Tesis Doctoral Internacional

International Doctoral Thesis

UNIVERSIDAD DE GRANADA

Carga obesogénica total efectiva: nuevo biomarcador de efecto combinado a obesógenos como factor de riesgo en obesidad

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MEMORIA DE TESIS PRESENTADA POR LA DOCTORANDA

Iris Reina Pérez

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Summary

The prevalence of overweight and obesity, as well as other metabolic diseases such as metabolic syndrome (MetS), is increasing worldwide. This continuous increase cannot be due to a single cause, but everything suggests that multiple risk factors are involved.

The environmental obesogens hypothesis suggests that human exposure to "obesogenic" EDCs may inappropriately interfere with lipid homeostasis and metabolism and promote adipogenesis, modifying both hormonal and neuronal signalling pathways, thereby predisposing exposed individuals to increased fat mass and excess weight. EDCs that not only increase adipose tissue mass but also result in other metabolic dysfunctions which are also referred to as metabolism disrupting chemicals (MDCs). Environmental obesogens are defined as chemicals capable of increasing the quantity of adipocytes (hyperplasia), promoting the fat storage in cells (hypertrophy), affecting preadipocyte differentiation or function, and initiating and/or deregulating basal metabolism and weight gain. Obesogens might also act indirectly by disrupting energy balance (promoting calorie storage), and altering appetite and satiety signals, besides the central mechanisms that keep the body's response to daily nutritional changes.

The list of chemicals with obesogenic potential has been growing steadily in recent years, with humans being exposed to this multitude of EDCs from a variety of sources. To date, current developments in environmental epidemiology and toxicological risk assessment are inadequate and unsatisfactory for evaluating the exposure to multiple obesogenic EDCs, and for knowing their impact on human development and health. Although there have been some attempts to consider the combined effect of several chemicals or compounds which

belong to the same chemical family, the methods available so far to quantify the combined effect of exposure to multiple residues are still scarce and not fully validated and conclusive.

Taking into account all of the above, the starting hypothesis of this International Doctoral Thesis was that exposure to mixtures of EDCs constitutes a risk factor for obesity, and that the development of methodologies based on the measurement of the combined effect of multiple obesogenic EDCs known to occur in human adipose tissue, through *in vitro* assays, would serve as biomarker of combined effect in metabolic diseases.

Therefore, the **main objective** of this International Doctoral Thesis was to investigate human exposure to specific endocrine disrupting chemicals (EDCs), specifically to those with obesogenic activities, by analysing both the presence of these contaminants in the human population and the individual and combined effect of residues (EDCs) present in human adipose tissue, through the development of *in vitro* biomarkers, which could be used as risk markers for obesity and other metabolic diseases.

The specific objectives defined for this purpose were the following:

1. To develop and validate an *in vitro* human mesenchymal stem cell model to assess the effect of obesogenic EDCs. This tool could serve as a way to compare the potency of different obesogens, as well as to identify new compounds with obesogenic activity.

2. To study *ad hoc* mixtures of environmental chemicals on the process of adipogenesis, investigating the possibility of synergistic, additive or even antagonistic effects of the individual components.

3. To deepen into the knowledge on mechanisms and signaling pathways through which obesogen-EDCs contribute to an obesogenic phenotype (analyzing gene and protein expression of specific adipogenic markers - PPARγ, C/EBPα, LPL and FABP4).

4. To evaluate the exposure to obesogens in adipose tissue samples from adult hospitalized patients in Southern Spain, with normal weight and obesity and to investigate the relationship between the accumulated concentrations in adipose tissue and the risk of clinical diagnosis of MetS.

5. To evaluate the developed total load biomarker ("*O-Screen*") in adipose tissue samples from adult patients with normal weight and obesity, and to investigate its possible association with obesity and other metabolic disorders.

To answer the first three objectives, hASCs (human adipose-derived stem cells) *in vitro* were exposed to different concentrations of bisphenol-F (BPF), bisphenol-S (BPS) and bisphenol-A (BPA) (0.01-25 μM), individually and in combination (equimolar mixture of the three bisphenols), during 7 and 14 days of adipocyte differentiation. The effect of exposure on lipid accumulation and adipogenesis (Oil Red O), in addition to gene (mRNA) and protein (WB, western blot) expression of the lipogenic markers PPARγ, C/EBPα, LPL and FABP4 were investigated.

The epidemiological study investigated the real exposure of the population to persistent EDCs using the biological matrix for which they have the greatest appetite, that is, adipose tissue; in particular, visceral adipose tissue was selected, as it is the tissue associated with the greatest risk of metabolic diseases. The possible associations

between pollutants and clinical diagnosis of MetS were also evaluated, using both exposure biomarker information and clinical effect biomarkers available for the study population.

The main results of this doctoral thesis are as follows:

Article #1 (Reina-Pérez et al., 2021): Exposure of hASCs to BPF and BPS promotes adipogenesis by altering adipocyte developmental programming and increasing intracellular lipid accumulation. In particular, cell exposure with BPF, at concentrations of 10 or 25 μM for 7 or 14 days, potentiates stem cell differentiation into adipocytes in a dose-dependent manner, which is confirmed by verifying an increase in the expression of selected adipogenic genes (*PPARγ, C/EBPα*, *LPL* and *FABP4*), as well as their corresponding proteins. Our results show for the first time the obesogenic potential of BPF on human adipocyte differentiation, and it corroborates the previous data obtained in murine preadipocytes.

Article #2 (Reina-Pérez et al., 2022): The equimolar ternary mixture of the three bisphenols (BPF, BPS and BPA), interferes with the programming of hASCs by increasing their ability to differentiate into adipocytes. However, the obesogenic effect of the mixture differed from that of the individual compounds by showing higher gene expression levels than those obtained for the individual components of the mixture. Exposure of the cells resulted in a dose-dependent effect on lipid accumulation, while gene expression showed a non-monotonic response, adopting a U-shape. This work is one of the few *in vitro* studies that have investigated the combined obesogenic activity of EDCs-obesogen mixtures on adipogenic differentiation, lipid accumulation, gene expression and protein synthesis, in an attempt to emulate real exposure situations in human population.

Article #3 (Reina-Pérez et al., 2023): The Spanish adult population continues to be exposed to persistent environmental pollutants (persistent organic pollutants, POPs), with significantly higher levels of organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) in the group of patients studied with MetS compared to individuals without this syndrome. Positive associations were found between certain POPs and the clinical diagnosis of MetS or some of its components. Specifically, higher levels of HCB and γ-HCH were associated with higher fasting glucose levels, higher blood pressure, and increased risk of MetS, both in linear regression models and in logistic models adjusted for age and sex. Exposure to the mixture of the 7 POPs found in adipose tissue is also positively and significantly associated with MetS risk.

The increasing prevalence of MetS in the world makes necessary the identification of preventable risk factors, besides stablishing intervention measures to stop and reverse the progression of this syndrome. Within this context, once the role of exposure to environmental-obesogenic pollutants in metabolic disease has been identified, more strictly regulations in their uses and applications should be implemented. The participation of clinical professionals in this area of research will be essential to deal with the environmental health challenges that we are facing and will face through precautionary measures.

Resumen

Cada vez es mayor la prevalencia de sobrepeso y obesidad, además de otras enfermedades metabólicas como el síndrome metabólico (MetS), en el mundo. Este incremento continuo no puede ser debido a única causa, sino que todo apunta a que serían múltiples los factores de riesgo implicados.

La hipótesis de los obesógenos ambientales sugiere que la exposición humana a compuestos químicos-disruptores endocrinos (EDCs)-"obesogénicos" puede interferir inadecuadamente en la homeostasis lipídica y el metabolismo y promover la adipogénesis, modificando las vías de señalización hormonal y neuronal, predisponiendo así a las personas expuestas a un aumento de la masa grasa y sobrepeso. Los obesógenos se definen como sustancias químicas capaces de aumentar la cantidad de adipocitos (hiperplasia), promover el almacenamiento de grasa en las células (hipertrofia), afectar a la diferenciación o función de los preadipocitos e iniciar y/o desregular el metabolismo basal y el aumento de peso. Los obesógenos también pueden actuar indirectamente alterando el equilibrio energético alterando las señales de apetito y saciedad, así como los mecanismos centrales que mantienen la respuesta del organismo a los cambios nutricionales diarios. Los EDCs que no solo aumentan la masa de tejido adiposo, sino que también provocan otras disfunciones metabólicas, son conocidos como sustancias químicas disruptoras del metabolismo.

La lista de sustancias químicas con potencial obesogénico no ha dejado de crecer en los últimos años, y los seres humanos están expuestos a esta multitud de compuestos desde diversas fuentes. Los desarrollos actuales en epidemiología ambiental y evaluación de riesgos toxicológicos son inadecuados e insatisfactorios para evaluar la exposición a múltiples EDCs obesogénicos, y para conocer su impacto

en el desarrollo y la salud humana. Aunque ha habido algunos intentos de considerar el efecto combinado de varias sustancias químicas pertenecientes a la misma familia química, los métodos disponibles hasta ahora para cuantificar el efecto combinado de la exposición a múltiples residuos siguen siendo escasos, no están totalmente validados ni son concluyentes.

Teniendo en cuenta todo lo anterior, la hipótesis de partida de esta Tesis Doctoral Internacional fue que la exposición a mezclas de EDCs constituye un factor de riesgo para la obesidad, y que el desarrollo de metodologías basadas en la medición del efecto combinado de múltiples EDCs obesogénicos almacenados en el tejido adiposo humano, mediante ensayos *in vitro*, serviría como biomarcador del efecto combinado en enfermedades metabólicas.

Así pues, el **objetivo principal** de esta Tesis Doctoral Internacional es investigar la exposición humana a varios EDCs, con actividad obesogénica, analizando tanto la presencia de estos contaminantes en el tejido adiposo humano, como el efecto individual y combinado de estos contaminantes, mediante el desarrollo de biomarcadores *in vitro*, que pudieran ser utilizados como marcadores de riesgo de obesidad y otras enfermedades metabólicas.

Los objetivos específicos definidos para este fin fueron los siguientes

1. Desarrollar y validar un modelo *in vitro* de células madre mesenquimales humanas para evaluar el efecto de EDC obesogénicos. Esta herramienta podría servir para comparar la potencia de diferentes obesógenos, así como para identificar nuevos compuestos con actividad obesogénica.

2. Estudiar mezclas *ad hoc* de sustancias químicas ambientales sobre el proceso de adipogénesis, investigando la posibilidad de efectos sinérgicos, aditivos o incluso antagónicos de los componentes individuales.

3. Profundizar en el conocimiento de los mecanismos y vías de señalización a través de los cuales los obesógenos-EDCs contribuyen a un fenotipo obesogénico, analizando la expresión génica y proteica de marcadores adipogénicos específicos (PPARγ, C/EBPα, LPL y FABP4).

4. Evaluar la exposición a obesógenos en muestras de tejido adiposo de pacientes adultos hospitalizados en el sur de España, con peso normal y obesidad e investigar la relación entre las concentraciones acumuladas en tejido adiposo y el riesgo de diagnóstico clínico de MetS.

5. Evaluar el biomarcador de carga total desarrollado ("*O-Screen*") en muestras de tejido adiposo de pacientes adultos con peso normal y obesidad e investigar su posible asociación con la obesidad y otros trastornos metabólicos.

Para dar respuesta a los tres primeros objetivos se utilizaron células hASCs (células madre derivadas del tejido adiposo humano) *in vitro* que se expusieron a diferentes concentraciones de bisfenol-F (BPF) y bisfenol-S (BPS) y bisfenol-A (BPA) (0,01-25 μM), de manera individual y combinada (mezcla equimolar de los tres bisfenoles), durante 7 y 14 días de diferenciación adipocitaria. Se investigó el efecto de la exposición sobre la acumulación lipídica y la adipogénesis (tinción *Oil Red O*, ORO), además de la expresión génica (mRNA) y proteica (*western blot*, WB) de los marcadores lipogénicos PPARγ, C/EBPα, LPL y FABP4.

En el estudio epidemiológico se investigó la exposición real de la población a EDCs persistentes empleando para ello la matriz biológica por la que tienen más apetencia, el tejido adiposo; de forma particular se seleccionó el tejido adiposo visceral, por ser éste el tejido asociado con un mayor riesgo de enfermedades metabólicas. Se evaluó también las posibles asociaciones entre los contaminantes y el diagnóstico clínico de MetS, utilizando tanto la información de biomarcadores de exposición como de los biomarcadores de efecto clínicos disponibles para la población de estudio.

Los principales resultados de esta tesis doctoral son los siguientes:

Artículo #1 (Reina-Pérez et al., 2021): La exposición de células hASCs a BPF y BPS promueve la adipogénesis como se comprueba al alterar la programación del desarrollo de los adipocitos y aumentar la acumulación de lípidos intracelulares. De forma particular, la exposición celular con BPF, a concentraciones de 10 o 25μM durante 7 o 14 días, potencia la diferenciación de las células madre en adipocitos de manera dosis-dependiente, lo que se confirma al comprobar un incremento de la expresión de los genes adipogénicos seleccionados (*PPARγ*, *C/EBPα*, *LPL* y *FABP4*), así como de sus proteínas correspondientes. Se muestra por primera vez el potencial obesogénico de BPF sobre la diferenciación de adipocitos humanos, corroborando datos previos obtenidos en preadipocitos murinos.

