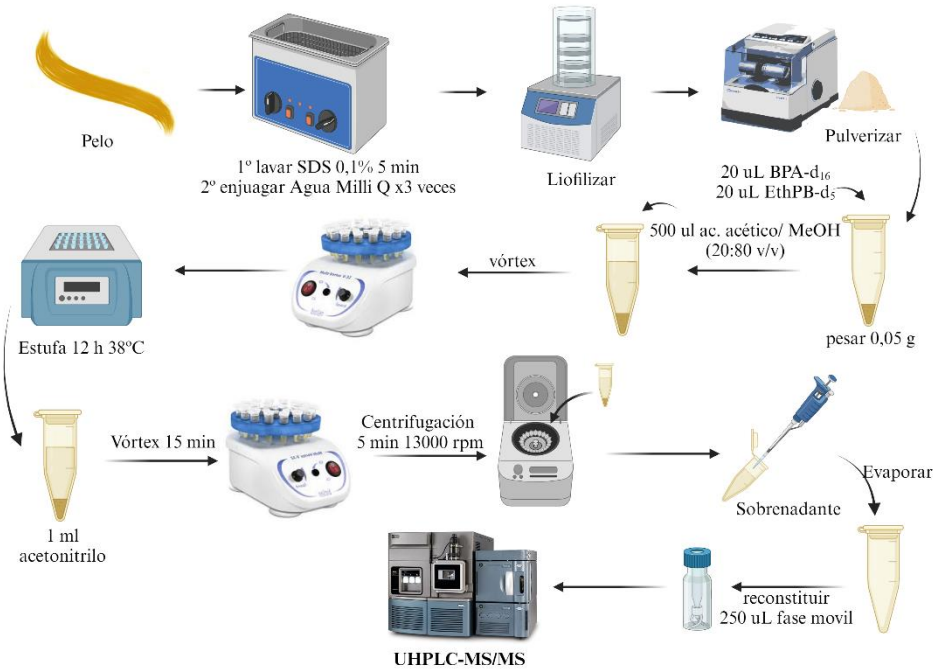


**Figura 18.** Protocolo pretratamiento, extracción y análisis de los bisfenoles y los parabenos en muestras de pelo. Elaborado con Biorender.com

### *4.2.5. Análisis estadístico*

#### 4.2.5.1. Análisis estadístico de la exposición dietética

Las características de los casos y los controles se resumieron utilizando la mediana y el rango intercuartílico (IQR, percentil 25-percentil 75) para las variables continuas no paramétricas, la media y desviación estándar (DS) para las variables continuas paramétricas, y se calcularon frecuencias para las variables categóricas. Para evaluar el nivel de significación de las diferencias observadas entre las variables categóricas se utilizó la prueba de Chi-cuadrado (para variables categóricas) y la U de Mann-Whitney o Kruskal-Wallis (para variables continuas).

Se utilizaron modelos de regresión logística para estimar las odds ratio (OR) y los intervalos de confianza del 95% (IC 95%) para evaluar la influencia de la exposición dietética al BPA, el BPS y los bisfenoles totales (BPA + BPS) sobre el sobrepeso/obesidad. A continuación, el IMC dicotomizado como normopeso y sobrepeso/obesidad fue la variable dependiente. La exposición dietética a los bisfenoles (BPA, BPS y bisfenoles totales) se categorizó según terciles y posteriormente se dicotomizó como baja (primer y segundo tercil) y alta (tercer tercil) exposición, estas fueron las variables independientes (categoría de referencia: primer y segundo tercil). Se utilizaron dos modelos: un modelo crudo y otro ajustado para potenciales factores de confusión identificados en estudios previos (edad, ingesta energética y calidad de la dieta) (Charisiadis et al., 2018; Robles-Aguilera et al., 2021; Song et al., 2014; Zhang et al., 2019), y aquellas variables que produjeron cambios > 10% en OR del modelo crudo (tabaquismo entre los miembros de la unidad familiar, actividad física y porcentaje de grasa corporal) se incluyeron en el modelo ajustado como factores de confusión. Además, se realizó estratificado por sexo debido a las diferencias biológicas, sociales y de comportamiento entre hombres y mujeres que pueden influir en la prevalencia de sobrepeso y obesidad (Kapoor et al., 2021). La justificación de este planteamiento se basa en la bibliografía publicada anteriormente, según la cual el sexo podría modificar el efecto de la exposición al bisfenol sobre el IMC (Robles-Aguilera et al., 2021; Moon et al., 2022)

#### 4.2.5.2. Análisis estadístico de las muestras biológicas

La distribución de las variables continuas y paramétricas se resumió mediante media y DS, mientras que la distribución de las variables continuas y no paramétricas se resumió mediante la mediana y el IQR. Se calcularon distribuciones de frecuencia para las variables categóricas. Para evaluar las diferencias entre casos y controles para todas las variables, se utilizaron la prueba *t*-Student (para variables contínuas paramétricas), la prueba U de Mann-Whitney (para variables contínuas

no paramétricas) y la prueba de Chi-cuadrado de Pearson (variables categóricas).

Se utilizó un modelo de regresión logística para analizar la influencia de las concentraciones de cada bisfenol (ng $g^{-1}$ o ng $mL^{-1}$) en las cuatro matrices biológicas (saliva, orina, uña y pelo) como variable independiente, y sobre el sobrepeso y la obesidad como variable dependiente, así como para los parabenos (ng $g^{-1}$) en el caso del pelo.

La variable dependiente fue dicotomizada de la misma manera que se describió para la exposición dietética. Las variables independientes se dicotomizaron según el valor de la mediana (categoría de referencia: concentración ≤ valor de la mediana). Cuando el porcentaje de concentraciones no detectadas de un analito era > 30%, el punto de corte para la dicotomización era el límite de detección (LOD)/√2 (categoría de referencia: concentración ≤ LOD/√2) (National Center for Environmental Health, 2021). El valor LOD para el bisfenol total en orina se consideró como la suma de cada LOD del bisfenol por separado. Se calcularon los OR y el error estándar (ES) y el IC al 95% para los modelos crudos y ajustado. El sexo, la edad, ingesta energética, fumar, actividad física, porcentaje grasa corporal (para saliva, orina, uña y pelo) y los niveles de creatinina (para orina) se consideraron factores de confusión en los modelos ajustados. En el caso de las variables ingesta energética, fumar, actividad física, porcentaje grasa corporal y los niveles de creatinina se consideraron potenciales factores de confusión cuando produjeron cambios > 10% en OR del modelo crudo.

Todos los análisis estadísticos de la presente Memoria de Tesis Doctoral se realizaron con IBM SPSS (versión 26.0, IBM® SPSS® Statistics, Armonik, NY, EE.UU.). La significación estadística se fijó en $p \leq 0,05$.

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# CAPÍTULO I.

## Determinación de disruptores endocrinos en muestras de alimentos

## Objetivos específicos capítulo I

1. Realizar una revisión exhaustiva de la literatura para conocer las técnicas analíticas más comúnmente utilizadas para la extracción y detección de los bisfenoles.
2. Determinar que alimentos son de habitual consumo en la población española.
3. Medir las concentraciones de bisfenoles en 98 muestras de alimentos de consumo habitual.
4. Estimar la ingesta dietética de bisfenoles en la población infantil.

## Introducción capítulo I

Desde mediados y finales del siglo pasado ha aumentado la preocupación por la presencia en el medio ambiente de compuestos químicos originados principalmente por la actividad humana. Este hecho ha generado un mayor interés por el desarrollo y el perfeccionamiento de las técnicas analíticas tanto para la extracción como para la detección de estos compuestos.

Estas técnicas han evolucionado considerablemente a lo largo de los años, comenzando inicialmente con métodos capaces de extraer e identificar un único compuesto. Sin embargo, el conocimiento de que nuestra exposición abarca a más de un único compuesto químico ambiental ha llevado consigo al desarrollo de métodos multirresiduo, que facilitan la extracción y detección simultáneas de múltiples compuestos químicos de diferentes características.

Entre las técnicas de extracción más extendidas y perfeccionadas para la evaluación de numerosos compuestos se encuentra el QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe). Esta técnica se emplea en el tratamiento de muestras para extraer los compuestos de interés y eliminar las interferencias que podrían afectar a los resultados de los análisis.

El QuEChERS es una técnica simplificada y eficiente que combina extracción y limpieza en un único proceso. Esta técnica es un tipo de extracción en fase sólida dispersiva (dSPE) que comprende dos etapas principales: una fase inicial de extracción directa seguida de una fase de limpieza mediante extracción en fase sólida dispersiva.

Con respecto a las técnicas de análisis se utilizan principalmente la cromatografía de líquidos y la de gases acopladas a diferentes tipos de detección. En los últimos años y dada su capacidad de identificación inequívoca de los analitos y su sensibilidad se ha empleado casi exclusivamente la espectrometría de masas en tándem. En este trabajo se ha empleado específicamente la cromatografía de líquidos. Esta técnica facilita la separación de los componentes de una mezcla mediante interacciones a tres bandas entre analito, fase estacionaria y una fase móvil líquida. La combinación de la cromatografía de líquidos con la espectrometría de masas ha transformado la capacidad de identificar y cuantificar múltiples compuestos químicos simultáneamente con elevada exactitud.

En resumen, los avances en el área de la química analítica han permitido mejorar significativamente la capacidad para seguir y estudiar la presencia de compuestos químicos ambientales en matrices complejas, contribuyendo a una mejor comprensión de su impacto en la salud y el medio ambiente.

A continuación, se muestra el trabajo científico publicado con relación a la temática desarrollada en este capítulo.

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# Presence of Parabens and Bisphenols in Food Commonly Consumed in Spain

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**Abstract:** Given the widespread use of bisphenols and parabens in consumer products, the assessment of their intake is crucial and represents the first step towards the assessment of the potential risks that these compounds may pose to human health. In the present study, a total of 98 samples of food items commonly consumed by the Spanish population were collected from different national supermarkets and grocery stores for the determination of parabens and bisphenols. Our analysis demonstrated that 56 of the 98 food samples contained detectable levels of parabens with limits of quantification (LOQ) between 0.4 and 0.9 ng $g^{-1}$. The total concentration of parabens (sum of four parabens: ∑parabens) ranged from below the LOQ to 281.7 ng $g^{-1}$, with a mean value of 73.86 ng $g^{-1}$. A total of 52% of the samples showed

detectable concentrations of bisphenols. Bisphenol A was the most frequently detected bisphenol in the food samples analysed, followed by bisphenol S and bisphenol E. Bisphenol AF, bisphenol B and bisphenol P were not found in any of the analysed samples. LOQ for these bisphenols were between 0.4 and 4.0 ng $g^{-1}$.

**Keywords:** parabens; bisphenols; food; Spain

## 1. Introduction

The antimicrobial activity of parabens has been known since 1924 and for this reason these alkyl esters of *p*-hydroxybenzoic acid have been extensively used as preservatives in many consumer products such as health and personal care product and foodstuffs [1–3]. Parabens are regulated as preservatives in Commission Regulation (EU) No 1004/2014 on cosmetic products that sets a maximum limit of 0.4% and 0.8% for single esters and mixtures of esters, respectively [4].

There are growing concerns about the presence of these preservatives in pharmaceuticals and cosmetic products associated with their estrogenic effects demonstrated by in vivo and in vitro studies [5]. This disrupting hormone activity seems to be linked to the length of the alkyl chain, with long-chain parabens like propyl 4-hydroxybenzoate (PropPB) and butyl 4-hydroxybenzoate (ButPB) being those of highest concern [6]. In 2010, the EU Scientific Committee on Consumer Safety (SCCS) considered that the use of methyl 4-hydroxybenzoate (MetPB) and ethyl 4-hydroxybenzoate (EthPB) at the maximum authorized concentrations is safe but due to the lack of scientific data, the Committee cannot ascertain that the use of PropPB and ButPB at the maximum concentrations is completely safe [7]. With respect to the use of MetPB and EthPB (food additives E218 and E214, respectively) and their sodium salts (E219 and E215, respectively) in foodstuffs, the maximum permitted levels (MPLs) are between 300 and 1000 mg $kg^{-1}$. No other parabens are approved for use in food. The European Food Safety

Authority (EFSA) concluded that an ADI (Acceptable Daily Intake) of 0–10 mg $kg^{-1}$ body weight (bw) $day^{-1}$ could be set for MetPB and EthPB and their sodium salts [8]. In 2015, the European Medicines Agency (EMA) reported evidence of adverse health effects related to the intake of PropPB and ADI at 1.25 mg $kg^{-1}$ bw $day^{-1}$ [9]. Despite having been used as food preservatives for many decades, little information is available about parabens concentration in certain foods and dietary exposure [10,11].

Bisphenol A (BPA) is also known to disrupt hormone function. This chemical is produced in large volumes, and it is used primarily to harden polycarbonate plastics and epoxy resins [12] used in a wide variety of consumer products including beverage bottles, food can coatings, plastic tableware, thermal paper, and medical devices. The most common route of human exposure is food and beverage consumption [13]. The harmful effects on the reproductive, cardiovascular, immune and metabolic systems related to human exposure to BPA have been extensively described [14]. In 2017, BPA was included in the European Chemical Agency (ECHA) Candidate List of substances of very high concern. In view of the recent limitations on the use of BPA in food contact materials [15–18], the food packaging industry is exploring alternatives to replace BPA in these materials [19–21].

In this regard, BPA substitutes such as BPS [4, 4′-sulfonyldiphenol], BPF [4, 4′-dihydroxydiphenylmethane], BPB [2,2-bis(4-hydroxyphenyl) butane], BPE [1,1-Bis (4-hydroxyphenyl) ethane], and BPAF [4,4′-(hexafluoroisopropylidene) diphenol] are being used as alternatives to BPA in some industrial applications for manufacturing polycarbonate resins [22–26]. As with BPA, these replacement chemicals have a structure similar to BPA and therefore also exhibit endocrine disrupting properties [19,27,28]. However, studies on the occurrence of bisphenols, other than BPA, in foodstuffs are limited. In 2015, the EFSA re-examined BPA exposure and toxicity issues, reducing the BPA tolerable dietary

intake (TDI), previously set at 50 µg $kg^{-1}$ bw $day^{-1}$ [18], to 4 µg $kg^{-1}$ bw $day^{-1}$ [29]. No specific limits were indicated for other types of bisphenols.

In children, the food chain is the main exposure route to parabens and bisphenols. In addition, children are especially vulnerable to developmental exposure, and it has been reported that exposure levels in infants and children in relation to their body weight are higher than in adults [30]. Recently, we have developed an analytical method to determine BPB, BPS, BPE and BPP concentration in food products for children [19].

We conducted an exhaustive literature review that showed that the analytical techniques most commonly used for the extraction of the analytes included in the present study are solid phase extraction (SPE) and QuEChERs (Quick, Easy, Cheap, Effective, Rugged, and Safe), and QuEChERs and liquid–liquid extraction (LLE) for the clean-up phase. The main separation techniques are liquid chromatography and gas chromatography coupled with mass spectrometry, used for analyte detection. Other studies have used an alternative method that uses liquid chromatography coupled with fluorescence detection ("Supplementary Material". Data available online at https://www.mdpi.com/2304-8158/10/1/92/s1, Figure S1: Chromatogram of the blank. Figure S2: Chromatogram from one of the samples (canned tuna in oil), which contains remarkable levels of BPA, BPS, MetPB, EthPB and ButPB.

Bisphenols have been the focus of extensive research over the last years; however, the available studies are mainly focused on BPA. Since BPA and its substitutes show similar endocrine disrupting properties and effects, further study of these compounds used to replace BPA is warranted. In this work, the concentration of parabens and bisphenols in 98 samples of food items commonly consumed by Spanish population is determined, which are collected from different supermarkets and grocery stores. Although several studies have addressed the presence of bisphenols in food items in Spain, they are very limited regarding the number of samples of food and food packaging analysed. In addition,

there are very few studies on the presence of parabens in food and to our knowledge, no studies have been conducted on paraben concentrations in such a wide range of food products commonly consumed by the Spanish population. Moreover, existing studies focus on one class of endocrine disruptors only and none of them include the analysis of both bisphenols and parabens.

## 2. Material and Methods

### *2.1. Chemicals*

All reagents were of analytical grade. Ultrapure water (18.2 MΩ-cm) was prepared with the in-house Milli-Q Plus® system from Merck Millipore. MetPB, EthPB, PropPB and ButPB (≥99% purity) were supplied by Alfa Aesar (company, city, MA, USA) (Figure 1). BPA, BPF, BPS, BPAF, BPP (≥99% purity), BPE, BPB (≥98% purity), and deuterium labelled bisphenol A (BPA-$d_{16}$, ≥99% purity) were supplied by Sigma-Aldrich (Madrid, Spain) (Figure 2). Ethyl-$d_5$-paraben (EthPB-$d_5$) was obtained from Toronto Research Chemicals (company, city, Canada). Stock solutions of bisphenols (100 mg $L^{-1}$), parabens (100 mg $L^{-1}$) and internal standards BPA-$d_{16}$ and EthPB-$d_5$ (10 mg $L^{-1}$) were prepared in methanol (MeOH). The working standard solution was prepared by diluting the stock solutions of the 11 analytes investigated (10 mg $L^{-1}$) with MeOH and was stored in amber glass vials at −20 °C until analysis. Calibration standards were prepared by spiking food matrix with working standard solution. Liquid chromatography-mass spectrometry (LC-MS) solvents methanol and acetonitrile were provided by VWR Chemicals (company, Barcelona, Spain). Ammonia solution 25% for LC-MS used as mobile phase modifier and sodium hydroxide (NaOH) pellets of reagent grade (≥98% purity) were from Sigma-Aldrich (Madrid, Spain). Sodium chloride (NaCl) and magnesium sulphate ($MgSO_4$) were from Panreac (Barcelona, Spain). Sorbents, silica functionalized with octadecyl groups (C18), and primary-secondary amine (PSA) were from Scharlab (Barcelona, Spain).

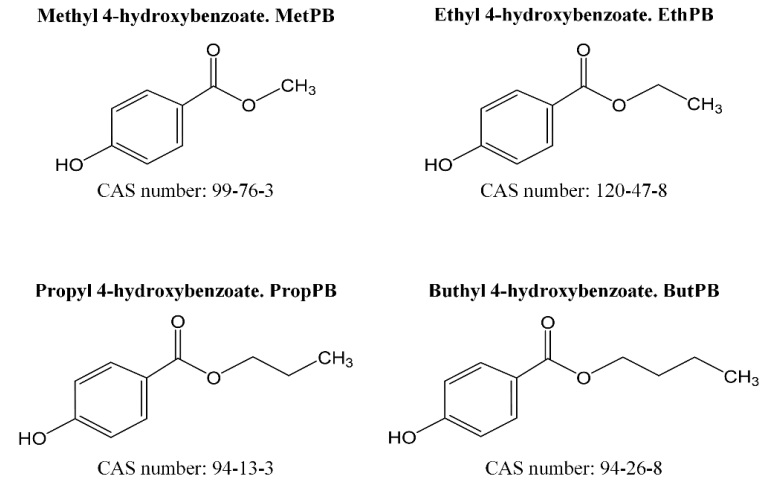


**Figure 1.** Parabens analysed in the present work.

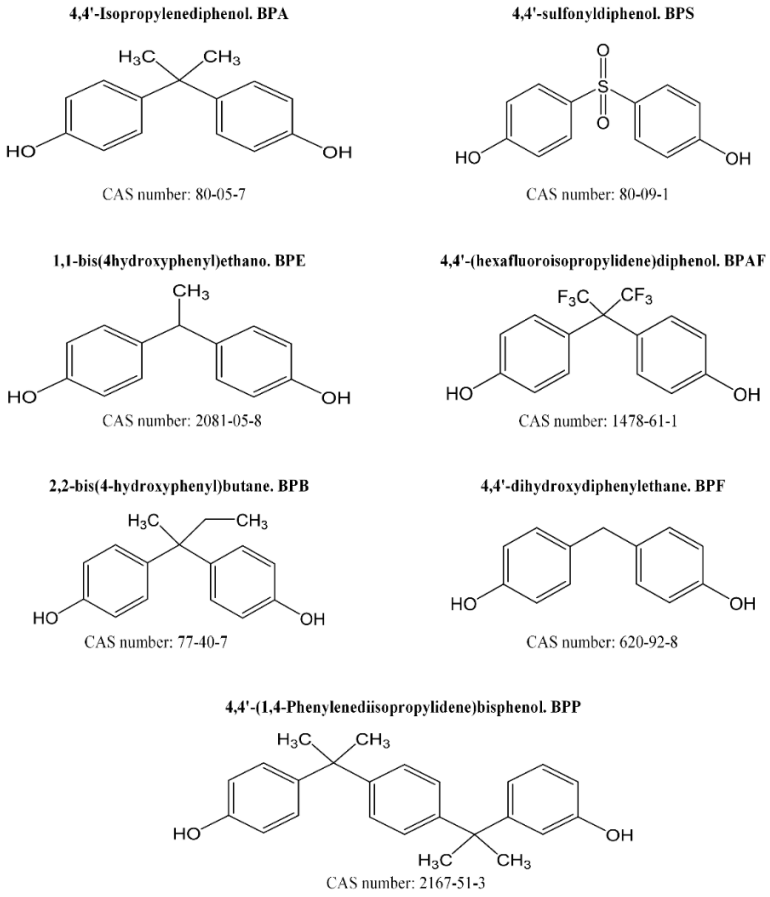


**Figure 2.** Bisphenols analysed in the present work.

### *2.2. Instrumentation and Software*

A Waters Acquity UPLC™I-Class system (company, city, state, country) was used for the determination of seven bisphenols and four parabens. A Waters Xevo TQ-XS (company, city, state, country) with an orthogonal Z-Spray™ electrospray ionization (ESI) source (company, city, state, country) was used for the spectrometric measurements. The column was a Waters UPLC® BEH $C_{18}$ (2.1 mm × 50 mm, 1.7 µm particle size). A ScanVac CoolSafe™ lyophilizer (Lynge, Denmark) was used for lyophilization of food samples. Other laboratory equipment was a vortex-mixer (IKA, Staufen, Germany), a GX400 laboratory balance (Mettler-Toledo, Columbus, OH, USA), a Universal 32 centrifuge (Hettich, Tuttlingen, Germany), a Spectrafuge™ 24D centrifuge (Labnet International, Inc., Edison, NJ, USA) and a SBHC0NC sample concentrator (Stuart, Staffordshire, UK), and an Ultrasons-HD series ultrasonic bath (Selecta, Barcelona, Spain). For the treatment and analysis of data and for equipment control, MassLynx 4.1 software (version, company, city, state, country) from Waters was used.

### *2.3. Food Sampling*

The food items included were selected among the most consumed items by the Spanish population. Most of the selected food was packaged in plastic, cans, paper trays, paperboard, foil and carton packages. The food items included in this study were collected from different national supermarkets and grocery stores and were segregated into the categories defined by the NOVA food classification system based on their nature, extent and purpose of the industrial processes they undergo (unprocessed or minimally processed foods, processed food and ultra-processed foods) [31]. A singular feature of the NOVA classification is the definition of ultra-processed food, which are not modified foods, but formulations obtained by the processing of cheap industrial ingredients that usually also include additives to make them more durable and tastier [31].

### *2.4. Analytical Methods*

Solid foods and dairy products were lyophilized prior to the treatment of the samples and their subsequent analysis. For sample treatment the method developed by García-Córcoles et al. (2018) [19] with some modifications was used. Briefly, 2 g of each sample were weighed into a 10 mL glass tube and 5 µL of a solution of internal standards in MeOH, BPA-$d_{16}$ and EthPB-$d_5$ (10 ppm) was added. Samples were homogenized in 2 mL ultrapure water and 6 mL acetonitrile in a vortex-mixer for one minute, and subsequently bath sonicated for 30 min at 15°C. NaCl 1 g was added to each food sample and centrifuged for 5 min at 4000 rpm (2594× *g*). The upper organic layer was transferred to a 10 mL glass tube and 600 mg $MgSO_4$ and 150 mg PSA were added to remove proteins, carbohydrates, and lipids. The mixture was stirred in a vortex-mixer for one minute and centrifuged for 5 min at 4000 rpm (2594× *g*). The supernatant was evaporated to dryness into a centrifugal evaporator (2000 rpm, 50 °C). For the chromagraphic analysis a new method based on ultrahigh performance liquid chromatography-tandem mass spectrometry was optimized. The extracts were reconstituted by adding 250 µL of a MeOH/$H_2O$ mixture (50:50, *v/v*) and stirring in a vortex mixer until homogenization. After centrifugation for 5 min at 4000 rpm (2594× *g*), the extract obtained was transferred to a glass vial and directly injected into the ultra-high performance liquid chromatography-tandem mass spectrometric (UHPLC-MS/MS) system.

### *2.5. Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectrometry Analysis*

The chromatographic conditions optimized for the analysis are summarized next. An Acquity UPLC® BEH C18 column (2.1 mm × 50 mm, 1.7 µm particle size) was used for analyte separation at 40 °C column temperature. Based on our previous experience, 0.025% (*v/v*) ammonium in water (solvent A) and MeOH (solvent B) were used as mobile phase [32]. Flow rate was 0.35 mL $min^{-1}$ and injection volume was 10 µL. The mobile phase gradient in the food samples was: 0.0 to 1.0 min, 10%

solvent B; 1.0 to 6.0 min, 10 to 90% B; 6.0 to 6.1 min, 90 to 100% B; 6.1 to 6.6 min back to 10% B (run time 10 min). For sensitive and selective quantification of the target compounds in food samples with the highest specificity and sensitivity, negative electrospray ionization in multiple-reaction-monitoring (MRM) mode was used. The most abundant transition monitored was used for quantification and the second to confirm identification. In two cases (BPA and BPE) only one transition was sensitive and useful. Table 1 shows the optimized parameters for the UHPLC-MS/MS analysis.

**Table 1.** Optimized parameters for the Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analysis of compounds.

| | $t_R$ (min) | Transitions | CV | CE | | $t_R$ (min) | Transitions | CV | CE |
|---|---|---|---|---|---|---|---|---|---|
| **BPS** | 0.5 | 249.1 → 107.5 [b] | −4 | −26 | **MetPB** | 0.9 | 151.0 → 91.4 [a] | −12 | −18 |
| | | 249.1 → 155.5 [a] | −4 | −20 | | | 151.0 → 135.5 [b] | −12 | −14 |
| **BPF** | 4.2 | 199.1 → 76.4 [b] | −14 | −24 | **EthPB** | 2.0 | 165.1→ 91.6 [a] | −14 | −22 |
| | | 199.1 → 92.4 [a] | −14 | −20 | | | 165.1 → 136.3 [b] | −14 | −14 |
| **BPE** | 4.6 | 213.1 → 197.7 | −46 | −18 | **PropPB** | 3.0 | 179.1 → 91.5 [a] | −26 | −22 |
| | | | | | | | 179.1 → 114.5 [b] | −26 | −16 |
| **BPA** | 4.9 | 227.2 → 132.9 | −50 | −26 | **ButPB** | 3.9 | 193.1 → 91.5 [a] | −18 | −26 |
| | | | | | | | 193.1 → 135.9 [b] | −18 | −16 |
| **BPAF** | 5 | 335.2 → 196.7 [b] | −4 | −36 | **BPA-$d_{16}$** | 4.8 | 241.3 → 141.7 | −24 | −26 |
| | | 335.2 → 264.9 [a] | −4 | −22 | | | | | |
| **BPB** | 5.2 | 241.2 → 211.8 [b] | −10 | −16 | **EthPB-$d_5$** | 2.0 | 170.2 → 91.3 [a] | −24 | −16 |
| | | 241.2 → 225.9 [a] | −10 | −20 | | | 170.2 → 137.6 [b] | −24 | −14 |
| **BPP** | 6.1 | 345.2 → 315.1 [b] | −18 | −40 | | | | | |
| | | 345.2 → 330.1 [a] | −18 | −24 | | | | | |

| | | | |
|---|---|---|---|
| **Voltage of capilar** | 3 kV | **Nebulizer gas pressure** | 7.0 bar |
| **Source temperature** | 150 °C | **Cone/desolvation gas** | $N_2$ (≥99.995%) |
| **Desolvation temperature** | 600 °C | **Collision gas** | Ar (99.999%) |
| **Cone gas flow** | 150 L $h^{-1}$ | **Dwell time** | 25 ms |
| **Desolvation gas flow** | 500 L $h^{-1}$ | **Inter-scan delay** | 3 ms |
| **Collision gas flow** | 0.15 mL $min^{-1}$ | | |

CV: Cone voltage (V); CE: Collision energy (eV). [a] SRM transition used for quantification. [b] SRM transition used for confirmation.

*2.6. Method Validation*

Validation and quality parameters of the method, selectivity, linearity, and sensitivity (limit of detection (LOD) and limit of quantification (LOQ)) and accuracy (precision and trueness), were evaluated for method validation. A non-contaminated sample was used as a blank to verify method selectivity, and after spiking to confirm a good sensitivity and accuracy. To minimize the influence of the matrix, quantitative analyses were performed based on matrix-matched calibration curves. The calibration curves were obtained from the analyses of each analyte in blank samples spiked with different concentrations of the analytes: 0 ng $g^{-1}$, 1 ng $g^{-1}$, 5 ng $g^{-1}$, 10 ng $g^{-1}$, 25 ng $g^{-1}$, 50 ng $g^{-1}$, 100 ng $g^{-1}$ and 250 ng $g^{-1}$. Figure 3 shows a chromatogram of a sandard (1 ng $g^{-1}$).

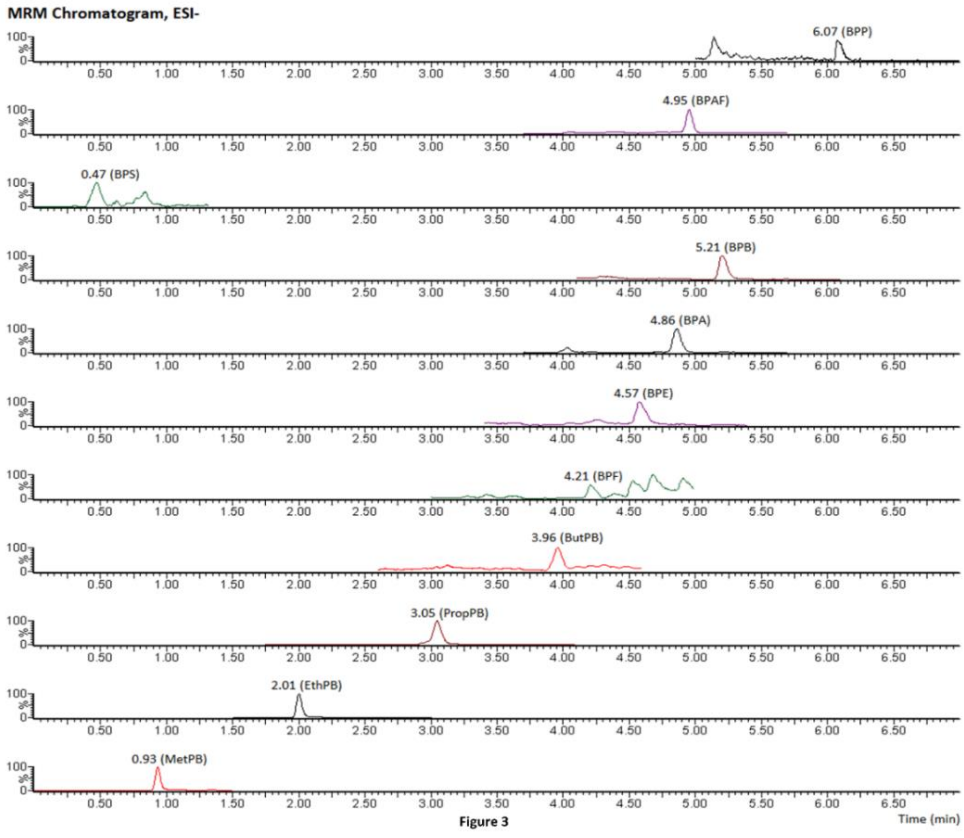


**Figure 3.** Chromatogram of the lower calibration level.

Method selectivity was determined by analysing the signal of the blanks and verifying the absence of peaks in the retention times of the target analytes (Figure S1). LODs and LOQs were defined as the analyte concentration producing an analytical signal of three (LOD) and ten (LOQ) times the signal-to-noise ratio. Table 2 shows the parameters evaluated for calibration, LODs, LOQs and linearity for each analyte. Finally, method accuracy in terms of precision and trueness was evaluated by spiking blank samples at three concentration levels (1, 100, 250 ng $g^{-1}$) of each compound investigated. Three replicates per day in six different days were obtained. Recovery data (trueness confirmation) were between 91% and 106% for all the analytes, with a standard relative deviation (precision confirmation) lower than 12% in all cases.

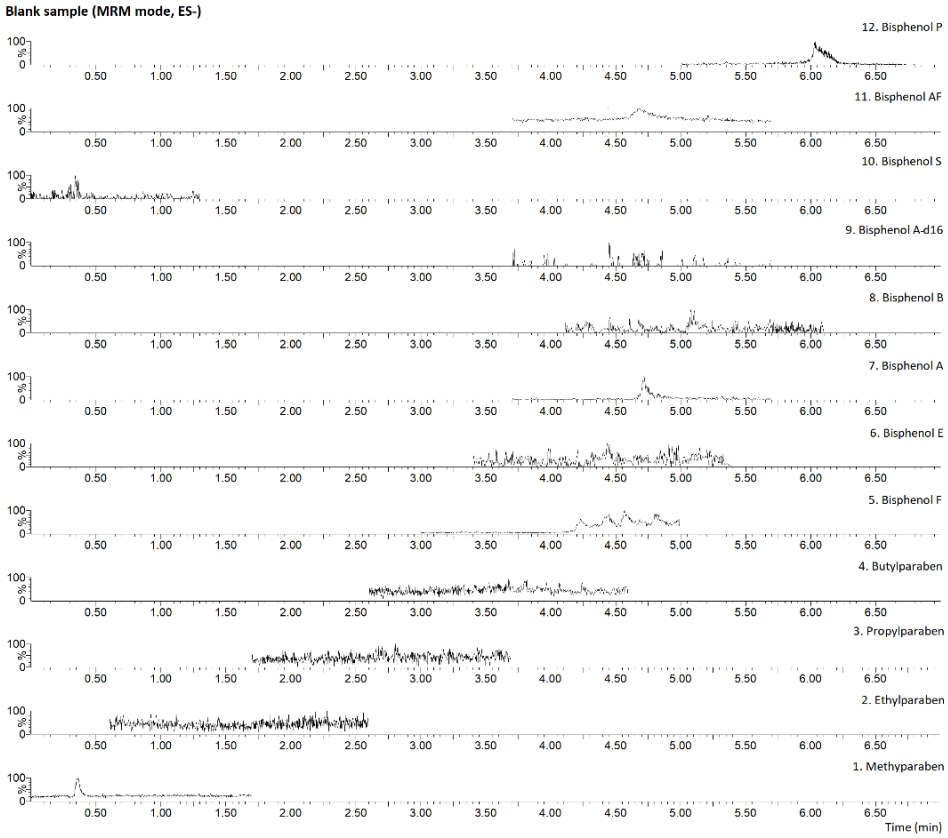


**Figure S1.** Chromatogram of the blank

**Table 2.** Validation and quality parameters of the method.

| | b | Linearity | | LOD | LOQ | Recovery Assay | | |
|---|---|---|---|---|---|---|---|---|
| | g ng$^{-1}$ | $R^2$ | % $P_{lof}$ | ng g$^{-1}$ | ng g$^{-1}$ | Added (ng g$^{-1}$) | % Rec | % RSD |
| **MetPB** | 0.8556 | 0.9535 | 15.2 | 0.1 | 0.4 | 1 | 95 | 10 |
| | | | | | | 100 | 102 | 5.1 |
| | | | | | | 250 | 96 | 6.3 |
| **EthPB** | 0.9615 | 0.9273 | 22.1 | 0.1 | 0.4 | 1 | 106 | 8.2 |
| | | | | | | 100 | 98 | 7.1 |
| | | | | | | 250 | 103 | 4.8 |
| **PropPB** | 0.1064 | 0.9742 | 15.3 | 0.3 | 0.9 | 1 | 94 | 12 |
| | | | | | | 100 | 98 | 6.2 |
| | | | | | | 250 | 96 | 4.1 |
| **ButPB** | 0.2612 | 0.982 | 35.4 | 0.2 | 0.7 | 1 | 96 | 7.2 |
| | | | | | | 100 | 103 | 3.2 |
| | | | | | | 250 | 98 | 4.6 |
| **BPS** | 0.0626 | 0.9665 | 22.1 | 0.3 | 1 | 1 | 91 | 11.1 |
| | | | | | | 100 | 92 | 7.4 |
| | | | | | | 250 | 103 | 9.4 |
| **BPE** | 0.1108 | 0.9945 | 60.2 | 0.3 | 1 | 1 | 93 | 8.2 |
| | | | | | | 100 | 105 | 2.3 |
| | | | | | | 250 | 97 | 6.0 |
| **BPF** | 0.1996 | 0.9915 | 48.1 | 0.1 | 0.5 | 1 | 103 | 8.6 |
| | | | | | | 100 | 104 | 7.2 |
| | | | | | | 250 | 94 | 5.6 |
| **BPAF** | 0.314 | 0.9851 | 20.3 | 0.1 | 0.4 | 1 | 95 | 8.5 |
| | | | | | | 100 | 103 | 4.1 |
| | | | | | | 250 | 96 | 10.3 |
| **BPA** | 0.0446 | 0.9755 | 12.1 | 0.3 | 0.9 | 1 | 104 | 7.2 |
| | | | | | | 100 | 104 | 4.5 |
| | | | | | | 250 | 94 | 10.7 |
| **BPB** | 0.0989 | 0.9848 | 20.3 | 0.3 | 0.9 | 1 | 104 | 6.2 |
| | | | | | | 100 | 97 | 7.0 |
| | | | | | | 250 | 93 | 5.5 |
| **BPP** | 0.0019 | 0.9723 | 16.2 | 1 | 4 | 5 | 104 | 8.6 |
| | | | | | | 100 | 92 | 5.2 |
| | | | | | | 250 | 94 | 4.2 |

b: Slope of the calibration curve; $R^2$: R-squared correlation coefficient; %P: p-value of the lack-of-fit test; LOD: Limit of detection; LOQ: Limit of quantification; %Rec: Recovery; %RSD: Relative standard deviation in percentage.

### *2.7. Estimation of Dietary Exposure in Spanish Children*

Dietary intakes of parabens and bisphenols were calculated for children aged 6–9 years. This age group is one of the most vulnerable groups to endocrine disrupting chemicals and extensive information about their food consumption, dietary habits and anthropometric measures is available from different surveys conducted by the Spanish Agency for Food Safety and Nutrition (*AESAN*) in collaboration with European Agencies such as EFSA.

Total daily exposure was calculated by multiplying the daily intake of food groups (g $day^{-1}$) by the mean bisphenol or paraben concentrations (μg $kg^{-1}$) and dividing this value by body weight in kg. The daily intake of food groups was obtained from the ENALIA study, a cross-sectional survey conducted under the umbrella of *AESAN* and EFSA on a nationally-representative sample of children and adolescents aimed at collecting data on food consumption. [33]. The bisphenol and paraben mean concentrations used were those calculated separating the analysed foods into the categories defined by ENALIA study (meat and derivatives, fish and derivatives, cereals and derivatives, vegetables and derivatives, fruits and derivatives, dairy and derivatives, eggs, salty snacks and pre-cooked foods) (https://www.aesan.gob.es/AECOSAN/web/seguridadalimentaria/subdetalle/enalia.htm#4).

The body weight used for the calculations of total daily exposure was estimated at 29.8 kg based on the data provided by a survey on weight and height conducted on a nationally-representative sample of Spanish children aged 6–9 years [34].

### *2.8. Statistics*

SPSS v.23 (version 23, IBM® SPSS® Statistics, Armonk, NY, USA) and Statgraphics plus 5.0 (version 5, Statpoint Technologies Inc., Warrenton, VA, USA) packages were used for the statistical treatment of analytical data. For calculations, the non-detected and non-quantified

values/compounds were excluded from data treatment. The strength of the association between bisphenol and paraben concentration in food samples was measured by Spearman's correlation. A $p < 0.05$ value was considered significant.

## 3. Results and Discussion

### *3.1. Parabens in Food Samples*

Our results found detectable levels of parabens in 56 out of the 98 food samples analysed. The most frequently detected paraben was MetPB (49%), followed by EthPB (15.5%), and PropPB (10.2%) (Table 3). The detection frequency of ButPB was lower than for the rest of parabens (8.1%) (Table 3). This frequency pattern found in our food samples is consistent with the fact that generally the lower esters are the are the preferred parabens in foods [35] and is similar to that reported in human urine, blood and breast milk from the US population, where the most frequently detected paraben is MetPB [36].

**Table 3.** Frequencies (%) and mean (ng $g^{-1}$) of parabens and bisphenols in food samples.

| Parabens | | | | | |
|---|---|---|---|---|---|
| | **MetPB** | **EthPB** | **ButPB** | **PropPB** | **∑PBs** |
| **Unprocessed or Minimally Processed Foods (*n* = 30)** | | | | | |
| **Frecuency (%)** | 70 | 23.3 | 3.3 | 10 | 73.3 |
| **Mean (ng $g^{-1}$)** | 106.9 (95.4) | 29.5 (40.1) | 2.5 (1) | 35 (48) | 80.7 (81.6) |
| **Processed Foods (*n* = 21)** | | | | | |
| **Frecuency (%)** | 42.9 | 23.8 | 9.5 | 4.8 | 52 |
| **Mean (ng $g^{-1}$)** | 142.1 (120.2) | 60.3 (56.2) | 55.4 (42.2) | 65.5 (26.2) | 130.7 (87.3) |
| **Ultra-Processed Foods (*n* = 47)** | | | | | |
| **Frecuency (%)** | 38.3 | 6.4 | 10.6 | 12.8 | 49 |
| **Mean (ng $g^{-1}$)** | 41.8 (47.2) | 28.6 (12) | 39.9 (60.1) | 3.2 (2) | 40.31 (48.5) |
| **All (*n* = 98)** | | | | | |
| **Frecuency (%)** | 49 | 15 | 8.1 | 10.2 | 57.1 |
| **Mean (ng $g^{-1}$)** | 84.6 (88.5) | 42.2 (44.7) | 39.1 (50.8) | 21.1 (31.5) | 73.9 (76.8) |

| Bisphenols | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **BPS** | **BPE** | **BPF** | **BPAF** | **BPA** | **BPB** | **BPP** | **∑BPs** |
| **Unprocessed or Minimally Processed Foods (*n* = 30)** | | | | | | | | |
| **Frecuency (%)** | 46.9 | 6.3 | 0 | 0 | 21.9 | 0 | 0 | 63 |
| **Mean (ng $g^{-1}$)** | 17.3 (13.7) | <LOQ | 0 | 0 | 6 (3.8) | 0 | 0 | 18.4 (15.5) |
| **Processed Foods (*n* = 21)** | | | | | | | | |
| **Frecuency (%)** | 38.1 | 0 | 0 | 0 | 38.1 | 0 | 0 | 67 |
| **Mean (ng $g^{-1}$)** | 39.5 (67) | 0 | 0 | 0 | 86.3 (158.6) | 0 | 0 | 35 (119.5) |
| **Ultra-Processed Foods (*n* = 47)** | | | | | | | | |
| **Frecuency (%)** | 6.4 | 4.26 | 0 | 0 | 27.7 | 0 | 0 | 36 |
| **Mean (ng $g^{-1}$)** | 47.5 (73.3) | <LOQ | 0 | 0 | 35.3 (42.4) | 0 | 0 | 38.3 (48.1) |
| **All (*n* = 98)** | | | | | | | | |
| **Frecuency (%)** | 26.5 | 4.1 | 0 | 0 | 28.6 | 0 | 0 | 52 |
| **Mean (ng $g^{-1}$)** | 29 (46.3) | <LOQ | 0 | 0 | 43.3 (91.5) | 0 | 0 | 30.4 (41.9) |

∑PBs. ∑Parabens; ∑BPs. ∑Bisphenols; LOQ. limit of quantification. (_). Standard deviation.

The total concentration of parabens (∑parabens) ranged from below the LOQ to 281.7 ng $g^{-1}$, with a mean value of 73.9 ng $g^{-1}$ (Tables 4–6). Some of the samples such as chicken burger (281.7 ng $g^{-1}$), frozen chopped onion (231.9 ng $g^{-1}$), eggs (229.9 ng $g^{-1}$), and milk bread with chocolate (145.3 ng $g^{-1}$), contained remarkably high concentrations of MetPB. The highest concentration of EthPB was found in canned tuna in oil (146.9 ng $g^{-1}$) and in anchovy stuffed olives (86.9 ng $g^{-1}$), and of PropPB in milk bread with chocolate (145.3 ng $g^{-1}$) and in olives (85.2 ng $g^{-1}$). ButPB was found in lower concentrations than the other parabens, with its highest levels found in pineapple in plastic packaging (68.9 ng $g^{-1}$). An example of a chromatogram of one of the food samples analysed in this study is shown in Figure S2.

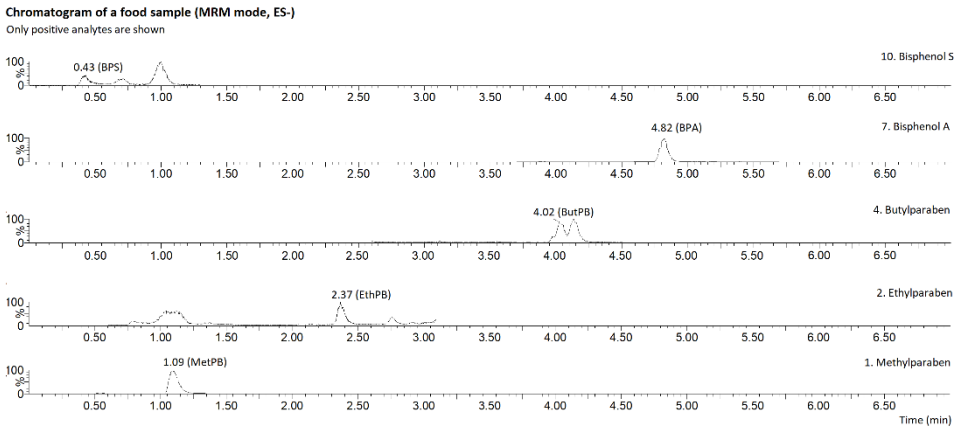


**Figure S2.** Chromatogram from one of the samples (canned tuna in oil), which contains remarkable levels of BPA, BPS, MetPB, EthPB and ButPB.

**Table 4a.** Concentrations (ng $g^{-1}$ fresh weight) of parabens in unprocessed or minimally processed foods.

| *Sample* | Packaging | MetPB | EthPB | ButPB | PropPB | ∑PBs |
|---|---|---|---|---|---|---|
| *Chicken* | Plastic and porex tray | ND | ND | ND | D | |
| *Eggs* | Plastic and paperboard | 229.9 (29.2) | ND | ND | ND | 229.9 (29.2) |
| *Whole milk* | Plastic | ND | ND | ND | ND | |
| *Whole milk* | Carton | ND | ND | ND | ND | |
| *Whole milk* | Carton | ND | ND | ND | ND | |
| *Whole milk* | Carton | ND | ND | ND | ND | |
| *Frozen hake* | Plastic and paperboard | ND | ND | ND | ND | |
| *Lentils* | Plastic | ND | ND | ND | ND | |
| *Grape* | Plastic | D | ND | ND | ND | |
| *Blueberries* | Plastic | ND | ND | ND | ND | |
| *Pineapple* | Plastic | D | 5.8 (4.2) | 2.5 (1.0) | 68.9 (4.0) | 77.2 (37.4) |
| *Raspberry* | Plastic | ND | ND | ND | ND | |
| *Melon* | Plastic | 125.9 | ND | ND | ND | 125.9 |
| *Apple* | Not packed | 7.4 (3.2) | ND | ND | ND | 7.4 (3.2) |
| *Apple* | Plastic | 12.1 (9.2) | ND | ND | ND | 12.1 (9.2) |
| *Pear* | Not packed | 101.6 (84.7) | ND | ND | ND | 101.6 (84.7) |
| *Frozen red fruit mix* | Plastic | D | 100.2 (8.2) | ND | ND | 100.2 (8.2) |
| *Frozen mango* | Plastic | 39.5 | ND | ND | ND | 39.5 |
| *Frozen chopped garlic* | Plastic | D | 13.6 (1.3) | ND | ND | 13.6 (1.3) |
| *Frozen chopped onion* | Plastic | 231.9 (18.9) | ND | ND | ND | 231.9 (18.9) |
| *Frozen chopped parsley* | Plastic | D | ND | ND | ND | |
| *Frozen spinach* | Plastic | D | ND | ND | ND | |
| *Tomato* | Not packed | D | ND | ND | ND | |
| *Tomato* | Plastic | D | ND | ND | ND | |
| *Striped carrot* | Plastic | D | D | ND | ND | |
| *Carrod* | Plastic | D | 6 (3.6) | ND | ND | 6 (3.6) |
| *Lettuce* | Plastic | D | D | ND | ND | |
| *Pumpkin* | Plastic | D | ND | ND | ND | |
| *Mushrooms* | Plastic | D | 22.1 (10.4) | ND | 1.0 (0.1) | 23.1 (14.9) |
| *Green pepper* | Not packed | D | ND | ND | ND | |
| *Mean* | | 106.9 (95.4) | 29.5 (40.6) | 2.5 (1.0) | 35 (48.0) | 73.17 (79.9) |

PBs. ∑Parabens; ND. not detected (<LOD); D. detected (>LOD and <LOQ). (_). Standard deviation.

**Table 4b.** Concentrations (ng $g^{-1}$ fresh weight) of bisphenol analogues in unprocessed or minimally processed foods.

| | | BPS | BPE | BPF | BPAF | BPA | BPB | BPP | ∑BPs |
|---|---|---|---|---|---|---|---|---|---|
| *Chicken* | Plastic and porex tray | ND | ND | ND | ND | 2.1 (1.0) | ND | ND | 2.1 (1.0) |
| *Eggs* | Plastic and paperboard | ND | ND | ND | ND | ND | ND | ND | |
| *Whole milk* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Whole milk* | Carton | ND | ND | ND | ND | ND | ND | ND | |
| *Whole milk* | Carton | ND | D | ND | ND | ND | ND | ND | |
| *Whole milk* | Carton | ND | D | ND | ND | ND | ND | ND | |
| *Frozen hake* | Plastic and paperboard | ND | ND | ND | ND | D | ND | ND | |
| *Lentils* | Plastic | ND | ND | ND | ND | D | ND | ND | |
| *Grape* | Plastic | D | ND | ND | ND | ND | ND | ND | |
| *Blueberries* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Pineapple* | Plastic | 44.3 (2.8) | ND | ND | ND | 11.3 (4.6) | ND | ND | 55.6 (23.3) |
| *Raspberry* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Melon* | Plastic | 4.2 (2.1) | ND | ND | ND | 7.9 (4.2) | ND | ND | 12.08 (2.6) |
| *Apple* | Not packed | 12.7 (1.6) | ND | ND | ND | 6.0 (9.1) | ND | ND | 18.7 (4.7) |
| *Apple* | Plastic | 8.5 (3.3) | ND | ND | ND | 2.9 (2.4) | ND | ND | 11.4 (4) |
| *Pear* | Not packed | ND | ND | ND | ND | ND | ND | ND | |
| *Frozen red fruit mix* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Frozen mango* | Plastic | D | ND | ND | ND | ND | ND | ND | |
| *Frozen chopped garlic* | Plastic | 1.4 (0.8) | ND | ND | ND | ND | ND | ND | 1.4 (0.8) |
| *Frozen chopped onion* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Frozen chopped parsley* | Plastic | 33.3 (13.9) | ND | ND | ND | ND | ND | ND | 33.3 (13.9) |
| *Frozen spinach* | Plastic | D | ND | ND | ND | ND | ND | ND | |
| *Tomato* | Not packed | 4.7 (2.2) | ND | ND | ND | ND | ND | ND | 4.7 (2.2) |
| *Tomato* | Plastic | 25.9 (10.7) | ND | ND | ND | ND | ND | ND | 25.9 (10.7) |
| *Striped carrot* | Plastic | 11.5 (5.3) | ND | ND | ND | ND | ND | ND | 11.5 (5.3) |
| *Carrod* | Plastic | D | ND | ND | ND | ND | ND | ND | |
| *Lettuce* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Pumpkin* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Mushrooms* | Plastic | 16.0 (6.9) | ND | ND | ND | ND | ND | ND | 16.0 (6.9) |
| *Green pepper* | Not packed | 27.5 (6.3) | ND | ND | ND | ND | ND | ND | 27.5 (6.3) |
| *Mean* | | 17.3 (13.7) | | | | 6 (3.8) | | | 18.4 (15.5) |

BPs. ∑Bisphenols; ND. not detected (<LOD); D. detected (>LOD and <LOQ). (_). Standard deviation.

**Table 5a.** Concentrations (ng $g^{-1}$ fresh weight) of parabens in processed foods.

| *Sample* | Packaging | MetPB | EthPB | ButPB | PropPB | ∑PBs |
|---|---|---|---|---|---|---|
| *Cooked ham* | Plastic | ND | ND | ND | ND | |
| *Spicy Sausage* | Plastic | ND | ND | ND | ND | |
| *Spicy Sausage* | Plastic | D | ND | ND | ND | |
| *Chicken burguer* | Plastic | 191.1(118.3) | ND | ND | ND | 191.1 (118.3) |
| *Chicken burguer* | Plastic | 281.7 (88.9) | ND | ND | ND | 281.7 (88.9) |
| *Sausage (Chorizo)* | Plastic | ND | ND | ND | ND | |
| *Serrano ham* | Plastic | ND | ND | ND | ND | |
| *Plain yogurt (sweetened)* | Plastic | ND | ND | ND | ND | |
| *Plain yogurt (sweetened)* | Plastic | ND | ND | ND | ND | |
| *Guacamole* | Plastic | D | ND | ND | ND | |
| *Olives* | Plastic | 5.2 (1.7) | 29.2 (14.5) | ND | ND | 34.4 (17) |
| *Olives* | Plastic | D | 13.6 (5.5) | 85.2 (39.5) | 65.5 (26.2) | 164.3 (37) |
| *Anchovy stuffed olives* | Can | ND | 86.9 (17.5) | ND | ND | 86.9 (17.5) |
| *Semi-cured cheese* | Plastic | ND | 24.8 (7.2) | ND | ND | 24.8 (7.2) |
| *Semi-cured cheese (slice)* | Plastic | D | ND | ND | ND | |
| *Pasta* | Plastic | ND | ND | ND | ND | |
| *Rice (for microwave)* | Plastic and paperboard | 90.2 (28.6) | ND | ND | ND | 90.2 (28.6) |
| *Canned tuna in oil* | Can | ND | ND | ND | ND | |
| *Canned tuna in oil* | Can | D | 146.9 (8.5) | 25.5 (14.9) | ND | 172.4 (85.8) |
| *Canned sweet corn* | Can | ND | ND | ND | ND | |
| *Cake* | Not packed | ND | ND | ND | ND | |
| *Mean* | | 142.1 (120.2) | 60.3 (56.2) | 55.4 (42.2) | 65.5 (26.2) | 130.7 (87.3) |

∑PBs. ∑Parabens; ND. not detected (<LOD); D. detected (>LOD and <LOQ). (_). Standard deviation.

**Table 5b.** Concentrations (ng $g^{-1}$ fresh weight) of bisphenol analogues in processed foods.

| *Sample* | Packaging | BPS | BPE | BPF | BPAF | BPA | BPB | BPP | ∑BPs |
|---|---|---|---|---|---|---|---|---|---|
| *Cooked ham* | Plastic | ND | ND | ND | ND | 6.6 (3.4) | ND | ND | 6.6 (3.4) |
| *Spicy Sausage* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Spicy Sausage* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Chicken burguer* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Chicken burguer* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Sausage (Chorizo)* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Serrano ham* | Plastic | 39.3 (21.3) | ND | ND | ND | 17.3 (14.9) | ND | ND | 56.6 (15.6) |
| *Plain yogurt (sweetened)* | Plastic | ND | ND | ND | ND | 29.9 (18.6) | ND | ND | 29.9 (18.6) |
| *Plain yogurt (sweetened)* | Plastic | ND | ND | ND | ND | 12.3 (6.0) | ND | ND | 12.3 (6.0) |
| *Guacamole* | Plastic | D | ND | ND | ND | ND | ND | ND | |
| *Olives* | Plastic | 8.5 (5.4) | ND | ND | ND | ND | ND | ND | 8.5 (5.4) |
| *Olives* | Plastic | 30.2 (7.7) | ND | ND | ND | ND | ND | ND | 30.2 (7.7) |
| *Anchovy stuffed olives* | Can | ND | ND | ND | ND | ND | ND | ND | |
| *Semi-cured cheese* | Plastic | ND | ND | ND | ND | D | ND | ND | |
| *Semi-cured cheese (slice)* | Plastic | 5.6 (1.9) | ND | ND | ND | ND | ND | ND | 5.6 (1.9) |
| *Pasta* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Rice (for microwave)* | Plastic and paperboard | 3.3 (1.4) | ND | ND | ND | ND | ND | ND | 3.3 (1.4) |
| *Canned tuna in oil* | Can | ND | ND | ND | ND | 409.0 (31.0) | ND | ND | 409.0 (31.0) |
| *Canned tuna in oil* | Can | 187.8 (14.2) | ND | ND | ND | D | ND | ND | 187.8 (14.2) |
| *Canned sweet corn* | Can | ND | ND | ND | ND | 42.7 (6.2) | ND | ND | 42.7 (6.2) |
| *Cake* | Not packed | 1.7 (0.7) | ND | ND | ND | ND | ND | ND | 1.7 (0.7) |
| *Mean* | | 39.49 (67.0) | | | | 86.3 (158.6) | | | 35 (119.5) |

∑BPs. ∑Bisphenols; ND. not detected (<LOD); D. detected (>LOD and <LOQ). (_). Standard deviation.

**Table 6a.** Concentrations (ng $g^{-1}$ dw) of parabens in ultra-processed foods.

| *Sample* | Package Type | MetPB | EthPB | ButPB | PropPB | ∑PBs |
|---|---|---|---|---|---|---|
| *Sausage (Hot dogs)* | Plastic | 6.8 (7.2) | ND | ND | ND | 6.8 (7.2) |
| *Turkey cold cut* | Plastic | D | ND | 2.05 (0.06) | ND | 2.1 (0.1) |
| *Sausage (Turkey cold)* | Plastic | D | ND | ND | ND | |
| *Mortadella (Bologna)* | Plastic | D | ND | 16.5 (11.2) | ND | 16.5 (11.2) |
| *Chocolate milkshake* | Carton | ND | ND | ND | ND | |
| *Chocolate milkshake* | Carton | D | ND | ND | ND | |
| *Chocolate milkshake* | Carton | D | ND | ND | ND | |
| *Chocolate milkshake* | Carton | ND | ND | ND | ND | |
| *Semi-fermented milk* | Plastic and foil | 6.1 (3.7) | ND | ND | ND | 6.1 (3.7) |
| *Semi-fermented milk* | Plastic and foil | 41.08 (12.6) | ND | ND | ND | 41.1 (12.6) |
| *Semi-fermented milk* | Plastic and foil | 88.4 (34.9) | ND | ND | ND | 88.4 (34.9) |
| *Flavoured Yogurt* | Plastic | 26.6 (6.1) | ND | ND | ND | 26.6 (6.1) |
| *Flavoured Liquid Yogurt* | Plastic | 145.7 (8.9) | ND | ND | ND | 145.7 (8.9) |
| *Flavoured Liquid Yogurt* | Plastic | 42.5 (20.5) | ND | ND | ND | 42.5 (20.5) |
| *Spread cheese* | Foil and paperboard | ND | ND | ND | ND | |
| *Melted cheese* | Plastic | ND | ND | ND | ND | |
| *Melted cheese* | Plastic | ND | ND | ND | ND | |
| *Breadsticks for cheese* | Plastic | ND | ND | ND | ND | |
| *Tuna dumplings* | Can | ND | ND | ND | ND | |
| *Battered hake sticks* | Plastic | ND | ND | ND | ND | |
| *Pizza (cooked ham and cheese)* | Plastic | ND | ND | ND | ND | |
| *Pizza (4 cheese)* | Plastic | ND | ND | ND | ND | |
| *Pizza (bolognese)* | Plastic | ND | ND | ND | ND | |
| *Ketchup* | Plastic | ND | ND | ND | D | |
| *Ketchup* | Plastic | ND | ND | ND | D | |
| *Tomato sauce* | Carton | ND | ND | ND | ND | |
| *Corn snacks* | Plastic | ND | ND | ND | 1.4 (0.3) | 1.4 (0.3) |
| *Corn snacks* | Plastic | ND | ND | ND | ND | |
| *Nachos* | Plastic | ND | ND | ND | ND | |
| *Chips* | Plastic | ND | ND | ND | ND | |
| *Chips* | Plastic | ND | ND | ND | ND | |
| *Chips* | Plastic | D | ND | ND | ND | |
| *Chips (Sour Cream & Onion)* | Plastic, foil and paperboard | 7.7 (0.7) | ND | ND | ND | 7.7 (0.7) |
| *Gummy candy* | Plastic | ND | ND | ND | ND | |
| *Gummy candy* | Plastic | ND | ND | ND | ND | |
| *Chocolate doughnuts* | Plastic | ND | D | ND | ND | |
| *Milk bread* | Plastic | ND | ND | ND | ND | |
| *Croissants* | Plastic | ND | ND | ND | ND | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Croissants* | Plastic | D | ND | 4.3 (2.6) | 1.5 (0.8) | 5.8 (2) |
| *Chocolate puff pastry* | Plastic | ND | ND | ND | ND | |
| *Cacao-filled roll* | Plastic | ND | ND | ND | ND | |
| *Muffins* | Plastic | ND | ND | ND | ND | |
| *Muffins* | Plastic | D | ND | ND | 4.2 (0.8) | 4.2 (0.8) |
| *Burger bun* | Plastic | 10.9 (9.6) | 20.1 (7.7) | ND | ND | 31.0 (6.5) |
| *Sandwich bread* | Plastic | ND | ND | ND | ND | |
| *Milk bread with chocolate chips* | Plastic | ND | ND | 145.3 (10.2) | ND | 145.3 (10.2) |
| *Puffed rice cake with chocolate* | Plastic | D | 37.1 (5.5) | 31.2 (8.3) | 5.5 (3.0) | 73.8 (16.8) |
| *Mean* | | 41.8 (47.2) | 28.6 (12.0) | 39.9 (60.1) | 3.2 (2.0) | 40.3 (48.5) |

∑PBs. ∑Parabens; ND. not detected (<LOD); D. detected (>LOD and <LOQ). (_). Standard deviation.

**Table 6b.** Concentrations (ng $g^{-1}$ dw) of bisphenol analogues in ultra-processed foods.

| *Sample* | Package Type | BPS | BPE | BPF | BPAF | BPA | BPB | BPP | ∑BPs |
|---|---|---|---|---|---|---|---|---|---|
| *Sausage (Hot dogs)* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Turkey cold cut* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Sausage (Turkey cold)* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Mortadella (Bologna)* | Plastic | 5.4 (3.3) | ND | ND | ND | ND | ND | ND | 5.4 (3.3) |
| *Chocolate milkshake* | Carton | ND | ND | ND | ND | ND | ND | ND | |
| *Chocolate milkshake* | Carton | ND | D | ND | ND | ND | ND | ND | |
| *Chocolate milkshake* | Carton | ND | ND | ND | ND | ND | ND | ND | |
| *Chocolate milkshake* | Carton | ND | D | ND | ND | ND | ND | ND | |
| *Semi-fermented milk* | Plastic and foil | ND | ND | ND | ND | ND | ND | ND | |
| *Semi-fermented milk* | Plastic and foil | ND | ND | ND | ND | ND | ND | ND | |
| *Semi-fermented milk* | Plastic and foil | ND | ND | ND | ND | ND | ND | ND | |
| *Flavoured Yogurt* | Plastic | ND | ND | ND | ND | 60.9 (17.2) | ND | ND | 60.9 (17.2) |
| *Flavoured Liquid Yogurt* | Plastic | ND | ND | ND | ND | 115.4 (66) | ND | ND | 115.4 (66) |
| *Flavoured Liquid Yogurt* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Spread cheese* | Foil and paperboard | ND | ND | ND | ND | ND | ND | ND | |
| *Melted cheese* | Plastic | 4.9 (0.9) | ND | ND | ND | D | ND | ND | 4.9 (0.9) |
| *Melted cheese* | Plastic | ND | ND | ND | ND | 2 (0.3) | ND | ND | 2 (0.3) |
| *Breadsticks for cheese* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Tuna dumplings* | Can | ND | ND | ND | ND | ND | ND | ND | |
| *Battered hake sticks* | Plastic | ND | ND | ND | ND | ND | ND | ND | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Pizza (cooked ham and cheese)* | Plastic | ND | ND | ND | ND | 4.3 (1.8) | ND | ND | 4.3 (1.8) |
| *Pizza (4 cheese)* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Pizza (bolognese)* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Ketchup* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Ketchup* | Plastic | ND | ND | ND | ND | D | ND | ND | |
| *Tomato sauce* | Carton | ND | ND | ND | ND | ND | ND | ND | |
| *Corn snacks* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Corn snacks* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Nachos* | Plastic | ND | ND | ND | ND | 42.1 (4.2) | ND | ND | 42.1 (4.2) |
| *Chips* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Chips* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Chips* | Plastic | 132.1 (21.2) | ND | ND | ND | ND | ND | ND | 132.1 (21.2) |
| *Chips (Sour Cream & Onion)* | Plastic, foil and paperboard | ND | ND | ND | ND | 8.8 (10.7) | ND | ND | 8.8 (10.7) |
| *Gummy candy* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Gummy candy* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Chocolate doughnuts* | Plastic | ND | ND | ND | ND | 82 (10.2) | ND | ND | 82 (10.2) |
| *Milk bread* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Croissants* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Croissants* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Chocolate puff pastry* | Plastic | ND | ND | ND | ND | 1 | ND | ND | 1 |
| *Cacao-filled roll* | Plastic | ND | ND | ND | ND | D | ND | ND | |
| *Muffins* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Muffins* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Burger bun* | Plastic | ND | ND | ND | ND | 1.2 (1.6) | ND | ND | 1.2 (1.6) |
| *Sandwich bread* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Milk bread with chocolate chips* | Plastic | ND | ND | ND | ND | D | ND | ND | |
| *Puffed rice cake with chocolate* | Plastic | ND | ND | ND | ND | ND | ND | ND | |
| *Mean* | | 47.5 (73.3) | | | | 35.3 (42.4) | | | 38.3 (48.1) |

∑BPs. ∑Bisphenols; ND. not detected (<LOD); D. detected (>LOD and <LOQ). (_). Standard deviation.

The literature review revealed only a small number of studies about the presence of parabens in food, maybe related to the fact that the source of parabens in food is not known with certainty (use as antimicrobial agents and in food packaging) [2,3,37]. We compared the concentrations of parabens detected in this study with those reported in other studies carried out in the European Union (EU) (Table S1) as the levels should comply with EU legislation on food safety assessment. Moreover, a LOD from 0.1 to 0.3 ng $g^{-1}$ and a LOQ from 0.4 to 0.9 ng $g^{-1}$ found for the studied analytes mean that this method can be consider sensitive as other authors have reported similar values for parabens (information available at "Supplementary Material", Table S1).

The concentration of parabens found in this study is higher than that found in other works (Table S1). A possible explanation for this is that the foods analysed in this study are different and come in different packages than those analysed in previous works, which makes comparison difficult. In addition, food categorization into specific groups is vague/unclear/unspecific in previous works. Lastly, most of the foods selected for the present study are processed foods, where parabens are extensively used as additives, and come in plastic packaging, from where parabens may leach into the food inside, hence the higher concentrations found. The concentrations of parabens varied widely even within the same category of foodstuff, and processed foods generally contained higher paraben concentrations than unprocessed/fresh foods [2,3].

As in this study, the most frequently detected paraben in European studies was MetPB, which is also the one detected in the highest concentrations in food. MetPB concentrations previously reported in European studies were similar to the concentrations found in our food samples, ranging from below the LOD to 85 ng $g^{-1}$ [38–44]. The European studies have also reported that other parabens frequently detected were EthPB (ranging from <LOD to 0.8 ng $g^{-1}$) and PropPB (ranging from <LOD to 7.4 ng $g^{-1}$) [38,40–44].

On the other hand, ButPB was not detected in the European studies (<LOD) [41,42], but we found detectable concentrations of this paraben (ranging from <LOD to 145.3 ng $g^{-1}$) although with the lowest frequency (8%) in the food samples analysed (Table 3).

Isopropylparaben and benzylparaben have been detected in European studies in food samples [38,40,41,43–45], but these parabens have not been analysed in the present study; therefore, they should be included in future studies for their determination in food.

Pearson's correlations revealed that EthPB concentration was positively associated with PropPB ($p$ = 0.0025; Spearman's coefficient 0.335) and ButPB ($p$ = 0.0001; Spearman's coefficient 0.506) concentrations. These correlations found in the food samples analysed show that EthPB and PropPB originate from the same sources. However, the source of parabens in foods is not completely understood but their use as broad-spectrum antimicrobial preservatives used in processed foods may be a potential source [2,3] as well as the use of certain food packaging materials where parabens are added as antimicrobials from where they can be released into the food inside [46–48]. We found no differences in the concentrations and frequency of paraben occurrence among the food in different packaging (Tables 4–6). We also found detectable concentrations of MetPB in two different brands of chocolate milkshake samples packed in carton. However, in a recent study conducted in Spain [49] reported MetPB concentrations ranging from nonquantifiable to 155.359 ng $g^{-1}$ in milk carton samples.

Parabens were also detected in eggs, which could be explained by the ingestion of paraben-contaminated feed or soil that penetrate into chicken tissues and are subsequently transferred into eggs [50]. Parabens were also detected in non-packaged fruit and vegetables as parabens may naturally occur in some fruits and vegetables and may contribute to disease resistance through their antimicrobial and antifungal properties [51–53]. Furthermore, EthPB has been reported to have allelopathic functions [54,55] and MetPB has been also found in a wide variety of

plant species and it could be applied in the preparation of bio-based poly (ether ester) materials [56].

*3.2. Bisphenols in Food Samples*

Diet accounts for up to 99% of BPA exposure [57]. Tables 4–6 show the concentrations of bisphenol analogues and the sum of their concentrations in the food samples analysed. A total of 52% of the samples showed detectable concentrations of bisphenols. BPA was the most frequently detected bisphenol in ultra-processed food (mean = 43.28 ng $g^{-1}$ fresh weight). BPS was the second most frequently detected bisphenol in the food samples (26.5%). BPE was found in 4.1% of food samples. However, BPF, BPAF, BPB and BPP were not found in any of the samples analysed.

The concentration of ∑bisphenols ranged from below LOQ to 409 ng $g^{-1}$. The highest ∑bisphenols were found in processed food, in canned tuna samples, with a mean value of 409 ng $g^{-1}$ of BPA and 187.8 ng $g^{-1}$ of BPS. A special concern is that canned and raw tuna is one of the most consumed fish products [25]. Moreover, bisphenol bioaccessibility is higher in canned seafood than in other food matrices with values ranging from 80 to 99% [25,58]. These results are consistent with other studies reporting higher concentrations of individual and total bisphenols in canned food than in foods sold in glass, paper, or plastic containers [21,59]. In an EFSA comprehensive report regarding the levels of BPA in foodstuff in the EU, the ratio of BPA concentrations between canned and non-canned foods ranged from 3 to 500 times, meaning that the contamination could also occur during food processing and manufacture [60].

This might be due to the leaching of bisphenols from the epoxy resins that line the cans into the food. The highest level of bisphenols (132.10 ng $g^{-1}$ BPS) in the processed food group was found in potato chips. High concentrations of BPS were also found in pineapple samples sold in plastic packaging (44.3 ng $g^{-1}$ BPS) included in the unprocessed or

minimally processed food categories. The lowest bisphenol concentrations were found in frozen chopped garlic (mean = 1.4 ng $g^{-1}$ BPS), cake (mean = 1.7 ng $g^{-1}$ BPS) and burger bun (mean = 1.4 ng $g^{-1}$ BPA). LODs and LOQs for these compounds are in the range of 0.1 to 1 ng $g^{-1}$ and 0.4 to 4.0 ng $g^{-1}$ respectively, which agrees with the results reported by similar works (information available at "Supplementary Material", Table S2).

However, relevant bisphenol concentrations were also found in nonpackaged food, which could be explained by the potential contamination during primary production activities [61,62]. In addition, the ubiquity of plastics could also be related to the unexpected presence of bisphenols in food [63].

In recent decades the harmful effects on human health related the use of BPA in food packaging have raised much controversy [64,65], with a large number of studies dealing with BPA determination in food [45,66–68]. More recently, there has also been an increase in research that focuses not only on BPA but also on BPA analogues, as they have been reported to exhibit similar adverse health effects to BPA [27,69,70].

There is extensive literature available about BPA levels in food, but the studies assess different types of food and different BPA analogues. For this reason, comparison between studies conducted in different countries can be difficult. We performed a literature review and selected those studies conducted in the EU that determined the presence of BPA and BPA analogues in food samples (Table S2). Large variations in bisphenol concentrations were found in the present study, similar to those reported in previous studies due to methodological differences [21,71–73]. The results published in the European literature show concentrations ranging from <LOD to 835 ng $g^{-1}$ (Table S2).

BPA is the most frequently detected bisphenol in the analysed food (Table S2), as in the present study (28.6%) (Table 3). Other bisphenols frequently detected in European studies were BPF (<LOD to 139.3 ng $g^{-1}$) [20,25,26,59,74–77], followed by BPB (<LOD to 183.20 ng $g^{-1}$)

[25,63,71,76,78–80]. However, BPAF, BPS and BPE have a lower detection frequency in the food samples analysed from EU countries [25,26,59,63,71,77]. In contrast, in our work BPS and BPE were detected in 26.5% and 4.1% of the food samples analysed, respectively (Table 3). González et al. (2020) found BPE in two of 40 samples analysed [63], which could be explained using BPE as a replacement for BPA in many products as a result of recent regulation limiting the presence of BPA. BPS is one of the most widely BPA substitutes used in the manufacturing of polycarbonate plastics and epoxy resins [81]. In addition, BPS has been frequently found in human biological samples, with detection rates of 81% [21], 65% and 30% [82,83], 70% [84], 40% [85], and 78% [86] in urine and 3% in breast milk [87]. Lastly, BPS has shown endocrine disrupting activity similar to BPA in in vivo and in vitro assays [88].

Other BPA analogues that have been analysed in EU studies are bisphenol AP (BPAP), bisphenol Z (BPZ), and bisphenol M (BPM), but their presence in foods is scarce [25,59,78]. In the present study, these bisphenols were not determined in our food samples, but given their reported estrogenic activity, it will be interesting to include them in future analyses.

In the present study, the association between BPA and BPS concentrations was measured with the Spearman's correlation and a significant correlation was found between the two bisphenols in the analysed food samples ($r = 0.825$, $p < 0.043$). These results suggest that BPS is one of the main BPA analogues used in food-contact material and that is used together with BPA in different food-contact materials.

We found no marked differences between the concentrations of bisphenols in food packed in different materials, but the concentration of bisphenols was higherin canned tuna in oil. In contrast, in other canned foodstuff such as anchovy stuffed olives and tuna dumplings no detectable levels of bisphenols were found. Surprisingly, only detectable concentrations of BPE were found in food samples in carton packages. In contrast, a recent study conducted in Spain [49] found BPA in milk

cartons at concentrations ranging from 0.0018 to 0.059 ng $g^{-1}$. It is surprising that the inner surface of carton packages is made of four layers of low-density polyethylene. More studies should be conducted to describe the presence of bisphenols other than BPA in this kind of packaging material. BPA was also detected in non-packed apples and pears, which that could be explained by contamination during the primary production [63].

Some of the analysed samples (pineapple, canned tuna in oil, yogurt and chips) exceeded the migration limit for BPA recently established by the European Commission at 50 μg $kg^{-1}$ [18]. However, this does not represent a health risk these food products are not consumed in excess in Spain [89]. Nonetheless, migration of bisphenols should be explored in other brands of these products.

### *3.3. Estimated Dietary Intake in Children*

Tables S3 and S4 show the estimated dietary bisphenol and paraben intake in children aged 6–9 years for each foodstuff category.

The estimated intake of ∑PBs was 2.3 μg $kg^{-1}$ bw $day^{-1}$ and 1.3 μg $kg^{-1}$ bw $day^{-1}$ for ∑BPs, which were higher than those calculated by Liao et al. [2,21] for United States children. However, these estimated intake values did not exceed the limit of 10 mg $kg^{-1}$ bw $day^{-1}$ and 4 mg $kg^{-1}$ bw $day^{-1}$ set by the EFSA for parabens and bisphenols, respectively [8,29]. The evaluation of other BPA substitutes and their TDI values was not possible because no limits have been set yet by international organizations.

This study has some limitations including the fact that several food items were not included and that the foods analysed were mainly packaged in plastic containers, hence higher concentrations of parabens and bisphenols, and therefore higher estimated daily intakes, are expected. Lastly, even though the estimated dietary intake of parabens and bisphenols are below the TDI, other routes of exposure such as household dust, air, and dental fillings must be considered. Lastly, the

cumulative effect of parabens and bisphenols together with other endocrine disruptors present in food such as heavy metals, pesticides, and polybrominated diphenyl ethers could pose a risk to human health and should be studied.

**Table S3.** Estimated dietary intake of parabens in Spanish children aged 6-9 years.

| | Daily intake g day$^{-1}$* | Mean MetPB ng g$^{-1}$ | Daily intake MetPB ng day$^{-1}$ | Mean EthPB ng g$^{-1}$ | Daily intake EthPB ng day$^{-1}$ | Mean ButPB ng g$^{-1}$ | Daily intake ButPB ng day$^{-1}$ | Mean PropPB ng g$^{-1}$ | Daily intake PropPB ng day$^{-1}$ | Mean ΣPBs ng g$^{-1}$ | Daily intake ΣPBs ng day$^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Meat and derivatives*** | 72 | 159.9 | 11504.0 | – | – | 9.3 | 667.4 | – | – | 99.6 | 7169.4 |
| ***Fish and derivatives*** | 41 | – | – | 146.9 | 6016.7 | 25.5 | 1044.4 | – | – | 172.4 | 7061.2 |
| ***Cereals and derivatives*** | 165.9 | 50.6 | 8387.8 | 28.6 | 4745.6 | 60.3 | 10000.2 | 3.7 | 619.4 | 58.4 | 9687.5 |
| ***Vegetables and derivatives*** | 132.2 | 86.7 | 11454 | 13.9 | 1837.0 | – | – | 1 | 132.2 | 68.7 | 9072.8 |
| ***Fruits and derivatives*** | 148.7 | 48.6 | 7229.5 | 47.1 | 7009.8 | 43.9 | 6520.6 | 67 | 9963.0 | 75 | 11145.2 |
| ***Dairy and derivatives*** | 443.1 | 58.4 | 25872.6 | 24.8 | 10989.8 | – | – | – | – | 37.4 | 16569.8 |
| ***Eggs*** | 18.7 | 229.9 | 4301.9 | – | – | – | – | – | – | 229.9 | 4301.9 |
| ***Salty snacks*** | 4.4 | 7.7 | 34.1 | – | – | – | – | 1.4 | 6.2 | 4.6 | 20.1 |
| ***Pre-cooked*** | 0.8 | – | – | – | – | – | – | – | – | – | – |
| ***μg kg$^{-1}$ bw day$^{-1}$*** | | | 2.3 | | 1.1 | | 0.6 | | 0.4 | | 2.3 |

*MetPB. Methylparaben; EthPB. Ethylparaben; ButPB. Butylparaben; PropPB. Propylparaben; ΣPBs. Σparabens; bw. Body weight.*

*(–). Not detected or below of limit quantification. *Data obtained from ENALIA study*

**Table S4.** Estimated dietary intake of bisphenols in Spanish children aged 6-9 years.

| | Daily intake g day$^{-1}$* | Mean BPS ng g$^{-1}$ | Daily intake BPS ng day$^{-1}$ | Mean BPA ng g$^{-1}$ | Daily intake BPA ng day$^{-1}$ | Mean ΣBPs ng g$^{-1}$ | Daily intake ΣBPs ng day$^{-1}$ |
|---|---|---|---|---|---|---|---|
| ***Meat and derivatives*** | 72 | 22.4 | 1609.4 | 8.7 | 623.7 | 17.7 | 1272.5 |
| ***Fish and derivatives*** | 41 | 187.8 | 7691.9 | 409 | 16751.8 | 298.4 | 12221.9 |
| ***Cereals and derivatives*** | 165.9 | 2.5 | 414.8 | 31.7 | 5264.2 | 22 | 3647.7 |
| ***Vegetables and derivatives*** | 132.2 | 15.5 | 2043.2 | – | – | 15.5 | 2043.2 |
| ***Fruits and derivatives*** | 148.7 | 18.1 | 2687.1 | 7 | 1043.1 | 21.3 | 3160.8 |
| ***Dairy and derivatives*** | 443.1 | 5.3 | 2326.5 | 44.1 | 19536.2 | 33 | 14619.1 |
| ***Salty snacks*** | 4.4 | 132.1 | 584.2 | 25.5 | 112.5 | 61 | 269.7 |
| ***Pre-cooked*** | 0.8 | | – | 4.3 | 3.3 | 4.3 | 3.3 |
| ***μg kg$^{-1}$ bw day$^{-1}$*** | | | 0.6 | | 1.5 | | 1.3 |

*BPS. Bisphenol S; BPA. Bisphenol A; ΣBPs. ΣBisphenols; bw. Body weight.*
*(–). Not detected or below of limit quantification. *Data obtained from ENALIA study*

## 4. Conclusions

Our findings confirm significant amounts of parabens and bisphenols detected in daily consumed products by the Spanish population. MetPB was the most frequently detected paraben in the analysed samples. Although BPA is being gradually replaced by its analogues in many food-contact materials, it is still the most frequently bisphenol detected in food, followed by BPS. The estimated dietary exposure to bisphenols and parabens did not exceed the TDIs established by the EFSA. However, because other bisphenols in addition to BPA are found in foods, a risk assessment of their presence and the establishment of limits such as TDIs for each bisphenol individually and for the sum of bisphenols are necessary. This study shows the importance of collecting more data on the occurrence of parabens and bisphenols in food to assess dietary exposure and possible health impact, especially for the more vulnerable populations.

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## Abbreviations

| | |
|---|---|
| BPA | Bisphenol A |
| AESAN | Spanish Agency for Food Safety and Nutrition |
| BPA-$d_{16}$ | Deuterium labelled bisphenol A |
| BPAF | Bisphenol AF |
| BPAP | Bisphenol AP |
| BPB | Bisphenol B |
| BPE | Bisphenol E |
| BPF | Bisphenol F |
| BPM | Bisphenol M |
| BPP | Bisphenol P |
| BPS | Bisphenol S |
| BPZ | Bisphenol Z |
| ButPB | Butyl 4-hydroxybenzoate |
| bw | Body weight |
| C18 | Octadecyl-functionalized silica |
| ECHA | European Chemical Agency |
| EFSA | European Food Safety Authority |
| ESI | Electrospray ionization source |
| EthPB | Ethyl 4-hydroxybenzoate |
| EthPB-$d_5$ | Deuterium labelled ethylparaben |
| LC-MS | Liquid chromatography-mass spectrometry |
| LLE | Liquid-liquid extraction |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| MeOH | Methanol |
| MetPB | Methyl 4-hydroxybenzoate |
| $MgSO_4$ | Magnesium sulphate |
| MPLs | Maximum permitted levels |
| MRM | Multiple-reaction-monitoring |

| | |
|---|---|
| NaCl | Sodium chloride |
| PropPB | Propyl 4-hydroxybenzoate |
| PSA | Primary Secondary Amine |
| QuEChERs | Quick, Easy, Cheap, Effective, Rugged, and Safe |
| SCCS | Scientific Committee on Consumer Safety |
| SPE | Solid phase extraction |
| TDI | Tolerable dietary intake |
| UHPLC-MS/MS | Ultra-high performance liquid chromatography - tandem mass spectrometry |

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# CAPÍTULO II.

## Exposición alimentaria a bisfenoles. Relación con el sobrepeso/obesidad

## Objetivos específicos capítulo II

1. Determinar la exposición alimentaria a los bisfenoles en la población infantil en función del IMC.
2. Conocer cuales son las principales fuentes alimentarias de exposición a los bisfenoles.
3. Ver como influye la calidad de la dieta en la exposición a los bisfenoles.
4. Estudiar la asociación entre la exposición alimentaria a los bisfenoles y el sobrepeso/obesidad infantil.

## Introducción capítulo II

La exposición dietética a los bisfenoles es motivo de gran preocupación debido a su presencia generalizada en diversos productos de consumo alimentario. Como se mencionó anteriormente, los bisfenoles se encuentran habitualmente en materiales en contacto con los alimentos, envases de plástico y resinas epoxi que recubren latas de metal. Estos compuestos pueden migrar a los alimentos y bebidas, por lo que la vía oral es la principal vía de exposición humana. Por ello, identificar las principales fuentes alimentarias de exposición a los bisfenoles es crucial para evaluar los posibles riesgos para la salud asociados a su consumo.

El impacto de estas sustancias en la salud, especialmente en los niños, ha despertado una gran atención debido a su presencia generalizada y a sus posibles efectos a largo plazo. En lo que respecta a la infancia, la exposición a los bisfenoles es especialmente preocupante, ya que puede afectar a procesos críticos del desarrollo.

Entre los posibles efectos de los bisfenoles sobre la salud, en los últimos años se ha estudiado la relación entre el BPA y sus análogos con el sobrepeso y la obesidad. Algunos de estos estudios indican que la exposición a los bisfenoles puede contribuir a aumentar la probabilidad

de sobrepeso y obesidad, especialmente en la población pediátrica, lo que subraya la necesidad de seguir investigando y de adoptar medidas reglamentarias para mitigar este riesgo y proteger la salud de los grupos de edad más vulnerables, cómo la infancia.

A continuación, se muestra el trabajo científico en fase de publicación con relación a la temática desarrollada en este capítulo.

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# Dietary bisphenols exposure as an influencing factor of body mass index

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**Abstract:** Over the past three decades, there has been a significant increase in the prevalence and incidence of overweight and obesity worldwide. The obesogen hypothesis suggests that certain external agents may affect pathways related to fat accumulation and energy balance by stimulating fat cell differentiation and proliferation. Previous research has indicated that exposure to bisphenol A (BPA) and some of its analogues has direct effects on fat accumulation, favouring the transformation of pre-adipocytes into adipocytes. This study aimed to assess the possible contribution of dietary bisphenol exposure to the odds of developing overweight and obesity in a sample of Spanish children

according to gender. Dietary and anthropometric data were collected from 179 controls and 124 cases schoolchildren aged 6-12 years. Dietary exposure to BPA and bisphenol S (BPS) was assessed using a food consumption frequency questionnaire. Logistic regression models were used to assess the influence of dietary exposure to bisphenols on overweight and obesity stratified by gender. For girls, cases had significantly higher exposure to BPA from meat and eggs compared to controls (median= 319.6, interquartile range (IQR) = 176.4-381 vs 231.8 (IQR)= 162.1-350.2, $p$= 0.046). Diet quality was higher for controls (6.2 (2.1) vs 4.8 (2.2) $p$<0.001) among boys independently of a high or low exposure to bisphenols. However, higher diet quality was observed for controls girls with a high exposure of total bisphenols (6.8 (2) vs 5.3 (2) $p$=0.031). Girls exposed to high levels of BPA from meat and eggs haved higher likelihood of being overweight and obese (Adjusted Odds Ratio = 2.70, 95% confidence interval = 1.00 – 7.32). However, no consistent associations were found in boys. High BPA levels from meat and eggs were positively associated with overweight and obesity in girls. The dietary intake of BPA in the schoolchildren in the present study was much higher than the acceptable daily intake established by EFSA for the last year.

**Keywords:** children, overweight, obesity, bisphenol A, bisphenol S, weight excess.

## 1. Background

The prevalence and incidence of overweight and obesity worldwide have increased significantly in the last three decades [1]. Diverse studies indicate that the etiology of this chronic disease is multifactorial and complex. The predisposing biological factors including genetic characteristics, prenatal determinants, pregnancy, intestinal microbiota and viruses [2]. In 2006, Grün and Blumberg postulated the obesogens hypothesis for the first time, where certain exogenous agents could alter adipogenic pathways and energy balance, promoting an increase in adipocyte differentiation and proliferation rates [3]. Some of the most

known obesogens are endocrine-disrupting chemicals (EDCs), exogenous agents that may interfere with the hormonal system function in different ways, by influencing hormone synthesis, metabolism and/or cellular actions [4]. EDCs include compounds to which the human population is exposed in daily life through their use in pesticides/herbicides, a large variety of household and medical products (food, containers foodstuffs, clothes, drugs, sanitizers, cosmetics, personal care products, toys, construction materials, furniture), and in plant-based products [5,6], becoming ubiquitous in our environment. They are considered as obesogenic compounds due to their capacity to alter lipid metabolism and inappropriately promote adipogenesis and fat accumulation [7,8]. The prenatal period, infancy, and childhood are most vulnerable periods for the effects of these environmental contaminants due to the immaturity in metabolic enzymes and lower capacity to eliminate toxic compounds. This fact suggest that metabolism and detoxification are not as efficient as they are in adults [9].

Bisphenol A (BPA) is among the highest production volume chemicals detected in ecosystems, human fluids, and tissues [10]. To protect against BPA exposure, the European Commission has taken actions by banning the use of BPA in infant feeding bottles and restricting the use of BPA in certain food-contact materials [11]. Common exposure pathways include epoxy resins in canned foods/beverages, polycarbonate plastics, thermal paper, dental materials and consumer goods [6,7,12] being their main exposure oral ingestion through diet [13,14]. As the use of BPA is decreasing, substitutes such as bisphenol S (BPS) is becoming more widely used. However, the current evidence shows that most alternative bisphenols are as hormonally active as BPA. Perinatal and chronic exposure to BPS induced obesogenic effects, even at low doses, and the obesogenic capacity of BPS was even higher than that of BPA in preadipocytes [15].

In vitro studies have shown also that exposure to BPA has direct adipogenic effects, promoting the conversion of preadipocytes into

adipocytes and increasing lipid accumulation [16–18]. In vivo studies suggest the influence of bisphenols on fatty mass development, mainly when exposure occurred in the prenatal phase [11,19,20]. In spite of epidemiological studies have shown a positive association between childhood obesity and bisphenol exposure [21–23], the cross-sectional nature of most of them makes that causal links may be complex and consequently difficult to interpret. Thus, despite the significance of environmental obesogens in the pathogenesis of metabolic diseases, the contribution of synthetic chemical exposure to obesity epidemic remains largely unrecognised. Hence, the aim of the present study was to evaluate a possible contribution of dietary bisphenols exposure on likehood of developing overweight and obesity in a sample of Spanish children.

## 2. Materials and methods

### *2.1. Study design and population*

The present research is a case-control study carried out to investigate the influence of environmental factors in the development of overweight and obesity in Spanish children. Both cases and controls were recruited from different primary care centers and schools randomly selected from the province of Granada, located in areas with different socioeconomic level. Participants were recruited from January 2020 to January 2022. Cases and controls must meet the following inclusion criteria: (1) prepuberal children aged between 6 - 12 years-old; (2) having resided continuously in the study areas for at least 6 months; (3) overweight or obesity diagnosis (only cases). The exclusion criteria were: obesity as a symptom of other pathologies, or as a side effect of pharmacological treatment. A total of 124 cases and 179 controls were recruited in this study.

### *2.2. Data collection*

Face-to-face interviews were performed at baseline by trained interviewers to the participant's parents or legal tutors. In this way, sociodemographic information such as gender and age of children, and

lifestyle data (smoking habits of family members, physical activity out-of-school and diet) were collected. In addition, anthropometric measurements such as height (in cm) and weight (in Kg) were obtained by qualified personnel. Concretely, participants with light clothing and without shoes were weighed using a portable Tanita scale (model MC 780-S MA). A stadiometer (model SECA 214 (20-207 cm) was used to measure the height in the sanding position. During height measurements, the participants' backs, buttocks, and heels should be in contact with the wall. Weight and height were used to obtain the body mass index (BMI) which was calculated as weight divided by height squared. Thus, subjects were classified as underweight, normal weight, overweight and obese as described by Cole et al., 2000, 2007 [24,25].