Artículo #2 (Reina-Pérez et al., 2022): La mezcla ternaria equimolar de los tres bisfenoles (BPF, BPS y BPA), interfiere en la programación de las células hASCs, aumentando su capacidad para diferenciarse en adipocitos. No obstante, el efecto obesogénico de la mezcla difirió del de los compuestos individuales, mostrando niveles de expresión génica mayores que los obtenidos para los componentes individuales de la

misma. La exposición de las células resultó en un efecto dosis-dependiente en la acumulación lipídica, mientras que la expresión de los genes mostró una respuesta no monotónica, adoptando forma de U. Este trabajo es uno de pocos estudios *in vitro* que han investigado la actividad obesogénica combinada de mezclas de EDCs-obesógenos sobre la diferenciación adipogénica, la acumulación de lípidos, la expresión génica y la síntesis proteica, en un intento de emular situaciones de exposición real en población humana.

Artículo #3 (Reina-Pérez et al., 2023): La población adulta española sigue expuesta a contaminantes ambientales persistentes (POPs), con niveles de plaguicidas organoclorados (OCPs) y bifenilos policlorados (PCBs) significativamente mayores en el grupo de pacientes estudiados con MetS (MetS+) respecto a los individuos sin este síndrome (MetS-). Se encontraron asociaciones positivas entre ciertos POPs y el diagnóstico clínico de MetS o de algunos de sus componentes. Concretamente, niveles más altos de HCB y γ-HCH se relacionan con niveles más altos de glucosa en ayunas, con una presión arterial más elevada y con un mayor riesgo de MetS, tanto en los modelos de regresión lineal como en los modelos logísticos ajustados por edad y sexo. La exposición de la mezcla de los 7 POPs encontrada en el tejido adiposo se asocia también de manera positiva y significativa con el riesgo de MetS.

La creciente prevalencia del MetS en el mundo, hace necesaria la identificación de factores de riesgo prevenibles, así como el establecimiento de medidas de intervención para detener y revertir la progresión de este síndrome. Dentro de este contexto se enmarca la exposición a contaminantes ambientales-obesogénicos, que una vez identificados y entendido su papel en la génesis de la enfermedad,

deberían ser regulados en sus usos y aplicaciones de forma más estricta. La participación de los profesionales clínicos en esta área de investigación será fundamental para acometer los desafíos de salud ambiental a los que hacemos y haremos frente con medidas preventivas.

Introducción

TEJIDO ADIPOSO: función y desarrollo

El tejido adiposo es uno de los tejidos más extensos del cuerpo humano, representando aproximadamente el 20% del peso corporal total en individuos sanos (Badimon et al., 2015); en torno al 10-20% en los varones, y entre el 20-30% de su peso en las mujeres (Frühbeck, 2008). La acumulación de grasa difiere entre hombres y mujeres. Así, se deposita generalmente en la zona abdominal central en los hombres, lo que se conoce como fenotipo androide u "obesidad tipo manzana", mientras que en la zona femoral y glútea lo hace en las mujeres, lo que se conoce como fenotipo ginoide u "obesidad tipo pera" (Lee et al., 2013).

El tejido adiposo es de origen mesenquimal y está constituido por diversos tipos de células, principalmente, adipocitos, además de por componentes intercelulares, esto es, células vasculares estromales que incluyen fibroblastos, preadipocitos, células endoteliales, células mesoteliales, pericitos, células inmunitarias (macrófagos, células T, neutrófilos, linfocitos), e incluso células madre multi- y pluri-potentes (Badimon et al., 2015; Lee et al., 2013; Longo et al., 2019). Los adipocitos se caracterizan por tener una gran vacuola citoplásmica donde almacenan lípidos, principalmente triglicéridos (TG) y ésteres de colesterol, y tienen un tamaño entre 20 y 200 μm (Badimon et al., 2015; Frühbeck, 2008; Lee et al., 2013).

Se distinguen varios tipos de tejido adiposo, principalmente el tejido adiposo *blanco* (visceral y subcutáneo) - el mayoritario -, el tejido adiposo *marrón* y el tejido adiposo *beige* o *brite* (Choe et al., 2016; Lee et al., 2013; Lustig et al., 2022). El tejido adiposo blanco visceral está localizado entre los órganos internos de la cavidad abdominal, rodeándolos, envolviéndolos y protegiéndolos. El tejido adiposo blanco

subcutáneo, sin embargo, está distribuido por todo el cuerpo, justo debajo de la piel (Choe et al., 2016; Frühbeck, 2008; Lee et al., 2013; Longo et al., 2019; Wajchenberg, 2000); mientras que el tejido adiposo *beige* se concentra principalmente en las regiones cervico-supraclavicular, perirrenal y paravertebral (Lee et al., 2013).

El tejido adiposo está considerado como un órgano endocrino que interviene en la regulación de diversas funciones biológicas, además de en la respuesta de diferentes tejidos (hipotálamo, páncreas, hígado, músculo esquelético, riñones, endotelio, sistema inmune, etc.), mediante una red de señales endocrinas, paracrinas y autocrinas (Frühbeck, 2008; Kershaw and Flier, 2004). Entre sus funciones principales destacan el almacenamiento de energía, el metabolismo de hormonas esteroides, la protección y el aislamiento de órganos, además de participar en la regulación del sistema nervioso central, la respuesta inmune, la respuesta de la presión arterial, inflamación, angiongénesis y apostosis, entre otras muchas funciones (Choe et al., 2016; Frühbeck, 2008). El tejido adiposo blanco, en concreto, además de actuar como reservorio de energía y de células madre mesenquimales (Badimon et al., 2015; Choe et al., 2016; Frühbeck, 2008; Wajchenberg, 2000), es conocido por su papel clave en el metabolismo (Papalou et al., 2019), al sintetizar, secretar y liberar sustancias químicas de diferente naturaleza (compuestos peptídicos y no peptídicos, como por ejemplo hormonas y proteínas) (Frühbeck, 2008; Wajchenberg, 2000). Al conjunto de citoquinas (citocinas), hormonas y péptidos secretados por los adipocitos se les denomina adipoquinas (o adipocinas) (Badimon et al., 2015; Choe et al., 2016; Frühbeck, 2008). Entre estas citoquinas destaca el factor de necrosis tumoral (TNFα, *tumor necrosis factor-α*), implicado en la regulación del tamaño de los adipocitos; la interleuquina 6 (IL-6, *interluekine-6*), una citoquina proinflamatoria, y

otros factores como el IGF-I (*insulin-like growth factor-1*), implicado en la proliferación y diferenciación adipocitaria; y la monobutirina, implicada en la vascularización del tejido adiposo (Frühbeck, 2008; Wajchenberg, 2000).

El tejido adiposo contiene también receptores específicos de hormonas. Los receptores nucleares son factores de transcripción [sustancias endógenas (proteínas) que inician, estimulan o finalizan la transcripción genética (Lambert et al., 2018; Latchman, 1993)] que, activados por ligandos (Zolezzi et al., 2017), intervienen mediando la respuesta transcripcional a las hormonas en las células, regulando la expresión de genes diana implicados en el metabolismo, desarrollo y reproducción (Kojima et al., 2019; McEwan, 2016). Entre estos receptores de hormonas nucleares se encuentran los que controlan la expresión de genes implicados en la diferenciación de los adipocitos, la adipogénesis, la distribución de la grasa, el peso corporal y el metabolismo (Grün, 2010; Heindel et al., 2022); entre los que se encuentran, por ejemplo, los receptores de glucocorticoides (GRs, *glucocorticoid receptors*), de andrógenos (ARs, *androgen receptors*), de estrógenos (ERs, *estrogen receptors*, ERα y ERβ), de hormona del crecimiento (GH), de hormonas tiroideas (TRs, *thyroid receptors*, TRα y TRβ), de adenosina, PPARγ (*peroxisome proliferator-activated receptor gamma*), RXR (*retinoid X receptor*,), PXR (*pregnane X receptor*) y CAR (*constitutive androstane receptor*), FXR (*farnesoid X receptor*), AhR (a*ryl hydrocarbon receptor*), e IR (*insulin receptor*) (Heindel et al., 2022; Lustig et al., 2022; Wajchenberg, 2000).

Entre los factores de transcripción que los adipocitos-requieren para promover su diferenciación (adipogénesis) y metabolismo están, además de algunos de los ya mencionados anteriormente, receptores

hormonales nucleares como el PPARγ (considerado el regulador principal de la adipogénesis), los GRs, las C/EBPs (*CCAAT/enhancer-binding proteins*, entre ellos C/EBPα), y las UCPs (*uncoupling proteins*), entre otros (Frühbeck, 2008; Heindel et al., 2022; Wajchenberg, 2000).

Entre las sustancias químicas sintetizadas y secretadas por el tejido adiposo blanco, encontramos también, algunas proteínas como la LPL (*lipoprotein lipase*), estimulada por los glucocorticoides, que ejerce un papel clave como reguladora del almacenamiento de TG (Frühbeck, 2008; Wajchenberg, 2000). También la ASP (*acylation-stimulating protei*n), que estimula la síntesis de TG; la CETP (*cholesteryl-ester transfer protein*), que promueve el intercambio de los ésteres de colesterol de los TG entre las proteínas plasmáticas; la RBP (*retinol-binding protein)*; la PAI-1 (*plasminogen activator inhibitor-1*), relacionada con la coagulación sanguínea y con la resistencia a la insulina; la leptina, implicada en la hipertrofia del tejido adiposo que controla el apetito (inhibiéndolo) y la saciedad (estimulándola) al actuar sobre los receptores hipotalámicos, además de estimular la oxidación lipídica, la biogénesis mitocondrial y aumentar el gasto energético; la adiponectina, que promueve la oxidación lípidica y respuestas antiinflamatorias; la resistina, que estimula la obesidad; la omentina que incrementa la acción de la insulina y, por tanto, regula los niveles de glucosa en sangre; y la nesfatina, que regula el apetito (Choe et al., 2016; Frühbeck, 2008; Wajchenberg, 2000). El tejido adiposo blanco secreta también otras proteínas que intervienen en el metabolismo de los lípidos, como por ejemplo la perilipina (*perilipin*) que mantiene estables las gotas de grasa y evita la hidrólisis de los TG, y la FABP4 (*fatty acid binding protein 4*), proteína que evita que los ácidos grados inhiban la hidrólisis que lleva a cabo la enzima HSL (*hormone sensitive lipase*) (Frühbeck, 2008).

Entre los factores con función endocrina, secretados por el tejido adiposo, destaca la enzima aromatasa P450, por su papel en la síntesis de estrógenos generados a partir de la aromatización de los andrógenos; el angiotensinógeno, por su implicación sobre la diferenciación del preadipocito a adipocito; además de la leptina y adiponectina ya mencionadas (Wajchenberg, 2000).

OBESIDAD y otras enfermedades metabólicas

La Organización Mundial de la Salud (OMS) (*World Health Organization*-WHO) define la obesidad y el sobrepeso como una acumulación anormal o excesiva de grasa que incrementa el riesgo dedesarrollar enfermedades no transmisibles (ENTs) (NCDs, *noncommunicable diseases*); es decir, enfermedades crónicas de larga duración que se originan como consecuencia de factores fisiológicos, conductuales, de comportamiento, ambientales y genéticos (World Health Organization, 2022, 2021).

Otras alteraciones del tejido adiposo como cambios en su localización, tamaño (mayor número de adipocitos, hiperplasia; mayor tamaño de los adipocitos, hipertrofia), o recambio lipídico, pueden conllevar también al desarrollo de obesidad y/o a resistencia a la insulina, entre otras enfermedades metabólicas (Mejhert and Rydén, 2020). Así, por ejemplo, es la localización de la grasa en la zona abdominal, también llamada obesidad central o visceral (grasa visceral), uno de los componentes principales del desarrollo de síndrome metabólico (MetS*, metabolic syndrome*); nombre con el que se conoce a un grupo de factores de riesgo que, cuando concurren juntos (tensión arterial elevada, glucemia elevada y dislipidemia), aumentan la probabilidad de sufrir enfermedades cardiovasculares (ECVs) y/o diabetes tipo 2,

entre otros problemas graves de salud (Badimon et al., 2015; Fahed et al., 2022; Lam and LeRoith, 2019; Lee et al., 2013; Lustig et al., 2022).

Además de la grasa visceral (obesidad central), otros de los factores de riesgo asociados a MetS son la hipertensión arterial, la hiperglucemia y la hiperlipidemia (Badimon et al., 2015; Diamanti-Kandarakis et al., 2009; Fahed et al., 2022; Grundy, 2016; Lam and LeRoith, 2019; World Health Organization, 2022). Los componentes del MetS incluyen, por tanto, un inadecuado perímetro de cintura [obesidad abdominal, que varía según el país y población (etnia); concretamente, ≥94 cm en hombres o ≥80 cm en mujeres según la Federación Internacional de Diabetes (IDF, *International Diabetes Federation*), o ≥102 cm en hombres o ≥ 88 cm mujeres según la AHA/NHLBI (*American Heart Association/National Heart, Lung, and Blood Institute*)]; una alta presión arterial (sistólica ≥130 mm Hg y/o diastólica ≥85 mm Hg), niveles altos de glucosa plasmática en ayunas (≥100 mg/dL), niveles altos de TG (≥150 mg/dL), y niveles bajos de colesterol de lipoproteínas de alta densidad (HDL, *high density lipoproteins*; concretamente <40 mg/dL en hombres o <50 mg/dL en mujeres) (Alberti et al., 2009; Grundy, 2016; Lam and LeRoith, 2019). Se establece que la presencia simultánea en un individuo de al menos 3 de estos 5 componentes conllevaría al diagnostico clínico de MetS (Alberti et al., 2009; Grundy, 2016; Lam and LeRoith, 2019). No obstante, otros autores como Liu y colaboradores, consideran que un individuo es metabólicamente insano cuando concurren al menos 2 de los componentes del MetS (Liu et al., 2022).

Hay suficiente evidencia también que demuestra que la hipertrofia de los adipocitos del tejido adiposo blanco se asocia con resistencia a la insulina y la diabetes tipo 2, mientras que la hiperplasia del tejido

adiposo blanco no estaría asociada con tal riesgo (Choe et al., 2016; Mejhert and Rydén, 2020). Esto se explicaría porque la hipertrofia produce en el adipocito un menor almacenamiento de lípidos al reducir la sensibilidad a la insulina, un menor recambio de lípidos, una mayor secreción de citoquinas inflamatorias, hipoxia, mayor muerte celular y mayor infiltración de leucocitos (Choe et al., 2016; Frühbeck, 2008; Longo et al., 2019; Lustig et al., 2022; Mejhert and Rydén, 2020).

Otro factor que también es causa del desarrollo de obesidad, resistencia a la insulina y diabetes tipo 2 es una lipólisis basal incrementada, es decir, mayor liberación de ácidos grasos en los adipocitos hipertrofiados (Choe et al., 2016; Longo et al., 2019), así como una lipogénesis alterada dando lugar a lipotoxicidad, como es el caso del exceso de grasa del tejido adiposo blanco subcutáneo que pasa al tejido adiposo blanco visceral y este a su vez a otros tejidos como hígado, músculo esquelético, corazón y páncreas (Badimon et al., 2015; Lee et al., 2013; Longo et al., 2019).

Un marcador simple, muy utilizado, para determinar sobrepeso u obesidad en la población es el índice de masa corporal (IMC) (BMI, *body mass index*), expresado en kg/m^2. En adultos, un IMC ≥25 kg/m^2, indica sobrepeso, y un IMC ≥30 kg/m^2 obesidad (World Health Organization, 2021). Se dispone, no obstante, de otros biomarcadores que permiten identificar más exhaustivamente el sobrepeso y la obesidad, como es por ejemplo la circunferencia de la cintura o la relación cintura/cadera, como medida de la grasa abdominal (Alberti et al., 2009; Badimon et al., 2015; Gore et al., 2015a; Lee et al., 2013; Longo et al., 2019; Noubiap et al., 2022a; Schneider et al., 2010; Wajchenberg, 2000).

Recientemente la OMS ha publicado un informe donde se estima que en Europa tienen sobrepeso u obesidad el 60% de los adultos y el 33%

de los niños mayores de 5 años; cifras de prevalencia de sobrepeso y obesidad entre las más altas del mundo (Boutari and Mantzoros, 2022). Concretamente en Europa se ha pasado de un 8,4% en 1980, a un 20% en 2019 (Boutari and Mantzoros, 2022). En general, la obesidad aumenta con la edad y alcanza su pico en el rango de los 50 a los 65 años. No obstante, la prevalencia de obesidad en el mundo, ajustada por edad, también se ha visto incrementada, pasando de un 4,6% en el año 1980, a un 14% en el 2019, siendo superior en las mujeres que en los hombres (Boutari and Mantzoros, 2022). Ward y colaboradores (2019) han estimado que, en el año 2030, la mitad de la población adulta estadounidense tendrá obesidad, y que 1 de cada 4 tendrá obesidad severa (IMC >35 kg/m^2) (Ward et al., 2019).

Se distinguen dos tipos de obesidad: la obesidad metabólicamente insana (*metabolically unhealthy obesity*-MUO) y la obesidad metabólicamente sana (*metabolically healthy obesity*-MHO). Esta última se caracteriza por una expansión normal del tejido adiposo (en particular del subcutáneo), una adipogénesis normal (reclutamiento y diferenciación de las células precursoras del tejido adiposo óptimos y pocas células inmunitarias asociadas con la inflamación), sensibilidad a la insulina, además de una reducida lipólisis basal, aumento de la angiogénesis, normal oxidación de las mitocondrias, y adecuada secreción de adiponectina y citoquinas antiinflamatorias (Choe et al., 2016; Longo et al., 2019; Lustig et al., 2022). Además, los individuos obesos metabólicamente sanos no suelen desarrollar resistencia a la insulina ni tienen alterados otros parámetros metabólicos (Choe et al., 2016; Lustig et al., 2022). Sin embargo, los individuos obesos "metabólicamente insanos" suelen desarrollar resistencia a la insulina, además de presentar algunos o todos los componentes del MetS, así como menor angiogénesis, mayor inflamación (elevada infiltración de

macrófagos y citoquinas proinflamatorias) y secreción reducida de adiponectina y aumentada de leptina y resistina (Badimon et al., 2015; Choe et al., 2016; Longo et al., 2019; Lustig et al., 2022). De modo general, no significa que la obesidad metabólicamente sana no suponga un problema, ya que su salud metabólica evolucionará a un perfil no saludable antes que en los individuos con un peso normal. Sin embargo, esta distinción nos indica que hay distintos tipos de obesidad, los cuales responden a diferentes características anatómicas y bioquímicas.

Los datos también indican que la prevalencia de adultos obesos, metabólicamente insanos (definidos como aquellos individuos que presentaban al menos 2 de los componentes del MetS), también ha aumentado en la población estadounidense (Lustig et al., 2022). La tendencia entre los periodos 1999-2000 y 2017-2018 ha sido de un aumento del 19% al 26,4% (Lustig et al., 2022). Otros trabajos indican que el incremento en la prevelencia de MetS en esta población podría haber sido incluso mayor, pasando de un 25,3% en el periodo 1988-1994, al 36,9% en 2015-2016 (Hirode and Wong, 2020; Moore et al., 2017).

Un reciente meta-análisis en el que se incluyeron 28 millones de sujetos reclutados entre 1990 y 2018, indica que la prevalencia mundial del MetS (y de sus componentes individuales), en población adulta de diferentes zonas geográficas y niveles socioeconómicos, es también alarmantemente alta, varíando entre el 12,5% y el 31,4% según la definición de MetS que se aplique, la zona geográfica o el nivel socieconómico del país (Noubiap et al., 2022a). El mismo grupo de investigadores que realizaron el meta-análisis también han informado de la prevalencia mundial de MetS en la población infantil (6-12 años)

y adolescente (13-18 años) en el año 2020, obtenida mediante otra revisión sistemática (Noubiap et al., 2022b), se estima en el 2,8% (25,8 millones) entre los niños y en el 4,8% (35,5 millones) en los adolescentes. Los niños en América Latina central presentan la prevalencia más alta, con un 8,2%, y los del noroeste de Europa la que tiene la prevalencia menor, con un 1,4%. Los adolescentes con la prevalencia más alta (6,7%) se situan en países angloparlantes de renta alta, mientras que los de prevalencia más baja (2,9%) lo hacen en Asia oriental (Noubiap et al., 2022b).

En relación a los componentes individuales del MetS, los resultados del meta-análisis de Noubiap et al. (2022a) indican que la prevalencia mundial de obesidad central (específica por etnia) sería del 45,1%; del 34,9% para un perímetro de cintura inadecuado (≥102 cm en hombres y ≥88 cm en mujeres); del 42,6% para una preesión arterial alterada (sistólica ≥130 mm Hg y/o diastólica ≥85 mm Hg); del 40,2% para altos niveles de colesterol HDL (<40 mg/dL o <1,0 mmol/L en hombres y <50 mg/dL o <1,3 mmol/L en mujeres); del 28,9% para niveles de TG séricos (≥150 mg/dL o ≥ 1,7 mmol/L); y del 24,5% para la glucosa plasmática en ayunas (≥5,6 mmol/L) (Noubiap et al., 2022a). Otros estudios recientes recogen también la elevada prevalencia de los factores de riesgo metabólicos del MetS, con resultados similares a los anteriormente descritos (Antonio-Villa et al., 2022; Palmer and Toth, 2019; Peters et al., 2019; Wang et al., 2021) y en la población mundial (Sun et al., 2022; B. Zhou et al., 2017; Zhou et al., 2021, 2016). En línea con el incremento en la prevalencia de obesidad observado en las últimas décadas. Así, por ejemplo, en EEUU, la prevalencia de diabetes estandarizada por edad ha pasado de un 9,8% en 1999-2000 a un 14,3% en 2017-2018 (Wang et al., 2021), y la prevalencia de hipertensión de un 31,8% en 1999-2000 a 48,5% en 2007-2008,

permaneciendo estable en 53,8% hasta 2013-2014 y diminuyendo ligeramente (43,7%) en el último lustro (2017-2018) debido fundamentalmente al uso de antihipertensivos (Muntner et al., 2020).

En resumen, es evidente que la prevalencia de sobrepeso y obesidad, además de las enfermedades asociadas al MetS es cada vez mayor en el mundo, lo que constituye un motivo de gran preocupación y relevancia en salud pública (Alberti et al., 2009; Boutari and Mantzoros, 2022; Noubiap et al., 2022a).

OBESIDAD: papel del medio ambiente y la exposición a xenobióticos

La obesidad está considerada como una enfermedad multifactorial (Heindel et al., 2022). Si bien se acepta que la causa principal de la obesidad es un desequilibrio energético entre las calorías que consumimos y las que gastamos mediante el ejercicio físico y las actividades de la vida cotidiana, los datos indican que la excesiva ingesta de calorías y la insuficiente actividad física asociadas al estilo de vida actual serían insuficientes para poder explicar las cifras de sobrepeso u obesidad actuales y sus complicaciones metabólicas (Heindel et al., 2015b; Heindel et al., 2022). Los expertos indican que otros factores, fundamentalmente ambientales y de estilo de vida, deben estar interviniendo en el desarrollo de estas enfermedades, y apelan al estudio de lo que se conoce con el término *exposoma* (Heindel et al., 2022).

El concepto exposoma implica un cambio de perspectiva al tener en cuenta los riesgos ambientales en la salud humana, incluyendo todas las exposiciones ambientales a sustancias químicas, biológicas y físicas, que ocurren como consecuencia de nuestro estilo de vida, de nuestra

dieta, y/o del entorno en el que vivimos y trabajamos (*factores extrínsecos*). El concepto exposoma incluye además factores biológicos internos del individuo (*factores intrínsecos*) como su microbiota intestinal, estado de inflamación y estrés oxidativo, o incluso la falta de sueño (Catalán et al., 2022; Gao and Snyder, 2021; Grün and Blumberg, 2009; Heindel et al., 2022; Papalou et al., 2019; Vermeulen et al., 2020; Wild, 2005).

En esta línea hay que tener en cuenta que el tejido adiposo es un órgano que además de sintetizar y excretar hormonas y otras moléculas, almacena sustancias de diversa naturaleza, fundamentalmente compuestos químicos lipofílicos y generalmente persistentes, con tiempos largos de vida media, entre los que encontramos contaminantes orgánicos persistentes (POPs, *persistent organic pollutants*). De hecho, se ha llegado a afirmar que el tejido adiposo posee un rol toxicológico, secuestrando POPs de la circulación, con objeto de reducir su llegada a órganos críticos, como por ejemplo el cerebro o el hígado (La Merrill et al., 2013; Lee et al., 2017).

Dentro de los POPs se incluyen diferentes compuestos químicos entre los que destacan los plaguicidas organoclorados (OCPs, *organochlorine pesticides*), los bifenilos policlorados (PCBs, *polychlorinated biphenyls*) y polibromados (PBBs, *polybrominated biphenyls*), además de dioxinas y furanos, entre otros compuestos químicos (Lee et al., 2017; Papalou et al., 2019). Muchas de estas sustancias químicas se comportan, además, como disruptores endocrinos (EDCs, *endocrine disrupting chemicals*) (Diamanti-Kandarakis et al., 2009).

Desde finales de los años 90 son varias las definiciones de compuestos químicos-disruptores endocrinos (EDCs) establecidas por diferentes organismos públicos (Darbre, 2019; Zoeller et al., 2012). El concepto

de disruptor endocrino surge por primera vez en la Conferencia de Wingspread organizada por el Fondo Mundial para la Naturaleza y celebrada en Wisconsin (USA) en 1991 (Colborn and Clement, 1992). En esta conferencia se define "disruptor endocrino", como "una sustancia química exógena, o mezcla, que altera la estructura o la(s) función(es) del sistema endocrino y causa efectos adversos en el organismo o en su progenie, además de en poblaciones o subpoblaciones" (Zoeller et al., 2012). Esta definición, se ha ido completando y modificando desde entonces, a medida que se ha ido teniendo más evidencia científica sobre el tema (Zoeller et al., 2012), como por ejemplo a partir de las conclusiones de la reunión de Weybridge (Inglaterra) en 1996 (European Commission et al., 1997). Este conocimiento científico fue el que impulsó, ese mismo año, la creación del Comité Consultivo de Cribado y Ensayo de Sustancias con actividad disruptora endocrina (*Endocrine Disrupting Screening and Testing Advisory Committee*, EDSTAC), con el encargo de asesorar a la Agencia de Protección Medioambiental de los Estados Unidos (*United States Environmental Protection Agency*, EPA), sobre estas "nuevas" sustancias químicas (Zoeller et al., 2012). También han proporcionado definiciones de EDCs, la EPA, la Unión Europea o la OMS (Zoeller et al., 2012). Así, por ejemplo, la EPA define EDCs como "agentes exógenos que interfieren con la síntesis, secreción, transporte, metabolismo, unión o eliminación de las hormonas naturales del organismo responsables del mantenimiento de la homeostasis, reproducción y regulación de los procesos de desarrollo" (Diamanti-Kandarakis et al., 2009; Zoeller et al., 2012). Por su parte la Unión Europea los definen como "sustancias exógenas que causan efectos adversos para la salud en un organismo intacto, o en su progenie, secundarios a cambios en la función endocrina"; y la OMS como "sustancias o mezcla exógena que

alteran la(s) función(es) del sistema endocrino y, en consecuencia, causa efectos adversos en un organismo intacto, o en su progenie, o en (sub)poblaciones" (Bergman et al., 2013; Zoeller et al., 2012). Actualmente, la definición más generalizada es la propuesta por la Sociedad de Endocrinología, establecida en la primera edición de la Declaración Científica de la Sociedad de Endocrinología sobre las sustancias químicas-disruptores endocrinos, esto es: "los EDCs son aquellas sustancias químicas exógenas, o mezclas de ellas, que interfieren con cualquier aspecto de la acción hormonal" (Zoeller et al., 2012); definición que se vio corroborada en la segunda Declaración Científica de la Sociedad de Endocrinología sobre estas sustancias químicas (Gore et al., 2015b).