Dietary information for the last 12 months prior to the interview was obtained through parents or legal tutors of participants using a semi-quantitative food frequency questionnaire (FFQ) state supervised by trained nutritionists. It collected information on the following 112 food items categorized in 13 groups: dairy products (11), eggs, meat and meat derivatives (9), fish and fish derivatives (7), vegetables (17), tubers (2), fruits and nuts (18), legumes (4), cereals (12), precooked or ultra-processed food (2), bakery products, pastries and sweets (13), fats and oils (5), non-alcoholic beverages (5) and miscellaneous (7). It was specified portion sized for each item and 8 consumption frequency options: never, 1-3 times for month, 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.

The Spanish version of KIDMED used in the study was taken from a previously performed research [26]. It is a self-administered instrument aimed at estimating adherence to the Mediterranean diet. This questionnaire consists of 16 questions, of which 4 questions reflected negative connotations associated with an adequate Mediterranean diet and scored negatively (-1 point), and 12 affirmative questions reflecting positive aspects related to the Mediterranean diet and scored positively (+1 point). Individuals are divided into three categories to follow: low

adherence or low diet quality (score less than or equal to 3), medium adherence or medium diet quality (score 4-7) and high adherence or high diet quality (score greater than or equal to 8).

*2.3. Estimation of bisphenols dietary exposure*

Bisphenol concentrations in the selected foodstuffs were described previously [27,28]. Bisphenol levels were quantified using ultra-high-performance liquid chromatography-tandem mass spectrometry. From total of food samples analyzed, a 52% of them had bisphenol concentrations above quantification level.

The method used for the selection and analysis of food items has been described elsewhere [29]. Daily dietary exposure to BPA, BPS and total bisphenol (ng/day) for each participant was calculated by multiplying their daily food consumption (g/day) by the corresponding bisphenol content in each food (ng/g food). Mean intake (g/day) of foodstuffs was calculated multypliying the consumption frequency (servings/day) with portion size using the standard servings (g/serving) establish for the Spanish population [30].

*2.4. Statistical analysis*

The characteristics of cases and controls were summarized using median and interquartile range (IQR, percentil 25-percentil 75) for the continuous variables and percentages for categorical variables. To assess the level of significance of the differences observed among categorical variables used Chi-squared and Mann-Whitney U test or Kruskal-Wallis for continuous variables.

Logistic regression models were used to estimate odds ratios (OR) and 95% confidence intervals (95% CI) to assess the influence of BPA, BPS, and total bisphenol (BPA + BPS) dietary exposure on overweight and obesity. Then, BMI dichotomized as normal weight and overweight/obesity was the dependent variable. Dietary bisphenols exposure (BPA, BPS and total bisphenols) categorized according to

tertiles (T) and later dichotomized as low (first and second T) and high (third T) exposure were the influencing factors, considering T1 and T2 as the reference category. Two models were used: (a) crude and (b) adjusted model for a priori potential confounders according to previous studies (age, energy intake, diet quality and parental education level) [22,32–34], and those variables which produced changes > 10% in OR crude (smoking among members of the family unit, physical activity and body fat percentage). Moreover, we performed gender-stratified due to biological, social and behavioural differences between men and women that may influence the prevalence of overweight and obesity [35]. Besides, it has been reported that gender may have an influence on the burden of overweight and/or obesity [22,36]. The rationale for these approach is based on previously published literature where gender could modify the effect of bisphenol exposure on BMI [22]. Statistical analyses were performed with IBM SPSS (version 26.0, IBM® SPSS® Statistics, Armonik, NY, USA). The statistical significance set to $p \leq 0.05$.

## 3. Results

Table 1 shows the main characteristics of cases and controls stratified by gender. Statistically significant differences were observed for most of the study variables, except for energy intake for both, boys and girls. Cases were older, less physically active, family members smoked more frequently, and parents' education level was lower. Body fat percentage was significant higher for cases, both in boys as girls.

**Table 1.** General characteristics of cases and controls according to gender.

| | | Boys (n=159) | | | Girls (n=144) | | |
|---|---|---|---|---|---|---|---|
| | | Controls (n=93) | Cases (n=66) | *p*-Value | Controls (n=86) | Cases (n=58) | *p*-Value |
| Age, years | Median | 7 | 9.1 | **<0.001[a]** | 7.1 | 9 | **0.002[a]** |
| | IQR | 5.1 – 8.6 | 7.06 – 11-01 | | 5.1 – 9 | 7.1 – 11 | |
| Energy intake, Kcal/day | Median | 2070.5 | 2218.5 | 0.383[a] | 2101.9 | 1889.1 | 0.120[a] |
| | IQR | 1765.5 – 2335.9 | 1740.1 – 2558.9 | | 1760.7 – 2451.2 | 1547.3 – 2277.6 | |
| Physical activity (out-of-school) (%) | No | 21.5 | 53 | **<0.001[b]** | 32.6 | 62.1 | 0.090[b] |
| | Yes | 78.5 | 47 | | 67.4 | 37.9 | |
| Smoking among members of the family unit (%) | No | 92.5 | 53 | **<0.001[b]** | 83.7 | 62.1 | **0.003[b]** |
| | Yes | 7.5 | 47 | | 16.3 | 37.9 | |
| Body fat percentage | Median | 18.2 | 33.6 | **<0.001[a]** | 21.7 | 33.7 | **<0.001[a]** |
| | IQR | 15.7 – 20.7 | 28.6 – 38.1 | | 19.7 – 23.5 | 30.2 – 39.1 | |

IQR: interquartile range (percentile 25th – percentile 75th); [a]U Mann-Whitney test; [b]Chi-square test; $p \leq 0.05$ are highlighted in bold.

Tables 2a and 2b show the daily food intake by food groups and mean exposure to bisphenols for case and controls, according to the gender. Among boys, exposure to BPA from foods processed and to BPA, BPS and total bisphenols from legumes were significantly higher for cases. However, controls had significantly higher exposure to BPS from fruits (Table 2a). For girls, cases had a significantly higher exposure to BPA from meat and eggs and foods processed and BPA, BPS and total bisphenols for legumes, while BPS and total bisphenols exposure from dairy products was significantly higher among controls (Table 2b). Non-significant differences among cases and controls were observed for total BPA, BPS and total bisphenols.

**Table 2a.** Dietary intake of bisphenols (ng/day) by foods groups according to cases and controls for boys.

| | Controls (n=93) | | | Cases (n=66) | | | |
|---|---|---|---|---|---|---|---|
| Food Group | n* | Food Intake (g/day), median (IQR) | Bisphenol Intake (ng/day), median (IQR) | n* | Food Intake (g/day), median (IQR) | Bisphenol Intake (ng/day), median (IQR) | *p*-Value |
| **BPA** | | | | | | | |
| Dairy products | 66 | 452.2 (207 – 635.6) | 3269.6 (1579 – 5727.5) | 55 | 306.5 (145.4 – 604) | 1945.9 (637.8 – 4434) | 0.226 |
| Meat and eggs | 87 | 109.4 (90.3 – 140.9) | 305 (166.2 – 369.1) | 63 | 128.7 (105 – 156.1) | 324.8 (173.8 – 410.3) | 0.174 |
| Fish | 79 | 21.5 (7.2 – 32) | 1530.3 (4.5 – 6510.9) | 60 | 7.2 (0 – 21.5) | 1529.5 (0 – 3255.7) | 0.440 |
| Vegetables | 81 | 168 (108.7 – 227) | 143.1 (21.2 -347.7) | 48 | 159 (121.2 – 243) | 37.71 (19.4 – 329.9) | 0.355 |
| Fruits | 86 | 198.1 (131.8 – 280) | 428.3 (224.9 – 685.4) | 55 | 133 (88.5 – 209.5) | 392.3 (136.9 – 678.8) | 0.250 |
| Legumes | 93 | 7.2 (4 – 8.6) | 3.2 (1.8 – 3.9) | 66 | 8.6 (7.2 – 10) | 3.9 (3.2 – 4.5) | **0.029** |
| Cereals | 90 | 39 (25.5 – 60.6) | 8.8 (5.4 – 12.7) | 57 | 41.5 (17.2 – 58.3) | 11.5 (3.1 – 15.3) | 0.410 |
| Pastries | 91 | 8.58 (4.02 – 22.7) | 48.9 (0.9 – 104.5) | 65 | 10.7 (4 – 25.7) | 50.2 (4.8 – 105.8) | 0.151 |
| Processed | 91 | 49.2 (28.0 – 70.0) | 83.1 (42.1 – 126.9) | 58 | 70 (42.7 – 96) | 127.8 (84.6 – 282.8) | **<0.001** |
| **Total BPA (ng/day), median (IQR)** | 93 | 6923.6 (4350.9 – 12926) | | 66 | 6141. (3889.3 – 11877.1) | | 0.499 |
| **BPS** | | | | | | | |
| Dairy products | 66 | 452.2 (207.1 – 636) | 133.5 (73.9 – 217.5) | 55 | 306.5 (145.4 – 604) | 143.8 (72.5 – 208.9) | 0.889 |
| Meat and eggs | 87 | 109.4 (90.3 – 140.9) | 527.3 (131.3 – 557.5) | 63 | 128.7 (105.4 – 156) | 528.8 (183.3 – 573.1) | 0.470 |
| Fish | 79 | 21.5 (7.2 – 32) | 317.4 (1.51 – 1347.7) | 60 | 7.2 (0 – 21.5) | 317.1 (0 – 673.9) | 0.440 |
| Vegetables | 81 | 168 (109 – 227.3) | 3734 (1866 – 14671) | 48 | 159 (121 – 242.6) | 3820.4 (1937.5 – 16202) | 0.727 |
| Fruits | 86 | 198.1 (131.8 – 280) | 626 (407.7 – 844.6) | 55 | 133 (88.5 – 209.5) | 501.7 (239.1 – 744.) | **0.047** |
| Legumes | 93 | 7.2 (4.0 – 8.6) | 1.1 (0.6 – 1.3) | 66 | 8.6 (7.2 – 10) | 1.3 (1.1 – 1.5) | **0.029** |
| Cereals | 90 | 39 (25.5 – 60.6) | 6 (3.8 – 9.5) | 57 | 41.5 (17.2 – 58.3) | 5.8 (1.8 – 9) | 0.296 |
| Pastries | 91 | 8.6 (4.0 – 22.7) | 6.3 (5.7 – 12) | 65 | 10.7 (4 – 25.7) | 5.6 (1.3 – 9.7) | 0.061 |
| Processed | 91 | 49.2 (28.0 – 70.0) | 208.7 (98.7 – 1724.5) | 58 | 70 (42.7 – 96) | 215.2 (206.1 – 630.2) | 0.171 |
| **Total BPS (ng/day), median (IQR)** | 93 | 9900.4 (4377.9 – 17097.2) | | 66 | 8200.6 (3711 – 19262.0) | | 0.912 |

IQR: interquartile range (percentile 25th – percentile 75th); *p*-Values show bisphenols intake significant differences between cases and controls, by U de Mann-Whitney test; $p \leq 0.05$ are highlighted in bold; BPA: bisphenol A; BPS: bisphenol S.
*: n for consumers

**Table 2b.** Dietary intake of bisphenols (ng/day) by foods groups according to cases and controls for girls.

| | Controls (n=86) | | | Cases (n=58) | | | |
|---|---|---|---|---|---|---|---|
| Food Group | n* | Food Intake (g/day), median (IQR) | Bisphenol Intake (ng/day), median (IQR) | n* | Food Intake (g/day), median (IQR) | Bisphenol Intake (ng/day), median (IQR) | *p*-Value |
| **BPA** | | | | | | | |
| Dairy products | 67 | 350.5 (188.5 – 646) | 2894.2 (1435.8 – 4418.2) | 43 | 169.1 (88.7 – 393.1) | 1878.4 (105.7 – 4310.6) | **0.037** |
| Meat and eggs | 80 | 113.7 (89 – 136.7) | 231.8 (162.1 – 350.2) | 46 | 130.1 (99.7 – 154.4) | 319.6 (176.6 – 381) | **0.046** |
| Fish | 77 | 20.5 (7.2 – 31.5) | 1540.6 (7.7 – 3283.9) | 51 | 21.5 (7.2 – 32) | 3249.7 (1527.1 – 9749) | 0.139 |
| Vegetables | 66 | 191 (114.4 – 272) | 161.3 (24.5 – 345.3) | 42 | 187.2 (114.9 – 262) | 53.5 (21.7 – 331.1) | 0.277 |
| Fruits | 77 | 188.7 (118.7 – 273) | 499.8 (227.9 – 684.5) | 49 | 155.7 (88.3 – 265.9) | 665.9 (110.3 – 829.3) | 0.729 |
| Legumes | 85 | 7.2 (4 – 8.6) | 3.2 (1.8 – 3.9) | 58 | 8.6 (4.7 – 10.0) | 3.9 (2.1 – 4.5) | **0.040** |
| Cereals | 82 | 33.4 (19.4 – 49.6) | 7.5 (4.9 – 11·5) | 56 | 29.9 (16.3 – 54.8) | 8.7 (4.8 – 15) | 0.270 |
| Pastries | 82 | 11.6 (4 – 21.6) | 48.9 (0.8 – 103.9) | 56 | 8.5 (3.4 – 17.8) | 48.9 (0.5 – 104) | 0.901 |
| Processed | 84 | 44.3 (27.6 – 63.2) | 83.2 (44.3 – 125.4) | 55 | 58.2 (38.6 – 81.8) | 103.3 (80.9 – 163.4) | **0.016** |
| **Total BPA (ng/day), median (IQR)** | 86 | 7146 (4897.6 – 11475.6) | | 58 | 7753.5 (4199.4 – 11572.3) | | 0.883 |
| **BPS** | | | | | | | |
| Dairy products | 67 | 350.5 (188.5 – 646) | 128.7 (84.5 – 199.7) | 43 | 169.1 (88.7 – 393.1) | 110.4 (60.5 – 171) | 0.333 |
| Meat and eggs | 80 | 113.7 (88.9 – 136.7) | 228.9 (118.4 – 550.9) | 46 | 130.1 (99.7 – 154.4) | 530.2 (126.1 – 570.8) | 0.211 |
| Fish | 77 | 20.5 (7.2 – 31.5) | 320.8 (2.6 – 683.3) | 51 | 21.5 (7.2 – 32) | 671.9 (316.3 – 2016) | 0.139 |
| Vegetables | 66 | 191 (114.4 – 272) | 4499.8 (2043.4 – 15007) | 42 | 187.2 (114.9 – 262) | 4154 (1879 – 14942) | 0.529 |
| Fruits | 77 | 188.7 (118.7 – 273) | 634 (339.4 – 815.6) | 49 | 155.7 (88.3 – 265.9) | 622.2 (223.5 – 833) | 0.447 |
| Legumes | 85 | 7.2 (4 – 8.6) | 1.1 (0.6 – 1.3) | 58 | 8.5 (4.7 – 10) | 1.3 (0.7 – 1.5) | **0.040** |
| Cereals | 82 | 33.4 (19.4 – 49.6) | 5 (2.9 – 8.8) | 56 | 29.9 (16.3 – 54.8) | 4.5 (2.4 – 8.5) | 0.419 |
| Pastries | 82 | 11.6 (4 – 21.7) | 6.2 (1.6 – 8.2) | 56 | 8.5 (3.4 – 17.8) | 5.7 (1.4 – 7.8) | 0.286 |
| Processed | 84 | 44.3 (27.6 – 63.1) | 209 (101.6 – 620) | 55 | 58.2 (38.6 – 81.8) | 211.1 (102.6 – 1678.1) | 0.339 |
| **Total BPS (ng/day), median (IQR)** | 86 | 8327.4 (4871.1 – 17848.2) | | 58 | 8018.7 (4006.5 – 18378.7) | | 0.896 |

IQR: interquartile range (percentile 25th – percentile 75th); *p*-Values show bisphenols intake significant differences between cases and controls, by U de Mann-Whitney test; p≤0.05 are highlighted in bold; BPA: bisphenol A; BPS: bisphenol S. *: n for consumers

According to table 3, diet quality was significantly higher for controls, only for boys (6.2 (2.1) vs 4.8 (2.2) $p<0.001$). Boys with a BMI higher than 25 Kg/m$^2$ had a significantly lower diet quality independently of a high or low exposure to BPA, BPS or total bisphenols. However, among girls,

significantly higher diet quality was observed for controls with a high exposure of total bisphenols (6.8 (2) vs 5.3 (2) $p$=0.031).

**Table 3.** Diet quality (KIDMED) according to hight and low BPA, BPS and total bisfenols exposure for controls and cases by gender.

| | | Boys (n=159) | | | Girls (n=144) | | |
|---|---|---|---|---|---|---|---|
| | | Controls (n=93) | Cases (n=66) | *p-value* | Controls (n=86) | Cases (n=58) | *p-value* |
| | | Mean (SD) | | | Mean (SD) | | |
| **KIDMED** | | 6.2 (2.1) | 4.8 (2.2) | **<0.001** | 6.1 (2.6) | 5.6 (2) | 0.243 |
| **BPA** | High exposure (Tertil 3) | 6.22 (2.04) | 4.9 (2.5) | **0.028** | 6 (2.5) | 5.2 (1.9) | 0.219 |
| | Low exposure (Tertil 1+2) | 6.2 (2.2) | 4.7 (2.1) | **0.002** | 6.2 (2.7) | 5.9 (2.1) | 0.669 |
| **BPS** | High exposure (Tertil 3) | 6.4 (1.7) | 5.36 (2.2) | **0.042** | 6.8 (2.3) | 5.60 (2.3) | 0.065 |
| | Low exposure (Tertil 1+2) | 6.1 (2.4) | 4.4 (2.3) | **0.001** | 5.7 (2.7) | 5.64 (2.1) | 0.950 |
| **Total bisphenols** | High exposure (Tertil 3) | 6.5 (1.9) | 5.3 (2.2) | **0.031** | 6.8 (2) | 5.3 (2.) | **0.031** |
| | Low exposure (Tertil 1+2) | 6 (2.3) | 4.5 (2.2) | **0.002** | 5.8 (2.8) | 5.7 (2.1) | 0.958 |

SD: standard deviation; BPA: bisphenol A; BPS: bisphenol S. H de kruskal wallis; *p*-Values show diet quality significant differences between cases and controls; p≤0.05 are highlighted in bold.

Tables 4a y 4b shows the influence of the highest (defined as T3) BPA, BPS and total bisphenols dietary exposure by food groups on overweight and obesity by gender. Higher odds to be overweight or obese was observed for boys whose had a high exposure to BPA from processed foods and cereals and high exposure to BPA and BPS (separately and together) from legumes, according to the results showed for crude model. Non-significant values were found for the adjusted model for boys. On the other hand, a high exposure to BPA from meat and eggs and to BPA and BPS (separately and together) from legumes increased the odds to having weight excess (overweight and obesity) in girls, according to crude model. Significance was only kept for BPA from meat and eggs in the adjusted model (OR adjusted by age, energy intake, diet quality, smoking among members of the family unit and body fat percentage). When low exposure was used as the first tertile and medium and high exposure as separate categories, the direction of the associations remained similar although the statistical significance was lost (Supplementary Material, Table S2, S3, S4).

**Table 4a**. Influencing of the highest (tertil 3 vs tertil 1+2) BPA, BPS and total bisphenols dietary exposure by food groups on overweight/obesity for boys.

| | Boys (n=159) | | | | | |
|---|---|---|---|---|---|---|
| | Crude model | | | Adjusted model | | |
| **Food Group** | **OR** | **95% CI** | ***p-Value*** | **OR** | **95% CI** | ***p-Value*** |
| **BPA** | | | | | | |
| Dairy products (Ref. Low exposure*) High exposure** | 0.79 | 0.42 – 1.50 | 0.469 | 1.30[b] | 0.52 – 3.27 | 0.581 |
| Meat and eggs (Ref. Low exposure) High exposure | 1.47 | 0.77 – 2.78 | 0.240 | 1.35[d] | 0.28 – 6.53 | 0.709 |
| Fish (Ref. Low exposure) High exposure | 0.75 | 0.39 – 1.48 | 0.412 | 0.13[c] | 0.01 – 1.36 | 0.089 |
| Vegetables (Ref. Low exposure) High exposure | 0.93 | 0.48 – 1.79 | 0.825 | 0.69[b] | 0.25 – 1.87 | 0.461 |
| Fruits (Ref. Low exposure) High exposure | 0.77 | 0.40 – 1.51 | 0.450 | 0.74[d] | 0.14 – 4.07 | 0.777 |
| Legumes (Ref. Low exposure) High exposure | 2.22 | 1.16 – 4.24 | **0.016** | 1.20[d] | 0.15 – 9.40 | 0.863 |
| Cereals (Ref. Low exposure) High exposure | 2.13 | 1.11 – 4.06 | **0.022** | 1.88[f] | 0.42 – 8.51 | 0.441 |
| Pastries (Ref. Low exposure) High exposure | 1.85 | 0.87 – 3.93 | 0.112 | 1.10[d] | 0.17 – 7.03 | 0.920 |
| Processed (Ref. Low exposure) High exposure | 3.19 | 1.65 – 6.18 | **0.001** | 1.66[b] | 0.60 – 4.59 | 0.327 |
| **BPS** | | | | | | |
| Dairy products (Ref. Low exposure) High exposure | 0.82 | 0.44 – 1.55 | 0.542 | 1.34[f] | 0.24 – 7.63 | 0.736 |
| Meat and eggs (Ref. Low exposure) High exposure | 1.24 | 0.65 – 2.36 | 0.509 | 0.83[a] | 0.35 – 2.01 | 0.686 |
| Fish (Ref. Low exposure) High exposure | 1.23 | 0.65 – 2.31 | 0.529 | 0.68[c] | 0.12 – 3.71 | 0.655 |
| Vegetables (Ref. Low exposure) High exposure | 1.00 | 0.53 – 1.90 | 0.995 | 2.81[f] | 0.59 – 13.35 | 0.194 |
| Fruits (Ref. Low exposure) High exposure | 0.70 | 0.36 – 1.38 | 0.307 | 0.84[d] | 0.17 – 4.14 | 0.825 |
| Legumes (Ref. Low exposure) High exposure | 2.08 | 1.02 – 4.26 | **0.044** | 1.05[d] | 0.10 – 11.16 | 0.967 |
| Cereals (Ref. Low exposure) High exposure | 10.12 | 0.59 – 2.12 | 0.739 | 2.24[f] | 0.47 – 10.59 | 0.310 |
| Pastries (Ref. Low exposure) High exposure | 0.85 | 0.44 – 1.63 | 0.620 | 2.35[c] | 0.40 – 13.80 | 0.343 |
| Processed (Ref. Low exposure) High exposure | 1.24 | 0.65 – 2.36 | 0.509 | 0.36[d] | 0.06 – 2.12 | 0.257 |
| **Total bisphenols** | | | | | | |
| Dairy products (Ref. Low exposure) High exposure | 0.79 | 0.42 – 1.50 | 0.469 | 1.13[a] | 0.48 – 2.65 | 0.778 |
| Meat and eggs (Ref. Low exposure) High exposure | 1.47 | 0.77 – 2.78 | 0.240 | 2.01[d] | 0.39 – 10.38 | 0.405 |
| Fish (Ref. Low exposure) High exposure | 0.75 | 0.39 – 1.48 | 0.412 | 0.13[c] | 0.01 – 1.36 | 0.089 |
| Vegetables (Ref. Low exposure) High exposure | 1.02 | 0.58 – 2.09 | 0.780 | 2.92[f] | 0.62 – 13.88 | 0.177 |
| Fruits (Ref. Low exposure) High exposure | 0.61 | 0.31 – 1.20 | 0.154 | 0.83[c] | 0.14 – 4.84 | 0.837 |
| Legumes (Ref. Low exposure) High exposure | 2.08 | 1.02 – 4.26 | **0.044** | 1.05[d] | 0.10 – 11.16 | 0.967 |
| Cereals (Ref. Low exposure) High exposure | 1.68 | 0.89 – 3.18 | 0.110 | 1.96[b] | 0.77 – 4.95 | 0.157 |
| Pastries (Ref. Low exposure) High exposure | 1.41 | 0.74 – 2.65 | 0.290 | 0.92[d] | 0.18 – 4.58 | 0.916 |
| Processed (Ref. Low exposure) High exposure | 1.30 | 0.68 – 2.47 | 0.425 | 0.37[d] | 0.06 – 2.18 | 0.270 |

[a]Analytes in foods were adjusted for age, energy intake and diet quality. [b]Analytes in foods were adjusted for age, energy intake, diet quality and smoking among members of the family unit. [c]Analytes in foods were adjusted for age, energy intake, diet quality, smoking among members of the family unit, physical activity and body fat percentage. [d]Analytes in foods were adjusted for age, energy intake, diet quality, smoking among members of the family unit and body fat percentage. [e]Analytes in foods were adjusted for age, energy intake, diet quality, physical activity and body fat percentage [f]Analytes in foods were adjusted for age, energy intake, diet quality and body fat percentage. *p*-Values ≤0.05 are highlighted in bold; BPA: bisphenol A; BPS: bisphenol S. *= low exposure (tertiles 1+2); **= high exposure (tertile 3).

**Table 4b.** Influencing of the highest (tertil 3 vs tertil 1+2) BPA, BPS and total bisphenols dietary exposure by food groups on overweight/obesity for girls.

| | Girls (n=144) | | | | | |
|---|---|---|---|---|---|---|
| | Crude model | | | Adjusted model | | |
| **Food Group** | **OR** | **95% CI** | ***p-Value*** | **OR** | **95% CI** | ***p-Value*** |
| **BPA** | | | | | | |
| Dairy products (Ref. Low exposure*) High exposure** | 0.58 | 0.28 – 1.17 | 0.127 | 0.34[f] | 0.06 – 2.03 | 0.236 |
| Meat and eggs (Ref. Low exposure) High exposure | 2.10 | 1.04 – 4.23 | **0.038** | 2.70[b] | 1.00 – 7.32 | **0.050** |
| Fish (Ref. Low exposure) High exposure | 1.46 | 0.73 – 2.92 | 0.281 | 1.28[f] | 0.24 – 6.76 | 0.775 |
| Vegetables (Ref. Low exposure) High exposure | 0.74 | 0.37 – 1.45 | 0.378 | 0.52[d] | 0.08 – 3.23 | 0.480 |
| Fruits (Ref. Low exposure) High exposure | 1.50 | 0.76 – 2.94 | 0.239 | 1.60[b] | 0.66 – 3.88 | 0.302 |
| Legumes (Ref. Low exposure) High exposure | 2.06 | 1.05 – 4.06 | **0.036** | 1.45[f] | 0.17 – 12.37 | 0.736 |
| Cereals (Ref. Low exposure) High exposure | 1.49 | 0.75 – 2.96 | 0.255 | 2.52[a] | 0.99 – 6.45 | 0.054 |
| Pastries (Ref. Low exposure) High exposure | 1.04 | 0.47 – 2.27 | 0.929 | 3.61[f] | 0.36 – 35.94 | 0.274 |
| Processed (Ref. Low exposure) High exposure | 2.13 | 1.06 – 4.26 | **0.033** | 2.14[a] | 0.87 – 5.31 | 0.099 |
| **BPS** | | | | | | |
| Dairy products (Ref. Low exposure) High exposure | 0.76 | 0.37 – 1.54 | 0.446 | 3.47[d] | 0.49 – 24.40 | 0.212 |
| Meat and eggs (Ref. Low exposure) High exposure | 1.46 | 0.73 – 2.92 | 0.281 | 1.64[d] | 0.26 – 10.33 | 0.596 |
| Fish (Ref. Low exposure) High exposure | 1.48 | 0.76 – 2.91 | 0.250 | 0.56[f] | 0.11 – 2.82 | 0.480 |
| Vegetables (Ref. Low exposure) High exposure | 0.77 | 0.38 – 1.53 | 0.452 | 0.75[f] | 0.13 – 4.29 | 0.744 |
| Fruits (Ref. Low exposure) High exposure | 0.85 | 0.43 – 1.67 | 0.638 | 0.26[f] | 0.04 – 1.63 | 0.150 |
| Legumes (Ref. Low exposure) High exposure | 2.48 | 1.16 – 5.32 | **0.019** | 0.80[f] | 0.06 – 10.77 | 0.867 |
| Cereals (Ref. Low exposure) High exposure | 1.12 | 0.55 – 2.30 | 0.748 | 1.15[e] | 0.11 – 12.01 | 0.907 |
| Pastries (Ref. Low exposure) High exposure | 0.75 | 0.37 – 1.50 | 0.409 | 0.70[f] | 0.13 – 3.85 | 0.683 |
| Processed (Ref. Low exposure) High exposure | 1.19 | 0.60 – 2.35 | 0.615 | 2.15[d] | 0.35 – 13.05 | 0.407 |
| **Total bisphenols** | | | | | | |
| Dairy products (Ref. Low exposure) High exposure | 0.58 | 0.28 – 1.17 | 0.127 | 0.34[f] | 0.06 – 2.03 | 0.236 |
| Meat and eggs (Ref. Low exposure) High exposure | 1.68 | 0.85 – 3.34 | 0.137 | 2.53[d] | 0.40 – 15.92 | 0.321 |
| Fish (Ref. Low exposure) High exposure | 1.46 | 0.73 – 2.92 | 0.281 | 1.28[f] | 0.24 – 6.76 | 0.775 |
| Vegetables (Ref. Low exposure) High exposure | 0.83 | 0.42 – 1.64 | 0.588 | 0.89[f] | 0.15 – 5.14 | 0.894 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Fruits (Ref. Low exposure) High exposure | 1.33 | 0.68 – 2.61 | 0.404 | 1.28[b] | 0.53 – 3.05 | 0.584 |
| Legumes (Ref. Low exposure) High exposure | 2.48 | 1.16 – 5.32 | **0.019** | 0.80[f] | 0.06 – 10.77 | 0.202 |
| Cereals (Ref. Low exposure) High exposure | 1.29 | 0.65 – 2.58 | 0.468 | 1.85[a] | 0.72 – 4.78 | 0.259 |
| Pastries (Ref. Low exposure) High exposure | 0.78 | 0.39 – 1.58 | 0.492 | 1.05[a] | 0.44 – 2.51 | 0.912 |
| Processed (Ref. Low exposure) High exposure | 1.19 | 0.60 – 2.35 | 0.615 | 2.15[d] | 0.35 – 13.05 | 0.407 |

[a]Analytes in foods were adjusted for age, energy intake and diet quality. [b]Analytes in foods were adjusted for age, energy intake, diet quality and smoking among members of the family unit. [c]Analytes in foods were adjusted for age, energy intake, diet quality, smoking among members of the family unit, physical activity and body fat percentage. [d]Analytes in foods were adjusted for age, energy intake, diet quality, smoking among members of the family unit and body fat percentage. [e]Analytes in foods were adjusted for age, energy intake, diet quality, physical activity and body fat percentage [f]Analytes in foods were adjusted for age, energy intake, diet quality and body fat percentage. *p*-Values ≤0.05 are highlighted in bold; BPA: bisphenol A; BPS: bisphenol S. *= low exposure (tertiles 1+2); **= high exposure (tertile 3).

## 4. Discussion

The present study aimed to assess the association between dietary exposure to bisphenols and the likelihood of developing overweight and obesity in school children. The effects of bisphenols on BMI depend on the food group and its consumption, independent of sex and age, among other factors. The results showed an increased likelihood of being overweight and obese in school children exposed to high levels of BPA from meat and eggs. This finding was observed only in girls and not consistent associations were found in boys.

To the best of our knowledge, no previous studies have supported the claim that girls are at higher odds of developing overweight or obesity due to exposure to BPA from meat and eggs. BPA is a chemical compound used in the production of plastics and resins, and its presence in food may occur due to certain packaging and storage processes [27,37]. It is important to note that research on the health effects of BPA is ongoing, and there are conflicting debates and findings in the scientific literature.

That the present research only found a consistent association in the females may be due to the sexual dysmorphic effect where girls may be more susceptible to the effects of BPA due to differences in hormonal response or greater sensitivity to hormonal changes that may be influenced by BPA exposure [22,38]. Previous epidemiological studies

also found a positive association between dietary exposure to BPA and overweight and obesity in girls, but not in boys [22]. A research [22] found that overweight/obese girls were 3.38 times more likely to have high BPA exposure compared to normal-weight girls. Other epidemiological studies also observed gender differences [39,40]. [39] observed a positive association between high urinary BPA levels and overweight in girls; but they found no association in boys. However, a work [40] found a negative association between urinary BPA and lower BMI and adiposity measures in girls.

Some studies have examined how exposure to BPA and some analogues may be associated with changes in metabolism, body fat distribution, adipose tissue function and other metabolic processes that could contribute to the development of obesity. In vitro studies show that BPA, bisphenol F (BPF), BPS and bisphenol AF (BPAF) promote preadipocyte to adipocyte proliferation, due to their ability to bind to nuclear receptor in the murine cell line 3T3-L1 and in human preadipocytes [17,18,41]. BPA is also associated with the induction of inflammatory responses, lipogenesis and decreased insulin sensitivity in adipose tissue cells, leading to a dysfunctional adipocyte [42,43]. In a recent in vitro study, we observed the combined effect of BPA, BPF and BPS on the differentiation of preadipocytes to adipocytes in human adipose tissue. Concretely, in cells exposed to a bisphenol mix (10 nM to 10 mM BPA, BPF and BPS) for 14 days, it was observed a promotion of intracellular lipid accumulation in a dose-independent manner that resulted in significant changes in gene expression of adipogenic markers, such as peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT/enhancer-binding protein (C/EBPα), lipoprotein lipase and fatty acid-binding protein 4 (FABP4) [44]. In animal models, exposure to bisphenols has also been shown to induce alterations in lipid metabolism. Several studies in zebrafish (Danio rerio) showed that chronic exposure to BPA and BPS induced dysregulation of genes involved in lipid metabolism, triggering hepatic steatosis [45–47]. In addition, exposure to environmental doses of BPA in zebrafish was found to cause obesity [48].

Studies in rodents show that exposure to BPA during developmental stages causes alterations in hormones involved in satiety and appetite, increased food intake, altered adipocyte numbers, glucose and insulin, leading to weight gain [49–51].

To our knowledge, we have not found any studies that have assessed the association between dietary factors of BPA and BPS exposure (by food source) and childhood overweight/obesity. Limited epidemiological research has studied the relationship between dietary bisphenol and obesity in childhood with not conclusive results. Thus, Heinsberg et al didn't found association between dietary BPA levels and adiposity in Samoan children [52] whereas in other study observed that Spanish adolescent girls with overweight and obesity had a more dietary BPA exposure compared to normal weight [22].

Biomonitoring studies have addressed the association between bisphenol levels and overweight/obesity with contradictory findings. In this sense, a study derived from the National Health and Nutrition Examination Survey (NHANES) in the United States, involving 745 children and adolescents, the findings showed a statistically significant positive association between urinary BPA and BPF levels with increased body fat. However, no significant association was found with BPS [53]. Another research performed in 212 children from the Health Outcomes and Measures of the Environment (HOME) Study showed no significant association between childhood urinary BPA and BPS concentrations with increased adiposity [54]. Another NHAMES-derived study in children and adolescents showed a modest positive association between urinary BPS levels and increased BMI and abdominal fat. However, urinary BPA concentrations were not significantly associated with any body mass findings [55]. A Korean study involving 2,351 children and adolescents who participated in the Korean National Environmental Health Survey (KoNEHS) found no statistically significant positive association between urinary BPA and obesity in Korean children [56].

In relation to dietary exposure, our findings are consistent with previous research that also highlights food intake as the main source of bisphenol exposure, with 90% of exposure estimated to come from diet [13,14,22,57,58]. Most of the fresh foods in the present study were found to have BPA and BPS, the selected foods are packaged foods, although some foods are fresh (Supplementary Materal, Table S1). Consumption of fresh food is considered a healthy dietary habit and is associated with lower exposure to bisphenols or other environmental chemical contaminants compared to other foods. However, studies show that exposure to bisphenols from these foods comes mainly from packaged and ready-to-eat foods [27,59]. The presence of bisphenols in food may be due to the presence of bisphenols in the environment in which they originate (air, dust, water, etc.) or due to the presence of bisphenols in the composition of food packaging [37,60,61]. In relation to contamination by the environment in which they are ingested, the presence of BPA has been detected in fresh foods such as meat, fish, eggs, cereals, vegetables and fresh fruit, demonstrating the possibility of contamination prior to processing and packaging [27,62]. The presence of these compounds in fresh foods points to the ubiquity of bisphenols throughout the food production chain, beyond packaging.

**Table S1**. Products included in each food group.

| Food Groups | Products included | Packaging |
|---|---|---|
| Dairy products | Cured and semi-cured cheese | Plastic |
| | Babybel | Plastic |
| | Cheese | Plastic |
| | Sliced cheese | Plastic |
| Cold Meat | Turkey. mortadella | Plastic |
| Fish | Canned seafood | Can |
| | Canned tuna | Can |
| Vegetables | Packaged carrot and pumpkin | Plastic |
| | Packaged onion | Plastic |
| | Packaged mushrooms | Plastic |
| | Frozen chopped garlic | Plastic |
| | Fresh raw tomato | Not packed |
| | Packaged raw tomato | Plastic |
| | Packaged peppers | Plastic |
| | Canned asparagus and corn | Can |

| | | |
|---|---|---|
| | Packaged parsley. basil. mint | Plastic |
| Fruits | Apple. pear | Plastic |
| | Mango | Plastic |
| | Fruit in syrup | Can |
| | Frozen berries | Plastic |
| | Olives | Plastic |
| Cereals | Hamburger bun | Plastic |
| | Rice (for microwave) | Plastic and paperboard |
| | Puffed rice cakes | Plastic |
| Pastries | Donuts. croissants. chocolate puff pastries | Plastic |
| | Homemade biscuits | Plastic |
| Miscellaneous | Pre-cooked pizza. chips. corn snacks. cheese balls snacks. | Plastic |

In the present study, cases of both genders showed slightly higher but significant exposure to BPA and BPS through intake of legumes and BPA from processed foods compared to controls due to their higher daily intake. On the other hand, exposure to BPS from fruit and BPA from dairy products was found to be higher in the control group in boys and girls respectively. These differences may be related to assimilation behaviour during childhood, as diet is a dietary pattern determined by direct food experience, imitation, food availability, economic income, emotional symbols and cultural traditions [63,64].

In our study, dietary exposure to BPA was below the limit of 4 μg/kg bw/day set by the European Food Safety Authority [65]; however, a new limit of 0.2 ng/kg bw/day has recently been set [66] which is lower than the dietary exposure of our study participants (average intake of BPA = 306.74 ± 263.64 ng/kg bw/day, data not shown). International organisations have not yet established a specific limit for BPS and the other analogues.

Dietary exposure to BPA and analogues is highest in early life. This is due to the unequal relationship between body weight and food consumption [67]. The effect of EDCs has been shown to be more intense, pronounced and at lower doses in early life. Since the detoxifying mechanisms present in adulthood are not fully functional in the

developmental stages. The metabolic rate during early life is higher than during adulthood, leading to an increase in their effects on the organism, such as their obesogenic effect [68]. Due to these findings, it is important to protect the most vulnerable groups from exposure to bisphenols and to obtain more evidence on the possible on weight gain or other adverse effects in these age groups.

Among our findings, diet quality (KIDMED) is not associated with exposure to BPA, BPS and total bisphenols in both genders. However, statistically significant differences by weight and diet quality were observed for boys, with the control group scoring higher on the KIDMED compared to the cases. On the other hand, the present study shows that exposure to total BPA and total BPS in both genders is slightly higher in the control group, although these differences do not reach statistical significance. A study published in 2022 by Melough et al. observed that healthy diets commonly recommended for disease prevention do not appear to reduce exposure to many EDCs, including bisphenols [69]. This may be due to the dietary intake of bisphenols from fresh produce such as fruits, vegetables, meats and fish among others [2,62].

The present study has two strengths. The first is that, to our knowledge it is the first study to evaluate the association between dietary factors of BPA and BPS exposure (according to food source) and childhood overweight/obesity. And the second strength is that qualified personnel were available to take the anthropometric measurements and to collect the data by means of questionnaires, thus achieving greater accuracy in obtaining the data. In relation to the limitations of the study, the main limitation is a relatively small sample size, that could contribute to the variability of the results, which is why most of the findings have not shown statistically significant associations. In addition, the analyses were not adjusted for multiple comparisons by the exploratory nature of our study. We are interested in detecting the greatest number of possible associations that need to be confirmed in further studies.

The results obtained in this explorative study can serve as a basis to confirm hypotheses in further research. Despite the fact that BPA remains the main bisphenol detected in food samples and it has been found to be the most important [27,70], the present study shows that the total daily dietary intake of BPS in schoolchildren is higher than that of BPA. This result reflects that analogues are replacing BPA and exposure to BPA is expected to continue to increase. The current lack of legal regulation of analogues and the failure to set toxicological limits are the reason why analogues are increasingly detected in both food and biological samples [27,70–73]. Since BPA analogues have a similar chemical structure to BPA, they can be said to exhibit similar endocrine disrupting and obesogenic activity [19,43,74,75].

## 5. Conclusions

The present investigation shows a statistically significant positive association between dietary exposure to BPA from meat and eggs and overweight and obesity in girls. Furthermore, it has been observed that the dietary intake of BPA in the schoolchildren in the present study was much higher than the acceptable daily intake established by EFSA for the last year.

The ubiquity of bisphenols and the results found in the present study represent a public health concern. However, further epidemiological studies are needed to assess the obesogenic activity of bisphenols in the most vulnerable age groups, as further studies are needed to confirm the present findings.

## Declarations

**Ethics approval and consent to participate:** All parents or legal tutors of the study participants were fully informed about the present study and signed the informed consent. The present study has been approved by the ethics committees of the University of Granada and of the Provincial

Biomedical Research of Granada (CEI), Spain (reference 1939-M1–22, Andalusian Biomedical Research Ethics Portal), and has been performed following the ethical standards.

**Availability of data and materials:** The data used in the current study are available from the corresponding author on reasonable request.

**Competing interests:** The authors declare that they have no competing interests

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**Authors' contributions:** YG-O: Data curation, Methodology, Formal analysis, Writing -original draft, Writing – review & editing, CM: Data curation, Methodology, Formal analysis, Writing -original draft, Writing – review & editing, MVG-M: Investigation, Writing – review & editing, JJM: Investigation, Writing – review & editing, VAFB: Investigation, Writing – review & editing, MAM-B: Investigation, Writing – review & editing, CS-S: Investigation, Writing – review & editing, IS-B: Formal analysis, Writing -original draft, Writing – review & editing, AR: Conceptualization, Project administration, Funding acquisition, Supervision, Writing – review & editing, AZ-G: Conceptualization, Project administration, Funding acquisition, Supervision, Writing – review & editing. All authors read and approved the final manuscript

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## List of abbreviations

| | |
|---|---|
| EDCs | Endocrine-Disrupting Chemicals |
| BPA | BisPhenol A |
| BPS | BisPhenol S |
| BMI | Body Mass Index |
| FFQ | Food Frequency Questionnaire |
| IQR | InterQuartile Range |
| OR | Odds Ratio |
| CI | Confidence Interval |
| T | Tertiles |
| BPF | BisPhenol F |
| BPAF | BisPhenol AF |
| PPARγ | Peroxisome Proliferator-Activated Receptor-γ |
| C/EBPα | CCAAT/Enhancer-Binding Protein |
| FABP4 | Lipoprotein Lipase and Fatty Acid-Binding Protein 4 |
| NHANES | National Health and Nutrition Examination Survey |
| HOME | Health Outcomes and Measures of the Environment |
| KoNEHS | Korean National Environmental Health Survey |

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# CAPÍTULO III.

## Determinación de bisfenoles en muestras biológicas y su relación con el sobrepeso/obesidad

## Objetivos específicos capítulo III

1. Determinar la presencia de bisfenoles en las cuatro matrices biológicas seleccionadas (saliva, orina, uña y pelo).
2. Analizar cómo influye la presencia de bisfenoles en cada muestra en función del IMC.
3. Estudiar la asociación entre la presencia de BPA y análogos con la probabilidad de presentar sobrepeso/obesidad.

## Introducción capítulo III

Este capítulo de la Memoria de Tesis Doctoral se va a centrar en la determinación de la presencia de bisfenoles en la población objeto de estudio, para ello se han recogido cuatro matrices biológicas (saliva, orina, uña y pelo).

Se han seleccionado estas cuatro matrices en base a que la elección de un solo tipo de ellas puede no reflejar correctamente el grado de exposición de la población, ya que cada una de ellas muestra diferentes vías de eliminación de contaminantes del organismo. Algunas muestras, como la saliva y la orina, muestran una exposición a corto plazo, mientras que otras, como la uña y el pelo, reflejan una exposición a largo plazo.

En el caso de la orina es la más utilizada habitualmente para evaluar compuestos químicos debido a su facilidad de recogida y su naturaleza no invasiva de su recolección en la población infantil. Sin embargo, confiar únicamente en la orina como indicador de la exposición a bisfenoles puede contribuir a las incoherencias entre los estudios que examinan la relación entre los bisfenoles y el sobrepeso/obesidad. Esta incoherencia surge de la variación intra-sujeto en las concentraciones de BPA y sus análogos en orina. Dado que, en un mismo individuo, las concentraciones urinarias pueden variar de un día para otro e incluso a lo largo del mismo día.

En el caso de la uña y el pelo, de forma similar a la orina, ofrecen las ventajas de una recogida fácil y no invasiva. Además, sirven como mejores indicadores de la exposición, ya que reflejan una exposición prolongada

sin fluctuaciones en los niveles de concentración. De hecho, la uña y el pelo se utilizan ampliamente en los análisis forenses para la determinación de drogas u otros tóxicos.

Por último, las muestras de saliva presentan una opción viable a las muestras de sangre para evaluar la exposición humana a químicos ambientales debido a su naturaleza no invasiva y a la falta de necesidad de personal especializado para su recogida. Además, la saliva constituye una alternativa adecuada a la sangre porque es secretada por glándulas rodeadas de capilares sanguíneos, lo que facilita la transferencia de sustancias tóxicas de la sangre a la saliva. En consecuencia, las concentraciones de tóxicos detectadas en la saliva reflejan de forma adecuada las concentraciones equivalentes en la sangre.