Los EDCs afectan al sistema endocrino interfiriendo con las hormonas, ya sea modificando su síntesis, secreción, transporte, metabolismo, eliminación, homeostasis o acción biológica, o alterando la unión de las hormonas a sus receptores u otras vías o mecanismos de señalización, generando efectos en diferentes sistemas como el endocrino o el reproductivo (Diamanti-Kandarakis et al., 2009; European Commission, 2018; Flynn, 2011). Recientemente La Merrill y colaboradores, han revisado la evidencia científica y resumido cuáles serían las características principales (KCs, *key characteristics*) que definen un EDC (La Merrill et al., 2020). Los investigadores han establecido en diez estas características: **1**: unión o activación de uno o varios receptores hormonales; **2**: antagonismo de los receptores de hormonas, pudiendo inhibir o bloquear los efectos de las hormonas endógenas y, por tanto, actuar como antagonistas de estos receptores; **3**: modulación de la expresión de distintos receptores hormonales; **4**: alteración de la transducción de señales, a través de receptores de membrana, intracelulares y/o nucleares, atenuando o potenciando la

acción hormonal; **5**: modificaciones epigenéticas en las células al interferir con la acción de las hormonas, entorpeciendo su capacidad para alterar la expresión y/o acción de un receptor hormonal o la transcripción de los genes que responden a las hormonas; **6**: alteración de la síntesis de hormonas; **7**: modificación del transporte de las hormonas; **8**: modulación de la distribución, circulación y concentración de las hormonas a los (o en los) órganos y tejidos diana; **9**: alteración del metabolismo y/o eliminación de las hormonas, y, en consecuencia, la concentración y actividad de las hormonas; y **10**: modificación del destino de las células que producen las hormonas o responden a las mismas, es decir, alteración del número, posición y/o estructura de las células que producen hormonas o que responden a estas, afectando a la estructura y organización de los tejidos, al interferir o estimular la proliferación, diferenciación, migración, y/o muerte celular (La Merrill et al., 2020).

La evidencia, como se comentaba anteriormente, muestra que la exposición a los EDCs implica cambios en la unión, actividad y acción sobre los receptores hormonales con repercusiones en la salud del individuo expuesto (Amir et al., 2021; Foulds et al., 2017; Gore et al., 2015a; Merrheim et al., 2020; Sakkiah et al., 2017). Entre los receptores nucleares que pueden verse afectados por la acción de los EDCs, encontramos ERs, ARs, progesterona, TRs, RXR, además de receptores no nucleares como los receptores de membrana de hormonas esteroideas, o receptores no esteroideos como los receptores de neurotransmisores (serotonina, dopamina y adrenalina), y de receptores "huérfanos" (*orphan receptors*) como el AhR (Diamanti-Kandarakis et al., 2009). Cuando los EDCs se unen a los receptores nucleares (NRs, *nuclear receptors*), pueden producir un

efecto que activa la acción de los mismos (actividad agonista), pero también inhibir su acción (antagonista), o incluso producir una acción parcial (Flynn, 2011).

Exposición humana a EDCs

La exposición humana a EDCs puede ocurrir de manera diaria por multiples vías de exposición, ya que muchos de estos compuestos químicos son vehiculizados a través de la dieta, mediante la ingesta de su residuo en alimentos y bebidas (vía oral), a través de la vía aérea al respirar aire contaminado, pero también a través de la piel (vía dérmica). Así, se han encontrado residuos de EDCs en la comida, en los envases alimentarios, diferentes plásticos, productos de higiene personal y cosméticos, además de en el aire exterior e interior de las viviendas (Diamanti-Kandarakis et al., 2009; Papalou et al., 2019).

Los EDCs son, además, un grupo sustancias químicas de muy diferente origen, estructura y uso. Indicábamos anteriormente que algunos EDCs presentan gran estabilidad e inercia para reaccionar químicamente, por lo que reúnen las características óptimas para haber sido, y ser, empleados en grandes cantidades y con gran libertad, sin especial protección medioambiental (Fernández and Olea, 2014). Estos EDCs son bien conocidos por su capacidad para acumularse y persistir en las cadenas tróficas, como es el caso de los POPs, sobre los que, en la mayoría de los casos, se han establecido medidas de control y/o prohibición (High Level Political Forum of United Nations on Sustainable Development., 2022). Sin embargo, entre los EDCs también es posible encontrar sustancias que parecen no bioacumularse, es decir, no persisten ni se acumulan en el organismo humano, pero su presencia como contaminantes en el entorno (agua, aire, alimentos, utensilios) es tan frecuente que la exposición diaria es inevitable

(Fernández and Olea, 2014). Entre estos compuestos se encuentran algunos fenoles, como bisfenol A (BPA, *bisphenol A*) utilizados en el plástico policarbonato y las resinas epoxi, ftalatos (*phthalates*) empleandos como plastificantes pero también en cosmética, hidrocarburos aromáticos policíclicos (PAHs, *polycylic aromatic hydrocarbons*) procedentes de procesos de combustión, parabenos (*parabens*) usados como conservantes en alimentos y cosméticos por su actividad como antimicrobianos y antifúngicos, así como algunas benzofenonas (*benzophenones*) o filtros UV presentes en diferentes cremas solares, cosméticos y otros productos de consumo (Heindel et al., 2022; Papalou et al., 2019). Estos EDCs no persistentes (*npEPs, non-persistent environmental pollutants*) no parecen bioacumularse, y alcanzan el organismo humano, fundamentalmente a través de una exposición ambiental «de fondo» (dieta, agua, polvo, aire, cosméticos, textiles), posiblemente en dosis bajas pero sostenidas en la mayor parte de los casos (Diamanti-Kandarakis et al., 2009; Gore et al., 2015a; Papalou et al., 2019; Vandenberg et al., 2012). Los npEPs son general y rápidamente metabolizados y excretados a través de la orina (Søeborg et al., 2014), aunque también pueden encontrarse en el tejido adiposo (Artacho-Cordón et al., 2019; Fernandez et al., 2007).

La exposición humana a EDCs puede jugar un papel importante en la etiología de enfermedades cada vez más frecuentes en nuestra sociedad, relacionándose con diferentes efectos perjudiciales para la salud humana, incluso aunque la exposición ocurra a bajas concentraciones (Vandenberg et al., 2012). No obstante, el momento en que ocurre esta exposición parece determinar las consecuencias sobre el individuo. Así, por ejemplo, si la exposición ocurre durante el embarazo tendrá resultados muy distintos a los esperados si tiene lugar durante la etapa adulta del individuo. Además, el embrión/feto y

el neonato son extremadamente sensibles a la exposición a EDCs, y los efectos adversos suelen ser más graves que cuando la exposición ocurre en el individuo adulto (ACOG Committee Opinion, 2013; Colborn et al., 1993; Gore et al., 2015a; Heindel et al., 2015b).

El concepto de salud y enfermedad de los adultos, con base etiológica y origen durante la etapa fetal o neonatal, surge a finales de los años 80, cuando estudios sobre las condiciones nutricionales y ambientales de las embarazadas se relacionaron con el aumento del riesgo de sufrir enfermedades no transmisibles (metabólicas y cardiovasculares) a lo largo de la vida; lo que se conoce como el origen fetal de las enfermedades (Barker, 1995; Sallout and Walker, 2003), también conocido como los Orígenes del Desarrollo de la Salud y la Enfermedad (DOHaD, *Developmental Origins of Health and Disease*) (Gore et al., 2015a; Heindel et al., 2015b).

Entre los efectos nocivos relacionados con la exposición a EDCs destacan los relacionados con el sistema endocrino, el sistema inmune, el sistema nervioso y el reproductivo, con enfermedades tan prevalentes como por ejemplo la obesidad y la diabetes, entre otras enfermedades metabólicas y cardiovasculares, e incluso algunos tipos de tumores hormonodependientes (Diamanti-Kandarakis et al., 2009; Gore et al., 2015a; La Merrill et al., 2020). Al mismo tiempo, diversos estudios en animales han confirmado el efecto que estas sustancias químicas antropogénicas-EDCs pueden ocasionar en el desarrollo, y en particular sobre los sistemas reproductivos y endocrinos (World Health Organization, 2012). Otro aspecto a destacar en la disrupción endocrina es la no existencia de un umbral de concentración preciso para el desarrollo del efecto, ya que éste es fundamentalmente dependiente del momento hormonal del individuo expuesto, lo que

contribuye a que el nivel de exposición pueda ser muy inferior al reconocido como límite de seguridad para otros efectos toxicológicos distintos de la disrupción endocrina (Fernández and Olea, 2014; Gore et al., 2015a).

Disruptores endocrinos obesogénicos y metabólicos

Desde principios del siglo XXI se viene investigando la implicación de algunos EDCs en el aumento de obesidad en la población. Fue Paula Baillie-Hamilton (2002) la que formuló, tras analizar la correlación entre el aumento de la frecuencia de sobrepeso en la población adulta y el aumento de la producción de sustancias químicas industriales, la hipótesis de la relación causal entre ambos acontecimientos (Baillie-Hamilton, 2002). Poco después se acuña el término obesógeno (*obesogens*), propuesto por Grün y Blumberg en 2006 (Grün and Blumberg, 2006), quienes definieron a los obesógenos como "compuestos que regulan de forma inapropiada el metabolismo lipídico y la adipogénesis promoviendo la obesidad" (Grün and Blumberg, 2006); es decir, sustancias que favorecen la acumulación y almacenamiento de lípidos, incrementan la adipogénesis, y alteran la diferenciación y función de los adipocitos, modificando puntos clave de la homeostasis metabólica que conducen a la obesidad (Grün, 2010; Grün and Blumberg, 2009; Janesick and Blumberg, 2012). Esta definición de obesógenos se ha ido ampliando con el paso de los años, a medida que ha ido creciendo la evidencia científica en este área de conocimiento. Actualmente se considera obesógenos a los EDCs que promueven el desarrollo de obesidad por diferentes mecanismos, como por ejemplo: a) aumentando el número y tamaño de los adipocitos (hiperplasia e hipertrofia), b) modificando el equilibrio energético, incrementando el almacenamiento de calorías y/o

alterando la tasa metabólica basal, c) modificando la microbiota intestinal, d) alterando el control hormonal del apetito y de la saciedad, y/o e) transformando los circuitos cerebrales que controlan la ingesta de alimentos y el gasto de energía (Heindel et al., 2015b). Por lo tanto, la exposición a EDCs obesógenos está considerada como un factor de riesgo de obesidad, pero también de enfermedades asociadas a ella, como el MetS (Grün, 2010; Heindel et al., 2015b).

Los obesógenos, de manera similar a otros EDCs, siguen sus mismas caraterísticas toxicológicas particulares; esto es, como por ejemplo su capacidad de actuar a bajas dosis, o generar curvas dosis-respuesta no monotónicas, dificultando la posibilidad de predecir lo que ocurre a bajas dosis, una vez conocida la respuesta a las altas concentraciones normalmente testadas en los ensayos toxicológicos regulatorios con animales de experimentación (Diamanti-Kandarakis et al., 2009; Grün and Blumberg, 2009; Heindel et al., 2022; Papalou et al., 2019; Vandenberg et al., 2012; Zoeller et al., 2012).

Dentro de los EDCs también encontramos a los "disruptores metabólicos" (MDCs, *metabolism-disrupting chemicals*). El término MDCs apareció con posterioridad al término obesógeno (Heindel et al., 2015b). La diferencia entre ambos conceptos es su efecto en el organismo. Así, mientras la exposición a MDCs se ha relacionado, en general, con enfermedades metabólicas (MetS o sus componentes, obesidad abdominal, diabetes, hipertensión, y/o hiperlipidemia), la exposición a obesógenos es más específica al asociarse únicamente con obesidad, enfatizando los períodos de desarrollo (Heindel et al., 2015b). El término MDCs fue introducido por primera vez en la Declaración (Consenso) de Parma, en 2014 (Heindel et al., 2015b), definiéndose como compuestos químicos ambientales capaces de

modificar el desarrollo del tejido adiposo al incrementar el número y/o tamaño de las células grasas, y/o modificar la ingesta de alimentos y el metabolismo, a través de efectos específicos, individuales o combinados, en diferentes órganos, como por ejemplo el cerebro, páncreas, tejido adiposo, hígado, tracto gastrointestinal e incluso el tejido muscular (Nadal et al., 2017).