A continuación, se muestra el trabajo científico en fase de publicación con relación a la temática desarrollada en este capítulo.

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# Levels of Bisphenol A and its Analogues in Nails, Saliva and Urine of Children: A Case Control Study

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**Abstract:** A growing number of studies link the increase in overweight/obesity worldwide to exposure to certain environmental chemical pollutants that display obesogenic activity (obesogens). Since exposure to obesogens during the first stages of life has been shown to have a more intense and pronounced effect at lower doses, it is imperative to study their possible effects in childhood. The objective here was to study the association of Bisphenol A (BPA) and 11 BPA analogues in children, using three biological matrices (nails, saliva and urine), and overweight and obesity (n= 160). In this case-control study, 59 overweight/obese children and 101 controls were included. The measuring of Bisphenols in the matrices was carried out by ultra-high performance liquid chromatography coupled with triple quadrupole tandem mass spectrometry (UHPLC-MS/MS). Logistic regression was

used to study the association between overweight/obesity and Bisphenol exposure. The results suggested that BPF in nails is associated with overweight/obesity in children (OR:4.87; $p$= 0.020). In saliva, however, the highest detected concentrations of BPAF presented an inverse association (OR: 0.06; $p$= 0.010) with overweight/obesity. No associations of statistical significance were detected between exposure to BPA or its other analogues and overweight/obesity in any of the biological matrices.

**Keywords:** Overweight and Obesity; Childhood Obesity; Obesogens; BPA and Analogues; Biological Samples.

## 1. Introduction

Overweight and obesity are defined by the World Health Organization (WHO) as "an abnormal or excessive accumulation of fat that may be harmful to health". The prevalence of overweight and obesity has tripled in most of the world's population since 1975 (1). Therefore, according to the WHO, obesity is one of the most important public health problems in the world today (2). Obesity is known to be a major risk factor in the development of cardiovascular disease, several types of cancer, diabetes and premature death, among other associated problems (1,3,4).

All the triggers and mechanisms involved in the development of obesity are not yet fully understood (5). The main cause of the onset of obesity is an imbalance between consumed and expended calories (1). Obesity has also been linked to genetic factors (6,7). However, in these last few decades evidence has mounted linking the increase of obesity worldwide to exposure to obesogens (8,9). Obesogens are understood to be environmental chemicals that promote inadequate fat storage through their interference with adipogenesis, as well as interfering with mechanisms controlling satiety, appetite and food preferences, among others (10-13). Bisphenol A (BPA) is among the most studied obesogens.

Bisphenols are produced in large quantities worldwide (>5 million tons per year) and their use has been increasing in the last decades. Due to this ubiquity, bisphenols have been detected in food, dust, sludge,

drinking water, etc (14). and the main route of human exposure to them is diet (15,16). Bisphenols are constituents of polycarbonate plastics and epoxy resins, used to make varnishes, lacquers, adhesives, plastics, dental sealants, water pipes and food packaging. However, their presence in the latter is not stable and over time can migrate from the packaging to the food (17,14). In 2011, the European Commission banned the use of BPA in the manufacture of polycarbonate infant feeding bottles (18). This is because the early stages of development and childhood are the most vulnerable to exposure to environmental chemical contaminants, as it has been shown that the effect is more intense and pronounced in children at lower doses. This is largely because certain protective mechanisms present in adulthood, such as detoxifying liver enzymes and the blood-brain barrier, are not fully developed in the foetal and postnatal stage. Additionally, metabolism is higher in these early stages of development than in the later stages, enhancing the effects of these environmental chemical pollutants (19). In 2015, the European Food Safety Authority (EFSA) reduced the tolerable daily intake (TDI) of BPA from 50 to 4 micrograms per kilogram of body weight per day. In 2018, the EU Regulation on the use of BPAs in varnishes and coatings in contact with food was adopted, prohibiting those specifically intended to come into contact with food for infants. Additionally, a tolerable limit of 0.05 mg BPA per kilogram of food was set for plastic materials in contact with food, and further guidelines were established to ensure that exposure to BPAs remains below the TDI (20). Currently, EFSA has re-evaluated the risks of BPA and has lowered the TDI from 4 micrograms per kilogram of body weight per day to 0.2 nanograms per kilogram of body weight. As a result of the alarm generated by the adverse effects associated with BPA exposure, BPAs have begun to be replaced by their analogues. Information on the toxic potential of these analogues is still very deficient, though since they have a very similar chemical structure to BPAs, they are expected to exhibit similar endocrine disrupting and obesogenic activity as BPA, as several studies have shown (12,21-24).

In vitro studies have shown that BPA and analogues (Bisphenol S (BPS) and Bisphenol F (BPF)) promote adipocyte differentiation, leading to excessive fat accumulation. This effect was observed from concentrations of 10 nM to 50 µM (22,23,25). Animal studies, at concentrations of 2.4 and 25 µg BPA per kilogram of body weight, have found that BPA exposure increases adipose tissue mass and promotes weight gain (26,27). Numerous epidemiological studies have focused on BPA as an obesogen, showing that BPA could exert effects on all organs involved in the regulation of energy homeostasis, such as adipose tissue and the brain, among others (8). In these studies, exposure to low-dose BPA and its analogues was associated with weight gain, disruption of carbohydrate and lipid homeostasis, as well as having an effect on brain regions involved in food intake (8,22,23).

Few epidemiological studies focused on the effects of BPA and its analogues on obesity/overweight in children, and all said studies were restricted to urine as the biological matrix. The focus of this research was thus to study the association between the presence of BPA and its analogues and obesity/overweight in children, using three distinct biological matrices (nails, saliva and urine).

## 2. Material and Methods

### *2.1 Study design and setting*

This case-control study was designed to evaluate environmental factors affecting the overweight and obesity in Spanish children and adolescents, funded by 'FEDER-Consejería de Salud y Familias' of the Junta de Andalucía PE-0250-2019. Recruitment of the study population was carried out between January 2020 and January 2022 in various health and educational centers in the province of Granada, Spain. The parents or legal guardians of the participants gave their written informed consent. Confidentiality was guaranteed with the deletion of participants personal data. The study was approved by the Ethics Committee of the University of Granada.

### *2.2. The study population*

Eligible cases met the following inclusion criteria: overweight or obesity diagnosis; between the ages of 6 and 12 years-old; having resided continuously in the study areas for at least 6 months. The same inclusion criteria were applied to the controls, with the exception of a diagnosis of overweight or obesity. Exclusion criteria included obesity as a symptom of other pathologies, or as a side effect of pharmacological treatment.

Of the 231 who agreed to participate, the selected subjects were those that correctly collected and submitted biological samples (saliva, urine and nails), amounting to 160 participants (53.5% male). After comparing total population with selected sample (subjects with the three biological samples correctly collected), non-significant differences were observed for gender, age, weight, height and urinary creatinine level, both for cases and for controls groups (Supplementary Table S1).

**Table S1.** Comparison between controls and cases included/not included subjects.

| | | Cases (n=94) | | | Controls (n=137) | | |
|---|---|---|---|---|---|---|---|
| | | Included (n=59) | Not included (n=35) | *p* | Included (n=101) | Not included (n=36) | *p* |
| Gender (%) | Male | 58.3 | 48.6 | 0.398 [a] | 49.5 | 50 | 0.999 [a] |
| | Female | 41.7 | 51.4 | | 50.5 | 50 | |
| Age, categorized (%) | ≤ 10 yrs | 75 | 57.1 | 0.108 [a] | 79.2 | 88.6 | 0.312 [a] |
| | > 10 yrs | 25 | 42.9 | | 20.8 | 11.4 | |
| Weight, kg | Median | 53.3 | 51.2 | 0.758 [b] | 25.5 | 26.6 | 0.813 [b] |
| | IQR | 21.9 | 25.7 | | 12.6 | 10.1 | |
| Height, cm | Mean | 140.4 | 141.7 | 0.682 [c] | 127.8 | 126.1 | 0.641 [c] |
| | SD | 12.9 | 17.8 | | 20.7 | 13.6 | |
| Energy Intake, kcal $day^{-1}$ | Mean | 2001.1 | 1900.9 | 0.409 [c] | 2011.9 | 1925.3 | 0.574 [c] |
| | SD | 453 | 441.3 | | 512.4 | 345.1 | |
| Physical Activity (out-of-school) (%) | No | 38.6 | 44.8 | 0.646 [a] | 41.4 | 38.1 | 0.999 [a] |
| | Yes | 61.4 | 55.2 | | 58.6 | 61.9 | |
| Parents' level of education (%) | Primary | 7 | 0 | 0.141 [a] | 1.1 | 0 | 0.882 [a] |
| | Secondary | 52.6 | 41.4 | | 24.7 | 23.8 | |
| | University | 40.4 | 58.6 | | 74.2 | 76.2 | |
| Parents' smoking habits (%) | No | 81.7 | 73.5 | 0.434 [a] | 78.6 | 86.1 | 0.461 [a] |
| | Yes | 18.3 | 26.5 | | 21.4 | 13.9 | |
| Parents' marital status (%) | Married | 77.2 | 65.5 | 0.354 [a] | 92.1 | 76.2 | 0.004 [a] |
| | Divorcee | 17.5 | 31.0 | | 3.4 | 23.8 | |
| | Single | 5.3 | 3.4 | | 4.5 | 0 | |
| Urinary creatinine, g $L^{-1}$ | Median | 0.9 | - | - | 0.9 | - | - |
| | IQR | 0.8 | - | | 0.6 | - | |

IQR: interquartile range; SD: standard deviation; *p*-Values <0.05 are highlighted in bold; [a]Chi-square test; [b]U Mann-Whitney test; [c]Student´s *t*-test

### *2.3. Data collection*

The variables taken into account in the study were anthropometry (weight and height), sociodemographic variables (gender and age), urine creatinine levels and levels of Bisphenols in biological matrices.

Anthropometric measurements were taken by qualified personnel. Height was taken with a measuring rod (model SECA 214 (20-207 cm), while weight was measured with a portable Tanita scale (model MC 780-S MA). Body mass index (BMI) was calculated as weight in kg divided by height squared, in meters. Participants were classified as underweight, normal weight, overweight and obese using the standards proposed by the International Obesity Task Force, as described by Cole et al. (2000, 2007) (28,29).

The determination of creatinine levels in the urine samples was analysed by the *Ángel Méndez Soto Clinical Analysis Laboratory*. The method used was the classical Jaffé method, based on photometric measurement of the reaction kinetics of creatinine with picric acid at 37°C (30,31). Biosystems provided a reagent kit (Barcelona, Spain).

*2.4. Determination of Bisphenols in biological samples*

The biological samples used were saliva, urine and nails. For saliva collection, each subject was given a wide-mouth glass bottle, and for the duration of a week, they had to passively collect saliva on an empty stomach until the bottle was approximately half-full. Urine was collected in a polypropylene bottle. A single urine sample is taken from the study subjects, this should be the first urine of the day. Saliva and urine were stored in the participants' homes under frozen conditions until collection. For the nail samples the participants were given a bottle to collect both finger and toenails, over a 3-month period. The nails are collected without nail polish. After collection, all samples were stored at -80°C until their laboratory analysis, with the exception of the nails that were kept at room temperature.

A total of 12 Bisphenols (BPA; BPF; BPS; Bisphenol AP, BPAP; Bisphenol AF, BPAF; Bisphenol B, BPB; Bisphenol E, BPE; Bisphenol C, BPC; Bisphenol FL, BPFL; Bisphenol M, BPM; Bisphenol P, BPP; Bisphenol Z, BPZ) were analysed for.

Following the completion of the questionnaire, all samples were collected within a 1 to 4 month-period. The validation parameters, LOQ, LOD, calibration range, recovery, etc., can be corroborated in our research group's previously published studies (32-34).

*2.4.1. Determination of Bisphenols in saliva*

The method followed for the determination of Bisphenols in saliva (n= 89) was developed by members of the research group (33). 1g of saliva was deposited in a 10 mL glass tube. Subsequently, 2 mL of acetonitrile and 150 µL of acetic acid solution (0.1 M) were added. This mixture was vortexed and centrifuged for 5min. The supernatant was recovered and transferred to a 10 mL glass tube, then evaporated to dryness. Subsequently, the first extraction was carried out by adding 1.5 mL of acetone extraction solvent to the dry residue. Then ultrasound-assisted extraction was carried out for 30min at a power setting of 35%. The mixture was then centrifuged for 5 min, and the supernatant was recovered by transferring it to another glass tube. Extraction was performed a second time, using 1.5 mL ethanol as the extraction solvent under the same conditions. The supernatant was evaporated to achieve complete dryness, then it was reconstituted with 20 µL of methanol (MeOH) and 80 µL of ultrapure water. Finally, it was centrifuged and analysed using ultra-high performance liquid chromatography coupled with a triple quadrupole tandem mass spectrometry (UHPLC-MS/MS) system (31).

*2.4.2. Determination of Bisphenols in urine*

The urine samples were submitted previously to an enzymatic treatment (ß-glucuronidase and ß-glucuronidase/arylsulfatase), the enzymatic treatment followed is described in the work of Moscoso-Ruiz et al. (2022) (34) and Vela-Soria et al. (2014).

For the determination of Bisphenols in the urine (n=149) we used the improved optimization of the extraction method for endocrine disrupting chemicals (EDCs) as described in Vela-Soria et al. (2014) (35) and further

developed as per Moscoso-Ruiz et al. (2022) (34). 4 mL of 10% (w/v) NaCl and 100 µL of HCl (6 N) were added to 4 mL of urine until pH 2 was reached. Subsequently, dispersive microliquid-liquid extraction was performed, with the addition of a mixture of 600 µL of chloroform and 400 µL of acetone, injected directly into the urine sample using a Hamilton syringe. The mixture was then vortexed gently and centrifuged for 5 min. The sedimented phase was recovered, then transferred to another 10 mL glass tube. This extraction process was repeated three times, and the resulting organic phase was then evaporated to dryness (sedimented phase). The dried residue was reconstituted with 20 µL of MeOH and 80 µL of ultrapure water, centrifuged and analysed on a UHPLC-MS/MS system (34).

*2.4.3. Determination of Bisphenols in nails*

The method for the determination of Bisphenols in nails (n= 74) was also developed by members of this research group as described in the article Martín-Pozo et al. (2020) (32). Firstly, the nails were washed following the protocol described in the work of Martin-Pozo et al. (2020) so that any external contamination was removed. Then 0.1g of lyophilized and shredded nails were weighed and 1 mL of sodium hydroxide/MeOH (0.04 mol $L^{-1}$) was added, shaken in a vortex for 2 min and incubated at 30°C for 15 h. After incubation, the digested nails were cooled to room temperature. Subsequently, they were centrifuged for 10 min, the organic phase was then recovered and evaporated to dryness. The residue was reconstituted, using 20 µL of MeOH and 80 µL of ultrapure water, then it was centrifuged and analysed on a UHPLC-MS/MS system (32).

*2.5. Statistical analysis*

The distribution of the continuous and parametric variables (height) was summarized via mean and standard deviation (SD), while the distribution of the continuous and non-parametric variables (weight, urinary creatinine levels and Bisphenol concentrations in the biological samples) was summarized via the median and interquartile range (IQR).

Frequency distributions were calculated for the categorical variables (sex and age).

To evaluate the differences between cases and controls for all variables, the Student's t-test (for parametric variables), Mann-Whitney U-test (for non-parametric variables) and Pearson's chi-square test (for categorical variables) were used.

A logistic regression model was used to analyse the influence of Bisphenol concentrations (ng $g^{-1}$ or ng $mL^{-1}$) in the three biological matrices as the independent variable, and on overweight and obesity as the dependent variable. The independent variables were dichotomized according to the median value (reference category: concentration ≤ median value). When the % of undetected concentrations of an analyte was > 30%, the cut-off point for the dichotomisation was the limit of detection (LOD)/√2 (reference category: concentration ≤ LOD/√2) (36). LOD value for total Bisphenol in urine was considered as the sum of each Bisphenol LOD separately.

Odds ratios (OR) and standard error (S.E.) were calculated for the initial and adjusted models. Gender, age (for nails, urine and saliva) and creatinine levels (for urine analysis) were considered confusion factors (CF) in the adjusted models.

SPSS v.23 (version 23, IBM® SPSS® Statistics, Armonk, NY, USA) was employed for all the statistical analyses. The significance was set to $p < 0.050$.

## 3. Results

Table 1 displays the study population's characteristics. Significant differences were observed for anthropometric variables, the values being higher for cases than for controls ($p < 0.001$) for all three parameters (weight, height and BMI). The population distribution according to gender and age, and median values for urinary creatinine levels, did not show significant differences between cases and controls.

**Table 1.** General characteristics of study population (N = 160).

| | | n | Controls (n= 101) | Cases (n= 59) | *p* |
|---|---|---|---|---|---|
| **Gender (%)** | Male | 85 | 58.8 | 41.2 | 0.505[b] |
| | Female | 76 | 67.1 | 32.9 | |
| **Age, categorized (%)** | ≤ 10 yrs | 125 | 64 | 36 | 0.280[b] |
| | > 10 yrs | 36 | 58.3 | 41.7 | |
| **Weight, kg** | Median | | 25.5 | 53.3 | **< 0.001[c]** |
| | IQR | | 12.6 | 21.9 | |
| **Height, cm** | Mean | | 127.8 | 140.4 | **< 0.001[a]** |
| | SD | | 20.7 | 12.9 | |
| **BMI, kg/m²** | Mean | | 16.1 | 24.5 | **< 0.001[a]** |
| | SD | | 2 | 3.8 | |
| **Urinary creatinine, g L⁻¹** | Median | | 0.9 | 0.9 | 0.439[c] |
| | IQR | | 0.6 | 0.8 | |

IQR: interquartile range; SD: standard deviation; *p*-Values <0.05 are highlighted in bold; [a]Student´s *t*-test; [b]Chi-square test; [c]U Mann-Whitney test

Table 2 shows the concentrations of Bisphenols determined in the nails, urine and saliva. BPA and BPAF were detected in all three matrices, followed by BPF which was detected in the nails and urine, BPS in the urine and saliva, and BPAP in the nails and saliva. The analogues BPE, BPB, BPC, BPZ, BPM, BPP and BPFL were only detected in the saliva. One of the most important findings of the study was that the nails were the biological matrix with the highest total concentrations of Bisphenol, BPA and BPF (149.2 ng $g^{-1}$, 136.3 ng $g^{-1}$ and 23.1 ng $g^{-1}$). The highest concentration was found for overweight and obese subjects, with significant differences for BPA and total Bisphenols. In the urine, BPA and total Bisphenol were found in higher concentrations in the control group than in the cases, but without significant differences. However, only BPA in the nails showed significant differences ($p$ = 0.005). In the saliva the highest detected value of BPA was determined in subjects with a BMI ≥ 25 kg/m². For total Bisphenols determined in the nails and

saliva, the highest values were detected in the cases. Nevertheless, significant differences were found only for total Bisphenols ($p = 0.011$) in the nails. In the urine, the highest concentration of total Bisphenols was found in the control group, but no significant differences were observed.

**Table 2.** Bisphenol Concentrations in nails, urine and saliva (ng $g^{-1}$ or ng $mL^{-1}$).

| | **Nails (ng $g^{-1}$)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Controls (n=52)** | | | | **Cases (n=22)** | | | | |
| | % detected | Median | $P_{25}$ | $P_{75}$ | % detected | Median | $P_{25}$ | $P_{75}$ | *p* |
| **BPF** | 92.3 | 7.8 | 5.5 | 17 | 81.8 | 12.1 | 8.4 | 23.1 | 0.062 |
| **BPE** | 0 | <LOD | <LOD | <LOD | 4.6 | <LOD | <LOD | <LOD | - |
| **BPA** | 100 | 21.2 | 13.2 | 32.7 | 100 | 43.7 | 18.5 | 136.3 | **0.005** |
| **BPS** | 0 | <LOD | <LOD | <LOD | 4.6 | <LOD | <LOD | <LOD | - |
| **BPB** | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |
| **BPC** | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |
| **BPZ** | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |
| **BPAP** | 1.9 | <LOD | <LOD | <LOD | 9.1 | <LOD | <LOD | <LOD | - |
| **BPAF** | 3.9 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |
| **BPM** | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |
| **BPP** | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |
| **BPFL** | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |
| **Bisphenols total** | 100 | 36.2 | 24.4 | 55.6 | 100 | 70.7 | 33.6 | 149.2 | **0.011** |
| | **Urine (ng $mL^{-1}$)** | | | | | | | | |
| | **Controls (n=97)** | | | | **Cases (n=52)** | | | | |
| | % detected | Median | $P_{25}$ | $P_{75}$ | % detected | Median | $P_{25}$ | $P_{75}$ | *p* |
| **BPF** | 2.1 | <LOD | <LOD | <LOD | 3.9 | <LOD | <LOD | <LOD | 0.522 |
| **BPE** | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |
| **BPA** | 58.8 | 0.70 | <LOD | 2.61 | 71.2 | 0.6 | 0.2 | 2 | 0.969 |
| **BPS** | 9.3 | <LOD | <LOD | <LOD | 7.7 | <LOD | <LOD | <LOD | 0.262 |
| **BPB** | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |
| **BPC** | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |
| **BPZ** | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **BPAP** | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |
| **BPAF** | 5.2 | <LOD | <LOD | <LOD | 7.7 | <LOD | <LOD | <LOD | 0.882 |
| **BPM** | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |
| **BPP** | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |
| **BPFL** | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |
| **Bisphenols total** | 59.8 | 2 | <LOD | 4.1 | 69.2 | 1.7 | <LOD | 3.1 | 0.594 |
| | | | | **Saliva (ng g$^{-1}$)** | | | | | |
| | **Controls (n=58)** | | | | **Cases (n=31)** | | | | |
| | % detected | Median | $P_{25}$ | $P_{75}$ | % detected | Median | $P_{25}$ | $P_{75}$ | *p* |
| **BPF** | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD | - |
| **BPE** | 3.5 | <LOD | <LOD | <LOD | 6.5 | <LOD | <LOD | <LOD | 0.650 |
| **BPA** | 34.5 | <LOD | <LOD | 0.7 | 41.9 | <LOD | <LOD | 0.8 | 0.333 |
| **BPS** | 6.9 | <LOD | <LOD | <LOD | 19.4 | <LOD | <LOD | <LOD | 0.499 |
| **BPB** | 1.7 | <LOD | <LOD | <LOD | 16.1 | <LOD | <LOD | <LOD | - |
| **BPC** | 1.7 | <LOD | <LOD | <LOD | 9.7 | <LOD | <LOD | <LOD | - |
| **BPZ** | 3.5 | <LOD | <LOD | <LOD | 3.2 | <LOD | <LOD | <LOD | 0.755 |
| **BPAP** | 12.1 | <LOD | <LOD | <LOD | 3.2 | <LOD | <LOD | <LOD | 0.106 |
| **BPAF** | 93.1 | 0.2 | 0.2 | 0.5 | 87.1 | <LOD | <LOD | 0.24 | 0.073 |
| **BPM** | 39.7 | <LOD | <LOD | 0.7 | 51.6 | <LOD | <LOD | <LOD | 0.913 |
| **BPP** | 27.6 | <LOD | <LOD | 0.7 | 16.1 | <LOD | <LOD | <LOD | 0.174 |
| **BPFL** | 8.6 | <LOD | <LOD | <LOD | 3.2 | <LOD | <LOD | <LOD | 0.313 |
| **Bisphenols total** | 100 | 2.7 | 2.3 | 3 | 100 | 2.7 | 2.2 | 3.2 | 0.455 |

LOD: limit of detection; *p*-Values <0.050 are highlighted in bold; U de Mann-Whitney test.

Tables 3, 4 and 5 show the influence of BPF, BPA, BPS, BPAF, and the total Bisphenol concentrations determined in the three biological matrices, on overweight and obesity in the study population. Crude and adjusted OR values were significant for BPF in the nails and BPAF in the saliva. Participants with higher BPF concentrations than the median value for nails had a higher likelihood of excess weight (OR = 3.64, $p$ = 0.020; OR = 4.87, $p$ = 0.012, crude and adjusted values, respectively) (Table 3). On the other hand, an inverse association was observed between BPAF and overweight and obesity. Subjects with concentration

of BPAF higher than LOD in the saliva had a lower likelihood to be overweight or obese (OR = 0.06, $p$ = 0.010; OR = 0.06, $p$ = 0.009, crude and adjusted values, respectively) (Table 5). A non-significant association was observed between Bisphenol concentrations in urine and overweight/obesity (Table 4).

**Table 3.** Bisphenol levels in nails as influencing factors on overweight/obesity (Logistic regression analysis).

| | Crude | | | Adjusted | | |
|---|---|---|---|---|---|---|
| | *p* | OR | S.E. | *p* | OR | S.E. |
| **BPF (Ref. BPF concentration ≤ median)** | **0.020** | 3.64 | 0.56 | **0.012** | 4.87 | 0.63 |
| **BPA (Ref. BPA concentration ≤ median)** | 0.131 | 2.21 | 0.52 | 0.157 | 2.14 | 0.54 |
| **Bisphenols Total (Ref. Bisphenols total concentration ≤ median)** | 0.131 | 2.21 | 0.52 | 0.107 | 2.44 | 0.55 |

*Ref.: reference category; OR: odds ratio; S.E.: Standard error; p-Values <0.050 are highlighted in bold; All analytes were adjusted for age and gender.*

**Table 4.** Bisphenol levels in urine as influencing factors on overweight/obesity (Logistic regression analysis).

| | Crude | | | Adjusted | | |
|---|---|---|---|---|---|---|
| | *p* | OR | S.E. | *p* | OR | S.E. |
| **BPF (Ref. BPF concentration ≤ LOD)** | 0.527 | 0.53 | 1.02 | 0.424 | 0.43 | 1.04 |
| **BPA (Ref. BPA concentration ≤ LOD)** | 0.653 | 0.85 | 0.35 | 0.551 | 0.78 | 0.42 |
| **BPS (Ref. BPS concentration ≤ LOD)** | 0.225 | 2.38 | 0.72 | 0.268 | 3.76 | 1.20 |
| **BPAF (Ref. BPAF concentration ≤ LOD)** | 0.886 | 0.90 | 0.75 | 0.616 | 0.62 | 0.94 |
| **Bisphenols Total (Ref. Bisphenols total concentration ≤ LOD)** | 0.267 | 0.66 | 0.38 | 0.602 | 0.79 | 0.46 |

*Ref.: reference category; OR: odds ratio; S.E.: Standard error; p-Values <0.050 are highlighted in bold; All analytes were adjusted for age, gender and urinary creatinine level.*

**Table 5.** Bisphenol levels in saliva as influencing factors on overweight/obesity (Logistic regression analysis).

| | Crude | | | Adjusted | | |
|---|---|---|---|---|---|---|
| | *p* | OR | S.E. | *p* | OR | S.E. |
| **BPA (Ref. BPA concentration ≤ LOD)** | 0.527 | 1.34 | 0.46 | 0.601 | 1.28 | 0.47 |
| **BPAF (Ref. BPAF concentration ≤ LOD)** | **0.010** | 0.06 | 1.10 | **0.009** | 0.06 | 1.10 |
| **Bisphenols Total (Ref. Bisphenols total concentration ≤ median)** | 0.823 | 1.11 | 0.45 | 0.884 | 1.07 | 0.45 |

*Ref.: reference category; OR: odds ratio; S.E.: Standard error; p-Values <0.050 are highlighted in bold; All analytes were adjusted for age and gender.*

## 4. Discussion

The aim of this study was to determine the presence of BPA and its analogues in nails, urine and saliva and to analyse the association between their concentrations and overweight and obesity in children. The findings suggest that a high concentration of BPF in nails is associated with an increased likelihood of overweight/obesity. However, the concentration of BPAF in saliva is inversely related to body weight. The most relevant results obtained in nails for BPF and in saliva for BPAF cannot be compared with previous studies, since the association in these two biological samples has not been studied to date, to the best of our knowledge.

The results of this study show that children who have a higher concentration of BPF, BPA, BPAF and total Bisphenols in urine have a lower likelihood of overweight/obesity, yet without statistically significant results. However, the highest detected concentrations of BPS in urine were associated with a higher likelihood of overweight/obesity, though also without statistically significant results. Previous similar studies show the following findings: Jacobson et al. (2019) observed in children and adolescents that urine BPS concentrations were associated with an increased likelihood of obesity (OR = 1.16; 95 % confidence intervals (CI): 1.02 - 1.32) (37); the findings obtained in the work of Liu et al. (2019) show that children in the highest quartile of urine BPS concentrations had a 1.36-fold increased risk (95 % CI: 0.53 - 3.51) of obesity compared to children in lower quartiles (38); Gajjar et al. (2021)

also showed that urinary BPS concentrations were directly associated with higher % body fat in children at 8 years of age (OR = 1.1; 95 % CI: - 0.6 - 2.7) (39); as for urinary BPA concentrations, no association was found with obesity in work by Seo et al. (2022) (40), Jacobson et al. (2019) (37), Xue et al. (2015) (41) and Okubo et al. (2019) (42). Regarding the concentrations of bisphenols detected in urine of schoolchildren and adolescents in previous studies, it is observed that the concentration range detected for BPA, BPS and BPF is higher than that detected in the present study, being 1.2 - 1.6 ng $mL^{-1}$ BPA, 0.3 - 0.4 ng $mL^{-1}$ BPS and 0.2 - 0.3 ng $mL^{-1}$ BPA (37,38,39). Whereas for the present study it is 0.58 - 0.70 ng $mL^{-1}$ BPA and <LOD BPS and BPF.

Conversely, other previous studies found a positive association between high urinary BPA concentrations and overweight/obesity. In the work of Amin et al. (2019) a statistically significant direct association between in urine BPA and the risk of obesity was observed, being 12.48 times higher in children found in the third tercile of BPA versus children in the first and second terciles (95% CI: 3.36 - 46.39, $p < 0.001$) (5). Liu et al. (2019) also found that for children in the highest quartile of Bisphenols (BPA and BPF) in urine, their risk of obesity was 1.74 times higher (95% CI: 0.92 - 3.31) for BPA and 1.54 times higher for BPF (95 % CI: 1.02 - 2.32) (38). A study in 63 prepubertal children with exogenous obesity found that obese children with metabolic syndrome had significantly higher urinary BPA levels than obese children without metabolic syndrome, and both obese groups had significantly higher urinary BPA levels than the control group (43). Five other studies also found that higher urinary BPA concentrations were directly associated with a greater likelihood of higher BMI in school-aged children (44-48).

Most related Bisphenol studies only focused on urinary BPA and reported that, in childhood, higher BPA concentrations were associated with higher body fat percentages (5,38,43-48). However, this work observed that higher detected concentrations of urinary BPA, BPF, BPAF and total Bisphenols show a trend towards a lower BMI (OR = 0.78; OR

= 0.43; OR = 0.62; OR = 0.79, respectively), this association was not statistically significant ($p$ = 0.551; $p$ = 0.424; $p$ = 0.616; $p$ = 0.602, respectively). Other recent studies on urinary BPA show the following results. Gajjar et al. (2021) observed an inverse association between urinary BPA concentrations and body fat % in children 8 years of age (OR = -1.2; 95 % CI: -3.4 - 1.0) (39). Silva et al. (2021) observed in children 6 to 10 years of age that higher concentrations of BPA and total Bisphenols in urine were associated with lower BMI (49). In the case of the work carried out by Malik et al., (2021), it was observed that the presence of BPA in urine was associated with both obesity and low weight in children, i.e., children who were found to have the highest BPA concentrations in urine (fourth quartile) were associated with both obese and underweight children (50). In relation to the results obtained in saliva in this study, it was observed that the highest BPAF concentrations were associated with lower BMI, the association being statistically significant (OR = 0.06, $p$ = 0.009). In the case of nails, BPF showed a direct and statistically significant association (OR = 4.87, $p$ = 0.012).

In this study biological samples analyses were used to measure bisphenols exposure. Although multiple sources of bisphenols exposure exist in children, the diet is considered to be one of the main sources of exposure as we have been demonstrated in other studies (51,52). Food contamination with these chemicals typically occurs during food processing, packaging, transportation, and storage (53).

The use of urine as the only indicator of exposure to Bisphenols may be responsible for the lack of consistency between the associations of Bisphenols and adiposity in the previous studies, given that there is a large within-subject variation in the concentrations of BPA and its analogues in urine due to the short half-life of Bisphenols; even within the same subject there are variations in urinary concentrations from day to day and even over the course of the same day. A key issue is that some BPA and BPA analogues are metabolized very rapidly in the body (21), therefore, spot urine samples are limited in their ability to reflect long-

term exposure levels. Urine is commonly used in studies because of the ease and non-invasive nature of its collection in the child population. Nails, however, share both these advantages in addition to being a better biomarker, since they display long-term exposure without fluctuations in concentration levels within the same day, or from one day to another. Therefore, the use of nails as a bioindicator is very promising since, by their nature they reflect long-term cumulative exposure, given their relatively slow growth rate (54). In fact, nails have been widely used in forensic analysis and as a bioindicator of the ingestion of drugs and heavy metals (55-57). Moreover, nail samples offer other advantages over other commonly used biological samples such as blood and urine, namely that their collection is simple and non-invasive, and do not require specialized personnel, transport or storage conditions (58). On the other hand, saliva samples are a good alternative to blood samples for assessing human exposure to toxicants because of their non-invasive nature and because they do not require qualified personnel to collect them (59). Another reason why saliva is an adequate alternative to blood is because saliva is secreted by glands surrounded by blood capillaries that allow the passage of toxicants from blood to saliva (60). Therefore, the concentration of toxicants detected in saliva are an accurate reflection of their concentrations in the blood.

Obesity in general is of great concern worldwide. Since increased dietary intake and sedentary lifestyles alone do not explain its increase globally, particular attention is being paid to a wide variety of environmental chemicals that may play an important role. Experimental models and epidemiological evidence suggest that BPA and some of its analogues (BPS and BPF) may act as environmental obesogens (12,22-24). It is true that various potential mechanisms of action of Bisphenols during adipogenesis have been reported, but there is no common consensus. Some of these mechanisms of action described in the scientific literature is the action of BPA on the modulation of key regulators of adipogenesis (peroxisome proliferator-activated receptor gamma of preadipocyte (PPARγ), CCAAT/enhancer-binding protein Alpha

(C/EBPα), dual leucine zipper-bearing kinase (DLK), lipoprotein lipase (LPL)) through interference with receptor signaling (61-63). In one in vitro study, it was observed that BPA enters adipose stem cells and interacts with the oestrogen receptor (ER), then translocating to the nucleus, where it increases transcription of key adipogenic genes such as DLK, C/EBPα, PPARγ and LPL, which in turn enhances and accelerates the pathway from human adipose stromal/stem cells to mature adipocyte (61). Another adipogenic effect resulting from BPA exposure in 3T3-L1 cells may also be mediated by increased glucocorticoid receptor and C/EBPδ transcriptional activity (64). Boucher et al., 2014 performed an in vitro study in which they demonstrated that BPA exposure induced differentiation of primary human preadipocytes through a non-classical ER pathway rather than via glucocorticoid activation (65). The results showed that BPA induced the differentiation of primary preadipocytes through increased expression of adipogenic markers at the mRNA level and increases the expression levels of factors involved in the transcriptional cascade responsible for the differentiation of primary preadipocytes to adipocytes (65). Another mechanism of action that has been studied is the effect of exposure to BPA and its analogues on perturbations in the synthesis and signaling of peripheral serotonin, especially in the intestine, that may contribute to obesity since serotonin also plays an important role in the energy balance of mammals (66). Thus, Barra et al. (2022) proposed that BPA and its analogues could increase the intestinal production of peripheral serotonin in the human organism and could contribute to its obesogenic effects (66). The study by Barra et al. (2022) (66) is based on additional experiments to support this hypothesis, in one of those studies it was observed that the exposure of mice with genetic or pharmacological inhibition of ryptophan hydroxylase 1 (Tph1) to BPA, evaluated whether the metabolic deficits induced by BPA, such as obesity, depend on the production of peripheral serotonin, since peripheral serotonin in adipose tissue functions as an obesity hormone that reduces energy expenditure and increases lipid accumulation (67).

The importance of bisphenols derives from their ubiquity, as these compounds are present in most commonly used plastic packaging (polycarbonates) and epoxy resins used in the coating of cans in contact with food (14,17). Exposure to bisphenols is daily. Bisphenols usually enters the bloodstream via the oral route. Its absorption is immediate and with a bioavailability of more than 70% (68). These are conjugated with glucuronic acid and almost entirely eliminated in the urine (69). Studies have also shown that bisphenols bioaccumulate in adipose tissue (12). It is true that, if adipose tissue were taken from an obese person, a higher load of these contaminants would be found than in a non-obese person. However, in this study, biological matrices that are not fatty in nature (urine, nails and saliva) have been taken. Therefore, it is not to be expected that in these matrices there will be a greater bioaccumulation behaviour in obese people, as they do not have this lipophilic character. Thus, an important question to raise is the relationship between bisphenol exposure and obesity. As mentioned previously, obese people can be expected to have higher concentrations of pollutants in their organism (70). For this reason, statistical models are adjusted for % fat and other predisposing factors (energy intake, physical inactivity, etc.) to obtain the probability of obesity due to bisphenols rather than other factors.

There were several limitations to this study. Firstly, the relatively small sample size could be a contributing factor to the non-significant findings for most of the Bisphenols. Also, it has prevented us to split the sample in order to analyze influencing factors on overweight and obesity separately. Secondly, complication in the collection of saliva and nail samples resulted in fewer samples being submitted of these two matrices. In the case of nails, many of the children participating in the study tended to bite their nails, being a considerable drawback in their acquisition. In relation to saliva, some children refused to collect saliva because they were disgusted by it. However, the main strength of this study is that, to the best of our knowledge, this case-control study in Spanish children is the first to evaluate the possible association between BPA and 11 of its

analogues, and overweight and obesity, analysed in three distinct biological matrices (urine, nails and saliva). By not exclusively using a single biological sample, such as urine, to assess exposure to BPA and its analogues, we eliminate the risk of having one measurement that can bias our results via a misclassification of exposure due to, for example, the great variability of urine composition within a single day, or from one day to another.

The adverse health effects of BPA analogues should continue to be monitored, since BPA has already begun to be replaced by them, and human exposure to these alternatives will continue to increase. In addition, the limited evidence on the association between Bisphenols and overweight/obesity calls for further epidemiological and toxicological studies to assess whether human exposure to BPA substitutes increases the risk of overweight/obesity in children.

## 5. Conclusion

The current study is the first to report on the association between BPA and 11 of its analogues, and childhood overweight and obesity, analysed in three distinct biological matrices (urine, nails and saliva). The results suggest a positive association between BPF exposure in nails and a negative association between BPAF exposure in saliva. No associations of statistical significance were found between BPA and its analogues and overweight/obesity.

However, the contradictions between the associations of Bisphenols and adiposity in the previous studies require further epidemiological and toxicological studies, ideally of a longitudinal design and including Bisphenol measurements in different biological matrices that show long-term exposure, assuring that the concentration of Bisphenols in the samples does not fluctuate within the same day, or from day to day.

## Declarations

**Ethics statement:** This study was approved by the ethics committees of the University of Granada, the Provincial Biomedical Research of Granada (CEI), Spain (reference 1939-M1–22, Andalusian Biomedical Research Ethics Portal). The study was performed in accordance with the corresponding ethical standards.

**Author contributions:** YG-O and IM-R: collected the data, performed the analyses, interpreted the data and wrote the manuscript. VAFB: provided the means for recruitment of the study population, collected the data, critically reviewed the manuscript and gave final approval of the version to be published. CM: collected the data, interpreted the data and revised the manuscript, critically reviewed the manuscript and gave final approval of the version to be published. LR: collected the data, supervised the writing, reviewed the manuscript and gave final approval of the version to be published. AZ-G: conceptualised and designed the study, critically reviewed the manuscript and gave final approval of the version to be published. RG-M: provided project administration and funding acquisition. AR: conceptualised and designed the study, coordinated, supervised data collection, reviewed the manuscript critically and gave final approval of the version to be published, as well provided project administration and funding acquisition. All authors have read and agreed on the published version of the manuscript.

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# Hair as an Indicator of Prolonged Exposure to Endocrine Disruptors and its Relation to Weight Gain in a Child Population

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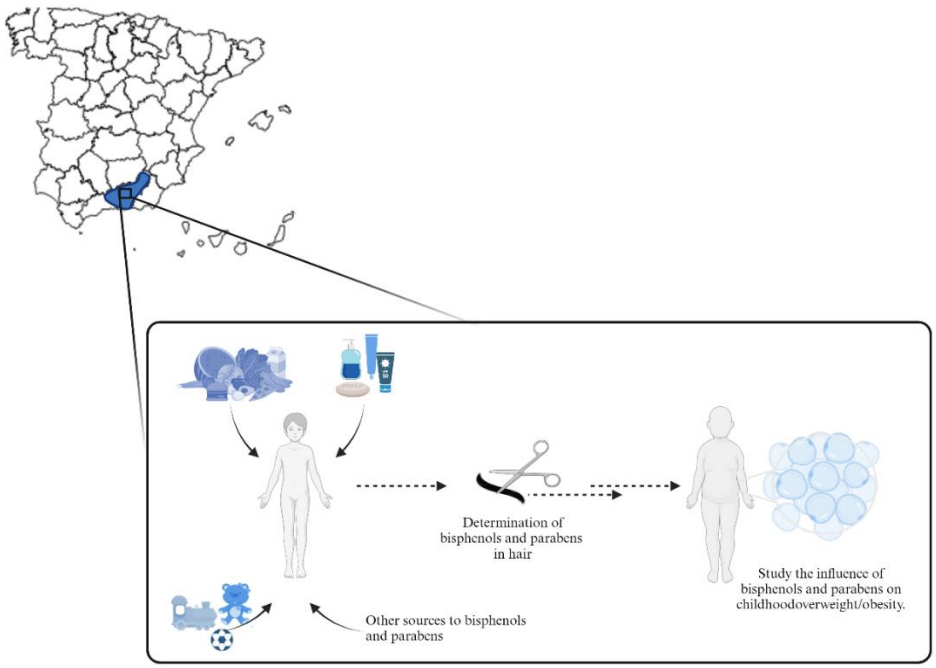
## Abstract

**Objective:** The aim of this research was to determine the presence of bisphenols and parabens in hair samples and to study the possible association with overweight or obesity in a Spanish school population. **Methods:** A total of 124 cases and 179 controls (aged 3 to 12 years) were recruited, from whom sociodemographic, lifestyle, hair and urine samples were obtained. By ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) technique, 12 bisphenols and 6 parabens were analyzed in the samples. The correlation between the concentration of bisphenols and parabens in hair and urine was studied using Spearman correlation coefficients. Finally, binary logistic regression models were used to evaluate their relationship with overweight and obesity. **Results:** Hair samples from 270 schoolchildren were analysed. Concentrations of bisphenols were higher in the control

group, with the exception of bisphenol A (BPA). Detected levels of parabens were higher in the cases. There was a low correlation between the concentrations detected in hair and urine, except for isopropylparaben (isoPropPB) - propylparaben (PropPB) in girls and isoPropPB - isoPropPB in boys, where moderate correlations were observed (0.715 and 0.652, respectively). Higher PropPB concentrations were shown to increase the probability of being overweight or obese by 4.67 times (p= 0.039) in boys, but not in girls. In addition, a higher concentration of total bisphenols in hair was inversely associated with overweight/obesity in boys (OR: 0.09, *p*= 0.039). **Conclusions:** High concentrations of PropPB and low concentrations of total bisphenols in the hair of males were found to be associated with overweight/obesity. No statistically significant associations were observed for the remaining analytes.

**Keywords:** Bisphenols; Parabens; Obesogens; Obesity; Schoolchildren; Biological samples

## Graphical Abstracts



## 1. Introduction

Endocrine disrupting chemicals (EDCs) are exogenous substances that can interfere with the hormonal system in animals and humans. These EDCs can disrupt normal endocrine functioning, affecting hormone synthesis and metabolism, among others. As a result, they may cause adverse health effects such as reproductive, developmental, neurological and immunological problems [1].

Among the most studied EDCs are bisphenols. Bisphenols, such as bisphenol A (BPA) and its analogues, are the base monomers for the manufacture of polycarbonate plastic and epoxy resin, present in a wide range of everyday products such as food and beverage containers (plastics and cans), water pipes, medical equipment and dental materials [2-4]. Exposure of food contact materials containing BPA or its analogues to certain environmental agents, such as light and heat, cleaning agents and the passage of time, can lead to leaching of BPA from the packaging into the food or beverage [5]. Thus, the oral route is the main route of

Parabens are commonly used preservatives in cosmetic, pharmaceutical and food products due to their antimicrobial activity and low allergenic potential [4, 7]. Among the most commonly used are methylparaben (MetPB), ethylparaben (EthPB), propylparaben (PropPB) and butylparaben (ButPB). The estrogenic activity of parabens increases as a function of alkyl chain length and branching (ButPB > PropPB > MetPB) [5]. Although MetPB has the lowest estrogenic power, it penetrates through the skin at a faster rate than other parabens and can accumulate in the various layers of the skin [5, 8]. The main sources of exposure to these compounds are diet, personal care products and the environment [7].

In recent decades, exposure to these EDCs has been associated with obesity, as a common effect of bisphenols and parabens is their obesogenic capacity. These compounds may alter lipid metabolism and energy balance, contributing to the development of obesity [9, 10]. Evidence for this effect comes from *in vitro*, *in vivo* and epidemiological studies. *In*

*vitro* studies have shown that bisphenols and parabens can induce the differentiation of pre-adipocyte cells into adipocytes [11-13], while *in vivo* studies have shown that exposure to these compounds is associated with increased body weight and fat accumulation in zebrafish and rodents [14-17]. Epidemiological studies have linked the presence of bisphenols and parabens in biological samples to overweight/obesity in humans [18, 19]. In addition, the effects of obesogens have been shown to be stronger and at lower concentrations in early developmental stages, such as infancy, due to the immaturity of the body's protective systems [9, 10].

A variety of biological samples, such as blood and urine, among others, have historically been used to determine EDC exposure [20, 21]. However, EDC analysis in the biological matrix of hair has been gaining popularity due to its advantages [20, 22]. Unlike urine, which reflects short-term exposure, hair can provide a measure of long-term exposure [20]. In addition, the collection of hair samples is non-invasive and easy to store, transport and handle.