Hasta el momento, la comunidad científica ha identificado más de veinte compuestos químicos obesógenos, que han demostrado su actividad tanto en modelos *in vitro*, en líneas celulares murinas (células 3T3-L1) y células multipotentes (células madre mesenquimales, MSCs-*mesenchymal stem cells*), como en modelos experimentales (Heindel et al., 2022; Kassotis et al., 2022). En la lista de obesógenos se incluyen sustancias químicas de diversa índole, como plastificantes, retardantes de llama, productos agrícolas (insecticidas, pesticidas), contaminantes encontrados en el aire, o ingredientes para uso alimentario y en cosmética (Heindel et al., 2022). Entre estos compuestos se incluye, por ejemplo, BPA y sus análogos como BPS (*bisphenol S*), TBBPA (*tetrabromobisphenol A*) y TCBPA (*tetrachlorobisphenol* A); ftalatos como el DEHP (*di-2-ethylhexyl phthalate*) y DBP (*di-n-butyl phthalate*); el TBT (tributyltin); los PBDEs (*polybrominated diphenyl ethers*) y sus sustitutos, los OPFRs (*organophosphate flame retardants*,); los POPs (*persistent organochlorined pollutants*), como por ejemplo DDT (*dichlorodiphenyltrichloroethane*) y su metabolito DDE (*dichlorodiphenyldichloroethylene*), βHCH (β-*hexachlorocyclohexane*), y HCB (*hexachlorobenzene*); los PCBs; los PFAS (*per- and poly-fluorinated alkyl substances*) como el PFOA (*perfluorooctanoic acid*) y el PFOS (*perfluorooctane sulfonate*); algunos parabenos como el MP (*methylparaben*) y BP (*butylparaben*).

Muchos obesógenos son también MDCs (Heindel et al., 2022). Como ejemplos de MDCs persistentes encontramos algunos POPs, como DDT, y algunos PCBs, PFAS (PFOS y PFOA), y el TBBPA. Como ejemplos de MDCs no persistentes (*npEPs*) se encuentran el DES (*diethylstilbestrol*), el BPA y algunos ftalatos como el DEHP (Nadal et al., 2017).

Las sustancias anteriores nombradas han sido catalogadas como obesógenos dado que, entre los mecanismos de acción moleculares que desencadenan, se incluye la activación o antagonismo de diferentes receptores nucleares y rutas de señalización metabólica, entre los que destacan, en mayor o menor medida, PPARγ, C/EBPα, GR, LPL, SREBP-1 (*sterol regulatory element-binding protein*), FAS (*fatty acid synthase*), SCD-1 (*stearoyl-CoA desaturase 1*,), AhR, FABP4, TNFα, IL-6, adiponectina, insulina, leptina, POMC (*pro-opiomelanocortin*), ERs, TRβ (Heindel et al., 2022). La activación o antagonismo de estos receptores tienen efectos en cualquier tejido implicado en el control del metabolismo como el tejido adiposo, hígado, páncreas, cerebro y músculo (Heindel et al., 2022; Zoeller et al., 2012), resultando en una mayor cantidad y tamaño de los adipocitos, estrés oxidativo, inflamación, alteración de los ritmos circadianos, cambios en la ingesta de alimentos, adicción por la comida, etc. (Heindel et al., 2022).

Un buen ejemplo de obesógeno es el caso del DES, un potente estrógeno sintético ampliamente utilizado para tratar el aborto espontáneo en los años 40-70 del siglo pasado (Hatch et al., 2010; Newbold et al., 2009, 2005; Papalou et al., 2019; Swan, 2000). Diferentes modelos experimentales en los que se ha analizado el efecto de la exposición prenatal a DES, indican que los animales expuestos a dosis bajas de DES no sufrían modificaciones de su peso corporal durante el tratamiento, pero sí evidenciaban aumento de peso en la

edad adulta (Hatch et al., 2010; Newbold et al., 2009, 2005). La administración de dosis altas de DES, en esa etapa del desarrollo, causaba un descenso significativo del peso durante el tratamiento, pero en la pubertad se observaba un incremento ponderal por aumento del depósito graso (Newbold et al., 2009), además del incremento en los niveles de leptina, adiponectina, IL-6, y TG (Newbold et al., 2009). Numerosos estudios epidemiológicos parecen avalar la relación directa entre la exposición a obesógenos y efectos adversos sobre el organismo humano. Así, por ejemplo, la exposición a OCPs, como HCB y DDE, durante la etapa de desarrollo fetal, se han relacionado con un mayor riesgo de que los hijos de las embarazadas expuestas tengan un mayor peso durante la edad adulta (Karmaus et al., 2009; Smink et al., 2008; Vilahur et al., 2013); aunque no todos los estudios han encontrado relación con la exposición a estos compuestos obesógenicos persistentes (Govarts et al., 2012).

Algunos bisfenoles, utilizados en la fabricación de polímeros plásticos, como el policarbonato y las resinas epoxi, y como aditivos en otras aplicaciones, también están considerados como obesógenos (Kurşunoğlu and Sarer Yurekli, 2022; Vandenberg et al., 2007). El bisfenol más conocido, producido y utilizado es el BPA, cuya producción mundial entre 2010 y 2016 aumentó de 5 a 10 millones de toneladas y se estima que haya alcanzado los 10,2 millones de toneladas en el 2022 (Kurşunoğlu and Sarer Yurekli, 2022). A pesar de que la actividad hormonal de BPA se conoce desde 1936 (Kurşunoğlu and Sarer Yurekli, 2022), no ha sido hasta muy recientemente cuando la ECHA (*European Chemicals Agency*) lo ha clasificado como disruptor endocrino (ECHA, 2017) y obesógeno (Heindel et al., 2022; Kurşunoğlu and Sarer Yurekli, 2022).

Aunque las concentraciones detectadas de BPA, en los estudios de biomonitorizacion humana, son cada vez menores, por las restricciones europeas impuestas, por ejemplo, en Europa (2011), la detección de algunos de sus sustitutos o análogos, como BPS y BPF (*bisphenol F*), no deja de crecer (Karrer et al., 2019; Kurşunoğlu and Sarer Yurekli, 2022). BPS ha sido también clasificado como obesógeno por algunas de las agencias reguladoras (Heindel et al., 2022), sin embargo, no ha ocurrido lo mismo con su homólogo BPF. Dada la similitud estructual de ambos sustitutos respecto a BPA, al igual que la semejanza en sus características fisicoquímicas, parecería plausible esperar que ambos compuestos no sean alternativas seguras para la población (Carvaillo et al., 2019; Karrer et al., 2019; Rochester and Bolden, 2015). Se requieren, no obstante, más estudios de biomonitorización que informen sobre la carga actual de estos y otros compuestos químicos ambientales obesógenos para conocer y evaluar si esta exposición humana se relaciona con algunas de las enfermedades metabólicas prevalentes que no dejan de aumentar en la población.

BIOMARCADORES de exposición y efecto

La biomonitorización humana (HBM, *human biomonitoring*) se define como el método para evaluar la exposición humana a sustancias químicas (biomarcadores de exposición) mediante la medición de éstas, o sus metabolitos, en diferentes muestras biológicas obtenidas de la población (Centers for Disease Control and Prevention, 2019). Tiene, por tanto, la finalidad de cuantificar la carga total de la exposición y analizar su impacto en salud. Los estudios de HBM proporcionan también información sobre la dosis interna de los contaminantes, al tener en cuenta diversas rutas y fuentes de

exposición (Ganzleben et al., 2017). Son, por tanto, la base para estimar la carga química corporal total. La carga corporal total viene, no obstante, determinada por diferentes factores de carácter general del compuesto químico (concentración, propiedades fisicoquímicas, o momento de la exposición), además de por factores individuales del mismo (absorción, metabolismo, tasa de excreción) (Sexton et al., 2004).

Los estudios de HBM permiten, por otra parte, i) abordar las tendencias espaciales y temporales de las exposiciones a contaminantes ambientales; ii) priorizar y regular los compuestos químicos; iii) verificar las dosis internas; iv) reducir, restringir, e incluso prohibir el uso de determinados compuestos; así como v) mejorar las evaluaciones de riesgo químico de las sustancias químicas de interés (Ganzleben et al., 2017).

En los estudios de HBM se pueden también utilizar biomarcadores de efecto y susceptibilidad para evaluar cómo y en qué medida afecta la exposición en la salud individual y colectiva (Mustieles et al., 2020; Zare Jeddi et al., 2021). Un biomarcador de efecto se define como una alteración bioquímica, fisiológica, conductual o de otro tipo, medible en un organismo que, dependiendo de su magnitud, puede reconocerse como asociada a una alteración de la salud o enfermedad establecida (National Research Council et al., 2006). Los biomarcadores de efecto permiten, por tanto, evaluar los cambios que se han producido en la composición bioquímica o fisiológica de un individuo, como consecuencia de la exposición humana a sustancias químicas (National Research Council et al., 2006), permitiendo una evaluación rápida de posibles efectos, sin la necesidad de esperar décadas hasta el desarrollo de enfermedades clínicas.

La mayoría de la información generada hasta el momento, tanto en el campo de la experimentación, como de la clínica y la epidemiología, e incluso de la regulación de las sustancias químicas se basa fundamentalmente en la evaluación del riesgo de compuestos químicos estudiados uno a uno, sin tener en consideración el efecto combinado de mezclas complejas (Kortenkamp, 2008). Sin embargo, no podemos olvidar, que la exposición humana ocurre de manera conjunta a múltiples compuestos (Kortenkamp, 2014; Vandenberg et al., 2012). El efecto combinado de mezclas de EDCs es una cuestión que aún no ha sido resuelta, fundamentalmente debido a su complejidad, así como a la existencia de lagunas de conocimiento (Diamanti-Kandarakis et al., 2009; Ganzleben et al., 2017; Kortenkamp, 2014, 2008, 2007; Papalou et al., 2019). Para resolver en parte algunas de estas lagunas, se ha propuesto el desarrollo de biomarcadores de exposición-efecto (Kortenkamp, 2008), como por ejemplo el uso de ensayos de levadura, u otros ensayos de transactivación *in vitro*, además de ensayos *in vivo* sobre efectos específicos (Christiansen et al., 2020; Kudłak et al., 2019; Repouskou et al., 2020; Skledar and Mašič, 2020; Yu et al., 2019; R. Zhou et al., 2017).

En este sentido, nuestro grupo de investigación ha sido pionero en el desarrollo de biomarcadores de efecto combinado que han permitido la estimación cuantitativa del efecto de mezclas de compuestos químicos extraídas de matrices biológicas humanas (tejido adiposo, placenta, sangre). Nuestra aproximación se ha basado en el desarrollo de ensayos biológicos que evaluan la capacidad de inducir proliferación en células estrógeno- o andrógeno-dependientes, mediada por la activiación-inhibición de receptores hormonales nucleares, de estrógenos (ER) (Ibarluzea et al., 2004; Pastor-Barriuso et al., 2016), o de andrógenos (AR) (Arrebola et al., 2015b). Estos

biomarcadores han sido aplicados con éxito en estudios epidemiológicos de muy diferente índole, en los que la exposición a EDCs, (anti)estrogénicos o (anti)androgéncios, se ha cuantificado tanto mediante la medida individual de residuos químicos específicos, como mediante la estimación de la actividad biológica (proliferativa) del extracto químico tisular.

Aunque el estudio de mezclas resulta un reto, otros equipos de investigación, además del nuestro, han demostrado que es posible predecir y analizar el efecto de las mezclas de EDCs, utilizando diferentes aproximaciones toxicológicas, distintas a las anteriormente comentadas (Kortenkamp, 2014, 2008, 2007). Ha sido demostrado, también, que la exposición a una mezcla de compuestos químicos induce un efecto adverso, incluso aunque la composición de la mezcla contenga concentraciones a las que, de manera individual, los compuestos no producirían ningún efecto biológico, efecto conocido como como "*something from nothing"*; escenario que simula mucho más la realidad de exposición química humana (Kortenkamp, 2014; Silva et al., 2002).

Es necesario, no obstante, seguir investigando tanto en el efecto individual como en el combinado de las mezclas de compuestos químicos-obesogénicos a los que la población humana está expuesta, ya que no hay aún estudios *in vivo* o *in vitro* que hayan analizado el efecto obesogénico resultante de la exposición a mezclas de EDCs. Es de máximo interés, por lo tanto, investigar la respuesta, de la exposición a EDCs obesógenos individuales, así como de mezclas, y analizar las implicaciones del efecto obtenido en diferentes tejidos y órganos (Diamanti-Kandarakis et al., 2009; Gore et al., 2015a; Kortenkamp, 2014, 2007; Papalou et al., 2019; Vandenberg et al., 2012;

Zoeller et al., 2012). Este fue el cometido que se quiso abordar en esta Tesis Doctoral Internacional.

Hypothesis and Rationale

The environmental obesogens hypothesis suggests that human exposure to "obesogenic" EDCs may inappropriately interfere with lipid homeostasis and metabolism and promote adipogenesis, modifying both hormonal and neuronal signalling pathways, thereby predisposing exposed individuals to increased fat mass and excess weight. EDCs that not only increase adipose tissue mass but also result in other metabolic dysfunctions are also referred to as metabolism disrupting chemicals (MDCs) (Heindel et al., 2017). Environmental obesogens are defined as chemicals capable of increasing the quantity of adipocytes (hyperplasia), promoting the fat storage in cells (hypertrophy), affecting preadipocyte differentiation or function, and initiating and/or deregulating basal metabolism and weight gain. Obesogens may also act indirectly by disrupting energy balance (promoting calorie storage), altering appetite and satiety signals, as well as central mechanisms that keep the body's response to daily nutritional changes (Gupta et al., 2020; Heindel et al., 2015a; Janesick and Blumberg, 2016).

The list of chemicals with obesogenic potential has been growing steadily in recent years, with humans being exposed to this multitude of EDCs from a variety of sources (Veiga-Lopez et al., 2018). To date, current developments in environmental epidemiology and toxicological risk assessment are inadequate and unsatisfactory for evaluating the exposure to multiple obesogenic EDCs, and for knowing their impact on human development and health (Legler et al., 2020). Although there have been some attempts to consider the combined effect of several chemicals or compounds belonging to the same chemical family, the methods available so far to quantify the combined effect of exposure to multiple residues are still scarce and not fully validated and conclusive (Kortenkamp, 2014; Völker et al., 2022).