Based on this information, it could be considered that chronic exposure to bisphenols and parabens in early life, as measured by hair analysis, could be associated with an increased risk of childhood overweight or obesity. Given that the most commonly used matrix to study this association is urine, which reflects short-term exposure, and taking into account that obesity is a chronic disease, this study is justified by the need for more accurate and less invasive monitoring methods. Hair is an ideal biological matrix to reveal long-term exposure to these EDCs. In this context, the present work aims to study the influence of the presence of bisphenols and parabens in hair (long-term exposure) on overweight or obesity in a child population.

## 2. Material and Methods

### 2.1. Study design and population

A case-control study was conducted in several health and educational centres in the province of Granada, Spain. Participants were recruited

over a two-year period, from 2020 to 2022. Inclusion criteria were: (i) diagnosis of overweight or obesity (for cases only); (ii) prepubertal children aged 3-12 years; (iii) continuous residence in the study area for at least six months. Exclusion criteria were: (i) obesity as a symptom of other pathologies, or as a side effect of a pharmacological treatment.

The protocol of the present study was approved by the ethical committees of the University of Granada and the Provincial Centre for Biomedical Research of Granada (CEI). The fathers, mothers or legal guardians of the participants were informed of the study and signed the informed consent form.

A total of 194 controls and 128 cases were recruited, with a total of 322 participants. Of these, only were included who correctly collected and submitted biological samples (hair and urine), resulting in a total of 270 subjects included in the research (166 controls and 104 cases).

### 2.2. Data collection

Trained interviewers conducted face-to-face interviews with parents or legal guardians. Through these interviews, socio-demographic information was collected (gender and age of children), as well as lifestyle data (smoking habits of household members, physical activity outside school and diet).

In addition, anthropometric measurements, such as height (cm) and weight (kg), were taken by qualified personnel. Study participants were weighed in light clothing and without shoes using a Tanita portable scale (model MC 780-S MA). Height was measured with a stadiometer (model SECA 214, range 20 - 207 cm). During height measurement, participants' backs, buttocks and heels were checked to ensure that they were in contact with the wall. Weight and height were used to calculate body mass index (BMI), which was determined as weight divided by height squared. Subjects were thus classified as underweight, normal weight, overweight and obese, according to the criteria of Cole et al. (2000, 2007) [23, 24].

### 2.3. Determination of bisphenols and parabens in biological samples

As mentioned above, the biological samples collected consisted of hair and urine. Hair samples were obtained on the day of the interview, cutting as close as possible to the root in the occipital area of the scalp, as this is the most vascularised area where a higher concentration of toxicants is eliminated. Urine was collected in a polypropylene bottle, preferably using the first urine of the day due to its higher concentration. After collection, urine samples were stored at -80°C until analysis in the laboratory, while hair samples were kept at room temperature.

A total of 12 bisphenols (BPA, bisphenol F, BPF; bisphenol S, BPS; bisphenol AP, BPAP; bisphenol AF, BPAF; bisphenol B, BPB; bisphenol E, BPE; bisphenol C, BPC; bisphenol FL, BPFL; bisphenol M, BPM; bisphenol P, BPP; bisphenol Z, BPZ) and 6 parabens (MetPB, EthPB, PropPB, isoPropPB, ButPB and isoButPB) were analyzed in both hair and nails. The analytical methods used for the extraction and determination of bisphenols and parabens in the selected samples have been previously published in different scientific articles of our own research group [22, 25]. These methodologies are briefly described below.

#### 2.3.1. Determination of bisphenols and parabens in hair samples

For the extraction and determination of bisphenols in hair, the protocol described by Rodríguez-Gómez et al. (2017) [22] was followed. First, the hair was washed following the protocol described in that work. Then, 0.05 g of the hair sample was weighed and incubated with 0.5 mL of acetic acid/MeOH mixture (20:80, v/v) at 38 °C for 12 h. After cooling to room temperature, 1 mL of acetonitrile was added for the extraction of the analytes. The mixture was vortexed for 15 min and centrifuged for 5 min at 13000 rpm. The organic phase containing the analytes was recovered and evaporated to dryness under $N_2$ stream. The residue was reconstituted using 250 µL of the initial mobile phase. Finally, it was centrifuged at 13000 rpm and analysed by ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) [22].

### 2.3.2. Determination of bisphenols and parabens in urine

The method that was carried out for the extraction and determination of the compounds of interest in urine is described in Moscoso-Ruiz et al. (2022) [25]. Four mL of the urine sample were taken and ß-glucuronidase and ß-glucuronidase/arylsulphatase enzymes were added. Then, 4 mL of 10% (w/v) NaCl and 100 µL of hydrochloric acid (HCl) (6 N) were added. At this point, a dispersive liquid-liquid microextraction was performed by adding 1 mL of the extraction solvents (60% chloroform and 40% v/v acetone). The mixture was shaken gently and centrifuged for 5 min at 4000 rpm. The sedimented phase was recovered and transferred to a glass tube. This extraction process was repeated three more times and the total final organic phase was evaporated to dryness. The dried residue was reconstituted with 100 µL of the initial mobile phase. Finally, it was centrifuged at 13000 rpm for 5 min and analysed by UHPLC-MS/MS [25].

On the other hand, creatinine levels were determined in urine samples. The analysis was performed by the Ángel Méndez Soto Clinical Analysis Laboratory. The classical Jaffé method was used for the determination, which is based on the photometric measurement of the reaction kinetics of creatinine with picric acid at 37°C [26, 27]. The reagent kit was purchased from Biosystems (Barcelona, Spain).

### 2.4. Statistical analysis

The distribution of continuous and parametric variables was summarised by mean and standard deviation (SD), while the distribution of continuous and non-parametric variables was summarised by median and interquartile range (IQR). Frequency distributions were calculated for categorical variables. To assess differences between cases and controls for all variables, Student's t-test (for continuous parametric variables), Mann-Whitney U-test (for continuous non-parametric variables) and Pearson's Chi-square test (categorical variables) were used. The correlation between the concentration of bisphenols and parabens in hair (ng $g^{-1}$) and urine (ng $mL^{-1}$) was determined by Spearman correlation coefficients.

Finally, a logistic regression model was used to analyze the influence of bisphenol and paraben concentrations (ng $g^{-1}$) on hair as the independent variable, and on overweight and obesity as the dependent variable. The independent variables were dichotomized according to the median value (reference category: concentration ≤ median value). When the percentage of undetected concentrations of an analyte was >30%, the cutoff point for dichotomization was the limit of detection (LOD)/√2 [28-31]. ORs and 95% confidence interval (95% CI) were calculated for the crude and adjusted models. Sex, age, energy intake, smoking, physical activity, and body fat percentage were considered confounders in the adjusted models [30-35]. Confounders were examined separately, and those that modified the OR of the crude model ± 10% were used in the fitted model.

All statistical analyses were performed with IBM SPSS (version 26.0, IBM® SPSS® Statistics, Armonik, NY, USA). Statistical significance was set at $p \leq 0.05$.

## 3. Results

Table 1a shows the characteristics of the total population, where significant differences were found in variables such as age, smoking habits of the members of the family unit, physical activity and percentage of body fat. It was observed that, in the case group, both smoking habit and body fat percentage showed higher values compared to the controls, while the control group showed higher scores in physical activity (median controls = 74.1 *vs* median cases = 53.8; $p = 0.001$). Table 1b shows the characteristics of the study population stratified by sex. The same differences and significance were observed as in Table 1a. Except in girls, where the physical activity variable did not show statistically significant differences between cases and controls ($p = 0.094$).

**Table 1a.** General characteristics of general population (n=270).

| | | Controls (n=166) | Cases (n=104) | *p*-Value |
|---|---|---|---|---|
| Gender (%) | Male | 49.4 | 48.1 | 0.833[a] |
| | Female | 50.6 | 51.9 | |
| Age, years | Mean | 7.21 | 8.89 | **<0.001[b]** |
| | SD | 2.48 | 2.54 | |
| Energy Intake, Kcal/day | Mean | 2092.2 | 2057.4 | 0.647[c] |
| | SD | 514.5 | 568.1 | |
| Smoking among family members (%) | No | 87.9 | 56.7 | **<0.001[a]** |
| | Yes | 12.1 | 43.3 | |
| Physical Activity (out-of-school) (%) | No | 25.9 | 46.2 | **0.001[a]** |
| | Yes | 74.1 | 53.8 | |
| Body fat percentage | Median | 20.3 | 33.6 | **<0.001[b]** |
| | IQR | 17.2 – 22.7 | 29.6 – 38.5 | |

*IQR: interquartile range (percentile 25$^{th}$ – percentile 75$^{th}$); SD: standard deviation; [a]Chi-square test; [b]U Mann-Whitney test; [c]Student test; p≤0.05 are highlighted in bold.*

**Table 1b.** General characteristics of boys and girls.

| | | Boys (n=132) | | | Girls (n=138) | | |
|---|---|---|---|---|---|---|---|
| | | Controls (n=82) | Cases (n=50) | *p*-Value | Controls (n=84) | Cases (n=54) | *p*-Value |
| Age, years | Mean | 7 | 8.9 | **<0.001[a]** | 7.4 | 8.9 | **0.001[a]** |
| | SD | 2.5 | 2.5 | | 2.5 | 2.6 | |
| Energy intake, Kcal/day | Mean | 2087.9 | 2226.1 | 0.212[b] | 2096.7 | 1901.4 | 0.059[b] |
| | SD | 473.1 | 654.0 | | 557.5 | 426.7 | |
| Physical activity (out-of-school) (%) | No | 19.5 | 46 | **0.001[c]** | 32.1 | 46.3 | 0.094[c] |
| | Yes | 80.5 | 54 | | 67.9 | 53.7 | |
| Smoking among members of the family unit (%) | No | 92.6 | 50 | **<0.001[c]** | 83.3 | 63 | **0.007[c]** |
| | Yes | 7.4 | 50 | | 16.7 | 37 | |
| Body fat percentage | Median | 18.1 | 33.6 | **<0.001[a]** | 21.7 | 33.7 | **<0.001[a]** |
| | IQR | 15.6 – 21 | 29 – 38 | | 20 – 24 | 30 – 39 | |

*IQR: interquartile range (percentile 25th – percentile 75th); [a]U Mann-Whitney test; [b]Student test; [c]Chi-square test; p≤0.05 are highlighted in bold.*

Tables 2a and 2b show the detected concentrations of bisphenols and parabens in hair samples from the general population and stratified by sex. Only the concentrations of those analytes that were detected in the present matrix (concentration > LOD) are shown. The results described here were not statistically significant for either the general or sex-stratified population.

**Table 2a.** Bisphenols and parabens concentration in hair (ng $g^{-1}$) in general population (n=270).

| | Controls | Cases | |
|---|---|---|---|
| | Median (IQR) | Median (IQR) | *p*-Value |
| **Bisphenoles** | | | |
| **BPA** | 206.9 (123.3 – 406.1) | 216.1 (94.2 – 472.3) | 0.820 |
| **BPS** | 96.6 (59.5 – 218.1) | 81.2 (5.7 – 161.5) | 0.065 |
| **BPAF** | 14.1 (10.1 – 20.5) | 12.4 (0.2 – 34.7) | 0.589 |
| **Total Bisphenols** | 373 (225 – 814.6) | 353 (192 – 730.7) | 0.790 |
| **Parabens** | | | |
| **MetPB** | 1411 (873.1 – 3460.3) | 1687.8 (866.8 – 4668) | 0.447 |
| **EthPB** | 66.1 (31 – 260.5) | 82.4 (37.1 – 299.8) | 0.490 |
| **PropPB** | 52.8 (5.8 – 259.1) | 77.9 (19.6 – 266.6) | 0.341 |
| **IsoPropPB** | 2.6 (0.2 – 8.3) | 2.6 (0.2 – 11.6) | 0.764 |
| **Total Parabens** | 1803.3 (1050.4 – 4016.8) | 1971.7 (999.1 – 6137.4) | 0.377 |

*IQR: interquartile range (percentile 25$^{th}$ – percentile 75$^{th}$); p-Values show bisphenols intake significant differences between cases and controls, by U de Mann-Whitney test; p≤0.05 are highlighted in bold; BPA: bisphenol A; BPS: bisphenol S; BPAF: bisphenol AF; MetPB: methylparaben; EthPB: ethylparaben; isoPropPB: isopropylparaben; PropPB: propylparaben*

Regarding bisphenols in Table 2b, it shows for BPA and total bisphenols in boys and for BPS in girls' higher concentrations in cases.

While for BPS and BPAF in boys and BPA, BPAF and total bisphenols in girls showed higher concentrations in the control group. As for parabens, most of them showed higher concentrations in the case group compared to controls. However, the detected concentrations of PropPB in girls and isoPropPB in both sexes were higher in the control group.

**Table 2b.** Bisphenols and parabens concentration in hair (ng $g^{-1}$) in boys and girls.

| | Boys (n=132) | | | Girls (n=138) | | |
|---|---|---|---|---|---|---|
| | Controls (n=82) | Cases (n=50) | | Controls (n=84) | Cases (n=54) | |
| | Median (IQR) | Median (IQR) | *p* Value | Median (IQR) | Median (IQR) | *p* Value |
| **Bisphenols** | | | | | | |
| **BPA** | 170.5 (122.7 – 380) | 284.7 (105.5 – 658) | 0.281 | 234.2 (122 – 427) | 205.2 (88.4 – 380) | 0.446 |
| **BPS** | 98.5 (59.2 – 239.9) | 76.4 (0.2 – 162.1) | 0.076 | 92.2 (60.5 – 210.8) | 95 (23.7 – 163) | 0.406 |
| **BPAF** | 12.8 (0.2 – 19.8) | 11.2 (0.2 – 23.5) | 0.358 | 14.8 (10.7 – 21.5) | 14.1 (0.2 – 44.3) | 0.893 |
| **Total Bisphenols** | 348.3 (225.3 – 834) | 361.9 (206 – 1010.3) | 0.894 | 392.7 (200.5 – 793) | 327.1 (182 – 649.4) | 0.664 |
| **Parabens** | | | | | | |
| **MetPB** | 1411 (748.1 – 3891) | 1707.4 (883 – 4106.5) | 0.466 | 1422.3 (887 – 3279.6) | 1462.6 (831 – 5856) | 0.834 |
| **EthPB** | 61.9 (29.6 – 355.8) | 91.1 (36.6 – 267.7) | 0.755 | 70.4 (32.6 – 245.5) | 81.8 (37.6 – 368) | 0.481 |
| **PropPB** | 33.6 (1 – 277.9) | 89.7 (23.6 – 273) | 0.119 | 84.1 (13.4 – 252.6) | 60.8 (18.5 – 221) | 0.722 |
| **IsoPropPB** | 2.1 (0.2 – 7.2) | 0.2 (0.2 – 12.1) | 0.866 | 3.4 (0.2 – 10.5) | 3.3 (0.2 – 10.8) | 0.877 |
| **Total Parabens** | 1672.5 (865.9 – 4371) | 1971.7 (1245 – 5239) | 0.327 | 1919.5 (1134 – 3996) | 2081.3 (980 – 7012) | 0.804 |

*IQR: interquartile range (percentile 25th – percentile 75th); p-Values show bisphenols intake significant differences between cases and controls, by U de Mann-Whitney test; p≤0.05 are highlighted in bold; BPA: bisphenol A; BPS: bisphenol S; BPAF: bisphenol AF; MetPB: methylparaben; EthPB: ethylparaben; isoPropPB: isopropylparaben; PropPB: propylparaben*

The correlations between the concentrations of bisphenols and parabens determined in hair and urine, both for the total population and stratified by sex, are shown below (Table 3). Six statistically significant and direct correlations were identified between the concentrations of bisphenols and parabens in both biological matrices. These correlations

were observed for isoPropPB in the total population, in boys and girls, for total parabens in girls and for BPAF in the total population and in boys. In most cases, the correlation coefficient was less than 0.600, indicating low dependence between variables with significant correlations. With the exception of isoPropPB in boys, with a coefficient of 0.652 ($p < 0.001$), indicating a moderate correlation between the presence of isoPropPB in hair and urine. The urine concentrations used to establish these correlations are given in Supplementary Tables S1a and S1b.

**Table 3.** Correlations between concentration of bisphenols and parabens in hair – urine in total populations and in boys and girls.

| | | MetPB | EthPB | PropPB | isoPropPB | Total Parabens | BPA | BPS | BPAF | Total Bisphenols |
|---|---|---|---|---|---|---|---|---|---|---|
| **Total populations** | **Spearman´s coefficient** | 0.007 | 0.144 | 0.016 | 0.496 | 0.100 | -0.035 | -0.183 | 0.217 | -0.005 |
| | ***p-Value*** | 0.938 | 0.102 | 0.855 | **<0.001** | 0.256 | 0.696 | 0.165 | **0.013** | 0.951 |
| **Boys** | **Spearman´s coefficient** | -0.144 | 0.078 | -0.113 | 0.652 | -0.104 | -0.032 | -0.043 | 0.321 | 0.025 |
| | ***p-Value*** | 0.263 | 0.534 | 0.376 | **<0.001** | 0.408 | 0.800 | 0.819 | **0.009** | 0.842 |
| **Girls** | **Spearman´s coefficient** | 0.166 | 0.177 | 0.178 | 0.326 | 0.308 | -0.032 | -0.290 | 0.112 | -0.049 |
| | ***p-Value*** | 0.193 | 0.159 | 0.160 | **0.008** | **0.013** | 0.798 | 0.134 | 0.376 | 0.699 |

BPA: bisphenol A; BPS: bisphenol S; BPAF: bisphenol AF; MetPB: methylparaben; EthPB: ethylparaben; isoPropPB: isopropylparaben; PropPB: propylparaben; $p \leq 0.05$ are highlighted in bold

**Supplementary Table S1.** Bisphenols and parabens concentration in urine (ng $mL^{-1}$) in general population.

| | Controls | Cases | |
|---|---|---|---|
| | Median (IQR) | Median (IQR) | *p*-Value |
| **Bisphenols** | | | |
| BPA | 0.3 (0.1 - 1.6) | 0.4 (0.1 - 1.3) | 0.825 |
| BPS | 0 (0 - 0.1) | 0 (0 - 0) | 0.266 |
| BPAF | 0 (0 - 0) | 0 (0 - 0) | 0.475 |
| Total Bisphenols | 1.4 (1.2 - 2.8) | 1.6 (1.2 - 2.8) | 0.659 |
| **Parabens** | | | |
| MetPB | 3.9 (2.1 - 8.3) | 4.7 (2.3 - 21.5) | 0.389 |
| EthPB | 0 (0 - 0.1) | 0 (0 - 0) | 0.356 |
| PropPB | 0 (0 - 0) | 0 (0 - 1.8) | 0.060 |
| IsoPropPB | 0 (0 - 0) | 0 (0 - 0) | **0.023** |
| Total Parabens | 4.3 (1.9 - 9.7) | 5 (2.7 - 27.9) | 0.058 |

*IQR: interquartile range (percentile 25th – percentile 75th); p-Values show bisphenols and parabens intake significant differences between cases and controls, by U de Mann-Whitney test; p≤0.05 are highlighted in bold; BPA: bisphenol A; BPS: bisphenol S; BPAF: bisphenol AF; MetPB: methylparaben; EthPB: ethylparaben; isoPropPB: isopropylparaben; PropPB: propylparaben.*

**Supplementary Table S2.** Bisphenols and parabens concentration in hair (ng $g^{-1}$) in boys and girls.

| | Boys (n=132) | | | Girls (n=138) | | |
|---|---|---|---|---|---|---|
| | Controls (n=82) | Cases (n=50) | | Controls (n=84) | Cases (n=54) | |
| | Median (IQR) | Median (IQR) | *p*-Value | Median (IQR) | Median (IQR) | *p*-Value |
| **Bisphenols** | | | | | | |
| BPA | 0.5 (0.1 - 2.1) | 0.3 (0.1 - 1.6) | 0.435 | 0.2 (0.1 - 0.8) | 0.5 (0.1 - 0.8) | 0.646 |
| BPS | 0 (0 - 0) | 0 (0 - 0) | 0.922 | 0 (0 - 0.1) | 0 (0 - 0) | 0.468 |
| BPAF | 0 (0 - 0) | 0 (0 - 0) | 0.420 | 0 (0 - 0) | 0 (0 - 0) | 0.985 |
| Total Bisphenols | 1.6 (1.2 - 3.7) | 1.6 (1.3 - 3) | 0.936 | 1.4 (1.2 - 2) | 1.7 (1.2 - 2.7) | 0.56 |
| **Parabens** | | | | | | |
| MetPB | 3.5 (2.3 - 7.2) | 4.7 (2.5 - 21) | 0.298 | 4 (1.7 - 10.1) | 4.5 (2.2 - 23.6) | 0.801 |
| EthPB | 0 (0 - 0.1) | 0 (0 - 0) | 0.334 | 0 (0 - 0.1) | 0 (0 - 0.1) | 0.809 |
| PropPB | 0 (0 - 0) | 0.8 (0 - 2.4) | **0.008** | 0 (0 - 1.6) | 0 (0 - 1.6) | 0.894 |

| | | | | | | |
|---|---|---|---|---|---|---|
| IsoPropPB | 0 (0 - 0) | 0 (0 - 0) | **0.009** | 0 (0 - 0) | 0 (0 - 0.1) | 0.700 |
| Total Parabens | 3.8 (1.9- 7.3) | 5 (2.8 - 19.5) | 0.148 | 4.9 (1.9 - 11.4) | 5.8 (2.6 - 38.2) | 0.197 |

*IQR: interquartile range (percentile 25th – percentile 75th); p-Values show bisphenols and parabens intake significant differences between cases and controls, by U de Mann-Whitney test; p≤0.05 are highlighted in bold; BPA: bisphenol A; BPS: bisphenol S; BPAF: bisphenol AF; MetPB: methylparaben; EthPB: ethylparaben; isoPropPB: isopropylparaben; PropPB: propylparaben.*

Tables 4a and 4b show the influence of the concentrations of bisphenols and parabens determined in hair on overweight and obesity in the total population and differentiated by sex. According table 4a, association between PropPB and overweight/obesity keeps the statistical significance in the crude and adjusted models, for boys. Furthermore, a negative relationship between exposure to total bisphenols and the probability of developing overweight or obesity was observed in male (p= 0.039; OR: 0.09). The rest of the analytes determined in the total population or according to sex, did not show statistically significant associations (Tables 4a and 4b).

**Table 4a.** Influence of higher concentration to bisphenols and parabens in hair on overweight/obesity in the total population (n = 270).

| | Crude | | | Adjusted | | |
|---|---|---|---|---|---|---|
| | *p* Value | OR | 95% CI | *p* Value | OR | 95% CI |
| **Bisphenols** | | | | | | |
| BPA (Ref. Low exposure*) High exposure** | 0.758 | 1.08 | 0.66 – 1.76 | 0.289 | 0.66[f] | 0.30 – 1.43 |
| BPS (Ref. Low exposure) High exposure | 0.356 | 0.79 | 0.49 -1.30 | 0.596 | 0.85[b] | 0.46 – 1.56 |
| BPAF (Ref. Low exposure) High exposure | 0.645 | 0.89 | 0.55 – 1.46 | 0.230 | 0.69[b] | 0.38 – 1.26 |
| Total Bisphenols (Ref. Low exposure) High exposure | 0.538 | 0.86 | 0.53 – 1.40 | 0.142 | 0.47[d] | 0.17 – 1.29 |
| **Parabens** | | | | | | |
| MetPB (Ref. Low exposure) High exposure | 0.218 | 1.36 | 0.83 – 2.23 | 0.791 | 1.14[e] | 0.43 – 3.07 |
| EthPB (Ref. Low exposure) | 0.442 | 1.21 | 0.74 – 1.98 | 0.683 | 1.11[a] | 0.66 – 1.87 |

| | | | | | | |
|---|---|---|---|---|---|---|
| High exposure | | | | | | |
| PropPB (Ref. Low exposure) | 0.218 | 1.36 | 0.83 – 2.23 | 0.164 | 1.53[b] | 0.84 – 2.80 |
| High exposure | | | | | | |
| isoPropPB (Ref. Low exposure) | 1.000 | 1.00 | 0.61 – 1.63 | 0.852 | 0.91[c] | 0.34 – 2.45 |
| High exposure | | | | | | |
| Total parabens (Ref. Low exposure) | 0.442 | 1.21 | 0.74 – 1.98 | 0.807 | 1.13[c] | 0.42 – 3.05 |
| High exposure | | | | | | |

*Ref.: reference category; OR: odds ratio; 95% CI: 95% confidence intervals. p-Values <0.05 are highlighted in bold*

*[a]Analytes in hair were adjusted for gender and age; [b]Analytes in hair were adjusted for gender, age and energy intake; [c]Analytes in hair were adjusted for gender, age, energy intake, smoking among family members and body fat percentage; [d]Analytes in hair were adjusted for gender, age, energy intake, physical activity and body fat percentage; [e]Analytes in hair were adjusted for gender, age, energy intake and body fat percentage; [f]Analytes in hair were adjusted for gender, age, physical activity and body fat percentage.*

**= low exposure (≤ median); **= high exposure (>median).*

*BPA: bisphenol A; BPS: bisphenol S; BPAF: bisphenol AF; MetPB: methylparaben; EthPB: ethylparaben; isoPropPB: isopropylparaben; PropPB: propylparaben*

**Table 4b.** Influence of higher concentration to bisphenols and parabens in hair on overweight/obesity in boys and girls.

| | Boys (n=132) | | | | | | Girls (n=138) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Crude | | | Adjusted | | | Crude | | | Adjusted | | |
| | *p*-Value | OR | 95% CI | *p*-Value | OR | 95% CI | *p*-Value | OR | 95% CI | *p*-Value | OR | 95% CI |
| **Bisphenols** | | | | | | | | | | | | |
| BPA (Ref. Low exposure*) High exposure** | 0.323 | 1.43 | 0.71 – 2.89 | 0.110 | 0.18[d] | 0.02 – 1.47 | 0.585 | 0.83 | 0.42 – 1.64 | 0.526 | 0.65[g] | 0.18 – 2.44 |
| BPS (Ref. Low exposure) High exposure | 0.108 | 0.56 | 0.27 – 1.14 | 0.067 | 0.18[e] | 0.03 – 1.13 | 0.785 | 1.10 | 0.55 – 2.18 | 0.876 | 1.11[g] | 0.28 – 4.37 |
| BPAF (Ref. Low exposure) High exposure | 0.991 | 1.00 | 0.49 – 2.04 | 0.117 | 0.20[g] | 0.03 – 1.49 | 0.494 | 0.79 | 0.40 – 1.56 | 0.957 | 1.03[i] | 0.40 – 2.65 |
| Bisphenols Total (Ref. Low exposure) High exposure | 0.931 | 0.97 | 0.48 – 1.96 | **0.039** | 0.09[d] | 0.01 – 0.88 | 0.439 | 0.76 | 0.38 – 1.52 | 0.622 | 0.84[a] | 0.41 – 1.71 |
| **Parabens** | | | | | | | | | | | | |
| MetPB (Ref. Low exposure) High exposure | 0.118 | 1.78 | 0.86 – 3.66 | 0.176 | 1.86[b] | 0.76 – 4.59 | 0.832 | 1.07 | 0.54 – 2.13 | 0.433 | 0.59[g] | 0.16 – 2.18 |
| EthPB (Ref. Low exposure) High exposure | 0.394 | 1.36 | 0.67 – 2.75 | 0.564 | 1.60[f] | 0.32 – 7.89 | 0.819 | 1.08 | 0.55 – 2.15 | 0.711 | 1.17[b] | 0.50 – 2.73 |
| PropPB (Ref. Low exposure) High exposure | **0.016** | 2.42 | 1.17 – 4.99 | **0.039** | 4.67[h] | 1.08 – 20.11 | 0.500 | 0.79 | 0.40 – 1.57 | 0.943 | 1.05[e] | 0.29 – 3.75 |
| isoPropPB (Ref. Low exposure) High exposure | 0.691 | 0.87 | 0.43 – 1.76 | 0.600 | 1.55[d] | 0.30 – 7.87 | 0.715 | 1.14 | 0.57 – 2.26 | 0.794 | 0.88[c] | 0.36 – 2.17 |
| Total parabens (Ref. Low exposure) High exposure | 0.245 | 1.52 | 0.75 – 3.10 | 0.527 | 1.36[c] | 0.52 – 3.56 | 0.952 | 0.98 | 0.49 – 1.94 | 0.715 | 0.79[e] | 0.21 – 2.89 |

Ref.: reference category; OR: odds ratio; 95% CI: 95% confidence intervals. *p*-Values <0.05 are highlighted in bold

[a]Analytes in hair were adjusted for age; [b]Analytes in hair were adjusted for age and energy intake; [c]Analytes in hair were adjusted for age, energy intake and smoking among family members; [d]Analytes in hair were adjusted for age, energy intake, smoking among family members, physical activity and body fat percentage; [e]Analytes in hair were adjusted for age, energy intake, smoking among family members and body fat percentage; [f]Analytes in hair were adjusted for age, energy intake, physical activity and body fat percentage; [g]Analytes in hair were adjusted for age, energy intake and body fat percentage; [h]Analytes in hair were adjusted for age, smoking among family members and body fat percentage; [i]Analytes in hair were adjusted for age and body fat percentage.

*= low exposure (≤ median); **= high exposure (>median).

BPA: bisphenol A; BPS: bisphenol S; BPAF: bisphenol AF; MetPB: methylparaben; EthPB: ethylparaben; isoPropPB: isopropylparaben; PropPB: propylparaben

## 4. Discussion

The present investigation focused on determining the influence of bisphenol and paraben concentrations in hair on overweight and obesity in a school population. The main findings were that, in boys, those with higher concentrations of PropPB were 4.67 times more likely to be overweight or obese. In addition, it was observed that a higher concentration of total bisphenols in hair was inversely associated with the probability of developing overweight or obesity in boys.

To our knowledge, no studies have been found that have investigated the influence of bisphenol and paraben concentrations in the hair of a child population and their role in overweight or obesity, except for one study that correlated BPA levels detected in the hair of adults and children with BMI [36]. This study examined exposure to BPA and other EDCs (triclosan and perfluorooctanoic acid) of 122 individuals (72 children and 50 adults) and found no associations between BPA and BMI [36], agreeing with our findings, as we were also unable to establish such an association between BPA and overweight/obesity in schoolchildren.

Currently, scientific evidence is scarce, being higher for BPA compared to its analogs and parabens. In relation to this, several studies have investigated the joint influence of bisphenols and parabens on childhood overweight and obesity, analyzing urinary levels during the prenatal, postnatal and/or childhood period [37-41]. In our study, there was a tendency to associate a higher concentration of parabens detected in hair with an increased likelihood of overweight/obesity, whereas bisphenols were negatively associated with weight gain in schoolchildren. In contrast to previous studies, a longitudinal cohort study by Berger et al. (2021) [39] analyzed the concentrations of BPA and several parabens in maternal urine, among other EDCs, and found that prenatal exposure correlated with children's BMI at five years of age. In this study, higher prenatal exposure to PropPB was found to be associated with an

increased likelihood of becoming overweight or obese during early childhood [39].

Another study, which also measured exposure to BPA and parabens during the prenatal period through maternal urine, found that the detected concentrations of the parabens (MetPB, EthPB, PropPB, and ButPB), as well as the sum, tended to be positively associated with higher weight [37]. However, no clear associations were observed between BPA and prenatal and postnatal obesity [37]. Ouidir et al. (2024) [41] conducted a longitudinal study and found an association between postnatal exposure to a mixture of EDCs (phenols and parabens) and increased BMI at 3 years of age. Furthermore, BPS exposure during the second trimester of pregnancy was found to be positively associated with all infant growth parameters [41].

On the other hand, a study conducted in India in 49 obese and 27 non-obese children (aged 2-14 years) found no statistically significant associations between childhood obesity and urinary concentrations of bisphenols and parabens [38]. Other study carried out in a Canadian childhood population evaluated possible associations between mixtures to various EDCs to better understand the combined role of these chemical compounds in childhood weight gain [40]. Dugandzic et al (2024) [40] showed that exposure to mixed BPA, parabens, and other EDCs increased the risk of childhood overweight or obesity by 45%, obesity by 109%, and abdominal obesity by 82%.

In relation to the sexual dysmorphic effect, some studies have found that these EDCs act differently according to sex. In the present work, it was observed that, in males, bisphenols tended to be negatively associated with overweight/obesity, whereas parabens showed a positive association. The most relevant findings indicated that higher concentrations of PropPB were positively associated with an increased likelihood of being overweight or obese, while higher exposure to total bisphenols was inversely associated with this likelihood. Similarly, several previous studies also observed isolated sex-specific associations

[34, 41-44]. In one of the studies, it was observed that MetPB exposure in the third trimester tended to be negatively associated with BMI at 3 years of age, with the association being stronger in boys [41]. In the case of girls, no statistically significant differences were found in our findings. However, in the study by Ouidir et al. (2024) [41], it was observed that BPA exposure during the third trimester and at 2 months of pregnancy tended to be associated with decreased weight in 3-month-old and 3-year-old girls, respectively [41]. In contrast, two studies in schoolchildren showed that urinary BPA concentrations were significantly associated with increasing BMI values in girls between 8 and 12 years of age [42, 43]. Furthermore, a study in an adolescent population observed a positive association between dietary exposure to BPA and overweight/obesity in adolescent girls [34], but not in boys. Regarding parabens, in the present work, the presence of total parabens in girls' hair was negatively associated with higher body weight. However, another study in adolescents observed a positive association between dietary exposure to total parabens and overweight/obesity in adolescent girls [44].

These findings, both stratified and not stratified by sex, demonstrate the growing concern for the role of bisphenols and parabens, as well as the combined role, in obesity and overweight. This supports the relevance of investigating these EDCs prenatally, postnatally, and in childhood, as well as by sex. However, it is crucial to highlight that the existing contrast between studies linking bisphenol and paraben exposure in children and their role in overweight/obesity may be due to several factors [45]: (i) differences in study design (methodology used, population sample size, type of biological sample and duration of follow-up); (ii) variability in exposure to these EDCs (source and dose of exposure); (iii) individual susceptibility (genetics and health status); (iv) differences in the populations studied (sociodemographic and socioeconomic). All these factors may contribute to discrepancies between studies.

Regarding the detection of EDCs in the present study, MetPB was the main compound detected in hair (100%, Supplementary Table S2),

with a concentration range from 866.8 - 4668.0 ng $g^{-1}$, followed by EtPB and BPA, whose levels ranged from 30.9 - 299.8 ng $g^{-1}$ and 94.2 - 472.3 ng $g^{-1}$, respectively. The total levels of parabens in hair were higher compared to bisphenols. These findings agree with those reported in previous studies [46, 47]. In these two studies MetPB was detected in 100% of the samples coinciding with our findings. However, the concentration levels were much higher in the work by Martin et al. (2019) [46] ranging from 68.3 - 14187.3 ng $g^{-1}$, while those determined by Robin et al. (2024) [47] were lower (1.0 - 648 ng $g^{-1}$). The high presence of MetPB or other parabens could be explained by the daily exposure to personal care, hygiene and food products, where they are widely used as preservatives due to their antimicrobial activity both in the cosmetic and pharmaceutical industry and in the food industry [4, 7]. As for their use as food additives, only MetPB and EthPB (E214 and E218), together with their salts (E215 and E219), are allowed [48].

**Supplementary Table S2.** Frecuency of detection (%) of parabens and bisphenols in hair.

| | Frecuency of detection (%) |
|---|---|
| **Parabens** | |
| MetPB | 100 |
| EthPB | 99 |
| PropPB | 81 |
| IsoPropPB | 54 |
| **Bisphenols** | |
| BPA | 98 |
| BPS | 82 |
| BPAF | 73 |

*BPA: bisphenol A; BPS: bisphenol S; BPAF: bisphenol AF; MetPB: methylparaben; EthPB: ethylparaben; isoPropPB: isopropylparaben; PropPB: propylparaben.*

In relation to bisphenols, some studies have analysed the presence of BPA in hair. The concentrations found were between 2.6 - 205.5 ng $g^{-1}$

[36], 13.1 - 72.8 ng $g^{-1}$ [49], and 24.4 - 1427.5 ng $g^{-1}$ [46], with the concentrations in our population being somewhat similar (94.2 - 472.3 ng $g^{-1}$), with the exception of the study by Tzatzarakis et al. (2015) [49], which showed the much higher concentration value (1427.5 ng $g^{-1}$). Other studies conducted in 2019 and 2024 analysed both the presence of BPA and BPS, BPF and BPB in hair, with BPA and BPS being the most frequently detected, with BPS having the highest concentrations [47, 50], with the opposite being observed in the present study. This may be because France is one of the pioneer countries in imposing restrictions on the use of BPA, which has led French industries to replace BPA with BPS before other European Union countries.

The reason why BPA remains the most frequently detected bisphenol in some biological samples [31] is its ubiquity in the environment, with the oral route through ingestion of food, water and beverages being the main route of entry [51–53]. These compounds are found in a wide variety of foods, including fresh foods, mainly due to environmental contamination and the use of bisphenol-containing materials in packaging [51, 52]. In recent years, the use of BPA in certain products has begun to be banned and its tolerable daily intake has recently been reevaluated, reducing it from 4 mg/kg bw/day to 0.2 ng/kg bw/day, which represents an approximately 20000-fold decrease [54, 55]. Due to these measures, it is to be expected and it is beginning to be observed that analogues are detected more frequently and in higher concentrations than BPA.

On the other hand, higher concentrations of bisphenols and parabens were found in hair samples compared to urine, a finding that was also observed in the studies of Robin et al. (2024) [47] and Fäys et al. (2021) [50]. Regarding correlations between MetPB concentrations in hair and urine samples, no correlations were observed, as in previous studies [47, 50]. Moderate-high or significant correlations were also not found in most of the compounds analysed, except in the cases of isoPropPB-isoPropPB in boys and isoPropPB-PropPB in girls, where moderate and significant

positive correlations were observed. This could be explained by the similarity of the chemical properties of the compound, being the same or very similar. The lack of correlation between both matrices has also been reported in two previous studies, which assessed exposure to other environmental chemical contaminants (pesticides) through hair and urine [56, 57]. A possible explanation could be due to differences in the time window covered by each of the samples [56, 57].

Human biomonitoring is an essential tool for assessing exposure to environmental chemical contaminants. Urine along with blood have been the main matrices used in human biomonitoring [21]. However, they are not the most suitable matrices for assessing long-term exposure to EDCs with a short elimination half-life [21, 58, 59]. Since many EDCs especially those to which we are exposed daily, can accumulate in the body [35, 60, 61], their accumulation in body tissues, such as adipose tissue [60, 61], or even in organs such as the brain [35], has been studied. The accumulation of these bisphenols and parabens may not be directly reflected in urine, as urine only shows what the body is eliminating at that specific time [38]. To minimize this limitation, if urine is chosen as the matrix, it would be desirable to analyse urine samples collected longitudinally at various times of the day and over an extended period, which could provide more accurate exposure estimates. It is important to highlight that, for the study of chronic diseases, the real impact on health comes from these accumulated substances, which can cause long-term negative effects [30, 51]. This may involve the use of other biological matrices, such as hair, nails, or specific tissues such as adipose tissue [22, 60 –62]. In the case of adipose tissue, it is invasive and difficult to collect. However, the use of faneras, such as hair and nails, is a very good alternative because its collection is non-invasive and painless [22, 46, 59], which is very important when working with child population. In addition, it is easy to store, transport and handle [22, 46, 62].

The present study presents several strengths. First, it highlights the use of a previously optimized method for the detection of EDCs in hair

[22]. Second, it uses a biological sample that reflects long-term exposure to EDCs, which is crucial to relate exposure to bisphenols and parabens to a chronic disease such as obesity. Finally, it is the first study that simultaneously determines bisphenols and parabens in hair and studies their relationship with overweight/obesity in children. However, our study is limited by its cross-sectional design, which may be the reason why it prevents us from establishing a causal relationship between the concentrations of bisphenols and parabens detected in hair and the probability of being overweight/obese during childhood. In addition, the small sample size may affect the interpretation and extrapolation of the findings.

Therefore, future longitudinal studies with larger population samples and more robust research designs are essential to assess the long-term effects of exposure to bisphenols and parabens on body composition in schoolchildren. Furthermore, the present research highlights the importance of considering simultaneous exposure to multiple contaminants during early childhood and their potential impact on child health, especially the risk of obesity. Additionally, more research is needed to better understand the underlying biological mechanisms and the complex interactions between various chemical compounds and environmental factors in relation to childhood obesity.

## 5. Conclusions

The present investigation showed that higher concentration levels of PropPB in hair were associated with a 4.67 times higher probability of being overweight/obese in boys. Likewise, it was observed that a lower concentration of total bisphenols in hair was correlated with overweight/obesity in boys. No statistically significant associations were shown for the rest of the analytes.

In conclusion, these results highlight the importance of investigating the effects of bisphenols and parabens on the most vulnerable populations and how these EDCs influence weight gain as a function of sex in

biological matrices reflecting long-term exposure. In addition, further longitudinal studies are needed to better understand the long-term impact of EDCs exposure in children. This highlights the need to implement public health strategies and policies that decrease EDCs exposure to reduce potential health risks.

## Declarations

**Ethics approval and consent to participate:** All parents or legal tutors of the study participants were fully informed about the present study and signed the informed consent. The present study has been approved by the ethics committees of the University of Granada and of the Provincial Biomedical Research of Granada (CEI), Spain (reference 1939-M1–22, Andalusian Biomedical Research Ethics Portal), and has been performed following the ethical standards.

**Author contributions:** YG-O: Data curation, Methodology, Formal analysis, Writing -original draft, Writing – review & editing, PG-P: Data curation, Methodology, Formal analysis, VR: Data curation, Methodology, Formal analysis, CM: Data curation, Methodology, Formal analysis, Writing -original draft, Writing – review & editing, CS-S: Investigation, Writing – review & editing, AR: Conceptualization, Project administration, Funding acquisition, Supervision, Writing – review & editing, AZ-G: Conceptualization, Project administration, Funding acquisition, Supervision, Writing – review & editing. All authors read and approved the final manuscript

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# CAPÍTULO IV.

## DEs en alimentos y su impacto en la microbiota y las enfermedades metabólicas

## Objetivos específicos del capítulo IV

1. Realizar una revisión de la literatura existente acerca del papel de los DEs sobre la disbiosis intestinal y las alteraciones en el metabolismo.
2. Desentrañar la compleja interacción entre los DEs, la microbiota humana y el desarrollo de enfermedades metabólicas.
3. Conocer los posibles mecanismos por los que los DEs influyen en la microbiota y contribuyen posteriormente en el desarrollo de enfermedades metabólicas.

## Introducción capítulo IV

El último capítulo de esta Memoria de Tesis Doctoral se centrará en una futura línea de investigación complementaria a la investigación realizada durante los años predoctorales. Este capítulo incluye una revisión de la literatura sobre del papel de los DEs en la microbiota y el desarrollo de enfermedades metabólicas.

Como ya se ha mencionado, los DEs pueden interferir en los sistemas hormonales, provocando numerosos efectos adversos sobre la salud. Sin embargo, en los últimos años, estudios recientes han empezado a desentrañar la compleja interacción entre los DEs, la microbiota humana y el desarrollo de enfermedades metabólicas. La microbiota humana es definida según el Instituto Nacional del Cáncer (NIH) como un «*conjunto de microorganismos y virus que viven en un ambiente dado, como el del cuerpo humano o en una parte de este, como es el aparato digestivo*». La microbiota desempeña un papel crucial en el mantenimiento de la homeostasis metabólica. Por ello, las alteraciones de los microorganismos que residen en el intestino se han relacionado con diversos trastornos metabólicos, como la obesidad, la diabetes y la enfermedad del hígado graso no alcohólico.

No obstante, los mecanismos precisos por los que los DEs influyen en la disbiosis intestinal y contribuyen posteriormente al desarrollo de las enfermedades metabólicas siguen siendo poco conocidos. Esta laguna en

el conocimiento pone de manifiesto la necesidad de futuras investigaciones para explorar esta compleja relación.

A continuación, se muestra una revisión de la literatura relacionada con la temática desarrollada en este capítulo.

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# Endocrine Disruptors in Food: Impact on Gut Microbiota and Metabolic Diseases

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**Abstract:** Endocrine disruptors (EDCs) have been associated with the increased incidence of metabolic disorders. In this work, we conducted a systematic review of the literature in order to identify the current knowledge of the interactions between EDCs in food, the gut microbiota, and metabolic disorders in order to shed light on this complex triad. Exposure to EDCs induces a series of changes including microbial dysbiosis and the induction of xenobiotic pathways and associated genes, enzymes, and metabolites involved in EDC metabolism. The products and by-products released following the microbial metabolism of EDCs can be taken up by the host; therefore, changes in the composition of the microbiota and in the production of microbial metabolites could have a major impact on host metabolism and the development of diseases. The remediation of EDC-induced changes in the gut microbiota might represent an alternative course for the treatment and prevention of metabolic diseases.

**Keywords:** endocrine disrupters; food; gut microbiota; metabolic diseases

## 1. Introduction

It has been widely reported that some exogenous compounds can interfere with the function of the endocrine system in the body. According

to the Endocrine Society, an Endocrine Disrupting Chemical (EDC) is "an exogenous [non-natural] chemical, or mixture of chemicals, that interferes with any aspect of hormone action" [1,2]. In this respect, the main source of human exposure to EDCs is food intake. These chemicals might pass into the food chain directly when they are used as pesticides, or they might be released from food packaging containing metals, bisphenol A, or phthalates. In addition, some plant-based compounds (the so-called phytoestrogens) found in dietary supplements also exhibit endocrine disrupting potential [3].

Endocrine disruptors have been associated with the increased incidence of metabolic disorders. It has been proposed that EDCs may increase the susceptibility to these disorders by altering the adipose tissue, pancreas, liver, gastrointestinal tract, muscle, and brain homeostatic and hedonic pathways [4]. However, few studies have reported that the effects of EDCs on the gut microbiota can increase the risk of metabolic disorders such as obesity and diabetes [5,6].