Taking into account all of the above, the starting hypothesis of this International Doctoral Thesis was that exposure to mixtures of EDCs constitutes a risk factor for obesity, and that the development of methodologies based on the measurement of the combined effect of multiple obesogenic EDCs known to occur in human adipose tissue, through *in vitro* assays, would serve as biomarker of combined effect in metabolic diseases.

Objective

The main objective of this International Doctoral Thesis was to investigate human exposure to specific endocrine disrupting chemicals (EDCs), specifically to those with obesogenic activities, by analysing both the presence of these contaminants in the human population and the individual and combined effect of residues (EDCs) present in human adipose tissue, through the development of *in vitro* biomarkers, which could be used as risk markers for obesity and other metabolic diseases.

The specific objectives defined for this purpose were the following:

1. To develop and validate an *in vitro* human mesenchymal stem cell model to assess the effect of obesogenic EDCs. This tool could serve as a way to compare the potency of different obesogens, as well as to identify new compounds with obesogenic activity.

2. To study *ad hoc* mixtures of environmental chemicals on the process of adipogenesis, investigating the possibility of synergistic, additive or even antagonistic effects of the individual components.

3. To deepen into the knowledge on mechanisms and signaling pathways through which obesogen-EDCs contribute to an obesogenic phenotype (analyzing gene and protein expression of specific adipogenic markers -PPARγ, C/EBPα, LPL and FABP4).

4. To evaluate the exposure to obesogens in adipose tissue samples from adult hospitalized patients in Southern Spain, with normal weight and obesity, and to investigate the relationship between the accumulated concentrations in adipose tissue and the risk of clinical diagnosis of MetS.

5. To evaluate the developed total load biomarker ("*O-Screen*") in adipose tissue samples from adult patients with normal weight and

obesity, and to investigate its possible association with obesity and other metabolic disorders.

Materials and Methods

Cell Culture

To carry out the *in vitro* studies that comprise an important part of this PhD thesis, commercial human adipose-derived stem cell (hASC) line was used; namely the Poietics™ Normal Human Adipose Derived Stem Cells [PT-5006, Lot 0F4505], from Lonza (Switzerland). The hASCs are cells capable of differentiating under controlled treatments into other cell lineages, including chondrogenic, osteogenic, adipogenic and neural lineages (Lim et al., 2014; Lonza, n.d.; Perrini et al., 2009). The hASCs used were isolated from subcutaneous lipoaspirates from a single non-diabetic adult, collected during elective liposuction surgical procedures and cryopreserved at primary passage (Lonza, n.d.).

hASCs were cultured and expanded following the manufacturer´s instructions together with published recommendations (Janderová et al., 2003; Ruiz-Ojeda et al., 2016). Briefly, cells were initially seeded in 25 cm^2 cell flasks (T25) at a density of one million, subsequently expanded in 75 and 175 cm^2 cell flasks, incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cell culture growth medium (GM) was comprised Advanced-Dulbecco´s Modified Eagle Medium (advanced-DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM GlutaMAX, 100 U mL-1 penicillin, and 100 μg/mL streptomycin, and replaced every 2-3 days. Cells were sub-cultured using a mixture of 0.25% trypsin-EDTA and passaged up to a maximum of 6 times. All products were supplied by Thermo Fisher Scientific (Waltham, MASS).

Cells were exposed to different concentrations of specific obesogen-EDCs (mainly bisphenols -BPA, BPF and BPS) and their ternary combination (MIX), as an example of an environmental exposure marker, to study their obesogenic potential and their effect on the

adipogenic phenotype. All cell cultures were repeated at least three times for each condition with multiple replicates.

Adipogenic Differentiation

Differentiation was induced by seeding hASCs in 24-well plates (Linbro, McLean, VA) at initial concentrations of 20,000 cells/cm^2 (40,000 cells per well) in GM. When confluence was reached (two days later), the GM was replaced by the differentiation medium (DM) consisting of GM supplemented with 1 μM dexamethasone (DEX), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1.7 μM human insulin (INS), and 0.1% dimethyl sulfoxide (DMSO) (vehicle) as negative control, or by the aforementioned DM plus 1 μM rosiglitazone (ROSI) as positive control (Janderová et al., 2003; Patel et al., 2003). Different concentrations of the selected obesogens (BPA, BPF, BPS) were added to DM, both individually or as mixture of these three bisphenols (MIX), in a 1:1:1 ratio, and tested throughout the progressive maturation of the adipocytes (7 and/or 14 days), constituting the experimental conditions. The final DMSO concentration in both the individual compounds and the mixture never exceeded 0.1% (v/v) of the culture medium. The DM was replaced every 2-3 days during the adipogenic differentiation. All differentiation products were supplied by Sigma (Sigma-Aldrich, St. Louis, MO, USA) except for ICI 182,780 (Tocris Bio-science, Bristol, UK).

hASCs were also used to elucidate possible mechanism of action of the selected obesogens; that is, to test whether BPA, BPF, BPS and the MIX induced adipogenesis through the regulation of adipogenic genes in an estrogen receptor (ER)-dependent manner (Rochester and Bolden, 2015). To do that, an antagonist of ERα (ICI 182,780) was used (Ohlstein et al., 2014). For this purpose, hASCs were cultured for 14

days in the experimental conditions described above, DM containing increasing concentrations of BPA, BPF, BPS and MIX with ICI 182,780 (100 nM), or in DM with 17β-estradiol (E2) at 1 μM, or in DM with E2 plus ROSI (E2 + ROSI).

hASCs were not exposed to other NR antagonists such as glucocorticoids receptor (GR) and PPARγ because the experimental condicions used to differentiate hASCs into adipocytes contained DEX, a glucocorticoid and ROSI that has affinity for PPARγ.

Cell Viability

Percentage of viable cells was determined using the trypan blue test following the protocol described by Strober (2015) ith slight modifications. Succinctly, after 7 or 14 days of culture under the aforementioned experimental conditions, cells were dissociated with trypsin, resuspended in phosphate buffered saline (PBS), mixed with trypan blue dye, and visually examined and counted in a Neubauer chamber, using an Olympus IX51 inverted microscope with Olympus TH4-200 lamp.

The viability assays were performed with multiple replicates for each experimental condition.

Analysis of the adipogenic phenotype in the *in vitro* studies:

To determine the adipogenic phenotype, as well as to study the obesogenic potential of the selected EDCs, the Oil Red O (ORO) staining, as well as gene and protein expressions of specific adipogenic markers (PPARγ, C/EBPα, LPL and FABP4) was used.

i) Quantitative Oil Red O (ORO) Staining Assay

The analysis of adipogenic phenotype was verified with the Quantitative Oil Red O Staining Assay. Thus, intracellular lipids, mainly

triglycerides, were visualized by ORO staining and quantified by spectrophotometric determination at day 7 and/or day 14 of adipogenic differentiation (Janderová et al., 2003; Ramírez-Zacarías et al., 1992). Briefly, after exposure at the experimental conditions, cells were washed with PBS and fixed in 4% paraformaldehyde for 1 h at room temperature. After washing with milliQ-water and 60% isopropanol, cells were stained with filtered ORO solution (0.5%, w/v) in a milli-Q water (60/40, v/v) for 45 min, followed by washing with 60% isopropanol and again with milliQ-water. Cells were first observed and photographed under a Leica microscope [Leica DMi8 microscope (Leica Microsystems, Wetzlar, Germany), with the Leica Application Suite (LAS) X software], and the retained dye was extracted with 100% isopropanol, measuring the optical density at a wavelength of 520 nm with a microplate reader (BioTek HTX, Fisher Scientific, USA). All aforementioned products were purchased from Thermo Fisher Scientific (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) or Sigma Aldrich (Sigma-Aldrich, St. Louis, MO, USA), except for paraformaldehyde (Electron Microscopy Science Hartfield, PA, USA).

The ORO staining assays were performed with multiple replicates for each experimental condition.

ii) RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells at the aforementioned experimental conditions on day 7 and/or 14 of adipogenic differentiation using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA was eliminated using the RNase-Free DNase kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The final RNA concentration

and quality (260/280 ratio) were determined with a NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1000 ng) was transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) in accordance with the manufacturer's instructions. qRT-PCR was used to measure the expression levels of genes involved in the adipogenic process [peroxisome proliferator-activated receptor gamma (*PPARγ*), CCAT/enhancer-binding protein (*C/EBPα*), lipoprotein-lipase (*LPL*), and fatty acid-binding protein 4 (*FABP4*)]. Hypoxanthine-guanine phosphoribosyltransferase-1 (*HPRT1*) and β-actin (*ACTB*) used as housekeeping genes (reference genes, internal control genes) for all experiments. Primer pairs for each target gene, purchased from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA), were *PPARγ* (Assay ID qHsaCED0044425), *C/EBPα* (Assay ID qHsaCED0019045), *LPL* (Assay ID qHsaCED0047106), *FABP4* (Assay ID qHsaCED0057474), *HPRT1* (Assay ID qHsaCID0016375), and *ACTB* (Assay ID qhsaLED0214042). qRT-PCR was carried out with an ABI Prism 7900HT instrument (Applied Biosystems, Foster City, CA) using SYBR Green PCR from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). Quantification was performed using the Qiagen Data Analysis Center (GeneGlobe Data Analysis Center, Qiagen, Hilden, Germany) and stability of reference genes was statistically validated for each sample in duplicate. The $2-\Delta\Delta Ct$ method was used to express the results as fold-changes and the negative control as reference (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

The analyses of gene expression were performed in duplicate for each sample. A ≥1.5-fold increase and decrease in gene expression was considered as up-regulation and down-regulation, respectively.

iii) Western Blot

Protein levels of selected genes were measured in cells cultured under the different experimental conditions after 7 and/or 14 days of adipogenic differentiation. Briefly, lysis was performed using cell lysis buffer [10 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 100% glycerol (Sigma-Aldrich, St. Louis, MO, USA)], a protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA), and β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). Samples were placed on ice for 20 min. After centrifugation for 30 min at 13,000× *g* and 4 °C, the protein content of the supernatant was quantified by the DC Protein Assay, using 50 µg of protein sample mixed with 4X Laemmli sample buffer containing 10% β-mercaptoethanol and milliQ-water. Samples were separated with SDS-PAGE using TGX Any kD gel and transferred onto a nitrocellulose membrane, which was incubated in blocking buffer [5% non-fat milk in 1X Tris-buffered saline (TBS) with 0.5% Tween 20 (TBS-T)] for 1 h at room temperature. Primary antibodies to incubate blotted membrane for PPARγ, C/EBPα, FABP4, LPL, and HSC-70 (B6) were used with appropriate horseradish peroxidase-labelled secondary antibodies (Table 1). Immunoreactive signals were detected using the Clarity Western ECL Substrate Kit, and membranes were digitally imaged with Image Reader LAS-4000 and quantified by densitometry using ImageJ software. Protein levels were represented as the fold-changes in expression relative to the control (HSC-70). All these products were acquired from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA) or Sigma Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

The analyses of protein expression were performed in duplicate for each sample.

Table 1. Brief description of primary and secondary antibodies used in western blot analysis

Peptide/Protein Target		Manufacturer, Catalog number	Species	Dilution
Primary Antibodies	PPARγ	Cell Signaling Technology, 2435	Rabbit	1:1000 (5% BSA in 1X TBS with 0.5% Tween 20)
	C/EBPα	Cell Signaling Technology, 2841		
	FABP4	Cell Signaling Technology, 3544		
	LPL	Abcam, ab172953		1:1000 (5% non-fat milk in 1X TBS with 0.5% Tween 20)
	HSC-70 (B6)	Santa Cruz Biotechnology, SC-7298	Mouse	
Secondary Antibodies	Goat Anti-Rabbit IgG (H+L)-HRP Conjugate	BIO-RAD, 1706515	Anti-Rabbit	
	Goat Anti-Mouse IgG (whole molecule)–Peroxidase Conjugate	Sigma, A4416	Anti-Mouse	

Study population

For the clinical study, a cross-sectional epidemiological study was carried out, which constituted the second part of this doctoral thesis, supported by two projects of the Instituto de Salud Carlos III and the European Regional Development Fund/ERDF (FIS-PI13/02406 and FIS-PI16/01812). The study population comprised 117 individuals selected among patients undergoing a non-cancer surgical intervention (hiatal hernia, umbilical hernia, inguinal hernia, para-oesophageal hernia, achalasia and cholecystectomy), from both the Endocrinology and Nutrition Unit and the Surgery Unit of the Hospital Universitario Virgen de la Victoria (Malaga, Spain). The recruitment

took place between 2013 and 2015. Exclusion criteria included patients receiving blood glucose-lowering treatment, major cardiovascular disease in the last 6 months before recruitment, evidence of acute/chronic inflammatory disease (such us chronic inflammatory bowel diseases, ulcerative colitis and Crohn's disease), cancer patients and infectious diseases, as well as those who did not signed the informed consent to participate in the study. The study was approved by the Ethics Committee of Virgen de la Victoria University Hospital and was conducted in accordance with the principles of the Declaration of Helsinki. All participants gave their signed consent after being fully informed of the goal and characteristics of the study.

Collection of human biological samples

Approximately 300 mg of visceral adipose tissue (VAT) and 10 mL of blood were collected during surgery under fasting conditions. Blood samples were immediately centrifuged for 5 min at 2,500 rpm and 4 °C to separate the serum. Both serum and adipose tissue samples were immediately coded and stored at −80 °C until chemical analysis. Once all the fat samples had been collected, they were sent to the chemistry laboratories of the ibs.GRANADA/University of Granada (Spain) for analysis.