Emerging evidence suggests interactions between EDCs and the microbiome, which may affect host health. A key triad between exposure to EDCs, the host genotype and phenotypic responses, and the gut microbiome has been suggested [7]. Exposure to EDCs has been shown to disrupt the microbiome, which may result in dysbiosis and the induction of pathways related to xenobiotics, microbiome-associated genes, enzymes, and the production of metabolites, which may play a crucial role in EDC biotransformation [8]. The products and by-products released following the microbial metabolism of EDCs can be taken up by the host, therefore having an impact on host health and on the development of diseases. In addition, the gut microbiota can modify the EDC profiles through different plausible mechanisms. Microbial enzymes (esterases, thiolases, azoreductases, nitroreductases, ß-glucoronidases, methylases, sulfatases, lipases, and ß-lyases) can be used to metabolize different types of EDC [9]. Dysbiosis and a reduced diversity of the gut microbiota may cause a reduction in the enzymatic activity, which in turn could result in

a decreased metabolization of EDCs to their circulating, active forms, thereby reducing the potential EDC toxicity to the host.

In this work, we conduct a systematic review of the literature in order to identify the current knowledge regarding the interactions between the EDCs in food, thye gut microbiota and metabolic disorders, in order to shed light on this complex triad.

## 2. Methods

The PubMed and Web of Sciences databases were searched to identify the relevant studies. The following keywords were used: “Gut microbiota”, “Diet”, “Obesity”, “Diabetes”, “Bisphenol A”, “Bisphenol A analogs”, “Microbiota”, “Pesticides”, “Parabens”, “Polychlorinated biphenyls”, “Phytoestrogens”, “Metals”, “Cadmium”, “Arsenic”, “Lead”, “Phthalates”, “Triclosan”, and “Triclocarban”. Data published between 2006 and 2020 were considered.

The literature review was conducted in compliance with the recommendations provided in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). Figure 1 shows the PRISMA flow diagram that maps out the number of studies identified, those included and excluded, and the reasons for exclusion ((a) papers not written in the English language, (b) no outcome of interest, and (c) not related to the subject of the study). A total of 107 studies were included for analysis [10].

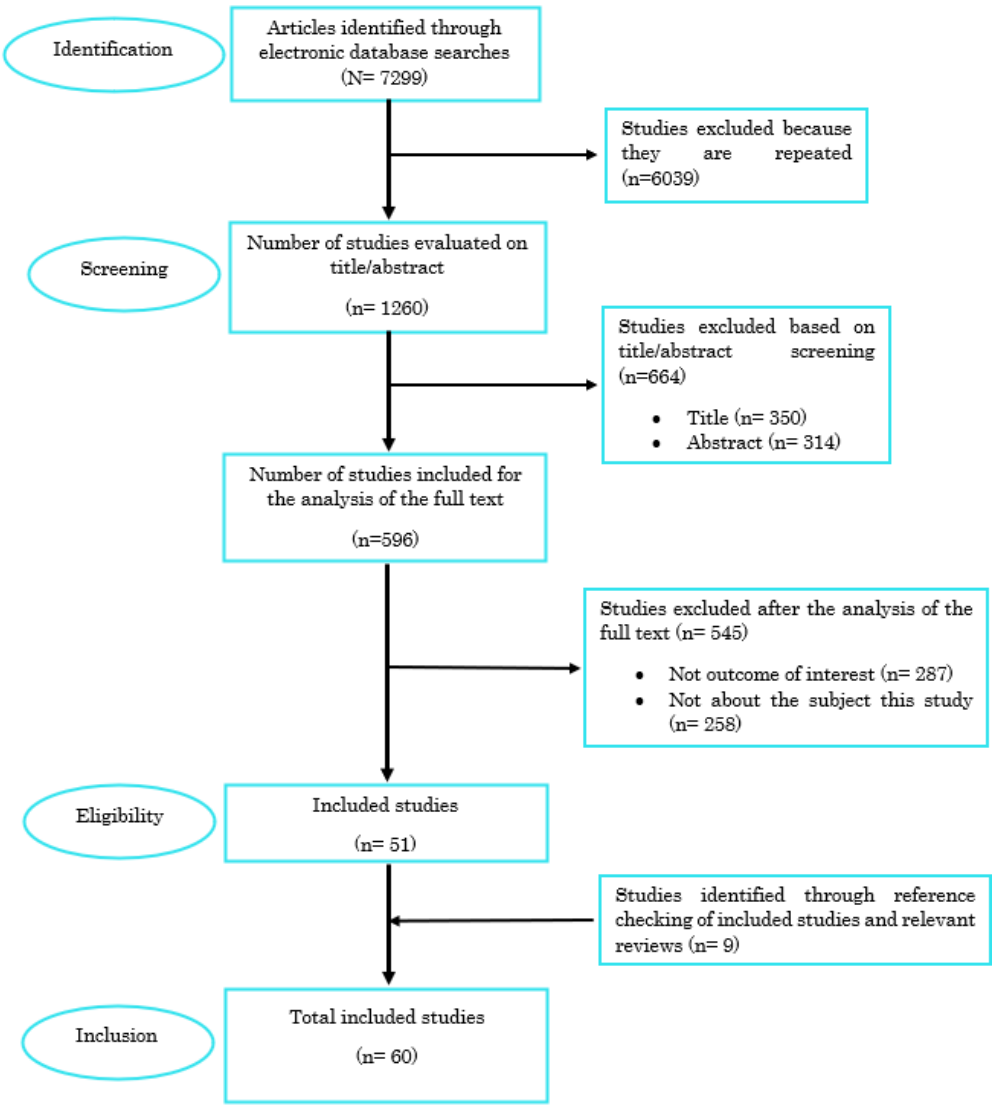


**Figure 1.** Flow diagram of the literature search.

## 3. Results and Discussion

### *3.1. The Gut Microbiota in Health and Metabolic Diseases*

A remarkable amount of evidence has emerged in recent years that strongly suggests that an essential role is played by the human microbiota in health and disease development via several mechanisms [11]. Variations in gut microbiota composition are considered to be

physiological from the perspective of healthy gut microbiota, and these changes are related to age; sex; and external factors such as dietary habits, exercise, and antibiotic use. Indeed, dysbiosis, defined as the alteration of gut microbiota communities, is often related to health disorders.

Firmicutes and Bacteroidetes are the two primary phyla in gut microbiota, accounting for 90% of the total composition [12]. Other phyla include Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia [13]. The Firmicutes phylum is composed of more than 200 different genera such as Enterococcus, Lactobacillus, Ruminicoccus, Bacillus, and Clostridium. The phylum Bacteroidetes is dominated by the genera Prevotella and Bacteroides. The Actinobacteria phylum is comparably less abundant and mostly represented by the Bifidobacterium genus [13].

In recent years, research has demonstrated that gut microbiota could play an important role in the pathophysiology of metabolic disorders, specifically in obesity and diabetes [14]. Animal studies have shown that obesity is related to changes in the gut microbiota composition, including a reduction in species variety and alterations in the genes involved in metabolism. By contrast, data related to the human microbiota are more variable [13]. When fed with a similar diet, the comparison of the gut microbiota of genetically obese (ob/ob) mice with that of lean mice showed a greater abundance of Firmicutes and lower relative abundance of Bacteriodetes (50%) in obese mice [15]. These changes in the microbiota have also been found in different human studies [16,17]. Moreover, low relative amounts of Bifidobacterium vulgatus and high abundances of Lactobacillus spp. are detected in the microbiota of obese children [18]. Other studies have reported on the relationship between the Proteobacteria phylum and obesity, by the identification of pro-inflammatory molecules such as lipopolysaccharides and increased fat storage in the host [19]. In addition, higher abundances of Rikenellaceae and Ruminococcaceae have been revealed in leptin-resistant obese

(leptin-promoting satiety) and diabetic (db/db) mice compared with in the lean members of the same litter [20]. Furthermore, other authors have demonstrated a relationship between Desulfovibrionaceae growth induction, obesity, and type 2 diabetes (T2D) [21,22].

There is also cumulative evidence from human and animal studies of an association between the development of diabetes and the existence of changes in the gut microbiota composition. Larsen et al. (2010) [23] showed that the Firmicutes phylum and Clostridia class decreased significantly in humans with T2D compared to in the healthy control group. Likewise, the ß-Proteobacteria class increased in diabetics compared to in the control group and was positively associated with plasma glucose levels. Murri et al. (2013) [24] reported increased Veillonella, Bacteroidetes, and Clostridium spp.—along with a decrease in Blautia, Lactobacillus, Prevotella, and Bifidobacterium—in children with diabetes type 1 diabetes (T1D) compared to in the healthy control group.

### *3.2. Role of EDCs in the Microbiota*

Table 1, Table 2 and Table 3 provide information concerning the effect of endocrine disruptors on gut microbiota in vitro, in animals, and in human studies.

**Table 1.** Effects of endocrine disruptors on gut microbiota in *in vitro* assays.

| References | Compound | Dose Exposure | Justification of exposure doce | Species Strain Mode | Methods | Outcomes | Conclusions |
|---|---|---|---|---|---|---|---|
| **Van de Wiele et al., (2010) [122]** | Metal (Arsenic). | 10 µg methylarsenical/g biomass/hr and 28 µg as-contaminated soils/g biomass/hr | Concentrations detected in arsenic contaminated soils in urban areas of the EEUU | Strains isolated from human feces. | HPLC. Plasma mass spectrometry. | High degree of methylation of Methylarsenical and As-contaminated soils in colon digestion. | Human microbiota has ability to actively metabolize As into methylated arsenicals and thioarsenicals. |
| **Wang et al., (2018) [28]** | BPA | 25 µg/L, 250 µg/L and 2500 µg/L | High human relevant exposure dose; EPA reference dose; 1% lowest observed adverse effect level | Humans | In vitro SHIME, 16s rRNA gene sequencing and PCR | BPA exposure decreased the diversity of gut microbioma (ascending colon and the transverse colon). Exposure to BPA of 25 µg/L decreased diversity of gut microbioma, but high-level exposures (250 and 2500 µg/L) increased diversity (descending colon). | Exposure to BPA significantly altered the microbiota and increased the proportion of shared microbes. |
| **Hoffman et al., (2019) [78]** | PCB126 | 20 or 200 µM | Concentrations physiologically relevant, especially in heavily exposed populations | C56BL6/J mice | 16s rRNA gene sequencing, PCR and HPLC | Significant reduction in bacterial growth after exposure to high concentrations of PCB 126 compared to control. Not significant reduction in bacterial growth at PCB concentrations below 20 µM. | Exposure to PCB126 can contribute to alterations in host metabolism through mechanisms dependent on the intestinal microbiota, specifically through bacterial fermentation or membrane disruption. |
| **Lei et al., (2019) [151]** | di (2- ethylhexyl) phthalate | 10 or 100 µM | The concentration mimics human exposure during adolescence by continually exposing mice to phthalate from ages 6 to 8 weeks | C57BL/6J mice | 16S rRNA gene sequencing and a triple-quadrupole time-of-flight instrument coupled to a binary pump HPLC system | Exposure of in vitro cecal microbiota to di (2-ethylhexyl)-phthalate increased the abundance of *Alistipes, Paenibacillus* and *Lachnoclostridium*. Non-directed metabolomics showed that di (2-ethylhexyl)-phthalate | Di (2-ethylhexyl)-phthalate can directly affect the production of bacterial metabolites related to neurodevelopmental disorders. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | greatly altered the metabolite profile in the culture. | |
| **Joly et al., (2013) [57]** | Chlorpyrifos | 1 mg/kg/day | NOAEL | Wistar rats | SHIME | Exposure to chlorpyrifos increased *Bacteroides spp.* and *Enterococcus spp.* and reduced *Bifidobacterium* spp. and *Lactobacillus* spp. | Chronic, low-dose exposure to chlorpyrifos causes gut dysbiosis. |
| **Shehata et al., (2013) [59]** | Glyphosate | 5.0, 2.40, 1.20, 0.60, 0.30, 0.15 and 0.075 mg/mL | To determine the minimal inhibitory concentration | Chickens | MALDI–TOF MS analysis, multiplex PCR | In vitro exposure to glyphosate showed resistance to glyphosate in highly pathogenic bacteria, but most beneficial bacteria showed susceptibility to glyphosate. | Glyphosate exposure showed differences in sensitivity between pathogenic and beneficial microbiota. Ingestion of glyphosate-contaminated food reduced the beneficial microbiota. |
| **Ackermann et al., (2014) [56]** | Glyphosate | 0, 1, 10 and 100 µg/mL | Concentrations lower than NOAEL. | Cows | DAISYII-incubators, FISH with 16S rRNA/23S rRNA-targeted | Exposure to 1 and 10 µg/mL glyphosate reduced abundances of all species except for *Isotricha* spp. and *Diplodinium* spp. Exposure to 100 µg/mL glyphosate reduced abundance of *Diplodinium* spp. | Glyphosate inhibits growth of beneficial bacteria, but increases the population of pathogenic bacteria |
| **Riede et al., (2016) [58]** | Glyphosate | 0.42 or 2.92 mg/L | The low dose reflects the estimated maximum dietary glyphosate intake of dairy cattle, according to model assumptions. The high dose is higher than residues found in beef cattle diet | Cows | RUSITEC experiments, LC-MS/MS method, 16s rRNA gene sequencing and PCR | Effects of glyphosate at concentrations of 0.42 or 2.92 mg/L. After the incubation period only observed subtle changes in the composition of ruminal bacteria. | No major changes were observed due to Glyphosate exposure to ruminal metabolism or the composition of bacterial communities. |

HPLC: high performance liquid chromatography; PCR: polymerase chain reaction; SHIME: simulator of the human intestinal microbial ecosystem; FISH: fluorescence in situ hybridation; MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; RUSITEC: rumen simulation technique; No observed adverse effects level: NOAEL

**Table 2.** Effects of endocrine disruptors on animal microbiota.

| References | Compound | Dose Exposure | Justification of exposure dose | Species Strain Mode | Methods | Outcomes | Conclusions |
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| **Ba et al., (2017) [127]** | Metals (Cadmium) | 100 nM | Tolerable Weekly Intake | C57BL/6J mice | 16s rDNA sequencing. Fecal microbiota transplant. | Early exposure to low dose of cadmium results in adiposities in adult male mice as well as in reduced diversity and altered composition of gut microbiota. | Early exposure to cadmium resulted in increased fat deposits in male but not in female mice. Low cadmium concentrations increased the expression of genes related to lipid metabolism. |
| **Wu et al., (2016) [126]** | Metals (Lead) | 32 ppm | Relevant concentration of Pb acetate in drinking water | Non-agouti (a/a) AVy mice offspring. | 16S rRNA gene sequencing. | Perinatal Pb exposure was significantly associated with increased bodyweight in adult males (P<.05) but not in females (P=.24). Perinatal Pb exposure altered gut microbiota composition in adult offspring, even after stopping exposure at 3 weeks (sex-independent). *Pseudomonas*, *Enterobacter*, and *Desulfovibrio* increased in adult mice perinatally exposed to Pb (P<.05) | Perinatal Pb exposure was associated with bodyweight (sex-dependent response) and with microbiota composition changes (sex-independent). |
| **Xia et al., (2018) [129]** | Metals (Lead) | 10 and 30 µg/L | Exposure dose is below the maximum allowable concentration of lead in water for zebrafish = 0.07 mg/L | zebrafish | 16S rRNA gene sequencing and GC/MS metabolomics analysis | Exposure to 30 µg/L Pb resulted in decrased *α-Proteobacteria* and increased *Firmicutes*. GC/MS metabolomics analysis showed that 41 metabolites were altered in the exposed group. Changes were related to glycolysis and lipid, amino acid metabolism, and nucleotide metabolism. | Pb exposure at 10 and 30 µg/L during 7 days was associated with changes in microbiota and in glucose, lipid, amino acid and nucleotide metabolism. |
| **Lu et al., (2014) [123]** | Metals (Arsenic) | 10 ppm as sodium arsenite | The exposure dose is above the maximum allowable concentration of arsenic in food. | C57BL/6 mice | 16S rRNA gene sequencing and MS–based metabolomics profiling. | The most abundant gut bacteria were *Firmicutes* (52.79%), *Bacteroidetes* (41.57%), followed by *Tenericutes* (3%), *Actinobacteria* (0.18%), *Cyanobacteria* (0.023%), and *Proteobacteria* (0.0042%) | Altered gut bacteria were strongly linked to changes microbiota metabolites. These changes increase the risk of tissue dysfunctions which might lead to obesity, insulin resistance, and cardiovascular disease. |

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| **Catron et al., (2019) [29]** | BPA, BPAF, BPB, BPF and BPS | BPA (0, 0.2, 0.6, 1.7, 2.9, 5.7, 11.5, 23.0, or 45.0 µM), BPAF (0, 0.2, 0.6, 1.8, 5.2, 15.3, or 45.0 µM), BPB (0, 0.6, 1.7, 5.1, 15.0, or 44.0 µM), BPF (0, 0.2, 0.6, 1.8, 5.2, 15.3, 45.0 µM), or BPS (0, 0.2, 0.6, 1.8, 5.2, 15.3, 45.0 µM) | Dose bases on zebrafish toxicity data available through the iCSS ToxCast dashboard and previous zebrafish studies | Zebrafish | 16s rRNA gene sequencing | Exposure to all the tested concentrations of BPS resulted in non-detectable levels of the *Neisseriaceae* family. Increasing BPS concentrations were associated with increased abundances of *Cryomorphaceae*. Increasing BPA or BPF concentrations were associated with increased abundances of *Chromatiaceae* and decreased abundances of *Neisseriaceae*. | BPS, BPA, or BPF exposure led to structural microbiota disruption during early development at concentrations not related to evident developmental toxicity. Results show that microbiota is very useful for characterization of health effects associated with exposure to environmental chemicals. |
| **Chen, Guo, et al., (2018) [30]** | BPA | 0, 2 and 20 µg/L | Environmental concentrations | Zefrafish (*Danio rerio*) | 16s rRNA gene sequencing | Nano-$TiO_2$ and BPA co-exposure led to altered composition of guy microbiota with increased *Proteobacteria* and *Actinobacteria* in males and females. *Hyphomicrobium* was the most abundant genus in males and females. | Co-exposure to nano-TiO 2 and BPA modifies gut microbiome dynamics, having toxicological effects on host health. |
| **DeLuca et al., (2018) [47]** | BPA | 50 µg/kg bw | Lowest observed adverse effect level | C57BL/6 mice | IMAC at Texas A&M University and triple quadrupole mass spectrometer coupled to LC | Exposure to BPA increased mortality, disease activity, scores of colonic inflammation colon after exposure to sodium dextran sulfate. | BPA exposure decreased microbiota metabolites derived from aromatic amino acids and associated with colon inflammation and inflammatory bowel disease. |
| **Javurek et al., (2016) [31]** | BPA | 50 mg/kg bw | Environmental exposure | California mice | 16s rRNA gene sequencing | BPA exposure increased growth of pathogenic bacteria (*Bacteroides*, *Mollicutes*, *Prevotellaceae*, among others) associated with inflammatory bowel disease, metabolic disorders and colorectal cancer. However, increased *Bifidobacterium* was also found after BPA exposure. | Gut microbiota disruption secondary to BPA exposure is associated with systemic effects, such as inflammatory bowel disease, metabolic disorders and colorectal cancer. |

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| **Koestel et al., (2017) [51]** | BPA | 52.2 ± 19.3 ng/food can or 36.2 ± 18.6 ng/food can | BPA levels identified in cans of diet .Serum dog concentrations 2.2 ng/mL, similar to that which has been reported in humans | Dogs | 16s rRNA gene sequencing | Exposure to high concentrations of BPA was associated with increased bicarbonate levels in plasma and with changes in fecal microbiota (increased *Clostrididiaceae*, *Bacteroides* spp., *Clostridiales*, *Ruminococcus* spp., *Lachnospiraceae*, *Roseburia* spp., *Clostridium hiranonis* and *Megamonas* spp.). | Exposure to high concentrations of BPA was associated with decreased *Bacteroides* spp., which is related to reduction in bacterial bisphenol degradation. This increases active BPA available for absorption in the gut. |
| **Lai et al., (2016) [48]** | BPA | Unknown concentration (BPA content in contaminated diet) | n.a. | CD-1 mice | LC-MS/MS, 16S rRNA gene sequencing and amplicon PCR | BPA and high-fat diet promoted growth of *Proteobacteria* (indicator of dysbiosis). Increased *Helicobacteraceae* proliferation and reduced *Firmicutes* and *Clostridia* were found in exposed mice. | Exposure to BPA in the diet led to structural changes in gut microbiota similar to those induced by high-fat diet and high-sucrose diet. |
| **Liu et al., (2016) [32]** | BPA | 2000 µg/L | Dose used to simulate environmental exposure for a short period of exposure | Zebrafish | 16S rRNA gene sequencing and amplicon PCR reaction | BPA exposure significantly modified gut microbiota composition with increased CKC4 phylum in male and female zebrafish. | BPA exposure altered gut microbiota composition. Gut dysbiosis may be related to changes in lipid metabolism of the host (increased triglycerides in the muscle). |
| **Malaise et al., (2017) [5]** | BPA | 50 µg/kg bw | Exposure dose is 100 times below the current NOAEL in mice. NOAEL= 5 mg/kg BW/day | Mice | 16S rRNA gene sequencing and Real time PCR | Perinatal oral exposure to 50 µg/kg BPA led to gut and systemic immune changes in post-natal day 45. These changes were linked to altered glucose sensitivity and secretion of IgA in feces and decreased fecal *Bifidobacteria* compared to mice in the control group. These effects appear before the infiltration with proinflammatory M1 macrophages in gonadal white adipose tissue that appears with aging, along with decreased insulin sensitivity (T1D) and weight gain. | The results explain the sequence of changes related to perinatal exposure to BPA which could also explain the development of metabolic diseases in adulthood (decreased insulin sensitivity and increased weight gain). |
| **Reddivari et al., (2017) [50]** | BPA | 200 µg/kg bw | To ensure gestational and lactational exposure of | Rabbits | 16S rRNA gene sequencing | BPA exposure induced significant decrease in *Oscillospira* and *Ruminococcaceae* and therefore in short-chain fatty acid | Perinatal exposure to BPA modified gut microbiota composition and decreased beneficial bacterial |

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| | | | pups, approximately 1/25 of the NOAEL dose | | | production. BPA exposure also reduced fecal levels of short-chain fatty acid, increased systemic lipopolysaccharide and gut permeability. | metabolites such as short-chain fatty acids. BPA exposure also increased chronic inflammation in colon and liver, and systemic lipopolysaccharides. |
| **Xu et al., (2019) [33]** | BPA | 30 or 300 µg/kg bw | Based on human exposure (30 µg/kg) and median human blood (300 µg/kg) levels | Mice | 16S rRNA gene sequencing and amplicon PCR reaction | BPA exposure-induced changes in gut microbiome composition are a potential mechanism of immunomodulation and T1D development. BPA at 30 or 300 µg/kg increased *Bacteroidetes*, and 300 µg/kg increased *Cyanobacteria* and TM7. The 30µg/kg dose decreased *Proteobacteria*, and 300 µg/kg decreased *Firmicutes* and *Tenericutes*. Females showed an increase in pro-inflammation factors, while males showed an increase in anti-inflammatory immune factors. | Altered gut microbiota and inflammation risk factors for T1D development associated with BPA exposure were sex-dependent. |
| **Xu et al., (2019) [34]** | BPA | 30 or 300 µg/kg bw | To accelerate Diabetes type I development (30 µg/kg bw) and alter the immunity (300 µg/kg bw) | Mice | 16S rRNA gene sequencing and amplicon PCR reaction | Adult females showed a higher risk of T1D and increased immune responses. However, female offspring showed lower risk of T1D and a shift towards anti-inflammation. In contrast, BPA exposure had little impact on DT1 and immunity in male offspring. | BPA effects on the development of T1D were related to host age and gender. Changes in gut microbiota and inflammation are responsible for T1D in juvenile exposure. Decreased inflammation is responsible for attenuated T1D in males and female offspring exposed during the perinatal period. |
| **Chen, Zhang, et al., (2018) [74]** | PCB126 and PCB153 | 1.0 µg/L | Concentration below the limit established by EPA. | Zefrafish | 16s rRNA gene sequencing | PCB126 exposure led to altered microbiota and deterioration of the intestinal and hepatic functions. PCB126 was associated with oxidative stress and with a sexual dysmorphic effect. Exposure to PCB126 was significantly associated with oxidative stress and exposure to PCB153 was associated with | Exposure to PCB126 showed a significant correlation between dysbiosis and fish health. |

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| | | | | | | lower body weight, higher hepatosomatic index in female zebrafish, but lower index value in exposed males PCB153. | |
| **Cheng et al., (2018) [75]** | PCBs | 6 or 30 mg/kg | To study dose-dependent effect of xenobiotics on the expression of hepatic drug metabolizing enzymes | C57BL/6 mice | 16 S Rrna sequencing, quantitative PCR and UPLC-MS/MS | Exposure to 6 mg/kg PCBs greatly increased the bacteria related to the metabolism of bile acids. This was associated with increased bile acids in serum and small intestine content in a microbiota-dependent fashion. However, at 30mg/kg PCBs, bile acid levels remained stable and were linked with increased hepatic flow transporters and ileal Fgf15. | Changes in microbiota promoted increase in taurine mediated by PCBs in the muricholic acids α and β conjugated with taurine in liver, large and small intestine. |
| **Chi et al., (2019) [76]** | PCB126 | 50 µg/kg bw | Dose environmentally relevant to concentrations historically reported in lake trout from the Great Lakes. | C57BL/6 mice | 16s rRNA gene sequencing, and qRT-PCR | Exposure to PCB126 induced gut dysbiosis (increased *Firmicutes* and *Bacteroidetes* and decreased *Erysipelotrichia*) as well as dyslipidemia and nonalcoholic fatty liver disease. | Exposure to low doses of PCB-126 in mice caused intestinal microbiota dysbiosis and multiple disorders in serum and liver |
| **Choi et al., (2013) [77]** | PCB153, PCB138 and PCB180 | 150 µmol/kg | PCB content in contaminated food | C57BL/6 mice | 16S rRNA gene sequencing and PCR | Exposure to PCBs in sedentary mice resulted in decreased abundance of *Proteobacteria*. Exercised mice showed a gut microbiome structure significantly different from sedentary mice. Exercise lessened PCB-induced changes in gut microbiome. | Exposure to PCBs promotes changes in gut microbiome, which can determine systemic toxicity. Physical exercise lessens changes in gut microbiome. |
| **Kohl et al., (2015) [80]** | PCB126 | 7.3 ng/g | The exposure concentration is below the maximum allowable concentration of PCBs in food. | Northern leopard frogs (*L. pipiens*) | 16S rRNA gene sequencing | Tadpoles exposed to PCB126 maintained increased *Fusobacteria* ($t = 2.95$; $df = 14$; $p = 0.01$). *Fusobacteria* was a very small portion of the tadpole (average of control and treated with PCB: 0.008%) and control frog ($0.3 \pm 0.1\%$) gut communities. Frogs exposed to PCB126 during larval stage had | Exposure to PCB126 results in changes in gut microbiota communities, which might affect health and fitness of the host. |

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| | | | | | | a relative Fusobacterial abundance of 3.5 ± 1.4%. | |
| **Petriello et al., (2018) [81]** | PCB126 | 1 µmol/kg | *This dose produces plasma PCB 126 levels that mimic human exposures of dioxin-like pollutants* | *Ldlr* −/− mice | 16S rRNA gene sequencing and regression modeling | PCB126 reduced α diversity ($p = 0.001$) in the colon and increased the *Firmicutes* to *Bacteroidetes* ratio ($p = 0.044$). Quantifiable amounts of PCB126 in the colon, upregulation of Cyp1a1 gene expression, and increased indicators of gut inflammation were found in exposed mice. | PCB126 exposure altered gut microbiota and metabolism and resulted in gut and systemic inflammation. |
| **Rude et al., (2019) [82]** | PCBs | 0.1, 1, or 6 mg/kg/day | FDA mandates tolerances of 0.2–3.0 ppm (200 – 3000 ng/g) for all foods | Mice | qPCR and 16S rRNA gene sequencing | PCB exposure resulted in epithelial permeability defects in the ileum and colon of juvenile mutated mice. PCB exposure also promoted intestinal inflammation dysbiosis in gut microbiota in juvenile mutated mice exposed to 1 mg/kg/d PCBs versus controls. | The results showed the interactions between PCBs and genetic susceptibility factors to impact individual risk. |
| **Horiuchi et al., (2017) [103]** | Phytoestrogen (S-equol) | 20 mg/kg, 2 times/d | Doses based on previous studies of the possible benefits of S-equol on diabetes | Mice | Immunochemistry. Insulin secretion assay. qRT-PCR. | Administration of S-equol resulted in reduction of the induction of blood glucose concentrations ($P<0.01$at 15min, $P<0.01$ at 30 min, $P < 0.05$ at 60 min, and $P < 0.01$ at 120min) | Gut microbiota-produced S-equol induced ß-cell growth in vivo and insulin secretion ex vivo. Administration of S-equol decreased *Streptozotocin*-induced hyperglycemia by promoting ß-cell function. |
| **Huang et al., (2018) [110]** | Phytoestrogens (Genistein) | 20 mg/kg bw | Dose physiologically relevant to obtain an accurate interspecies extrapolation | Mice | 16S Rrna gene sequencing and QRT-PCR | Perinatal genistein exposure caused increased incidence of DT1 in female offspring. Fecal microbiota from female offspring at postnatal day 90 showed increased Enterobacteriales (suggesting a proinflammatory response). In contrast, perinatal genistein administration caused a shift in microbiota towards anti-inflammation in males at postnatal day 90. | Perinatal administration of genistein resulted in strongly sex-dependent changes in microbiota. T1D exacerbation in non-diabetic females was related to immunomodulatory mechanisms associated with an altered gut microbiota. |
| **López et al., (2018) [108]** | Phytoestrogens (Genistein) | 3 mg/kg bw/day | Equivalent dose of 1.5 g of genistein per day for a 65 kg | C57BL/6 mice | 16S Rrna gene | Mice fed with a high-fat diet with genistein exhibited changes in the gut microbiota linked to lower circulating levels of | Genistein exposure through diet can modulate gut microbiota, decreasing metabolic endotoxemia and |

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| | | | adult person, approximately | | sequencing and QRT-PCR | lipopolysaccharides, improved glucose metabolism and reduced expression of pro-inflammatory cytokines in the liver compared to mice in the high-fat diet alone group. | neuroinflammatory response despite consumption of a high-fat diet. |
| **Marshall et al., (2019) [112]** | Phytoestrogens (Genistein) | 250 mg/kg | The dose is above the maximum allowable concentration of genistein in food. EFSA LOAEL of 35 mg/kg bw/day for males and 44 mg/kg bw/day for females | california mice | GC/MS, 16S rRNA sequencing Social behavior testing using the three-chamber test | When male offspring from genistein-supplemented dams were compared with genistein-free offspring, audible calls above 20 kHz correlated with daidzein, α-tocopherol, *Flexispira* spp. and *Odoribacter* spp. Results suggest that early genistein exposure can induce a disruption in the offspring normal socio-communicative behaviors. | Perinatal exposure to genistein may detrimentally affect the offspring "microbiome-gut-brain axis". |
| **Piccolo et al., (2017) [114]** | Phytoestrogens | Phytoestrogens naturally present in diet (pigs fed with soy-based infant formula). | Food exposure levels | White Dutch Landrace pigs | 16S rRNA gene sequencing and LC-MS | Sow-fed piglets exhibited higher α-diversity in the duodenum than formula-fed piglets ($P < 0.05$). No differences were found in the ileum. *Firmicutes* was the most abundant phylum in the duodenum in all tested diets, followed by *Proteobacteria* in the sow- and milk-fed piglets and *Cyanobacteria* in soy-fed piglets. | Neonatal diet can impact small intestine microbiome in pigs resulting in disturbances in the metabolism and development of intestinal tissue in the postnatal period. |
| **Williams et al., (2019) [115]** | Phytoestrogens | Phytoestrogens naturally present in diet | Food exposure levels | Southern White Rhinoceros (*Ceratotherium simum simum*) | 16S rRNA amplicon sequencing and MS | Composition and structure of fecal microbiota significantly differ by rhino species as well as at the phylum, family, and OUT levels. | Results suggest differences in receptor sensitivity to phytoestrogens related to the species and metabolism of dietary phytoestrogens by gut microbiota might have an impact on fertility of captive female rhinos. |
| **Yeruva et al., (2016) [113]** | Phytoestrogens | Phytoestrogens naturally present in diet (pigs were fed | Food exposure levels | White Dutch, Landrace Duroc pigs | 16S rRNA amplicon sequencing, qRT-PCR, ELISA and | In soy-fed piglets increased *Lactobacillaceae* spp. and *Clostria* spp. but decreased *Enterobacteriaceae* spp. were observed. | Neonatal diet promotes disturbances in microbiome of the small intestine in pigs, particularly in the duodenum. |

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| | | soy or milk formula). | | | UHPLC-HRAM | | |
| **Zhou et al., (2018) [104]** | Phytoestrogens (Genistein) | 0.25 and 0.6 g/kg | To analyze the effects of low and high exposure doses | C57BL/6 mice | Oral glucose tolerance tests, ELISA kit and 16S rRNA amplicon sequencing | Perinatal maternal consumption of a high-fat diet with genistein resulted in increased birth weight, improved glucose tolerance and decreased fasting insulin, as well as lower levels of triacylglycerol and total cholesterol in serum in the offspring. | The intake of genistein during pregnancy improves the metabolism of the offspring preventing the transgenerational effects of maternal high-fat diet on diabetes. |
| **Obadia et al., (2018) [97]** | Methylparaben (MPB) | 0.0, 0.1, 0.2 and 0.3% | *High levels of MPB (~0.5%) can affect the microbiota diversity, the exposure dose was selected to test lower levels* | *Drosophila melanogaster* | - | Concentrations > 0.1% MPB disrupt the growth of some species of yeast and *Acetobacter*, but even at 0.3% *Lactobacilli* growth was less affected. | Exposure to MPB probably alters the composition and amount of gut bacteria and yeasts in laboratory fly. |
| **Hu et al., (2016) [93]** | diethyl phthalate (DEP), methylparaben (MPB), triclosan (TCS) and their mixture (MIX) | 0.050 mg TCS/kg bw, 0.1050 mg MPB/kg bw and 0.1735 mg DEP/kg bw | NOAEL | Sprague-Dawley rats | 16s rRNA gene sequencing and PCR | Exposure to these chemicals produced an increase in *Bacteroidetes* (*Prevotella*) and decreased *Firmicutes* (*Bacilli*) in all the exposed rats. Increased *Elusimicrobia* was found in DEP and MPB exposed rats, Betaproteobacteria in MPB and MIX exposed rats, and *Deltaproteobacteria* in the TCS group. In adulthood, these differences decreased between cases and controls despite continued exposure, suggesting that contaminant exposure have a greater impact on gut microbiome of adolescent rats. | Exposure at doses similar to environmental human exposure can disturb the gut microbiota in adolescent rats. This disturbance mainly affects the health of the youngest. |
| **Fan et al., (2019) [150]** | di (2-ethylhexyl) - phthalate | 0.2, 2, and 20 mg/kg/day | Based on the EPA reference dose and previous studies | mice | LC-HRMS, 16S rRNA gene sequencing and qPCR | Prenatal exposure to low doses of di (2-ethylhexyl) phthalate (0.2 mg/kg/day) resulted in metabolic syndrome and gut dysbiosis. Thiamine liver metabolism was disrupted in the offspring, which | Exposure to low doses of di (2-ethylhexyl) phthalate during the early stages of life might increase the risk of obesity and metabolic syndrome. |

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| | | | | | | caused disturbances in glucose metabolism. | |
| **Lei et al., (2019) [151]** | di (2-ethylhexyl) - phthalate | 1 or 10 mg/kg bw/day | The concentration mimics human exposure during adolescence by continually exposing mice to phthalate from ages 6 to 8 weeks | C57BL/6J mice | 16S rRNA gene sequencing and a triple-quadrupole time-of-flight instrument coupled to a binary pump HPLC system | Oral probe di (2-ethylhexyl) phthalate exposure increased the abundance of *Lachnoclostridum*, while decreasing *Akkermansia*, *Odoribacter* and *Clostridium* sensu stricto. | Di (2-ethylhexyl) phthalate exposure directly alters microbiota therefore modifying the production of bacterial metabolites related to neurodevelopmental disorders. |
| **Gao, Bian, Mabub, et al., (2017) [68]** | diazinon | 4 mg/L | According to previous studies, dose which did not elicit discernible AChE inhibition | C57BL/6 mice | 16S rRNA gene sequencing and mass spectrometry –based metabolomics | Diazinon exposure significantly disturbed the intestinal microbiome, and the RNA sequencing revealed that diazinon exposure disrupts the functional metagenome. These changes were more pronounced male mice. | Diazinon exposure disturbed the structure of the gut microbiome, the functional metagenome and also had a sexual dysmorphic effect. |
| **Jin et al., (2015) [60]** | Carbendazim | 100 or 500mg/kg bw | The exposure concentration is above the maximum allowable concentration of carbendazim in food. | ICR mice (*Mus musculus*) | Real time PCR sequencing and 16s rRNA gene sequencing and HPLC | Carbendazim exposure at 100 and 500 mg kg led to histopathological changes in the liver, disturbed lipid metabolism and intestinal gut dysbiosis. During the first three days of exposure to carbendazim the most abundant constituents of microbiomes, *Firmicutes* and *Bacteroidetes*, tend to decrease. From the fifth day of treatment with carbendazim, *Bacteroidetes* maintained the decreasing tendency, but *Firmicutes* started to increase | Exposure to carbendazim disturbs microbiota and can lead to inflammation which results in altered lipid metabolism and triggers obesity in exposed mice. |
| **Liang et al., (2019) [61]** | Chlorpyrifos | 5 mg/kg | Concentration higher than NOAEL | C57Bl/6 and CD-1 mice | 16s rRNA gene sequencing and qPCR | Exposure to chlorpyrifos in mice induced changes in microbiota, increased body, a lower insulin sensitivity. Chlorpyrifos also resulted in disruption of the intestinal barrier and more, which led to the entry of lipopolysaccharides in the body | Chlorpyrifos exposure might contribute to the worldwide epidemic of inflammatory diseases. |

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| | | | | | | which promote the release of pro-inflammatory factors. | |
| **Liu et al., (2017) [65]** | p, p'-dichlorodiphenyldichloroethylene (p, p'-DDE) and ß-hexachlorocyclohexane (ß-HCH) | 1 mg p, p'-DDE/kg bw/day and 10 mg ß-HCH/kg bw/day | These doses mimic the chronic exposure in human | C57BL/6 mice | 16S rRNA gene sequencing, real time PCR and UPLC-M. | Exposure to organochlorines disturbed the abundance and composition of gut microbiota (increased *Lactobacillus* capable of deconjugating bile salts). This affects the hydrophobicity and composition of bile acids, down-regulates the expression of genes involved in the reabsorption of bile acids in the distal ileum and up-regulates the expression of genes involved in the hepatic synthesis of bile acids. | Chronic exposure to low doses of organochlorines increases the risk of dysfunction in bile acid metabolism. |
| **Tu et al., (2019) [66]** | 2,4-Dichlorophenoxyacetic acid (2, 4-D) | < 15 mg/kg bw/ day | Concentration lower than NOAEL | C57BL/6 mice | 16S rRNA gene sequencing and LC-MS | Metagenomic results revealed a distinct intestinal microbiota with changes in various microbial metabolic pathways, including urea degradation, amino acid and carbohydrate metabolism. | 2,4-D exposure resulted in changes in the composition and activity of gut microbiota. The metabolic profile of host plasma samples showed changes in the metabolic profiles indicative of 2,4-D toxicity at low doses. |
| **Yang et al., (2019) [62]** | Organophosphorus: diethyl phosphate (DEP) | 0.08 or 0.13 mg/kg | 1/500 LD50 | Wistar rats | NanoDrop spectrophotometer and 16S rRNA gene sequencing | Exposure to high dose of DEP promotes the growth of butyrate-producing bacterial genera Alloprevotella and Intestinimonas, which induced an increase in estradiol and a decrease in total triglycerides and low density lipoprotein cholesterol. Exposure to DEP also increased tyrosine-tyrosine peptide and ghrelin, attributed to the enrichment of *Clostridium* sensu stricto 1 and *Lactobacillus*, producers of short-chain fatty acids. | Chronic exposure to DEP affected the gut microbiota, serum hormones and proinflammatory cytokines in rats, with stronger responses observed at high doses. |
| **Wu et al., (2018) [63]** | Propamocarb | 0, 0.5, 5, 50 mg/kg bw/day | Dosages set according to the highest residue from the EU- | mice | 16S rRNA gene sequencing | Exposure to propamocarb disturbed the transcription of liver genes related to regulation of lipid metabolism. The | Exposure to high dose propamocarb changes the metabolism of mice by |

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| | | | MRLs and the NOAEL or long term toxicity | | | microbiota in the cecal content and feces changed at the phylum or gender level. | altering the gut microbiota and microbial metabolites. |
| **Gao et al., (2017) [137]** | Triclosan (TCS) | 2 ppm | The concentration used is 100 times lower than that which can promote liver carcinogenesis | C57BL/6 mice | 16S rRNA gene sequencing and shotgun metagenomics sequencing | Exposure to TCS produced significant changes in mouse gut bacterial community assembly. Metagenomic sequencing showed an increase in gut bacterial genes related to triclosan resistance, stress response and antibiotic resistance, and others. | Exposure to TCS alters the intestinal microbiome of mice by inducing changes at the compositional and functional levels. |
| **Gaulke et al., (2016) [139]** | TCS | 100 µg/g | Dose to cause endocrine disruption in fish | Zebrafish | 16S rRNA gene sequencing | Operational taxonomic units of the *Enterobacteriaceae* family are susceptible to TCS exposure, but operational taxonomic units of the *Pseudomonas genus* are more resistant to exposure. | Exposure to TCS promotes changes in the composition and ecological dynamics of gut microbial communities. |
| **Kennedy et al., (2016) [136]** | TCs (TCS and Triclocarban (TCC)) | 0.1% (v/v) | According to blood human level | Timed-pregnant Sprague Dawley (SD) rats | 16s rRNA gene sequencing | TCC exposure reduced the diversity of fecal microbiota in exposed rats versus controls at 7 days after exposure. This continued throughout perinatal exposure. | α-diversity was reduced in exposed animals at all sampling time points after baseline. Differences in ß-diversity were found between gestational day 18 and post-delivery day 16 in exposed versus control dams. |
| **Ma et al., (2019) [141]** | TCS | 0, 10 or 50 mg/kg | Doses referenced previous toxicity studies in rats (Lowest toxic dose in rats is 50 mg/kg/day) | Rats | 16s rRNA gene sequencing | Exposure to TCS reduced diversity and altered the microbiota composition at doses of 50 mg/kg/day in adult rats and at two doses in old rats. These changes were long-lasting even after the exposure was terminated and taccumulated over time inducing metabolic disorders in old rat offspring. | Exposure to TCS early in life results in long-lasting changes in the metabolism and intestinal microbiota and they accumulate over time. |
| **Narrowe et al., (2015) [138]** | TCS | 100 to 1,000 ng/l | Environmentally relevant concentrations | larval fathead minnows (Pimephales promelas) | High-throughput 16s rRNA sequencing | TCS resulted in an increase of all members of the order *Pseudomonadales*, in five *Acinetobacter* OTUs, and in 26 OTUs (*Flavobacterium*, *Chryseobacterium*, and *Shewanella*) at day 7. | Short-term, low-level environmental exposure to TCS is sufficient to disrupt gut microbiome in minnows. |

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| **Zang et al., (2019) [143]** | TCS | 0.002% (v/v) | Based on previous studies | Zebrafish (*Danio rerio*) | 16S rRNA gene sequencing and qRT-PCR | TCS exposure led to severe structural and morphologic damage to the intestines, spleen and kidney observed in histopathologic studies. *Lactobacillus* was able to mitigate this damage. | *Lactobacillus plantarum* ST-III increases gut microbial biodiversity in zebrafish and mitigates the damages associated to TCS exposure. |

No observed adverse effects level: NOAE; Lowest observed adverse effect level: LOAEL; MRL: Maximum residues levels

**Table 3.** Effects of endocrine disruptors in human's microbiota.

| References | Compound | Exposure route | Species Strain Mode | Methods | Outcomes | Conclusions |
|---|---|---|---|---|---|---|
| **Eggers et al., (2019) [130]** | Metals (lead) | Environmental (food) | Adult humans | DNA sequencing of the 16S rRNA V4 region | Increased urine Pb levels were associated with the presence of *Proteobacteria*, increased α-diversity ($p = 0.071$) and wealth ($p = 0.005$). Changes in ß-diversity were significantly associated ($p = 0.003$) with differences in Pb levels. | Pb exposure is associated with diversity and composition changes of intestinal microbiota in adults. |
| **Wu el al., (2016) [116]** | Phytoestrogen | Oral (diet) | Adult humans | 16S rRNA-tagged sequencing and plasma and urinary metabolomic platforms | Consumption of fermentable substrates was not associated with higher levels of short-chain fatty acids in fecal samples in vegans. | Despite the differences in plasma metabolome between vegans with high soy consumption and omnivores, the gut microbiota in the two groups was similar. |
| **Yang et al., (2019) [151]** | Phthalates [Di(2-ethylhexyl) phthalate] | Intravenous (plastic) | Newborns | Water ACQUITY UPLC and MS/MS. 16s rRNA sequencing | Biota differences were found between meconium samples and fecal samples collected later. Di(2-ethylhexyl) phthalate-exposed microbiota showed higher variability of bacteria taxa. | Short-term di(2-ethylhexyl) phthalate exposure led to temporary gut dysbiosis. This suggests that long-term exposure may result in permanent gut dysbiosis. Di(2-ethylhexyl) phthalate levels did not alter the dominant bacterial phyla composition, but the *Firmicutes-Bacteroidetes* ratio changed over time in both exposed and unexposed newborns. |
| **Stanaway et al., (2016) [69]** | azinphos-methyl | Oral and inhalation | Adult men | Isotope dilution GC-HR-MS, 16S rRNA gene DNA sequenced and Agencourt AMPure XP PCR purification system | Disturbances in *Streptococcus, Micrococcineae, Gemella, Haemophilus, Halomonas, Actinomycineae, and Granulicatell* were observed, and decreased oral bacterial genus *Streptococcus*. | Human exposure to agricultural pesticides is associated with the alteration of oral microbiota, but future research is needed to support these findings. |

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|---|---|---|---|---|---|---|
| **Bever et al., (2018) [144]** | TCS | Oral (breast milk) | Infants and Mothers | 16s rRNA sequencing and GC-MS | Diversity in fecal microbiome of TCS-exposed infants versus unexposed infants differed | Exogenous chemicals are correlated with disturbances in microbiome diversity in the intestinal community of infants during the early developing period. |
| **Ribado et al., (2017) [145]** | TCs (TCS and TCC) | Dermal (personal care products) | Infants and Mothers. | 16s rRNA sequencing | TC exposure was not associated with a reduction of gut microbiota diversity in mothers and their infants at any of three time points after birth. Shannon's diversity index did was not decreased in infants randomized to TC-containing products. | After 10 months, chronic TC exposure from household products does not contribute to recovery of gut microbiomes in mothers or their infants. The most abundant species in the unexposed infants, *B. fragilis*, is associated with direct maturation of the immune system and production of anti-inflammatory polysaccharides |

### 3.2.1. BPA and Analogs

Bisphenol A (BPA) is an environmental chemical widely used in industry for the manufacture of polycarbonate plastics and epoxy resins, with well-known endocrine disrupting activity [25,26]. The public concern about the safety of BPA has resulted in the imposition of a ban on its use in some products and the emerging market entry of BPA analogs such as bisphenol S (BPS), bisphenol F (BPF), bisphenol AF (BPAF), and bisphenol B (BPB). However, their structural similarity to BPA has also raised concerns about their endocrine disrupting potential. Several studies have reported on the association of BPA and BPA analogs with an increased risk of developing metabolic diseases such as obesity [25,27]. However, this association is not well demonstrated, and it is challenging to find evidence for direct causality between BPA and analog exposure and metabolic diseases using epidemiological studies.

Wang et al. (2018) [28] studied the changes in metabolism and accessibility of BPA in different parts of the gastrointestinal tract using an in vitro Simulator of the Human Intestinal Microbial Ecosystem (SHIME) model. Three different BPA concentrations were investigated, which provided information regarding an extensive range of BPA daily intake values, from the human relevant exposure dose (25 µg/L) and the EPA (Environmental Protection Agency) reference dose (250 µg/L), to the 1% lowest observed adverse effect level (2500 µg/L) [28]. The toxicity of BPA, in terms of the effects on the hepatic gene expression profiles, was compared with that of the SHIME effluents, using the human hepatocellular carcinoma (HEPG2) cell line. The findings showed that BPA exposure modified the microbial composition of the colon, increasing the amount of microbes in the ascending, transverse, and descending colon. The upregulation of BPA-degrading bacteria, such as Microbacterium and Alcaligenes, was also reported.

Exposure to BPA and BPA analogs in animal models such as rodents, zebrafish, rabbits, and dogs can affect the gut microbiota and have an

impact on the development of metabolic diseases. Some studies have reported that there is a sexual dysmorphic effect [6,29,30,31,32,33].

Xu et al. (2019) [33] determined the impact of BPA exposure on the development of T1D and the involvement of the host immune system and gut microbiota in a model of non-obese diabetic (NOD) mice. Adult male and female NOD mice were orally exposed to BPA at environmentally relevant doses (30 or 300 µg/kg). These doses were selected because they had previously been shown to modify the immune system and to be relevant for human exposure (30 BPA/kg body weight (bw) is within the range of human exposure levels, and 300 BPA/kg bw is also appropriate for human exposure levels based on BPA concentrations in human blood) [34]. In addition, the current EPA reference dose is 50 µg/kg/day. However, exposure to low-dose BPA also seems to have harmful effects, and as a consequence, after careful examination, the European Food Safety Authority (ESFA) has lowered the total dietary intake to 4 µg/kg bw/day.

Exposure to BPA resulted in a fast onset of T1D in female mice and slow onset in male mice. Subacute BPA exposure in female mice resulted in increased Bacteroidetes and Cyanobacteria and decreased Firmicutes, Tenericutes, and Proteobacteria. Chronic exposure to BPA in females also resulted in a proinflammatory gut microbiota, with a decrease in Bacteroidales and Lactobacillus. These results are consistent with human epidemiological studies, which show that the gut microbiota in individuals with T1D is dominated by Bacteroidetes at the phylum level and in turn with a reduction in Firmicutes in relation to control [87]. In male mice, BPA exposure results in a slow onset of T1D. Subacute exposure caused a decrease in Bacilli at the class level, which is in accordance with the findings reported in human epidemiological studies on the association between the the development of T1D and gut microbiota composition [88]. Additionally, chronic exposure to BPA in males resulted in decreased Lachnospiraceae, which has been shown to promote T1D in NOD mice models [89].

Using their NOD mouse model, Xu et al. (2019) [6] showed that BPA's effects on the development of T1D were related to host age and gender, following various windows of exposure. Exposed juvenile NOD females (starting postnatal day (PND) 28 to PND56) and NOD offspring (starting gestation day 5 to PND28) were exposed perinatally to BPA by dosing the dams to 0 or 30 μg/kg BW. Adult NOD females were exposed to 0 or 300 μg/kg bw. Interestingly, BPA increased the risk of developing T1D in adult and juvenile females, which was related to changes in the gut microbiota, but the female offspring showed a reduced risk of developing T1D. In contrast, BPA had insignificant effects on the development of T1D in the male offspring. The changes in the gut microbiota of juvenile females associated with BPA exposure included, at the genus level, an increase of Turicibacter, Oscillospira, Ruminococcus, Jeotgalicoccus, and Lachnospiraceae, which increase the risk of T1D and inflammation, as shown in several animal models [89,90,91].

Malaisé et al. (2017) [5], in their longitudinal study, found that the perinatal exposure of C3H/HeN mice to BPA at 50 μg/kg bw/day (100 times lower the no observed adverse effect level (NOAEL), 5 mg/kg bw/day) induced dysbiosis and systemic immune imbalances at PND45. These effects were associated with a rise in glucose intolerance and a decrease in IgA and Bifidobacteria in the feces. Some strains of the Bifidobacterium genus have been shown to have anti-inflammatory properties [92].

Javurek et al. (2016) [31] explored the changes in gut microbiota related to BPA exposure in parents and their offspring in California mice (Peromyscus californicus). Female and male monogamous and biparental mice were exposed to BPA (50 mg/kg feed weight) from periconception to weaning. The dose selected has been shown to induce metabolic alterations and it is below the diet-administered maximum non toxic dose for rodents (200 mg/kg BW/day). This dose is within the presumptive NOAEL and results in concentrations similar to those reported in human serum. They demonstrated for the first time that parental exposure to

concentrations of BPA environmentally relevant causes changes in the microbiota structure in non-exposed offspring. These changes were generational- and sex-dependent. In this respect, they reported that BPA (and ethinyl estradiol) exposure induced an increase in Akkermansia, Mollicutes, Prevotellaceae, Bacteroides, Erysipelotrichaceae, Methanobrevibacter, Sutterella in parents and offspring. These species have been associated to inflammatory bowel disease, obesity and metabolic disorders [93,94], autism spectrum disorders [95], colon cancer [96], and other conditions. However, Bifidobacterium was also found in higher amounts in fecal samples of female offspring. Some Bifidobacterium strains have been shown to exert health-promoting effects and is included in a number of probiotic foods and supplements [97]. DeLuca et al. (2018) [46] used a dextran sulphate sodium-induced colitis model in female C57BL/6 mice and found that BPA exposure at 50 µg/kg/day negatively affects gut physiology by reducing microbiota metabolites derived from aromatic amino acids, which might be associated with autoimmune diseases, specifically with inflammatory bowel disease. This dose was selected because it is the EPA reference dose for BPA.

Lai et al. (2016) [48] used 16S rRNA gene sequencing of cecal microbiota of CD-1 male mice to analyse the effects of dietary BPA intake on microbiota composition and physiology. Mice on high-fat high-sucrose diet were the positive controls. The findings showed that dietary BPA exposure was related to a decrease in the diversity of microbiota species. The structural changes of the gut microbiota exposed to dietary BPA were similar to those found in mice on high-fat high-sucrose diets. Additionally, the comparison between BPA and high-fat diet revealed an increase in Proteobacteria in both groups. The increased abundance of Proteobacteria has been related to different conditions such as metabolic disorders and inflammatory bowel disease [98]. Lastly, exposure to dietary BPA produced a decrease in the phylum Firmicutes, with most of the 16SRNA sequencing corresponding to the class Clostridia. Interestingly, Larsen et al. (2010) [23] demonstrated a significant

reduction in Firmicutes and Clostridia in the feces of human male adults with T2D compared to the healthy group.

Dietary exposure to BPA also modified the gut microbiota in zebrafish [30,32]. In a study conducted by Liu et al. (2016) [32], the authors concluded that exposure to BPA resulted in an increase of the phylum CKC4 in both sexes probably connected to changes in the host lipid metabolism (increased triglycerides in the muscle). However, one of the limitations of this study was that the functional study of CKC4, a phylum included in the SILVA database, was very incomplete.

Chen et al. (2018) [30] exposed adult zebrafish at BPA doses of 0, 2 and 20 µg/L, titanium dioxide nanoparticles (nano-TiO2) and their binary mixtures for three months. Exposure to both compounds resulted in changes in the gut microbiota. An antagonistic interaction was observed at low BPA concentrations, but a synergistic interaction was observed at high BPA concentrations. Zebrafish growth and gut health (oxidative stress, barrier function, inflammation) were associated with sex and concentration of chemicals. Additionally, zebrafish weight was found to be positively associated with the presence of Bacteroides, closely related to the Anaerococcus, Finegoldia, and Peptoniphilus genera.

The work by Reddivari et al. (2017) [49] showed that perinatal exposure to BPA in Dutch-Belted rabbits (200 µg/kg bw/day) induced an increase in the Methanobrevibacter spp community leading to inflammation of the colon and liver, and increased gut permeability in the offspring demonstrated by increased levels of serum lipopolysaccharide. This study used rabbits as an animal model because this species has an extensive infantile period of development similar to that of humans and utilized BPA at a relatively low dose level of 200 g/kg bw/day (approximately 1/25 of the NOAEL dose). Significant positive correlations were observed between increased Methanobrevibacter spp. in the colon and systemic concentrations of lipopolysaccharide ($r2 = 0.67$; $p = 0.023$). This species can metabolize dietary substrates that lead to increased host energy intake and weight gain [93]. Lastly, perinatal

exposure to BPA led to a reduction of the diversity of the microbial communities and their metabolites (short chain fatty acids) and to an increase in intestinal permeability.

The work by Koestel et al. (2017) [47] showed that the circulating BPA levels in dogs fed with canned dog food for two weeks (2.2 ± 0.15 ng/mL) were similar to the levels found in humans [99], with higher BPA concentrations related to modifications in the composition of the microbiome. These changes may lead to modifications in metabolic pathways, including the capacity to metabolize bisphenols. In this study, higher serum BPA levels were associated with decreasing relative abundances of Bacteroides spp., which may result in a reduction of BPA bacterial degradation.

We found only one study of the effects of BPA analogs on the microbiota. Catron et al. (2019) [29] exposed zebrafish during their developmental period to BPA, BPAF, BPB, BPF, and BPS at different concentrations. These concentrations were selected based on zebrafish toxicity data available through the ICSS ToxCast dashboard and previous zebrafish studies. 16S rRNA gene sequencing showed that structural microbiota disruption was highly dependent on concentration and that exposure to BPS, BPA, or BPF caused the enrichment of the microbial functions, but this did not occur with exposure to BPB or BPAF. Lastly, microbial disruption was inversely associated with host developmental toxicity and estrogenicity. The main finding of this work was that BPS, BPA, and BPF produced disruptions in microbial composition, occurring throughout the critical window of early development, at concentrations that did not cause evident developmental toxicity.

### 3.2.2. Pesticides

The adverse effects of agricultural pesticides derived from their endocrine disruptive activity that may affect the thyroid and the

reproductive, nervous, and adipose systems were investigated [100,101,102].

In vitro studies have demonstrated that different pesticides can cause gut dysbiosis [38,39,40,41], using the chicken microphrobiome [39] and the Rumen Simulation Technique (RUSITEC) system [40,41]. Joly et al. (2013) [38] studied the impact of in vitro exposure to low doses of organophosphorus chloropyrifos in a SHIME system, and the effects in vivo in pregnant Wistar rats. Their results showed that exposure to chlorpyrifos induces the proliferation of Bacteroides spp. and Enterococcus spp. and reduces the proliferation of Bifidobacterium spp. and Lactobacillus spp.

Three studies in rodents (in ICR, C57Bl/6, and CD-1 mice and Wistar rats) showed that exposure to pesticides (carbendazim, chlorpyrifos, and organophosphorus pesticides) induces dysbiosis in the microbiota and inflammation, leading to an alteration of lipid metabolism and triggering obesity in exposed rodents [69,70,73].

Wu et al. (2018) [74] evaluated the effects of exposure to propamocarb (3, 30, and 300 mg/L for 28 days) in five week-old male Institute of Cancer Research mice. These concentrations were established based on the highest residue from the EU-Maximum Residues levels and the long-term toxicity NOAEL (20 mg/kg bw/day) [103]. The authors found that exposure induced the disruption of the transcription of hepatic genes involved in the regulation of lipid metabolism. The cecal and fecal microbiota changed at the phylum or genus levels.

Liu et al. (2017) [71] exposed adult male C57BL/6 mice, over eight weeks, to low dose p,p'-dichlorodiphenyldichloroethylene (1 mg/kg body weight/day) and ß-hexachlorocyclohexane (10 mg/kg body weight/day), which are similar to the levels found in chronic exposure in humans. The authors found that exposure induced changes in the composition of the gut microbiota, particularly leading to higher abundances of Lactobacillus that are capable of deconjugating bile acids by bile salt

hydrolases. These transformation reactions affect the hydrophobicity and composition of bile acids, and down-regulate the expression of genes involved in the reabsorption of bile acids in the distal ileum, but up-regulate the expression of genes involved in the hepatic synthesis of bile acids.

Tu et al. (2019) [72] evaluated the toxic effects of exposure to 2,4-dichlorophenoxyacetic acid, at an occupationally important dose, on the intestinal microbiota of specific-pathogen-free C57BL/6 male mice. Metagenomic sequencing showed a distinct gut microbial community with disturbances in the pathways of amino acid and carbohydrate metabolism and urea degradation. These findings are of particular interest, as evidence has showed that modifications of microbiome-related pathways and metabolites would produce an alteration of gut-host homeostasis, which may increase the risk of diseases [104].

The sexual dysmorphic effects of pesticide exposure have been described in animal models. Gao et al. (2017) [68] showed that exposure to a low dose of organophosphorus pesticide diazinon (4 mg/L in drinking water) modifies the gut microbiota composition and functionality in C57BL/6 mice, with more impact in male mice. At the phylum level, Bacteroidetes increased by 1.8-fold, while Firmicutes decreased by 1.8-fold, in diazinon exposed males compared to controls. As shown previously, a high Firmicutes/Bacteroidetes ratio is related to obesity, which is consistent with the observed decrease of body weight in male mice. By contrast, no effects of diazinon exposure on body weight and the Firmicutes/Bacteroidetes ratio were observed in female mice.

Finally, an epidemiological study in humans carried out by Stanaway et al. (2017) [84] found that exposure to agricultural pesticides can cause dysbiosis of the human oral microbiota. A cohort of 65 agricultural workers and 52 non-agricultural workers was studied. The results showed that workers exposed to azinfos-methyl had a decrease in common genera found in the human oral microbiome (Streptococcus, Micrococcineae, Gemella, Haemophilus, Halomonas, Actinomycineae,

and Granulicatella). Although more studies are needed to confirm the results of Stanaway et al. (2017) [84], the data obtained indicate that the oral microbiome could be used as a simple biomarker for assessing pesticide exposure in epidemiological studies.

3.2.3. Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) are well-known EDCs that were massively used until the mid-1970s as insulators for electrical equipment such as transformers, switches, capacitors, and thermostats. Despite the current ban on manufacturing, PCBs continue to be a common environmental contaminant because of their accidental and intentional release in large quantities due to their long-term stability [105,106]. The main route of exposure to PCBs is the consumption of contaminated foods such as fish and shellfish. PCBs have been related to hormone-dependent cancers, an impaired reproductive system, and cognitive and metabolic disorders (such as impaired glucose metabolism and adipocyte inflammation) [107,108].

The available evidence has shown the detrimental effects of PCB exposure on the microbiota that can trigger host metabolic disorders [36,50,51,52,53,54,55,109]. Exposure to PCBs promotes an altered microbiome, with reduced Proteobacteria [53], decreases in microbial diversity, and an increased Bacteroidetes-to-Firmicutes ratio, usually linked to intestinal and systemic inflammation [55]. In addition, changes in bile acid homeostasis that result in dysbiosis have been described in adult mice exposed to a PCB mixture [51]. Other studies have also reported the impact of exposure to PCB on amphibian [54] and zebrafish microbiomes [50]. Recently, Rude et al. (2019) [56] reported that developmental exposure to PCBs induces dysbiosis and epithelial permeability defects in the ileum and colon that result in changes to the microbial ß-diversity of juvenile mice.

Petriello et al. (2018) [55] found that exposure to PCB126 (1 mmol/kg) induces disruption of the gut microbiota and host metabolism in seven

week-old male Ldlr −/− mice, which are a model of cardiometabolic disease. This PCB126 dose was selected because it results in plasma concentrations similar to those found in human exposure [110]. The 16S rRNA sequencing revealed changes at the phylum and genus levels, and consistent increases in intestinal and systemic inflammation. The Firmicutes/Bacteroidetes ratio was increased after PCB126 exposure. This increase has been consistently linked to obesity, insulin resistance, inflammation, and other alterations of host metabolism [111]. Additionally, a strong PCB-dependent association was found between Bifidobacterium and circulating glucagon-like peptide-1.

Chi et al. 2019 [52] exposed adult female C57BL/6 mice to environmentally relevant low-dose PCB126 (50 µg/kg bw) once per week over six weeks and found that chronic low-dose exposure promoted dysbiosis, with changes to the microbiota's structure and composition. In addition, PCB126 promoted dyslipidemia, hepatic damage, and a fatty liver. Lastly, metabolic indicators of these conditions seem to positively correlate with specific bacterial taxa.

The study by Chen et al. (2018) [50] showed that the exposure of zebrafish to model pollutants with different mechanisms of action and different affinities to estrogen and aryl hydrocarbon receptors (atrazine, estradiol, PCB126, and PCB153) at 1.0 µg/L for seven days resulted in changes to the microbiota and in the deterioration of intestinal and hepatic functions. The authors reported that aryl hydrocarbon and estrogen receptor signaling regulate gut microbiota physiology. Data showed that impaired Aeromonas reproduction was significantly related to oxidative damage, especially in the PCB126 groups. Previous research has linked Aeromonas spp. to intestinal inflammation and soft tissue infection [112].

#### 3.2.4. Parabens

Parabens are widely used as preservatives and bactericides in pharmaceuticals, personal care products, and some food products

[113,114,115]. Several studies have reported on the adverse health effects of parabens, including their endocrine disrupting and obesogenic activities [116,117,118,119]. However, there are few data available regarding the effects of parabens on the microbiota.

In this respect, Hu et al. (2016) [66] analyzed the effect of low-dose exposure to methylparaben, triclosan, and diethylphthalate and their mixtures, as well as the window of susceptibility, in Sprague-Dawley rats. The doses investigated result in urinary biomarker levels similar to those observed in humans [120]. The Bacteroidetes phylum was increased, while the growth of Firmicutes was decreased in all exposed rats compared to controls. However, Betaproteobacteria was increased only in the methylparaben and mixture groups, suggesting that exposure to paraben mixtures produces a distinct microbiome shift different from that resulting from individual chemicals or from simple additive effects. Surprisingly, these differences decreased in adulthood. In addition, a reduction in body weight in rats exposed during adolescence was observed [66]. This is consistent with other studies reporting a decrease in the Bacteroidetes phylum and an increase in Firmicutes, linked to weight gain [121,122]. These studies highlight i) the importance of studying the critical window of exposure such as adolescence; ii) the effect of low dose exposure, similar to a human exposure scenario; and iii) the need to evaluate the combined effect of multiple exposures. Obadia et al. (2018) [65] observed that exposure to methylparaben (0.1% and 0.3%) in fly medium reduces microbiota growth and modifies the composition and amount of the bacteria and yeasts in the intestine of the Drosophila fly.

The effects of parabens on the microbiota need further research, as their interactions are poorly understood.

### 3.2.5. Phytoestrogens

Phytoestrogens are compounds naturally occurring in plants that have estrogenic/antiestrogenic effects. Phytoestrogens can modulate and be metabolized by the gut microbiota [123]. Phytoestrogen activity is

strongly dependent on the microbiome. Their metabolites have stronger estrogenic activity than the natural compounds themselves, and because of the variability in microbiomes, there are large differences in the effects of phytoestrogens among individuals [124,125].

Daidzen, a phytoestrogen present in soy-based foods, can be metabolized to O-desmethylangolensin (ODMA) and equol by gut microbial communities in 80–95% and 25–60% of the population, respectively. In relation to this, Frankenfeld et al. (2011, 2014) [126,127] evaluated the presence of ODMA- and equol-metabolizing phenotypes in obese, overweight, and normal-weight individuals and found that the ODMA-metabolizing phenotype, but not the equol-phenotype, was linked to obesity in adulthood.

Another study investigated the effects of S-equol on pancreatic ß-cell growth and insulin secretion in male mice. The results showed that S-equol boosts ß-cell function and prevents hypoglycemia in mice, suggesting that S-equol may act as a potential preventive agent against type 2 diabetes mellitus [57]. Zhou et al. (2018) [64] investigated whether genistein intake by C57BL/6 female mice can reduce the negative impact of a maternal fat-high diet on glucose and lipid metabolism in their offspring. Female mice were placed on a high-fat diet alone, a high-fat diet supplemented with genistein at low (0.25 g/kg diet) and high doses n (0.6 g/kg diet), or a genistein-free control diet, for three weeks prior to pregnancy and throughout gestation and lactation. After weaning, female offspring from the high-fat group had lower weight at birth, as well as glucose intolerance and higher insulin, triacylglycerol, and total cholesterol levels in the serum compared with the control group. Offspring from the low-dose genistein group showed an increased weight at birth, improved glucose tolerance, and decreased fasting insulin. Offspring from the high-dose genistein group showed decreased serum triacylglycerols and total cholesterol compared with the offspring from the low-dose genistein mothers. The high abundances of Bacteroides and Akkermansia in the offspring from the genistein-fed female parents

might be key to the improvement of glucose metabolism. A decrease of Bacteroides has been shown in diabetes patients in comparison to in healthy controls [128]. In addition, it has been reported that Akkermansia could preserve the mucus layer thickness and is correlated with an improved metabolic profile [129]. Similarly, the increased Rikenella in the offspring from the high genistein group might be linked to the decreased triacylglycerols and total cholesterol in the serum. In addition, it has been reported that the abundance of Rikinella contributes to a lean body type phenotype [130].

Lopez et al. (2018) [59] also found, in nine-week-old male C57/BL6 mice, lower serum triglycerides, improved glucose metabolism, and lower weights in the high-fat diet and genistein group (3 mg/kg/day) compared to in mice fed the high-fat diet alone. In addition, the presence of genistein in the high-fat diet resulted in changes to the gut microbiota (increases in the Prevotella and Akkermansia genera), linked to lower circulating levels of lipopolysaccharides and the reduced expression of pro-inflammatory cytokines in the liver, compared to in mice in the high-fat diet alone group. It has been showed that the reduction of lipopolysaccharides can decrease neuroinflammation [131].

Recently, Huang et al. (2018) [58] investigated, in non-obese diabetic mice, perinatally exposed to physiological doses of genistein (20 mg/kg body weight), whether there is a sex-dependent effect on type 1 diabetes (T1D). In female offspring, perinatal exposure to genistein resulted in a higher incidence of early-onset T1D. In addition, increased Enterobacterials were found in the fecal microbiota from the PND90 female offspring, which is indicative of a pro-inflammatory response. These changes were not found in the PND30 females. However, perinatal genistein exposure in PND90 males induced changes in the gut microbiota linked to an anti-inflammatory response. The authors conclude that a strong sex-specific effect was found in the perinatal genistein exposure window and that the mechanism of T1D in non-obese diabetic females is induced by immune system modulation of the gut

microbiota. These results must be taken into account, since soy milk formula consumption during infancy was related to type 1 diabetes [132].

In California mice (Peromyscus californicus), Marshall et al. (2019) [60] also determined whether perinatal exposure to genistein (250 mg/kg feed weight) promoted dysbiosis and altered gut metabolites. Female mothers were fed a diet with genistein or a genistein-free control diet. Their results showed that exposure to genistein resulted in sex-related disturbances of the gut microbiota and metabolites in the offspring. Positive associations between the gut microbiome, metabolome, and disruption of social and vocalization behaviors (audible calls above 20 kHz) were also found in the offspring of exposed dams. When male offspring from genistein-supplemented dams were compared with genistein-free offspring, calls above 20 kHz correlated with daidzein, α-tocopherol, Flexispira spp., and Odoribacter spp. The effects secondary to genistein exposure may result from disturbances to neurobehavioral programming or from changes in the microbiota linked to changes in gut metabolites. These results suggest that the gut microbiome and its metabolites can induce a disruption in the offspring's neurobehavioral programming, known as the "microbiome gut–brain axis".

The effect of soy intake on microbiota composition and diversity has been studied in other animal models, such as porcine models [61,63] and the Southern white rhinoceros [62]. Yeruva et al. (2016) [63] determined the influence of diet on the development of the immune system in neonates, using a porcine model. Two-day old piglets were fed soy or milk formula until day 21 and compared to a sow-fed group, and the results showed that the formula diets induced changes in the small intestine microbiome, particularly in the duodenum. Significant increases in Lactobacillaceae spp. and Clostria spp., as well as a decrease in Enterobacteriaceae spp., were found in soy-fed piglets.

There is, however, little information regarding the impact of phytoestrogens on the human microbiota. Wu et al. (2016) [82] compared the plasma metabolites in omnivores versus vegans that consume

soyfoods and found significant differences in the metabolomes between the two groups, but the gut microbiota was very similar in both groups [82].

3.2.6. Metals

Metals have been considered EDCs because of their ability to bind to hormone receptors [133]. Metals are ubiquitous environmental pollutants, with the primary sources of human exposure being the inhalation of dust or direct ingestion of contaminated food and water. Metal exposure has been related to obesity, diabetes, and metabolic syndrome [134,135,136,137].

Metals can be metabolized by the colonic microbiota in humans. Van de Wiele et al. (2010) [35] reported the ability of this microbiota to methylate Arsenic (As), which suggests that the role played by microbiota metabolism should be considered when assessing the toxic effects on human health of ingested As.

Lu et al. (2014) [45] reported that the composition of the gut microbiome in C57BL/6 mice markedly alters after exposure to 10 ppm arsenic in the drinking water over 4 weeks, resulting in a decrease in four Firmicutes families. These results are in agreement with the As antiobesogenic properties described in several articles [138]. They also reported a significant association between this microbiota disruption and changes in microbiota metabolites. This suggests that exposure to As not only induces disturbances in the abundance and composition of bacterial communities but also affects their metabolomic profile, which subsequently results in the disturbance of host metabolite homeostasis. These changes in metabolite homeostasis are important risk factors involved in tissue dysfunctions, which may cause diseases such as obesity and diabetes [139].

Wu et al. (2016) [43] reported that perinatal lead (Pb) exposure (32 ppm) in the drinking water, in wild-type non-agouti (a/a) mice of the Avy strain isogenic mouse model of perinatal environmental exposure,

induced changes in the adult offspring gut microbiota. These changes were sex-independent, but a strong association was found between male offspring and increased body weight. Interestingly, the quantities of the two predominant phyla (Bacteroidetes and Firmicutes) shifted inversely with Pb exposure. In addition, reduced aerobic bacteria and increased anaerobic bacteria were observed in the exposed offspring. Lastly, Pseudomonas, Enterobacter, and Desulfovibrio were found in higher abundances in exposed adult mice than in controls ($p < 0.05$) [43].

Ba et al. (2017) [42] demonstrated the sex-specific effects of low-dose exposure to cadmium and found that early exposure to 100 nM induced fat accumulation in adult male C57BL/6J mice. In this work, 100 nM cadmium was present in the drinking water, which is equivalent to ~ 2.5 µg/kg bw per week and corresponds to the tolerable weekly intake and the mean intake by humans [140]. They also found an increased metabolism of fatty acids and lipids as well as a decrease in the composition and diversity of the gut microbiota. At eight weeks, the gut microbiota was found to be particularly vulnerable to low-dose cadmium exposure, and exposure during this period may induce adiposity in adult mice, even if the microbiota is later restored. The role played by the gut microbiota in adiposity related to cadmium exposure was also demonstrated by microbiota transplantation and removal experiments.

Xia et al. (2018) [44] observed that short-term exposure to 10 and 30 µg/L Pb increased the volume of intestinal mucus in the adult male zebrafish. They also found decreased α-Proteobacteria and increased Firmicutes after exposure to 30 µg/L Pb for seven days. In addition, 16S rRNA sequencing demonstrated an altered gut microbiota, in terms of composition and diversity, after exposure to 30 µg/L Pb. At this dose, 52 gut microbes and 41 metabolites underwent significant changes, particularly those related to the pathways of glucose, lipid, amino acid, and nucleotide metabolism. Lastly, they also found a marked reduction in the transcription of some genes related to glycolysis and lipid metabolism after seven-day exposure to 30 µg/L Pb.

A recent study showed that the urinary concentrations of Pb in adult humans were related to changes in gut microbial composition, even at low Pb levels [81], and so an association between increased urinary Pb concentrations and increased microbiota α-diversity and richness was found. Changes in ß-diversity were significantly associated with changes in urinary Pb concentrations, and Proteobacteria, including members of the Burkholderiales (a wide variety of bacterial species that perform a plethora of metabolic functions), were also associated with increased urinary Pb [81].

3.2.7. Triclosan and Triclocarban

Triclosan (TCS) and triclocarban (TCC) (TCs) are chlorinated, broad-spectrum antimicrobial endocrine disrupting chemicals found in thousands of consumers and industrial products [141], as well as in contaminated food [142,143]. These EDCs have been related to metabolic disorders such as obesity and diabetes [144,145].

It has been shown that TCS exposure induces changes in the gut microbiota of rats [66,77], mice [75], and fish [76,79]. Narrowe et al. (2015) [79] showed that even low but environmentally relevant levels of triclosan exposure (100–1000 ng/mL) can result in the disturbance of the juvenile fish gut microbiome. Seven-day exposure to triclosan in larval fathead minnows (P. promelas) resulted in significant changes, as measured by α- and ß-diversity, in the gut microbiome immediately after triclosan exposure; however, the microbiome rapidly recovered following two weeks of depuration. This demonstrates the sensitivity and resilience of the gut flora to the toxic effects of environmental contaminants.

Kennedy et al. (2016) [77] found that Sprague Dawley rats with ad libitum access to commercial Harlan ground 2020X supplemented with 0.1% w/w triclocarban during gestation and lactation exhibited significant changes in the community structure of the fecal microbiota, as well as finding decreased phylogenetic diversity in exposed dams and neonatal rats. Marked differences in ß-diversity were found in exposed

animals compared with controls in dams at 18 days of gestation and 16 day old neonates. This dose was chosen as it has been demonstrated that the serum TCC concentration of pregnant rats after oral exposure to 0.2% w/w TCC was similar to the concentrations reported in the human serum [146].

Ma et al. (2020) [78] studied the long-term effects in adult and old rats of perinatal exposure to TCs and found that 50 mg/kg/day (the lowest toxic oral dose in rats) resulted in disturbances of the metabolism and gut microbiota that were long-lasting and persisted even after the exposure had been terminated. They also accumulated over time, inducing metabolic disorders in old rat offspring. Exposure to TCs induced an increased growth of Bacteroidetes, which has been related to lipid accumulation [42]. Additionally, a reduction in Akkermansia muciniphila, a species linked to improved metabolism in diabetic and obese mice, was observed [147].

Interestingly, probiotics have been used to modulate the microbiota and palliate intestinal metabolic disorders due to triclosan exposure in animal models [80]. In this respect, Lactobacillus plantarum ST-III has been found to increase the diversity of the gut microbiota in zebrafish, thereby reducing the toxicity of chronic exposure to triclosan. Additionally, a probiotic-rich diet reduced the risk of lipid-metabolism disorders such as increased triglyceride and total cholesterol levels. Histopathological studies demonstrated severe structural damage to the intestines, spleen, and kidney after triclosan exposure; however, this damage can be reduced by the presence of Lactobacillus.

Bever et al. (2018) [85] compared the fecal microbiome of infants fed with breast milk that had measurable levels of TCS versus infants fed with breast milk that had no detectable concentrations of TCS and found that early life exposure to exogenous contaminants induces changes to microbiome diversity. Because of the impact of a healthy infant gut microbiome on phenotypes later in life, understanding how EDCs

influence the infant gut microbiome is critical to identifying and correcting problematic changes in infant gut health.

However, Ribado et al. (2017) [86] did not find that exposure to household TC-containing products induces changes to or a loss of microbial diversity, but they found increased Proteobacteria spp. in infants and mothers exposed to higher TC levels. Interestingly, increased Proteobacteria has been proposed as a potential diagnostic marker for dysbiosis and an increased risk of diabetes and colitis [148].

3.2.8. Phthalates

Phthalates are EDCs used as plasticizers in food processing and packaging, adhesives, personal care products, and cosmetics. A major source of phthalate exposure is the diet, primarily due to contamination during processing and packaging [149]. Phthalates have been considered obesogens, therefore contributing to overweightness and obesity. It has been shown that exposure to phthalates alters glucose and lipid metabolism, which increases the risk of developing insulin resistance [150,151].

A mouse model of prenatal di(2-etilhexil) ftalato (DEHP) exposure (0.2, 2, and 20 mg/kg/day) was used to study the long-term metabolic disturbances in offspring. In an ICR mouse model of prenatal DEHP exposure (0.2, 2, and 20 mg/kg/day), Fan et al. (2020) [67] showed that exposure to low-dose phthalate (0.2 mg/kg/day) in mice induced changes in glucose metabolism, energy expenditure, adipogenesis, and gut dysbiosis in a sex-dependent manner. The level of DEHP exposure was selected based on the EPA reference dose. Their findings strengthen the hypothesis that connections between the host and gut microbiota alter energy metabolism.

As mentioned above, Hu et al. (2016) [66] reported that postnatal, low-dose exposure to diethyl phthalate (DEP) in Sprague-Dawley rats from birth through adulthood induced changes in the composition of the gut microbiota, but these changes were seen only in adolescent rats. The

changes include an increased relative abundance of Bacteroidetes (Prevotella) Elusimicrobia and decreased Firmicutes (Bacilli) in exposed rats versus controls. Surprisingly, these DEP-induced changes decreased in adulthood despite continuous exposure, which suggests that the effects of exposure to environmental chemicals are more severe in adolescents. They also observed a small but consistent reduction of body weight in exposed adolescent rats, which is consistent with their findings of a reduced Firmicutes/Bacteroidetes ratio.

Lei et al. (2019) [37] conducted in vivo and in vitro experiments in female C57BL/6J mice to determine the effects of low or high dose DEHP (1 or 10 mg/kg bw/day) exposure on the gut microbiota composition and metabolite profile. The authors observed an increased abundance of Lachnoclostridium and decreased Clostridium sensu stricto after DEHP exposure. The addition of DEHP to the cultured cecal microbiota enhanced the abundance of Lachnoclostridium, which is able to produce p-hydroxyphenylacetic acid, the precursor of p-cresol, a bacterial metabolite linked to neurodevelopmental disorders.

Regarding human epidemiological studies, the information is scarce. Yang et al. (2019) [83] demonstrated in a recent epidemiological study showing that DEHP exposure in newborns resulted in changes to the microbiota composition, with a decrease in Rothia sp. and Bifidobacterium longum. The presence of Rothia in human milk has been associated with a minor incidence of asthma [152], and B. longum, considered a probiotic, seems to have positive effects on infants, particularly in reducing the risk of obesity and celiac disease.

## 4. Conclusions

The incidence of metabolic diseases such as obesity and T2D are increasing worldwide. Exposure to EDCs related to food intake induces a series of changes including microbial dysbiosis and the induction of xenobiotic pathways and associated genes, enzymes, and metabolites involved in EDC metabolism. The products and by-products released

following the microbial metabolism of EDCs can be taken up by the host and could have a major impact on host metabolism and the development of metabolic diseases. However, data regarding the effects of EDCs on the human gut microbiota are limited. The increasing EDC exposure via dietary intake requires the identification of the compounds and of the specific responses of the different species of the gut microbiome. In addition, the characterization of the common mechanisms of action of the different EDCs—such as the binding of the same hormone receptors, their possible cumulative and combined effects, and the indicative bacteria underlying the toxicity of EDCs on the gut microbiota—is also essential. In addition, the effect of exposure to low EDC levels on microbiota disruption should also be considered. The impact of other parameters such as host age and sex on the gut microbiota, described in this review, make necessary further research with broader dose ranges and analyses with more time points. This will help to determine the origin of sex-dependent effects using additional exposure windows. Lastly, the remediation of EDC-induced changes in the gut microbioma might represent an alternative for the treatment and prevention of metabolic diseases.

## Declarations

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# 5. CONCLUSIONES / CONCLUSIONS

## 5.1. Conclusiones

La presente Tesis Doctoral se ha centrado en el estudio de los DEs, especialmente los bisfenoles y su relación con el sobrepeso/obesidad infantil. Se ha observado que el BPA sigue siendo predominante en los alimentos y muestras biológicas, pero otros análogos como el BPS, el BPAF, el BPF y el BPAP también merecen especial atención. En relación con su asociación con el sobrepeso/obesidad, la presente Tesis Doctoral sugiere que se requieren más estudios longitudinales para una evaluación más exhaustiva sobre su impacto en la ganancia de peso durante la infancia.

Además, dado que lo hallazgos reflejan que los análogos están reemplazando al BPA, la exposición a estos seguirá aumentando. La ausencia actual de un marco legal sobre los análogos y el no establecimiento de IDT, son el motivo por el cual cada vez son más los análogos detectados tanto en alimentos como en muestras biológicas. Por ello, es crucial la realización de más estudios que evalúen los principales riesgos potenciales de los análogos sobre la salud y el establecimiento de límites toxicológicos para estos y la suma de todos los bisfenoles (efecto cóctel). Por otro lado, aunque los parabenos no han sido los compuestos químicos de interés principal de estudio de la presente Tesis Doctoral los hallazgos encontrados en dos de los trabajos de la Tesis Doctoral subrayan la necesidad de revisar la regulación y uso de parabenos (MetPB y EthPB junto con sus sales) en productos de consumo como conservantes en la industria alimentaria, así como de promover investigaciones adicionales para comprender mejor sus efectos sobre la ganancia de peso en población infantil.

### Conclusiones específicas de la presente Memoria de Tesis Doctoral.

1. Nuestros hallazgos confirman la detección de cantidades significativas de DEs (parabenos y bisfenoles) en productos alimentarios de consumo diario de una población infantil española.

Se detectó un porcentaje mayor de parabenos en comparación con los bisfenoles en los alimentos analizados.

2. Pese a que el BPA está siendo reemplazado gradualmente por sus análogos en muchos materiales que entran en contacto con los alimentos, sigue siendo el bisfenol que se detecta con mayor frecuencia en estos, seguido del BPS.

3. Teniendo en cuenta el nuevo límite de IDT del BPA establecido por la EFSA en abril de 2023 es de 0,2 ng/kg pc/día, la exposición dietética estimada para nuestra población de estudio superó dicha IDT. Además, debido que en los alimentos se han detectado los análogos del BPA, es necesaria una evaluación de riesgos de su presencia y el establecimiento de nuevos límites de IDT para cada bisfenol individualmente y para la suma de los bisfenoles.

4. Esta Tesis demuestra la importancia de recopilar más datos sobre la presencia de los DEs en los alimentos para evaluar la exposición dietética y el posible impacto en la salud, especialmente para las poblaciones más vulnerables.

5. Los alimentos frescos son considerados más saludables y con una menor exposición a los DEs. Sin embargo, tras analizar los alimentos más consumidos en nuestra población de estudio, observamos que los alimentos frescos, son las principales fuentes de exposición a los bisfenoles (BPA, BPS y bisfenoles totales) a través de la dieta, en comparación con los alimentos procesados. Esto se debe a la presencia de los bisfenoles en el medio que nos rodea (agua, polvo, suelos, entre otros), llegando a la cadena trofica y terminando por llegar a nosotros a través de multiples fuentes, siendo la dieta la principal.

6. La presente Tesis Doctoral reveló la presencia del BPA, el BPAF, el BPF, el BPS y el BPAP en diversas matrices biológicas, siendo el pelo seguido de la uña las que mostraron las mayores concentraciones de bisfenoles en general. Los sujetos con sobrepeso/obesidad mostraron

los niveles más elevados del BPA en pelo, uña y saliva y los bisfenoles totales en uña y saliva. Sin embargo, el grupo control mostró en general concentraciones más elevadas del BPA y los bisfenoles totales en la orina, mientras que el pelo mostró concentraciones más elevadas del BPS, el BPAF y los bisfenoles totales en este grupo. Por otro lado, se analizaron los parabenos en el cabello, encontrándose en concentraciones más altas que los bisfenoles. El grupo de los casos mostró una mayor presencia de los parabenos en el cabello en comparación con el grupo control.

7. Nuestros resultados indican que basarse únicamente en la orina para medir la exposición a los bisfenoles u otros químicos DEs podría dar lugar a incoherencias a la hora de relacionar los niveles de estos con el sobrepeso/obesidad, debido a la importante variabilidad dentro de un mismo individuo en un mismo día y de un día a otro. Dado que la orina muestra solo la presencia de estos compuestos químicos a corto plazo, es necesario analizar estos contaminantes en otras matrices biológicas para evaluar la exposición a largo plazo, especialmente en el contexto de enfermedades crónicas como la obesidad.

8. En la presente Memoria de Tesis Doctoral, se ha observado que el pelo y la uña, por su naturaleza reflejan una exposición acumulativa a largo plazo sin fluctuaciones en las concentraciones, ofreciendo un mayor potencial como matrices biológicas indicadoras de exposición. Considerando que la obesidad es una enfermedad crónica, el pelo y las uñas son matrices biológicas prometedoras para revelar la exposición a estos DEs a largo plazo. Con respecto a la saliva, también es una alternativa prometedora a la sangre para evaluar las exposiciones a compuestos químicos, dada su naturaleza no invasiva y su capacidad para reflejar las concentraciones de tóxicos presentes en la sangre.

9. Se ha estudiado la posible asociación entre la exposición al BPA y 11 análogos y el sobrepeso/obesidad infantil, además de 6 parabenos en el caso del cabello. Los resultados sugiren una asociación positiva

entre una mayor presencia del BPF en las uñas y la probabilidad de presentar sobrepeso u obesidad infantil. Con respecto a las diferencias entre sexos, se observó una asociación negativa entre una mayor presencia a los bisfenoles totales en pelo y el sobrepeso/obesidad en el sexo masculino. Asimismo, se asoció una mayor presencia del PropPB en el cabello de los chicos con una mayor probabilidad de sobrepeso u obesidad. En lo que respecta al resto de los DEs, no se encontraron asociaciones estadisticamente significativas entre estos y el sobrepeso/obesidad en la población general o estratificada por sexos. Por último, dado que la evidencia científica es limitada sobre la asociación entre los bisfenoles y el sobrepeso/obesidad se requiere más estudios epidemiológicos y toxicológicos para evaluar si la exposición humana al BPA y sus análogos aumenta la probabilidad de sobrepeso/obesidad en los escolares.

## 5.2. Conclusions

The present Doctoral Thesis has focused on the study of DEs, especially bisphenols and their relationship with childhood overweight/obesity. It has been observed that BPA is still predominant in food and biological samples, but other analogues such as BPS, BPAF, BPF and BPAP also deserve special attention. In relation to their association with overweight/obesity, the present Doctoral Thesis suggests that further longitudinal studies are required for a more comprehensive assessment on their impact on weight gain during childhood.

Furthermore, as the findings reflect that analogues are replacing BPA, exposure to BPA analogues is expected to continue to increase. The current lack of a legal framework on analogues and the non-establishment of TDIs are the reason why analogues are increasingly detected in both food and biological samples. Therefore, further studies assessing the main potential health risks of the analogues and the establishment of toxicological limits for the analogues and the sum of all bisphenols (cocktail effect) are crucial. On the other hand, although

parabens have not been the main chemical compounds of interest of study in this Doctoral Thesis, the findings found in two of the works of the Doctoral Thesis underline the need to review the regulation and use of parabens (MetPB and EthPB together with their salts) in consumer products as preservatives in the food industry, as well as to promote further research to better understand their effects on weight gain in children.

**Specific conclusions of this Doctoral Thesis Memory.**

1. Our findings confirm the detection of significant amounts of DEs (parabens and bisphenols) in food products of daily consumption in a Spanish children's population. A higher percentage of parabens compared to bisphenols was detected in the foods analyzed.

2. Although BPA is gradually being replaced by its analogues in many food contact materials, it is still the most frequently detected bisphenol in food, followed by BPS.

3. Taking into account the new TDI limit for BPA set by EFSA in April 2023 of 0.2 ng/kg bw/day, the estimated dietary exposure for our study population exceeded this TDI. In addition, because BPA analogues have been detected in food, a risk assessment of their presence and the establishment of new TDI limits for each individual bisphenol and for the sum of bisphenols is necessary.

4. This Thesis demonstrates the importance of collecting more data on the presence of DEs in food to assess dietary exposure and potential health impacts, especially for the most vulnerable populations.

5. Fresh foods are considered healthier and with lower exposure to DEs. However, after analysing the most consumed foods in our study population, we observed that fresh foods are the main sources of dietary exposure to bisphenols (BPA, BPS and total bisphenols) compared to processed foods. This is due to the presence of bisphenols in the environment around us (water, dust, soil, etc.), reaching the

trophic chain and eventually reaching us through multiple sources, diet being the main one.

6. The present Doctoral Thesis revealed the presence of BPA, BPAF, BPF, BPS and BPAP in various biological matrices, with hair followed by nail showing the highest concentrations of bisphenols overall. Overweight/obese subjects showed the highest levels of BPA in hair, nail and saliva and total bisphenols in nail and saliva. However, the control group showed overall higher concentrations of BPA and total bisphenols in urine, while hair showed higher concentrations of BPS, BPAF and total bisphenols in this group. On the other hand, parabens were analyzed in the hair, being found in higher concentrations than bisphenols. The case group showed a higher presence of parabens in the hair compared to the control group.