Adipose tissue sample extraction and chemical analysis (*exposure markers*)

Concentrations of 17 POPs as markers of environmental exposure were determined in 150 mg of adipose tissue as previously described (Martínez Vidal et al., 2002; Moreno Frías et al., 2004). Adipose tissue samples were spiked with p-chlorobenzophenone as an internal standard, and extracted following a two-step methodology, consisting on a mechanical homogenization in n-hexane followed by a filtration

through a glass column with 200 mg of alumina. Extracts were dried under a continuous nitrogen stream and fractionated in duplicate by high-performance liquid chromatography (HPLC). Residues of *o,p´*-DDT, *p,p´*-DDE, HCB, α-HCH, β-HCH, γ-HCH, aldrin, isodrin, endrin, dieldrin, heptachlor, vinclozolin, endosulfan I and II and three PCBs congeners (-138, -153, and -180) were quantified by gas chromatography and mass spectrometry (GC-MS/MS) with an Agilent 7890A system and MS Agilent 7000 GC/MS (triple quadrupole) mass spectrometer (Agilent Tehcnologies, Santa Clara, CA) after reconstitution in 200 µL n-hexane (Arrebola et al., 2014). Procedural laboratory blanks with solvents alone were tested and always yielded a negative result. Laboratory fortified matrix samples at different concentrations were used for quality control. Inter- and intra-day variabilities were calculated by analyzing fortified samples within the same day (repeatability) and on different days (intermediate precision), respectively, always yielding values <20%. The limit of detection (LOD) was determined as the smallest amount of the analyte that gave a signal-to-noise ratio ≥3 and was set at 4 ng/mL for *o,p´*-DDT and *p,p´*-DDE, and 2 ng/mL for the rest of POPs. Concentrations below theLOD were assigned half of the LOD.

Assessment of Metabolic Syndrome (MetS) and its components in clinically diagnosed middle-aged adults (*effect markers*)

The height and weight of the participants were recorded, calculating the BMI as weight/height squared (Kg/m^2). Waist circumference (WC) was measured at the nearest 0.1 cm using an inelastic tape (in a standing position and after a gentle exhalation by trained staff using standardized protocols. Systolic and diastolic blood pressures (SBP

and DBP, respectively) were also measured. Blood pressure was measured twice in the sitting position after a 5-min rest at 5-min intervals, and the mean of the measurements was used.

Fasting glucose, TG and HDL cholesterol were quantified in serum samples by using a Dimension autoanalyzer, Dade Behring Inc. (Deerfield, IL) in the analytical unit of Virgen de la Victoria Hospital.

Following the International Diabetes Federation (IDF) working group criteria, it was considered as clinical variables under study the 5 metabolic syndrome (MetS) components and their respective cut-off points: fasting glucose (≥100 mg/dL), TG (≥150 mg/dL), HDL cholesterol (<40 mg/dL in males and <50 mg/dL in females), blood pressure (SBP ≥130 mmHg and/or DBP ≥85 mmHg), and WC (≥94 cm for males and ≥ 80 cm for females in European populations).

Participants were classified as MetS+ when the presence of any 3 out of these 5 MetS components was detected, while those participants with less than 3 MetS components were included in the MetS- group (Alberti et al., 2009).

Statistical Analysis

The statistical analyses performed are described in each of the publications included in the results section.

Briefly, results of *in vitro* assays were expressed as means ± standard error of the mean (SEM). Significant differences in ORO staining results and in gene and protein expression were evaluated with the non-parametric Mann-Whitney U test.

In the clinical study, continuous variables were expressed using arithmetic mean ± standard deviation (SD), median and 25th-75th

percentiles, and categorical variables as percentages. MetS components were considered both as continuous (log-transformed) and categorical variables, considering the cut-off points established in the IDF working definition for the clinical diagnosis of MetS. POP concentrations were natural-log transformed to reduce the skewness of the distributions and the influence of extreme values, especially in the context of a modest sample size. Spearman's correlation test was used to assess relationships between POPs concentrations.

Associations between concentrations of POPs and MetS components were examined using both linear and logistic regression analyses when outcomes were coded as continuous or categorical variables, respectively. The linearity of the association between POP exposure and MetS components was examined using generalized additive models (GAMs), supporting the modeling of POP concentrations as continuous variables. Also, linear regression models were performed using dichotomized exposure variables (exposed *vs*. not exposed) for those POPs with ~50% below LOD (HCB, *o,p'*-DDT, γ-HCH) in the adipose samples. Logistic regression analyses were performed to explore the influence of adipose tissue levels of selected POPs and the risk of clinically-diagnosed MetS. All models were adjusted for age (years) and sex (male/female) as potential confounders. In addition, a sensitivity analysis was performed further adjusting models by BMI.

The potential of POP mixture effect on MetS was assessed using Weighted Quantile Sum Regression (WQS) (Carrico et al., 2015), which combines the individual associations into a weighted index, and estimates the specific weight of each chemical on the mixture. Further, associations between each WQS index and the outcome were also studied by using multivariable logistic regression adjusting for age, sex

and BMI. The WQS analyses were performed with log-transformed continuous pollutant concentrations, using a training set defined as a 40% random sample of the dataset, being the remaining 60% used for model validation. The final weights were calculated using a total of 1000 bootstrap steps.

The found results (association between the specific POPs and MetS or individual components of MetS) were interpreted taking into their internal validity, consistency and coherence, the existing epidemiological and toxicological evidence, and not exclusively considering the statistical significance (Amrhein et al., 2019). Given the hypothesis investigated in the epidemiological (cross-sectional) study and the moderate number of comparisons tested, a post-hoc correction for multiple comparisons did not perform to avoid a disproportionate increase in the frequency of type II errors (Rothman, 2014). A diagnosis of the models was conducted in order to ensure the goodness-of-fit and the fulfilment of implementation conditions.

SPSS (IBM SPSS, Armonk, NY, USA) was used. Statistical significance was defined as $p < 0.05$.

Results

Article #1

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Bisphenol F and bisphenol S promote lipid accumulation and adipogenesis in human adipose-derived stem cells

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Highlights

• BPF and BPS promote intracellular lipid accumulation in hASCs.

• Expression of key adipogenic genes increases in response to BPF and BPS.

• Our findings suggest that BPF and BPS are not safe alternatives to BPA.

• The obesogenic potential of BPF in human adipogenesis is reported for the first time.

Abstract

Bisphenol F (BPF) and bisphenol S (BPS) are increasingly used as substitutes for bisphenol A (BPA), an endocrine disrupting chemical (EDC) with obesogenic activity. We investigated the *in vitro* effects of BPS and BPF on the adipogenesis of human adipose-

derived stem cells (hASCs) exposed to different doses (0.01, 0.1, 1, 10 and 25 μM), stopping the adipogenic process at 7 or 14 days. Intracellular lipid accumulation was quantified by the Oil Red O assay, gene expression of *peroxisome proliferator-activated receptor gamma* (*PPARγ*), *CCAT/enhancer-binding protein* (*C/EBPα*), *lipoprotein-lipase* (*LPL*) and *fatty acid binding protein 4* (*FABP4*), by quantitative real-time polymerase chain reaction (qRT-PCR) and protein levels by Western Blot. hASCs with BPF or BPS produced a linear dose-response increase in intracellular lipid accumulation and in gene expression of the adipogenic markers, confirmed by protein levels. Co-treatment ICI 182,780 significantly inhibited BPF- but not BPS-induced lipid accumulation. Given the affinity of bisphenols for diverse nuclear receptors, their obesogenic effects may result from a combination of pathways rather than a single mechanism. Further research is warranted on the manner in which chemicals interfere with adipogenic differentiation. To our best knowledge, this report shows for the first time the obesogenic potential of BPF in hASCs.

Keywords

Endocrine disruptors; Adipogenesis; Bisphenol F (BPF); Bisphenol S (BPS); Obesity; Bisphenol A (BPA)

Abbreviations

ACTB, β-actin; advanced-DMEM, Advance-Dulbecco Modified Eagle Medium; BPA, bisphenol A; BPF, bisphenol F, 4,4′-methylenediphenol; BPS bisphenol S, 4,4′-sulfonyldiphenol; C/EBPα, CCAT/enhancer-binding protein; DEX, dexamethasone; DM, differentiation medium; DMSO, dimethyl sulfoxide; EDC, endocrine disrupting chemical; FABP4, fatty acid binding protein 4; FBS, fetal bovine serum; GM, growth medium; hASCs, human adipose-derived stem cells; INS, human insulin; HPRT1, hypoxanthine-guanine phosphoribosyltransferase-1; IBMX, 3-isobutyl-1-methylxanthine; LPL, lipoprotein-lipase; μg, micrograms; μM, micromolar; mM, millimolar; ml, millilitre; min, minutes; ng, nanograms; nm, nanometers; C-, negative control; ORO, Oil Red O; PPARγ, peroxisome proliferator-activated receptor gamma; PBS, phosphate buffered saline; C+, positive control; qRT-PCR, quantitative real-time polymerase chain reaction; ROSI, rosiglitazone; SEM, standard error of the mean; TBS, tris-buffered saline

1. Introduction

The prevalence of obesity is increasing at an alarming rate worldwide and poses a major public health challenge (Williams et al., 2015). Diet, physical activity, and genetic predisposition are considered the main triggers of the obesity pandemic; however, there is mounting evidence that other factors, such as exposure to obesogenic endocrine disrupting chemicals (EDCs), contribute to the development of obesity and related metabolic disorders (Frank, 2016; Petrakis et al., 2017).

The obesogen hypothesis, based on *in vitro*, *in vivo,* and epidemiological data, proposes a link between EDC exposure, mainly at specific developmental stages, and increased body weight and adiposity (Darbre, 2020; Grün and Blumberg, 2006; Heindel, 2019). Obesogens were defined as synthetic chemical compounds that might interfere with endogenous hormones, thereby favoring obesity *via* several possible pathways: increasing the number of adipocytes and/or fat storage/content of existing fat cells; altering the energy balance towards calorie storage, reducing thermogenesis; and/or disrupting signals that regulate appetite and satiety mechanisms (Janesick and Blumberg, 2011). The concept of obesogens was later extended to include "metabolic disruptors", *i.e.,* all synthetic chemical compounds with potential to increase susceptibility to obesity, diabetes, and/or related metabolic disorders (Heindel et al., 2017).

Bisphenol A (BPA) is currently used as a primary raw material in the production of polycarbonate and epoxy resins, and food and beverages are among the main sources of human exposure to this compound (Ahmed and Atlas, 2016; Vandenberg et al., 2010). Evidence of the health risks associated with BPA led to the proposal of structural analogs such as BPF and BPS. BPF and BPS are used in the manufacture of epoxy resins, polycarbonates, thermal papers, and infant feeding bottles, among other products (Björnsdotter et al., 2017; Usman and Ahmad, 2016). Their concentration in food varies among countries, among different foods, and among types of containers (Russo et al., 2019). The increased use of BPF and BPS in consumer products has been followed by the detection of their residues and metabolites in human biological matrices (Liu et al., 2018; Luo and Liu, 2016; Rochester and Bolden, 2015; Ye et al., 2015). According to the most recent data from the USA, urinary concentrations of BPA, BPF, and BPS range from 0.57 to 2.49 µg/L BPA, 0.14–1.11 µg/L BPF, and

0.14–0.88 µg/L BPS in adults, and from 0.64 to 2.42 µg/L BPA, 0.14–0.99 µg/L BPF, and 0.12–0.7 µg/L BPS in children and adolescents (Vandenberg et al., 2007; Lehmler et al., 2018).

Various authors have suggested that some of the biological activities of BPF and BPS are of a similar or even higher magnitude in comparison to those of BPA (Eladak et al., 2015; Rochester and Bolden, 2015; Usman and Ahmad, 2016). Thus, BPS and BPF have been shown to promote adipogenesis and intracellular lipid accumulation *in vitro*, mainly in murine cell lines such as mouse 3T3-L1 preadipocytes (Ahmed and Atlas, 2016; Drobna et al., 2019; Ramskov Tetzlaff et al., 2020). Both BPA analogs were also found to stimulate adipogenesis in murine preadipocytes by disrupting the regulation of some crucial adipogenesis-related genes (e.g., *PPARγ* and *C/EBPα*) and adipogenic markers (e.g., *LPL* and *FABP4)* (Ahmed and Atlas, 2016; Drobna et al., 2019; Ramskov Tetzlaff et al., 2020).

Nevertheless, no consensus has yet been achieved on the action mechanisms underlying the effect of BPA and its structural analogs on murine and human adipogenesis. Identification of a predominant mechanism is difficult because: a) these bisphenols bind to several receptor families with distinct affinities and activities (Mustieles et al., 2015); b) *in vitro* models of adipogenesis in stem cells/preadipocytes express various specific families of nuclear receptors with related functions (Kassotis et al., 2017); and c) the effect of EDCs on this process is influenced by the hormone cocktail selected for adipogenic differentiation, among other methodological factors (Kassotis et al., 2017).

Despite the increasing use of these BPA substitutes, there has been very little research on the obesogenic effect of BPF and BPS on human adipogenesis (Boucher et al., 2016a, 2016b; Peshdary et al., 2020). The objectives of this study were to investigate the effects of different concentrations of BPF and BPS, some of them representing human exposure, on adipogenesis in cultured human adipose-derived stem cells (hASCs) and to evaluate the expression of genes and proteins related to adipogenesis and lipid metabolism. A secondary objective was to investigate certain mechanistic aspects underlying the obesogenic effects of BPS and BPF in relation to the estrogen receptor.