7. Our results indicate that relying solely on urine to measure exposure to bisphenol or other DEs chemicals could lead to inconsistencies in relating levels to overweight/obesity, due to significant intra-individual variability on the same day and from day to day. Since urine only shows the presence of these chemicals in the short term, it is necessary to analyse these pollutants in other biological matrices to assess long-term exposure, especially in the context of chronic diseases such as obesity.

8. In this Doctoral Thesis Memoir, it has been observed that hair and nails, by their nature, reflect a long-term cumulative exposure without fluctuations in concentrations, offering a greater potential as biological exposure indicator matrices. Considering that obesity is a chronic disease, hair and nails are promising biological matrices to reveal long-term exposure to these DEs. With respect to saliva, it is also a promising alternative to blood for assessing exposures to chemical compounds, given its non-invasive nature and its ability to reflect the concentrations of toxicants present in the blood.

9. The possible association between exposure to BPA and 11 analogues and childhood overweight/obesity has been studied, in addition to 6 parabens in the case of hair. The results suggest a positive association between an increased presence of BPF in nails and the likelihood of childhood overweight/obesity. With regard to gender differences, a negative association was observed between a higher presence of total bisphenols in hair and overweight/obesity in males. Likewise, a higher presence of PropPB in the hair of boys was associated with a higher probability of being overweight or obese. Regarding the rest of the DEs, no statistically significant associations were found between these and overweight/obesity in the general population or stratified by sex. Finally, given the limited scientific evidence on the association between bisphenols and overweight/obesity, further epidemiological and toxicological studies are needed to assess whether human exposure to BPA and its analogues increases the likelihood of overweight/obesity in school children.

## 6. CÓMO PREVENIR LA EXPOSICIÓN A BISFENOLES

La relevancia, una vez adquiridos los conocimientos científicos, radica en nuestra capacidad para enseñar y demostrar de forma práctica lo que podemos hacer para minimizar la exposición al BPA y análogos, dados sus posibles efectos negativos sobre la salud, y especialmente en poblaciones vulnerables como en las primeras etapas de la vida.

Como se mencionó en la introducción en el **apartado 1.4.1** los bisfenoles se van a encontrar como constituyente del plástico. La reducción de la exposición a los bisfenoles se puede lograr siguiendo los siguientes consejos prácticos:

- Reducir o evitar materiales plásticos principalmente aquellos en contacto con productos alimentarios.
- Sustituir los envases de plásticos por otras alternativas más seguras como, por ejemplo, recipientes de vidrio, acero inoxidable u otros materiales seguros para almacenar y calentar alimentos.
- Evitar calentar alimentos en envases de plástico.
- Evitar dejar botellas de agua embotelladas en plástico o alimentos envasados en plástico al sol, ya que esto puede provocar la migración de los bisfenoles hacia el alimento o bebida.
- Elegir productos etiquetados como libres de bisfenoles, actualmente podemos encontrar opciones más seguras en juguetes, utensilios de cocina y envases de alimentos que estén certificados como libres de bisfenoles.
- Saber leer correctamente el significado de los símbolos de los envases plásticos.

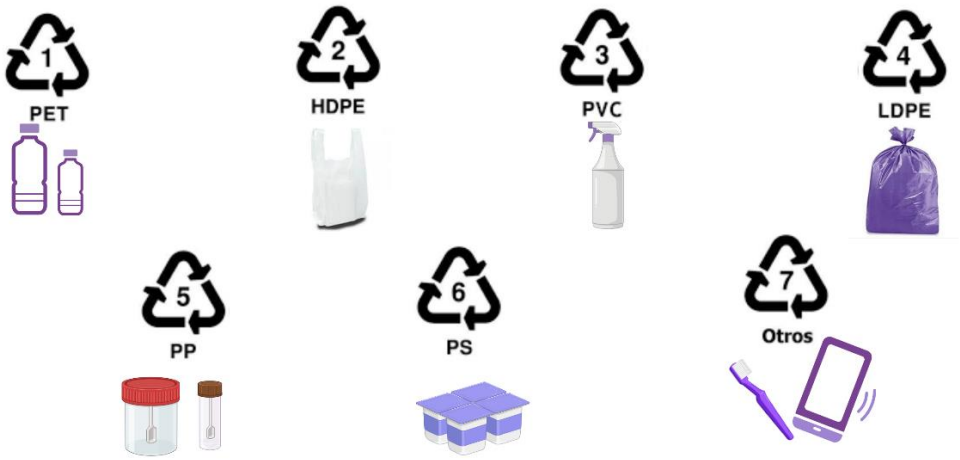


**Figura 19.** Símbolo de los envases plásticos. Elaborado con Biorender.com

- Evitar productos con PVC, ya que pueden liberar bisfenoles presentes en su composición.
- Revisar las etiquetas de los productos para identificar aquellos que contienen policarbonato, dado que puede contener bisfenoles.
- Optar por alimentos frescos sin envasar frente a alimentos envasados.
- Fomentar la conciencia pública sobre que son los bisfenoles y los riesgos asociados a su exposición.

Basándonos en resultados previos y los hallazgos de esta Tesis Doctoral, hemos observado que estos compuestos están ampliamente distribuidos en nuestro medio y se han detectado en alimentos frescos. Por lo tanto, actualmente es imposible evitar por completo la exposición. Sin embargo, al reducir la dosis de exposición a los bisfenoles presentes en el medio mediante la aplicación de estas sugerencias, se puede minimizar la exposición, preservar el bienestar y mejorar la calidad de vida a largo plazo, especialmente entre los grupos de edad más vulnerables.

# ANEXO I.

## Artículos de la memoria de tesis

foods

MDPI

Article

# Presence of Parabens and Bisphenols in Food Commonly Consumed in Spain

**Yolanda Gálvez-Ontiveros [1,†], Inmaculada Moscoso-Ruiz [2], Lourdes Rodrigo [3], Margarita Aguilera [4,†], Ana Rivas [1,*,†] and Alberto Zafra-Gómez [2,†]**

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**Abstract:** Given the widespread use of bisphenols and parabens in consumer products, the assessment of their intake is crucial and represents the first step towards the assessment of the potential risks that these compounds may pose to human health. In the present study, a total of 98 samples of food items commonly consumed by the Spanish population were collected from different national supermarkets and grocery stores for the determination of parabens and bisphenols. Our analysis demonstrated that 56 of the 98 food samples contained detectable levels of parabens with limits of quantification (LOQ) between 0.4 and 0.9 ng $g^{-1}$. The total concentration of parabens (sum of four parabens: ∑parabens) ranged from below the LOQ to 281.7 ng $g^{-1}$, with a mean value of 73.86 ng $g^{-1}$. A total of 52% of the samples showed detectable concentrations of bisphenols. Bisphenol A (BPA) was the most frequently detected bisphenol in the food samples analysed, followed by bisphenol S (BPS) and bisphenol E (BPE). Bisphenol AF (BPAF), bisphenol B (BPB) and bisphenol P (BPP) were not found in any of the analysed samples. LOQ for these bisphenols were between 0.4 and 4.0 ng $g^{-1}$.

**Keywords:** parabens; bisphenols; food; Spain



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## 1. Introduction

The antimicrobial activity of parabens has been known since 1924 and for this reason these alkyl esters of *p*-hydroxybenzoic acid have been extensively used as preservatives in many consumer products such as health and personal care product and foodstuffs [1–3]. Parabens are regulated as preservatives in Commission Regulation (EU) No 1004/2014 on cosmetic products that sets a maximum limit of 0.4% and 0.8% for single esters and mixtures of esters, respectively [4].

There are growing concerns about the presence of these preservatives in pharmaceuticals and cosmetic products associated with their estrogenic effects demonstrated by in vivo and in vitro studies [5]. This disrupting hormone activity seems to be linked to the length of the alkyl chain, with long-chain parabens like propyl 4-hydroxybenzoate (PropPB) and butyl 4-hydroxybenzoate (ButPB) being those of highest concern [6]. In 2010, the EU Scientific Committee on Consumer Safety (SCCS) considered that the use of methyl 4-hydroxybenzoate (MetPB) and ethyl 4-hydroxybenzoate (EthPB) at the maximum authorized concentrations is safe but due to the lack of scientific data, the Committee cannot ascertain that the use of PropPB and ButPB at the maximum concentrations is completely safe [7]. With respect to the use of MetPB and EthPB (food additives E218 and E214, respectively) and their sodium salts (E219 and E215, respectively) in foodstuffs, the

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# Dietary bisphenols exposure as an influencing factor of body mass index

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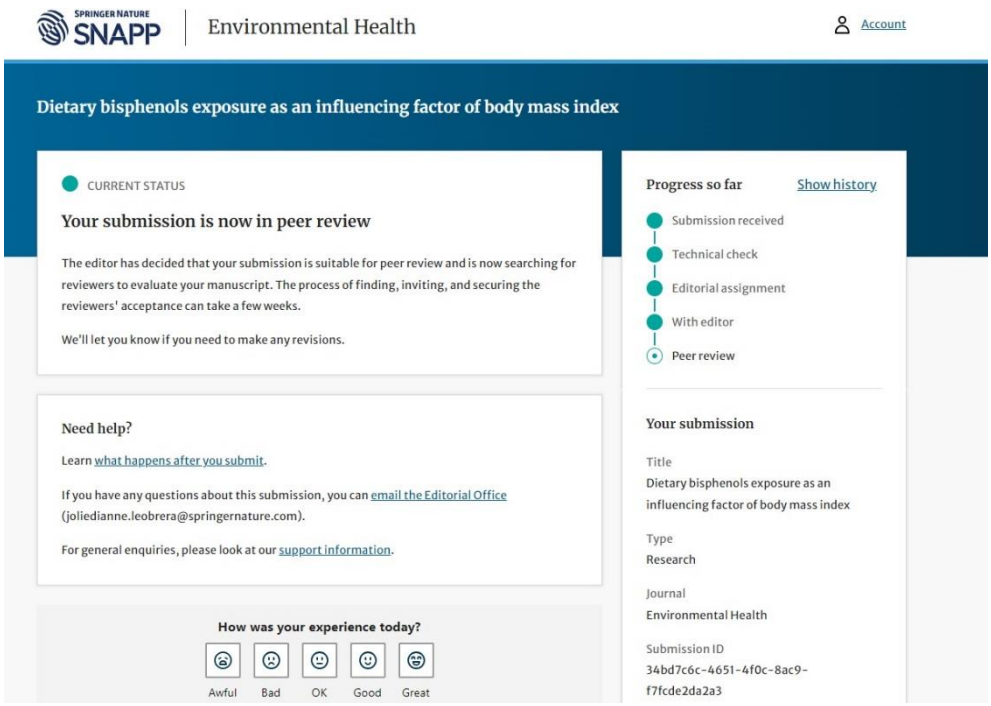
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# Levels of Bisphenol A and its analogs in nails, saliva, and urine of children: a case control study

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**Introduction:** A growing number of studies link the increase in overweight/obesity worldwide to exposure to certain environmental chemical pollutants that display obesogenic activity (obesogens). Since exposure to obesogens during the first stages of life has been shown to have a more intense and pronounced effect at lower doses, it is imperative to study their possible effects in childhood. The objective here was to study the association of Bisphenol A (BPA) and 11 BPA analogs in children, using three biological matrices (nails, saliva and urine), and overweight and obesity ($n$ = 160).

**Methods:** In this case–control study, 59 overweight/obese children and 101 controls were included. The measuring of Bisphenols in the matrices was carried out by ultra-high performance liquid chromatography coupled with triple quadrupole tandem mass spectrometry (UHPLC-MS/MS). Logistic regression was used to study the association between overweight/obesity and Bisphenol exposure.

**Results:** The results suggested that BPF in nails is associated with overweight/obesity in children (OR:4.87; p = 0.020). In saliva, however, the highest detected concentrations of BPAF presented an inverse association (OR: 0.06; $p$ = 0.010) with overweight/obesity. No associations of statistical significance were detected between exposure to BPA or its other analogs and overweight/obesity in any of the biological matrices.

KEYWORDS
overweight and obesity, childhood obesity, obesogens, BPA and analogs, biological samples

## 1. Introduction

Overweight and obesity are defined by the World Health Organization (WHO) as "an abnormal or excessive accumulation of fat that may be harmful to health." The prevalence of overweight and obesity has tripled in most of the world's population since 1975 (1). Therefore, according to the WHO, obesity is one of the most important public health problems in the world today (2). Obesity is known to be a major risk factor in the development of cardiovascular disease, several types of cancer, diabetes and premature death, among other associated problems (3, 5, 6).

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# Hair as an Indicator of Prolonged Exposure to Endocrine Disruptors and its Relation to Weight Gain in a Child Population

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## Abstracts

### Objective

The objective of the present investigation was to determine the presence of bisphenols and parabens in hair samples and to study the possible association with overweight or obesity in a Spanish school population.

### Methods

A total of 124 cases and 179 controls (3 and 12 years) were recruited, from whom sociodemographic, lifestyle, hair and urine samples were obtained. By ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) technique, 12 bisphenols and 6 parabens were analyzed in the samples. The correlation between the concentration of bisphenols and parabens in hair and urine was studied using Spearman correlation coefficients. Finally, binary logistic regression models were used to evaluate their relationship with overweight and obesity.

### Results

Hair samples from 270 schoolchildren were analyzed. Concentrations of bisphenols were higher in the control group, with the exception of bisphenol A (BPA). Detected levels of parabens were higher in the cases. There was low correlation between detected concentrations of in hair and

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# Hair as an Indicator of Prolonged Exposure to Endocrine Disruptors and its Relation to Weight Gain in a Child Population

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| Keywords: | Obesity, Pediatrics, Childhood Obesity, Overweight, Children |
| | |

nutrients

MDPI

Review

# Endocrine Disruptors in Food: Impact on Gut Microbiota and Metabolic Diseases

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**Abstract:** Endocrine disruptors (EDCs) have been associated with the increased incidence of metabolic disorders. In this work, we conducted a systematic review of the literature in order to identify the current knowledge of the interactions between EDCs in food, the gut microbiota, and metabolic disorders in order to shed light on this complex triad. Exposure to EDCs induces a series of changes including microbial dysbiosis and the induction of xenobiotic pathways and associated genes, enzymes, and metabolites involved in EDC metabolism. The products and by-products released following the microbial metabolism of EDCs can be taken up by the host; therefore, changes in the composition of the microbiota and in the production of microbial metabolites could have a major impact on host metabolism and the development of diseases. The remediation of EDC-induced changes in the gut microbiota might represent an alternative course for the treatment and prevention of metabolic diseases.

**Keywords:** endocrine disrupters; food; gut microbiota; metabolic diseases

## 1. Introduction

It has been widely reported that some exogenous compounds can interfere with the function of the endocrine system in the body. According to the Endocrine Society, an Endocrine Disrupting Chemical (EDC) is "an exogenous [non-natural] chemical, or mixture of chemicals, that interferes with any aspect of hormone action" [1,2]. In this respect, the main source of human exposure to EDCs is food intake. These chemicals might pass into the food chain directly when they are used as pesticides, or they might be released from food packaging containing metals, bisphenol A, or phthalates. In addition, some plant-based compounds (the so-called phytoestrogens) found in dietary supplements also exhibit endocrine disrupting potential [3].

Endocrine disruptors have been associated with the increased incidence of metabolic disorders. It has been proposed that EDCs may increase the susceptibility to these disorders by altering the adipose tissue, pancreas, liver, gastrointestinal tract, muscle, and brain homeostatic and hedonic pathways [4]. However, few studies have reported that the effects of EDCs on the gut microbiota can increase the risk of metabolic disorders such as obesity and diabetes [5,6].

Emerging evidence suggests interactions between EDCs and the microbiome, which may affect host health. A key triad between exposure to EDCs, the host genotype and phenotypic responses, and the gut microbiome has been suggested [7]. Exposure to EDCs has been shown to disrupt the microbiome, which may result in dysbiosis and the induction of pathways related to xenobiotics, microbiome-associated genes, enzymes, and the production of metabolites, which may play a crucial role in EDC biotransformation [8]. The products and by-products released following the microbial

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# ANEXO II.

## Artículos colaboraciones durante la Tesis Doctoral

*nutrients*

MDPI

*Review*

# Bisphenol A Analogues in Food and Their Hormonal and Obesogenic Effects: A Review

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**Abstract:** Bisphenol A (BPA) is the most well-known compound from the bisphenol family. As BPA has recently come under pressure, it is being replaced by compounds very similar in structure, but data on the occurrence of these BPA analogues in food and human matrices are limited. The main objective of this work was to investigate human exposure to BPA and analogues and the associated health effects. We performed a literature review of the available research made in humans, in in vivo and in vitro tests. The findings support the idea that exposure to BPA analogues may have an impact on human health, especially in terms of obesity and other adverse health effects in children.

**Keywords:** bisphenol A analogues; food; obesogenic effect

## 1. Introduction

Endocrine disruptors are compounds that alter the normal functioning of the endocrine system, and their bioaccumulation in humans may cause adverse health effects [1–3]. Bisphenol A (BPA) is a well-known endocrine disruptor, industrially produced, largely used as a component of epoxy resins and polycarbonate plastics [4,5]. BPA-based plastics and resins are used in the manufacturing of food contact material such as packaging, crockery, and thermic paper. Human exposure to BPA occurs mainly through diet (food and food contact materials) [6]. Moreover, BPA has also been found in plastic food containers, epoxy coatings in metal cans, kitchenware toys, medical devices, and dental composites and sealants [4,7–10].

In humans, BPA has proven to have developmental, reproductive, cardiovascular, immune, and metabolic effects [11]. In 2017, BPA was listed in the substances of very high concern list of the European Chemical Agency (ECHA). In view of the recent regulations that further restrict the use of BPA in food contact materials [12–16], food packaging companies are exploring substitutes to gradually eliminate BPA from their products [10,17,18].

Commercialization of BPA-free labeled products is increasing, while BPA analogues are being increasingly used in the manufacturing of consumer products [10]. BPA analogues share the basic

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# Impact of oxidative stress SNPs and dietary antioxidant quality score on prostate cancer

M. Pascual-Geler, I. Robles-Fernandez, C. Monteagudo, O. Lopez-Guarnido, L. Rodrigo, Y. Gálvez-Ontiveros, J. M. Cozar, A. Rivas & M. J. Alvarez-Cubero

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# Endobolome, a New Concept for Determining the Influence of Microbiota Disrupting Chemicals (MDC) in Relation to Specific Endocrine Pathogenesis

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Endogenous steroid hormones and Endocrine Disrupting Chemicals (EDC) interact with gut microbiota through different pathways. We suggest the use of the term "endobolome" when referring to the group of gut microbiota genes and pathways involved in the metabolism of steroid hormones and EDC. States of dysbiosis and reduced diversity of the gut microbiota may impact and modify the endobolome resulting at long-term in the development of certain pathophysiological conditions. The endobolome might play a central role in the gut microbiota as seen by the amount of potentially endobolome-mediated diseases and thereby it can be considered an useful diagnostic tool and therapeutic target for future functional research strategies that envisage the use of next generation of probiotics. In addition, we propose that EDC and other xenobiotics that alter the gut microbial composition and its metabolic capacities should be categorized into a subgroup termed "microbiota disrupting chemicals" (MDC). This will help to distinguish the role of contaminants from other microbiota natural modifiers such as those contained or released from diet, environment, physical activity and stress. These MDC might have the ability to promote specific changes in the microbiota that can ultimately result in common intestinal and chronic or long-term systemic diseases in the host. The risk of developing certain disorders associated with gut microbiota changes should be established by determining both the effects of the MDC on gut microbiota and the impact of microbiota changes on chemicals metabolism and host susceptibility. In any case, further animal controlled experiments, clinical trials and large epidemiological studies are required in order to establish the concatenated impact of the MDC-microbiota-host health axis.

**Keywords: microbiota, endocrine disrupting chemicals, endobolome, hormones, endocrine pathogenesis, microbiota disrupting chemicals**

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*nutrients*

MDPI

*Article*

# Factors Associated with Exposure to Dietary Bisphenols in Adolescents

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**Abstract:** Obesogenic endocrine-disrupting chemicals, such as bisphenol A (BPA) and its analogue bisphenol S (BPS), seem to play an important role in the development of obesity, although contradictory results have been reported. The aim of the present study was to conduct a gender analysis of the factors associated with exposure to dietary bisphenols in 585 Spanish adolescents. Dietary BPA and BPS exposure was assessed using a food frequency questionnaire. Foods and macronutrients accounting for more than 95% of energy intake were selected for analysis. Stepwise regression was used to estimate the foods that most contributed to dietary bisphenol exposure in the sample. Gender-related factors associated with greater dietary bisphenol exposure were evaluated using multivariate logistic regression models. Canned tuna was the main dietary source of BPA and BPS in both adolescent boys and girls. Overweight/obese girls showed a higher risk of high dietary exposure to BPA (odds ratio (OR): 3.38, 95% confidence interval (CI): 1.25–9.07) and total bisphenols (OR: 2.81, 95% CI: 1.03–7.67) in comparison with girls with a BMI lower than 25 kg/m$^2$. Present results indicate a positive association of dietary exposure to both total bisphenols and BPA with being overweight/obese in adolescent girls.

**Keywords:** bisphenol A; bisphenol S; dietary exposure; adolescents; body mass index



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## 1. Introduction

According to the World Health Organization, obesity is one of the most important current public health issues around the world [1]. Obesity is a complex condition with serious environmental, genetic, psychological, social, and economic dimensions. However, all of the causes and mechanisms involved in the development of obesity are not yet completely understood [2,3]. In addition, obesity prevalence in children and adolescents aged 2–18 years is rapidly growing [4], placing them at higher risk of adulthood obesity and of suffering metabolic disorders, cardiovascular diseases, and cancer at earlier ages [5,6]. Environmental factors have also been proposed as contributors to obesity, and there is a growing concern over obesogens. Obesogens are environmental chemicals with potential obesity-related endocrine-disrupting properties [7].

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Review article

# Metabolic pathways, alterations in miRNAs expression and effects of genetic polymorphisms of bisphenol a analogues: A systematic review

Viviana Ramírez [a,1], Yolanda Gálvez-Ontiveros [b,c,1], Patricia Porras-Quesada [a], Luis Javier Martinez-Gonzalez [d], Ana Rivas [b,c,1,*], María Jesús Álvarez-Cubero [a,c,d,1]

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ABSTRACT

Bisphenol A (BPA) is one of the most common endocrine disruptors found in the environment and its harmful health effects in humans and wildlife have been extensively reported One of the main aims of this review was to examine the metabolic pathways of BPA and BPA substitutes and the endocrine disrupting properties of their metabolites. According to the available literature, phase I and phase II metabolic reactions play an important role in the detoxification process of bisphenols (BPs), but their metabolism can also lead to the formation of highly reactive metabolites. The second part of this work addresses the associations between exposure to BPA and its analogues with the alterations in miRNAs expression and the effects of single nucleotide polymorphisms (SNPs). Available scientific evidence shows that BPs can dysregulate the expression of several miRNAs, and in turn, these miRNAs could be considered as epigenetic biomarkers to prevent the development of a variety of BP-mediated diseases. Interestingly, genetic polymorphisms are able to modify the relationship of BPA exposure with the risk of adverse health effects, suggesting that interindividual genetic differences modulate the susceptibility to the effects of environmental contaminants.

## 1. Introduction

Endocrine disrupting chemicals (EDCs) are exogenous compounds, natural or synthetic, with hormonal activity that can thereby interfere with the correct functioning of the endocrine system. This disruption can occur by mimicking endogenous hormones, antagonizing hormonal functions, inhibiting or stimulating the hormonal production, or modifying the expression pattern of specific receptors (Schug et al., 2011).

Bisphenol A [2,2-bis(4-hydroxyphenyl)propane, BPA] has been produced in large quantities since the 1960s. BPA is used to produce polycarbonate plastics and epoxy resins, which are used in the manufacturing of a huge variety of consumer products such as food packaging, lining of food cans, hard plastic bottles, thermal paper, personal care products, kitchenware, toys, electronic equipment and others (Akash et al., 2020; Andujar et al., 2019; Garcia-Corcoles et al., 2018) The capacity of BPA to migrate from food packaging materials into food has been extensively reported and it has been detected in biological human samples such as urine, blood, saliva, placenta, breast milk and umbilical cord serum (Berge et al., 2017; Gonzalez et al., 2019; Lee et al., 2018, 2019; Schonfelder et al., 2002).

The consumption of food stored in BPA-containing containers is the primary source of exposure to BPA. In humans, BPA exposure has been linked to a variety of adverse health effects including reproductive, developmental, cardiovascular, metabolic and immune system disorders in *vitro* and *in vivo* assays (Chen et al., 2016; Vandenberg et al., 2007). Furthermore, increasing evidence suggests that BPA exposure during pregnancy and early childhood poses a greater risk given that the normal development of tissues is controlled by the endocrine system and any disruptions can increase the risk of several diseases and behavioural disturbances (prostate and breast cancer, reproductive disorders, anxiety, attention deficiency, autism and hyperactivity) (Bergman et al., 2012; Garcia-Corcoles et al., 2018; Moghadam et al., 2015; Thayer et al., 2015).

The harmful effects of BPA exposure in humans health have led to its

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# Dietary exposure to parabens and body mass index in an adolescent Spanish population

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ABSTRACT

Parabens are alkyl esters of p-hydroxybenzoic acid which are extensively used in cosmetics, pharmaceuticals and foodstuffs due to their antimicrobial properties. The most commonly used parabens are methyl-(MeP), ethyl-(EtP), propyl-(PrP) and butyl-(BuP) paraben. Most human exposure to parabens is achieved through the consumption of food or pharmaceutical products and the use of personal care products. However, studies on dietary parabens exposure and the associated factors are very scarce. The main aim of the present study was to explore factors associated with dietary exposure to parabens in Spanish adolescents according to gender. Dietary data and anthropometric measures were collected from 585 adolescents (53.4% boys) aged 12–16 years. Parabens exposure through diet was assessed using a food frequency questionnaire with food products providing more than 95% of energy and macronutrient intake being included in analysis. Stepwise regression was used to identify the foods that most contributed to parabens intake. Logistic regression was used to evaluate factors predicting higher dietary exposure to parabens. The main contributors to dietary MeP, EtP, PrP and BuP exposure in adolescent boys were eggs (41.9%), canned tuna (46.4%), bakery and baked goods products (57.3%) and pineapple (61.1%). In adolescent girls, the main contributors were apples and pears (35.3%), canned tuna (42.1%), bakery and baked goods products (55.1%) and olives (62.1%). Overweight/obese girls were more likely to belong to the highest tertile of overall parabens intake (odds ratio [OR]: 3.32; 95% confidence interval [95% CI]: 1.21–9.15) and MeP (OR: 3.05; 95% CI: 1.14–8.12) than those with a body mass index lower than 25 kg/m$^2$. These findings suggest a positive association between dietary exposure to parabens and overweight/obesity in adolescent girls.

## 1. Introduction

Parabens are alkyl esters of p-hydroxybenzoic acid, being the most widely used methyl- (MeP), ethyl- (EtP), propyl- (PrP) and butyl- (BuP) parabens (Jiménez-Díaz et al., 2016). They are especially found in cosmetics, pharmaceuticals, foodstuffs and beverages where they are used as antimicrobials (Halla et al., 2018; Liao et al., 2013a, 2013b; Ursino et al., 2011). Processed food products that contain parabens for preservation include cookies, cooking oils, seasonings, meat, dairy products, snacks, cereal (Liao et al., 2013a, 2013b; Maher et al., 2020) and cereal-based foodstuffs (Azzouz et al., 2020), although parabens have also been found in mussels (Álvarez-Muñoz et al., 2018), clams and

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# Role of endocrine disrupting chemicals in children's neurodevelopment

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ABSTRACT

Environmental stressors, like endocrine disrupting chemicals (EDC), are considered important contributors to the increased rates of neurodevelopmental dysfunctions. Considering the cumulative research on adverse neurodevelopmental effects associated with prenatal exposure to EDC, the purpose of this study was to review the available limited literature about the effects of postnatal exposure to EDC on child neurodevelopment and behaviour. Despite widespread children's exposure to EDC, there are a limited number of epidemiological studies on the association of this exposure with neurodevelopmental disorders, in particular in the postnatal period. The available research suggests that postnatal EDC exposure is related to adverse neurobehavioral outcomes in children; however the underlying mechanisms of action remain unclear. Timing of exposure is a key factor determining potential neurodevelopmental consequences, hence studying the impact of multiple EDC co-exposure in different vulnerable life periods could guide the identification of sensitive subpopulations. Most of the reviewed studies did not take into account sex differences in the EDC effects on children neurodevelopment. We believe that the inclusion of sex in the study design should be considered as the role of EDC on children neurodevelopment are likely sex-specific and should be taken into consideration when determining susceptibility and potential mechanisms of action.

## 1. Introduction

Neurodevelopmental disorders such as autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), dyslexia, and intellectual disability (ID) seem to have increased in the paediatric population over the past decades (Landrigan et al., 2012; Grandjean and Landrigan, 2014). Environmental stressors, like endocrine disrupting chemicals (EDC), are considered important contributors to the increased rates of neurodevelopmental dysfunctions and are therefore classified as developmental neurotoxicants (Grandjean and Landrigan, 2014; Nesan and Kurrasch, 2020). Data from the US National Academy of Sciences (NAS) reveal that 3% of the neurodevelopmental disturbances result directly from the exposure to environmental toxic agents, whereas 25% are due to an interaction between environmental risk factors and genetic predisposition (National Research Council, 2000; Landrigan et al., 2012).

Endocrine-disrupting chemicals (EDC) are defined by the Endocrine Society as: "an exogenous chemical, or mixture of chemicals, that can interfere with any aspect of hormone action (Zoeller et al., 2012). EDC are capable of disrupting the endocrine system by: 1) mimicking or antagonizing endogenous hormones, 2) inhibiting or stimulating hormone production, 3) altering their metabolic pathways, or 4) modifying hormone receptor binding, which lead to impaired growth and functioning (Braun, 2017). In addition to neurodevelopmental dysfunctions, exposure to these environmental pollutants has also been associated to other harmful health outcomes such as cancer (Birnbaum and Fenton, 2003), reproductive (Sifakis et al., 2017), metabolic (Heindel et al., 2017; Andujar et al., 2019; Gálvez-Ontiveros et al., 2020), and immune system disorders (Casas and Gascon, 2020) as well as disturbances in gut microbiota (Aguilera et al., 2020) In fact, EDC like bisphenol A (BPA)

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# Effects of genetic polymorphisms in body mass index according to dietary exposure to bisphenols and parabens

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## HIGHLIGHTS

- *LEPR* rs9436303 variant allele G significantly contributes to increased BMI.
- Combined effect of *LEPR* rs9436303 and dietary exposure to bisphenols/parabens in BMI variability is proposed in present work.
- *LEPR* rs9436303 variant proposed as a genetic marker for susceptibility to obesity in exposed population.

## GRAPHICAL ABSTRACT

## ARTICLE INFO

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## ABSTRACT

A growing body of evidence supports that more than 900 single nucleotide polymorphisms (SNPs) and exposure to endocrine disrupting chemicals, such as bisphenols and parabens, are important contributors to the development of obesity. The aim of this study was to evaluate the way in which fat mass and obesity-associated gene (*FTO*) rs9939609 and leptin receptor (*LEPR*) rs9436303 variants contribute to variability in body mass index (BMI) according to estimated dietary exposure of bisphenols and parabens. This cross-sectional study included 101 Spanish participants (16–24 years). SNP genotyping assays were performed through quantitative PCRs (qPCRs) using Taqman® probes. Dietary exposure to bisphenols and parabens was calculated from food frequency questionnaire and chemical determination in food samples by ultra-high performance liquid chromatography-tandem mass spectrometry system. Linear regression models were conducted to address the association of genetic variants and BMI according to levels of bisphenols/parabens exposure. Risk G allele of

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of genome-wide association studies for height and body mass index in similar to 700 000 individuals of European ancestry. Hum. Mol. Genet. 27, 3641–3649.

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# Optimization of an ultrasound-assisted extraction method for the determination of parabens and bisphenol homologues in human saliva by liquid chromatography-tandem mass spectrometry

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ABSTRACT

Parabens and bisphenols are endocrine disrupting chemicals (EDCs) widely used in our daily lives. The main route of human exposure to these compounds is through the diet. This makes human saliva an important matrix for identification of these contaminants and evaluation of human exposure. In this work a multiresidue method to determine the presence of methyl-, ethyl-, propyl-, isopropyl-, butyl- and isobutylparaben; and bisphenol A, B, C, E, F, M, P, S, Z, AP, AF and FL in human saliva samples has been developed. Sample treatment involves an initial step of protein precipitation in acidic medium and a second step of analyte extraction. Extraction parameters were optimized using univariant and multivariant strategies. Microwave assisted extraction (MAE) and ultrasound assisted extraction (UAE) were compared and UAE was chosen the optimal extraction technique. The compounds were analyzed by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The calibration in matrix was applied and the limits of detection and quantification were from 0.1 to 0.4 ng $g^{-1}$ and from 0.3 to 1.0 ng $g^{-1}$, respectively. Accuracy was evaluated in terms of recovery (85.6 to 113.5%) with a relative standard deviation < 15% in all cases. The analytical method was successfully applied to quantify the target EDCs in ten human saliva samples, with some parabens being the most frequently detected compounds.

## 1. Introduction

Endocrine disrupting chemicals (EDCs) are compounds that can interfere with the hormone equilibrium of the body, thereby increasing the risk of harmful health effects on the nervous system, on both male and female reproductive system and thyroid function, which is involved in metabolism regulation and therefore can contribute to overweight and obesity. This group of compounds is found in pesticides, dust and water, and since they are used as plastic additives they can be found in food and personal care products (PCPs) from where they can enter our body via inhalation, ingestion, or dermal exposure [1,2].

Bisphenol A (BPA) and its analogues are a family of compounds mainly used for the manufacture of plastics and resins. They are aromatic compounds whose structure contains two phenolic rings joined by a group bridge. The most studied bisphenol is BPA, used since 1930 for its excellent properties for the fabrication of epoxy resins and polycarbonate plastics commonly used in food-contact material [3]. The EFSA (European Food Safety Authority) stablished a limit of 50 μg kg weight$^{-1}$ day$^{-1}$ in 2006, and since then it has been decreasing this amount over the years to 4 μg kg weight$^{-1}$ day$^{-1}$ [4]. BPA can migrate from the packaging to food and then enter the body, being the diet the main route of exposure to this contaminant. Due to European regulations on BPA, industries have started to use BPA analogues (bisphenols, BPs) as a replacement in plastics and epoxy resins, with no specific regulation yet. However, evidence is showing that exposure to some of these analogues, such as bisphenol E (BPE), bisphenol B (BPB), bisphenol F (BPF), and bisphenol S (BPS) might also result in endocrine disrupting activity leading to lipid accumulation and therefore obesity,

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# Association between dietary exposure to bisphenols and body mass index in Spanish schoolchildren

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## Abstract

The increase in children obesity worldwide has been of particular concern in recent decades. Environmental factors have been proposed as contributors to obesity, and there is a growing concern over obesogens, environmental chemicals with potential obesity-related endocrine-disrupting properties. In this regard, bisphenol A (BPA) and its analogues are suspected to have obesogenic properties. Current document report on the activities of the fellow, undertaken during the fourth, 2020–2021 cycle of the EU-FORA programme at the University of Granada, Institute of Nutrition and Food Science, in Spain. The work programme offered by the hosting site was related to the extrapolation of bisphenols exposure following the determination of these compounds in food frequently consumed by children and in their biological samples. The fellow has participated in the recruitment of the study population in the health centres. In addition, she has participated in the collection of the children biological samples, anthropometric measurements and dietary surveys and in the optimisation of the laboratory methodology for the extraction of bisphenols in biological samples. All these activities also provided the fellow an opportunity to develop her data science related skills, which will benefit her professional development. In addition, the fellow gained an overview of various topics related to food safety risk assessment by attending the EU-FORA dedicated training modules.



**Keywords:** bisphenols, obesity, food consumption, schoolchildren daily intake, biomonitoring

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## Appendix C – Exposure to Bisphenols Food Questionnaire

Link:

https://docs.google.com/document/d/1Ryq2WwbnJRZgGP5qaHsCuJ1UQo62LqDR/edit?usp=sharing&ouid=112625456176696250428&rtpof=true&sd=true

EXTERNAL SCIENTIFIC REPORT

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# OBEMIRISK-Knowledge platform for assessing the risk of bisphenols on gut microbiota and its role in obesogenic phenotype: looking for biomarkers

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## Abstract

"Obemirisk – Knowledge platform for assessing the risk of Bisphenols on gut microbiota and its role in obesogenic phenotype: looking for biomarkers" was a knowledge transfer project funded by the European Food Safety Authority (EFSA) that integrated a multidisciplinary team from Spain, France, Belgium, Slovakia and Poland. This project aimed to strengthen the knowledge capacity to assess the risk of bisphenol A and several structural analogues on gut microbiota that could mediate the obesogenic phenotype in childhood. Protocols and methodologies from different fields such as chemical analysis (food and biosamples), nutrition (surveys and questionnaires), microbiology (culturomics and metagenomics), and gene reporter assay (AhR-Ligand) have been applied and shared. Several data generated under the project are available under open publications and databases for the Consortium and scientific community. Common documents and publications integrating data from endocrine disrupting chemicals (EDCs), bisphenols, microbiota dysbiosis and obesity were elaborated. A networking and specific capacity-building programmes have been implemented to produce and share the new data on bisphenols data food composition, microbiota and its impact on obesity between providers and recipients' partners. Scientific exchanges and specific designed courses provided training for students in the risk characterization related domains. The project was mainly focused on the bisphenols´ presence in consumed foods by Spanish children and in several children biosamples (saliva, urine, nails, and hair). Moreover, a pilot project on obese *vs.* normal-weight children allowed to determine the obesity-linked microbiota dysbiosis through metagenomics and specific biomarkers of the dysregulated microbiota-immune system axis (AhR-Ligands). The Obemirisk project applied a collaborative and multidisciplinary approach to establish scientific data compilation for harmonising risk assessment and to perform trainings on next generation of risk assessment where microbiome disruption might become a robust biomarker to be used in food safety. Several aspects of the process of capacity building have been mainly conceptual due to the COVId-19 pandemic and will be further implemented through presential exchanges. Moreover, the consortium work strategy can also propose further EU collaborations for refining and elucidating the impact and mechanisms of bisphenols altering human microbiomes and triggering obesity. The knowledge, analyses and the integrative approach will be extrapolated for other foods, age ranges, geographical areas, and other biomatrices.

**Annexes A-C** are available on the file online article under "Supporting information":

**Annex A – Manuscript published in the scientific journals on Bisphenols data (File Zip 1)** https://drive.ugr.es/index.php/s/YMDkOQD5cysJII7/download?path=%2F&files=OBEMIRISK-BISPHENOLS.rar

**Annex B – Manuscript published in the scientific journals on Microbiota data (File Zip 2)**

https://drive.ugr.es/index.php/s/YMDkOQD5cysJII7/download?path=%2F&files=OBEMIRISK-MICROBIOTA%20Papers.rar

**Annex C – Internal Workshop, Granada, 14-15 October 2021** https://drive.ugr.es/index.php/s/YMDkOQD5cysJII7

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RESEARCH PAPER

# Improved method for the determination of endocrine-disrupting chemicals in urine of school-age children using microliquid–liquid extraction and UHPLC-MS/MS

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## Abstract

The presence of endocrine-disrupting chemicals in our daily life is increasing every day and, by extension, human exposure and the consequences thereof. Among these substances are bisphenols and parabens. Urine is used to analyze the exposure. The determination of 12 bisphenol homologues and 6 parabens is proposed. A procedure based on a method previously developed by our research group in 2014 is improved. The extraction yield is higher, because the new protocol is 5 times more efficient. Also, a comparison between calibration with pure standards and matrix calibration, to calculate the matrix effect, was also made. A high grade of matrix effect for all analytes was observed. In terms of validation, the limits of detection (LOD) were between 0.03 and 0.3 ng $mL^{-1}$ and limits of quantification (LOQ) 0.1 to 1.0 ng $mL^{-1}$, respectively, and the recovery is higher than 86.4% and lower than 113.6%, with a RSD lower than 13.5% in all cases. A methodology for accurate and sensitive quantification of bisphenol homologues together with parabens in human urine using UHPLC-MS/MS was developed. The method was successfully applied to 30 urine samples from children.

**Keywords** UHPLC-MS/MS · Dispersive liquid–liquid microextraction · Parabens · Bisphenols · Endocrine-disrupting chemicals · Urine samples

## Introduction

Endocrine-disrupting chemicals (EDCs) are compounds that exert actions mainly through nuclear hormone receptors and cause adverse effects on the health of an organism or its progeny [1]. People are constantly exposed to these chemicals in daily life from packaging to fungicides, and they are found abundantly in our environment. Scientific studies have demonstrated that a high exposition to EDCs can alter the hormonal balance and the endocrine system function [2–4].

Bisphenols (BPs) are a family of synthetic compounds with two hydroxyphenyl functional groups. Bisphenol A (BPA) is the most important compound of this family. The use of BPA is well-extended for food packaging, infant bottles, reusable water bottles, or microwaveable products [5]. Several studies have demonstrated that BPA can migrate from plastics to food, being the diet one of the main routes of exposure. Because of the lipophilic behaviour of BPA, it is accumulated in fat tissues, and it causes effects related to alterations in the endocrine system function, alterations in neurodevelopment, and cognitive disorders such as Alzheimer or the development of obesity [6–8]. Although, once in the body, a high percentage of BPA is metabolized by conjugation through glucuronidation and sulfatation, resulting in hydrophilic metabolites that are water-soluble and lack of estrogenic activity, an important amount of the compound is accumulated by the organism as a free form [9, 10]. For those reasons, its use in children's articles was banned by the European Commission in 2011. The European Food Safety Authority (EFSA) recommended 0.04 ng $kg^{-1}$ $day^{-1}$ as tolerable daily intake (TDI) [11], and the European Commission

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*nutrients*

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*Article*

# Presence of Parabens in Different Children Biological Matrices and Its Relationship with Body Mass Index

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**Abstract:** Parabens have been accepted almost worldwide as preservatives by the cosmetic, food, and pharmaceutical industries. Since epidemiological evidence of the obesogenic activity of parabens is weak, the aim of this study was to investigate the association between parabens exposure and childhood obesity. Four parabens (methylparaben/MetPB, ethylparaben/EthPB, propylparaben/PropPB, and butylparaben/ButPB) were measured in 160 children's bodies between 6 and 12 years of age. Parabens measurements were performed with ultrahigh-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS). Logistic regression was used to evaluate risk factors for elevated body weight associated with paraben exposure. No significant relation was detected between children's body weight and the presence of parabens in the samples. This study confirmed the omnipresence of parabens in children's bodies. Our results could be a basis for future research about the effect of parabens on childhood body weight using nails as a biomarker due to the ease of its collection and its non-invasive character.

**Keywords:** parabens; obesogens; children; urine; nail; saliva



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## 1. Introduction

Overweight and obesity are defined as abnormal or excessive fat accumulation that may cause health disorders such as cardiovascular diseases, musculoskeletal conditions, alterations in spinal posture and mobility, metabolic syndrome, and gastrointestinal and pulmonary conditions [1,2]. Recent data from World Health Organization showed that 39 million children under the age of 5 were overweight or obese in 2020, and nearly tripled since 1975 [3]. Overweight/obesity can be attributed to several factors, but traditional risk factors such as diet, physical activity, and genetics cannot completely explain the increase [4]. In 2006 Grün and Blumberg postulated the obesogens hypothesis, where certain environmental pollutants could induce adipogenesis [5]. Some endocrine-disrupting chemicals (EDCs) have been catalogued as obesogens due to the effect they induce in adipose tissue deposition via mimicking endogenous endocrine hormones or activating lipidogenesis-related nuclear receptors [6].

Parabens are EDCs widely used as preservatives in the cosmetic, pharmaceutical, and food industries because of their antimicrobial properties and low allergenic potential [7–9]. Additionally, their chemical stability, low toxicity, and allergenicity lead parabens to almost worldwide acceptance [7–10]. The most commonly used parabens are methylparaben (MetPB), ethylparaben (EthPB), propylparaben (PropPB) and butylparaben (ButPB) [11].

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*nutrients*

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*Review*

# The Role of Endocrine Disrupting Chemicals in Gestation and Pregnancy Outcomes

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**Abstract:** Endocrine disrupting chemicals (EDCs) are exogenous substances widely disseminated both in the environment and in daily-life products which can interfere with the regulation and function of the endocrine system. These substances have gradually entered the food chain, being frequently found in human blood and urine samples. This becomes a particularly serious issue when they reach vulnerable populations such as pregnant women, whose hormones are more unstable and vulnerable to EDCs. The proper formation and activity of the placenta, and therefore embryonic development, may get seriously affected by the presence of these chemicals, augmenting the risk of several pregnancy complications, including intrauterine growth restriction, preterm birth, preeclampsia, and gestational diabetes mellitus, among others. Additionally, some of them also exert a detrimental impact on fertility, thus hindering the reproductive process from the beginning. In several cases, EDCs even induce cross-generational effects, inherited by future generations through epigenetic mechanisms. These are the reasons why a proper understanding of the reproductive and gestational alterations derived from these substances is needed, along with efforts to establish regulations and preventive measures in order to avoid exposition (especially during this particular stage of life).

**Keywords:** endocrine disrupting chemical; pregnancy; gestation; complications; maternal-fetal health; fertility; bisphenols; phthalates; pesticides; advanced maternal age



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## 1. Introduction

Several chemicals, both natural and synthetic, can interfere with different pathways of the endocrine system, altering its functioning and leading to detrimental consequences for human health. These compounds are known as endocrine-disrupting chemicals (EDCs), and can be found in a variety of products, including metals, pesticides, and daily products such as plastic food-packaging, flame retardants, toys, cosmetics, detergents, etc. [1]. They can interact with the endocrine system homeostasis in many ways, altering the synthesis, secretion, binding, transportation, metabolisms and/or elimination of key hormones. Their ubiquitous presence of these exogenous substances and their demonstrated or potential negative impact on health has turned them into an object of profound investigation by the scientific community, showing the risk that these compounds represent [2]. As a matter of fact, several institutions have been developing policies related to EDCs since the 1990s, with the objective of regulating, reducing, or even banning some of these chemicals' applications.

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