2. Materials and methods

2.1. Cell culture

Commercial hASCs were purchased from Lonza (Poietics™ Normal Human ADSCs, PT-5006, Lot 0F4505, Lonza, Switzerland); they are isolated from normal (non-diabetic) adult subcutaneous lipoaspirates collected during elective surgical liposuction procedures and can be differentiated under controlled treatments into many different lineages, including chondrogenic, osteogenic, adipogenic, and neural cells (Fraser et al., 2008; Janderová et al., 2003).

hASCs were pooled, cultured, and expanded following the manufacturer's instructions and published recommendations (Ruiz-Ojeda et al., 2016). Briefly, hASCs were grown and expanded in sterile plastic dishes in growth medium (GM) of Advanced-Dulbecco's Modified Eagle Medium (advanced-DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM GlutaMAX, 100 U ml^{-1} penicillin, and 100 µg/ml streptomycin. Cells were initially seeded in 25 cm^2 cell flasks (T25) at a density of one million and subsequently expanded in 75 and 175 cm^2 cell flasks incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cell culture medium was replaced every 2–3 days, and cells were passaged up to a maximum of 6 times. Cells were sub-cultured using a mixture of 0.25% trypsin-EDTA. All products were supplied by Thermo Fisher Scientific (Waltham, MASS).

2.2. Adipogenic differentiation

Differentiation was induced by seeding hASCs in 24-well plates (Linbro, McLean, VA) at initial concentrations of 20,000 cells/cm^2 (40,000 cells per well) in GM. When confluence was reached, the GM was replaced by a differentiation medium (DM) consisting of GM supplemented with 1 µM dexamethasone (DEX), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1.7 µM human insulin (INS), and 0.1% dimethyl sulfoxide (DMSO) (vehicle) as negative control, or by the aforementioned DM plus 1 µM rosiglitazone (ROSI) as positive control (Janderová et al., 2003). Different concentrations of BPF or BPS (0.01, 0.1, 1, 10 and 25 µM) were added to the DM and tested throughout the progressive maturation of the adipocytes, constituting the experimental conditions.

hASCs were also cultured for 14 days in the DM containing increasing concentrations of BPS and BPF (0.1, 1, 10 and 25 µM) with the ER

antagonist ICI 182,780 (100 nM), with 17β-estradiol (E_2) at 1 μM, or with E_2 plus ROSI (E_2 + ROSI) at the aforementioned concentrations. All differentiation products were acquired from Sigma (Sigma-Aldrich, St. Louis, MO) except for ICI (Tocris Bioscience, Bristol, UK). The DM was replaced every 2–3 days during the adipogenic differentiation.

2.3. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells at days 7 or 14 of adipogenic differentiation at the aforementioned experimental conditions (0.01, 0.1, 1, 10 and 25 μM of BPF or BPS) using the RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's protocol. Genomic DNA was eliminated using the RNase-Free DNase kit (Qiagen, Germany), and the final RNA concentration and quality (260/280 ratio) were determined with a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1000 ng) was transcribed into cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, CA) according to the manufacturer's instructions.

Differential gene expression levels of *PPARγ*, *C/EBPα*, *LPL*, and *FABP4* were determined by qPCR using specific primer sequences. Hypoxanthine-guanine phosphoribosyltransferase-1 (*HPRT1*) and β-actin (*ACTB*) served as reference genes for all experiments. The primer pairs for each target gene, obtained from Bio-Rad (Bio-Rad Laboratories, CA), were *PPARγ* (Assay ID qHsaCED0044425), *C/EBPα* (Assay ID qHsaCED0019045), *LPL* (Assay ID qHsaCED0047106), *FABP4* (Assay ID qHsaCED0057474), *HPRT1* (Assay ID qHsaCID0016375), and *ACTB* (Assay ID qhsaLED0214042). qRT-PCR was carried out with an ABI Prism 7900HT instrument (Applied Biosystems, Foster City, CA) using SYBR Green PCR from Bio-Rad (Bio-Rad Laboratories). Quantification was performed using the Data Analyze Center (GeneGlobe Data Analysis Center, Qiagen), and statistical validation of the stability of the reference genes was calculated for each sample in duplicate. Results are expressed as fold-changes calculated using the 2^−ΔΔCt method.

2.4. Western blot

After 7 and 14 days of adipogenic differentiation, protein samples from cells cultured with BPF or BPS at 10 μM and 25 μM were lysed with cell

lysis buffer [10 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 100% glycerol (Sigma-Aldrich, St. Louis, MO)], a protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA), and β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Samples were placed on ice for 20 min. Next, after centrifugation for 30 min at 13,000 g and 4 °C, the protein content of the supernatant was measured using the DC Protein Assay (Bio-Rad Laboratories, CA). Samples were prepared with 50 µg of protein sample mixed with 4X Laemmli sample buffer (Bio-Rad Laboratories, CA) containing 10% β-mercaptoethanol and milliQ-water. Samples were separated with SDS-PAGE using TGX Any kD gel (Bio-Rad Laboratories, CA) and were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, CA). Membranes were incubated in blocking buffer [5% non-fat milk in 1X Tris-buffered saline (TBS) with 0.5% Tween 20 (TBS-T)] for 1 h at room temperature. Primary antibodies to incubate blotted membranes for PPARγ, C/EBPα, FABP4, LPL, and HSC-70 (B6) were used with appropriate horseradish peroxidase-labeled secondary antibodies (Supplementary Table 1). Immunoreactive signals were detected using the Clarity Western ECL Substrate Kit from Bio-Rad Laboratories, and membranes were digitally imaged with Image Reader LAS-4000 and quantified by densitometry using ImageJ software. Protein levels were represented as the fold-change in expression relative to the control (HSC-70).

2.5. Cell viability

The trypan blue test was employed to determine the number of viable cells following the protocol described by Strober with slight modifications (Strober, 2015). After 7 or 14 days of adipogenic differentiation at the aforementioned experimental conditions (0.01, 0.1, 1, 10 and 25 µM of BPF or BPS), cells were dissociated with trypsin, resuspended in PBS, mixed with trypan blue dye, and then visually examined and counted in a Neubauer chamber to determine the percentage of viable cells under an Olympus IX51 inverted microscope with Olympus TH4-200 lamp.

2.6. Quantitative Oil Red O staining assay

After 7 or 14 days of adipogenic differentiation, cells were fixed with paraformaldehyde (Electron Microscopy Science Hartfield, PA) and stained with Oil Red O (ORO) (Sigma-Aldrich, St. Louis, MO) to

detect intracellular lipids following the method of Janderová et al. with slight modifications (Janderová et al., 2003).

Mature adipocytes accumulate lipids, mainly triglycerides, which can be visualized by ORO stain and quantified by spectrophotometric determination of washed ORO staining (Ramírez-Zacarías et al., 1992). Briefly, after exposure at the different experimental conditions, cells were washed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 1 h at room temperature. After washing with milliQ-water and 60% isopropanol (Sigma-Aldrich, St. Louis, MO), cells were stained with filtered ORO (0.5%, w/v) in a milli-Q water solution (60/40, v/v) for 45 min followed by washing with 60% isopropanol and again with milliQ-water. Cells were first observed and photographed under an Olympus IX71 inverted microscope with Olympus TH4-200 lamp, and the retained dye was extracted with 100% isopropanol, measuring the optical density at a wavelength of 520 nm with a microplate reader (BioTek HTX, Fisher Scientific, USA).

2.7. Statistical analysis

Cell cultures were repeated at least three times for each condition with multiple replicates. Analyses of gene and protein expression were performed in duplicate for each sample. The viability assay and ORO staining were performed with multiple replicates for each experimental condition. Data were expressed as means ± standard error of the mean (SEM). Significant differences in ORO staining results and in gene and protein expression were evaluated with the non-parametric Mann-Whitney *U* test. SPSS version 22 (IBM SPSS, Armonk, NY) was used for statistical analyses. Statistical significance was defined as $*p < 0.05$.

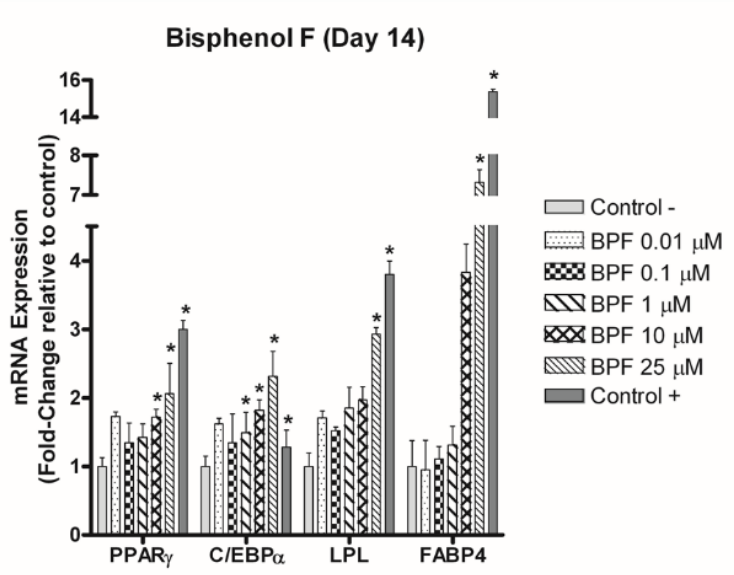
3. Results

3.1. BPF and BPS alter the expression of adipogenesis-related genes

We first evaluated several genes related to adipogenesis and lipid metabolism at day 14 of adipogenic differentiation in the presence of 0.1% DMSO vehicle (negative control), 1 µM ROSI (positive control), or BPF or BPS at 0.01, 0.1, 1, 10 or 25 µM. RNA was extracted and amplified for RT-qPCR analysis, and the selected genes were quantified and expressed as fold-changes, taking the negative control as reference.

BPF and BPS treatments affected hASCs differentiation, enhancing adipogenic effects. At day 14 of adipocyte differentiation, the results showed a dose-dependent increase in both BPF and BPS treatments (Fig. 1A–B). PPARγ mRNA expression was significantly increased with BPF treatments at 10 µM and 25 µM (1.72- and 2.06-fold, respectively), and with BPS at 1 µM, 10 µM, and 25 µM (2.35-, 2.43- and 2.92-fold, respectively) (Fig. 1A–B). In addition, both BPF and BPS treatments produced a significant increase in C/EBPα expression at 1 µM, 10 µM, and 25 µM (1.49-, 1.82- and 2.31-fold, and 1.46-, 2.13- and 2.18-fold, respectively) (Fig. 1A–B). LPL gene expression was significantly increased with 25 µM BPF (2.93-fold) and with 1 µM, 10 µM, and 25 µM BPS (1.93-, 2.19-, and 2.98-fold, respectively) (Fig. 1A–B). Both BPF and BPS produced a non-statistically significant increase in FABP4 expression except for 10 µM BPF (7.32-fold) (Fig. 1A–B).

A



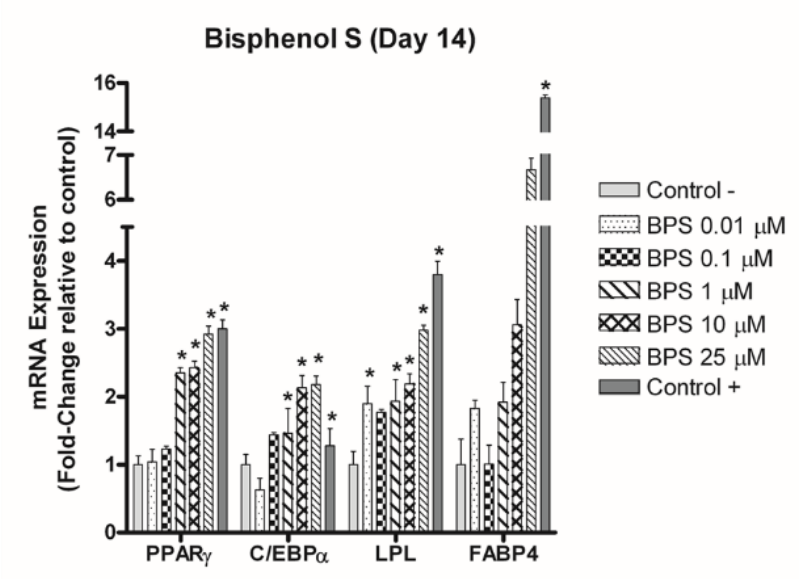


Fig. 1. Effect of BPF and BPS on the expression of adipogenic marker genes at day 14 of adipogenic differentiation. hASCs were differentiated into adipocytes, and the gene expressions of *PPARγ*, *C/EBPα*, *LPL,* and *FABP4* were determined. mRNA levels were normalized to *HPRT1* and *ACTB* levels and expressed as fold-changes calculated using the 2^-ΔΔCt method. Data were expressed as the means ± SEM of three independent experiments with multiple replicates for each condition. Significant differences were analyzed using the Mann-Whitney *U* test and were defined as *p < 0.05.

The same genes related to adipogenesis and lipid metabolism were then evaluated at day 7 during adipogenic differentiation in the presence of 0.1% DMSO vehicle (negative control), 1 µM ROSI (positive control), or BPF or BPS at 10 or 25 µM. After 7 days of adipocyte differentiation, only treatments (10 µM and 25 µM) with BPF or BPS at either concentration displayed a similar pattern, with a significantly increase in mRNA expression of *PPARγ*, *C/EBPα*, *LPL,* and *FABP4* (Fig. 2A–B). Specifically, BPF at 10 µM and 25 µM produced an increase in mRNA expression that was, respectively, 3.62- and 4.12-fold higher for *PPARγ*, 3.05- and 2.83-fold higher for *C/EBPα*, 3.25- and 4.25-fold higher for *LPL*, and 5- to 10-fold higher for *FABP4* (Fig. 2A). Likewise, BPS at 10 µM and 25 µM produced an increase in mRNA expression that was, respectively, 3.19- and 3.91-fold for *PPARγ*, 2.68- and 3.2-fold for *C/EBPα*, 3.02- and 3.87-fold for *LPL*, and 2.35- and 5.25-fold for *FABP4*, respectively (Fig. 2B).

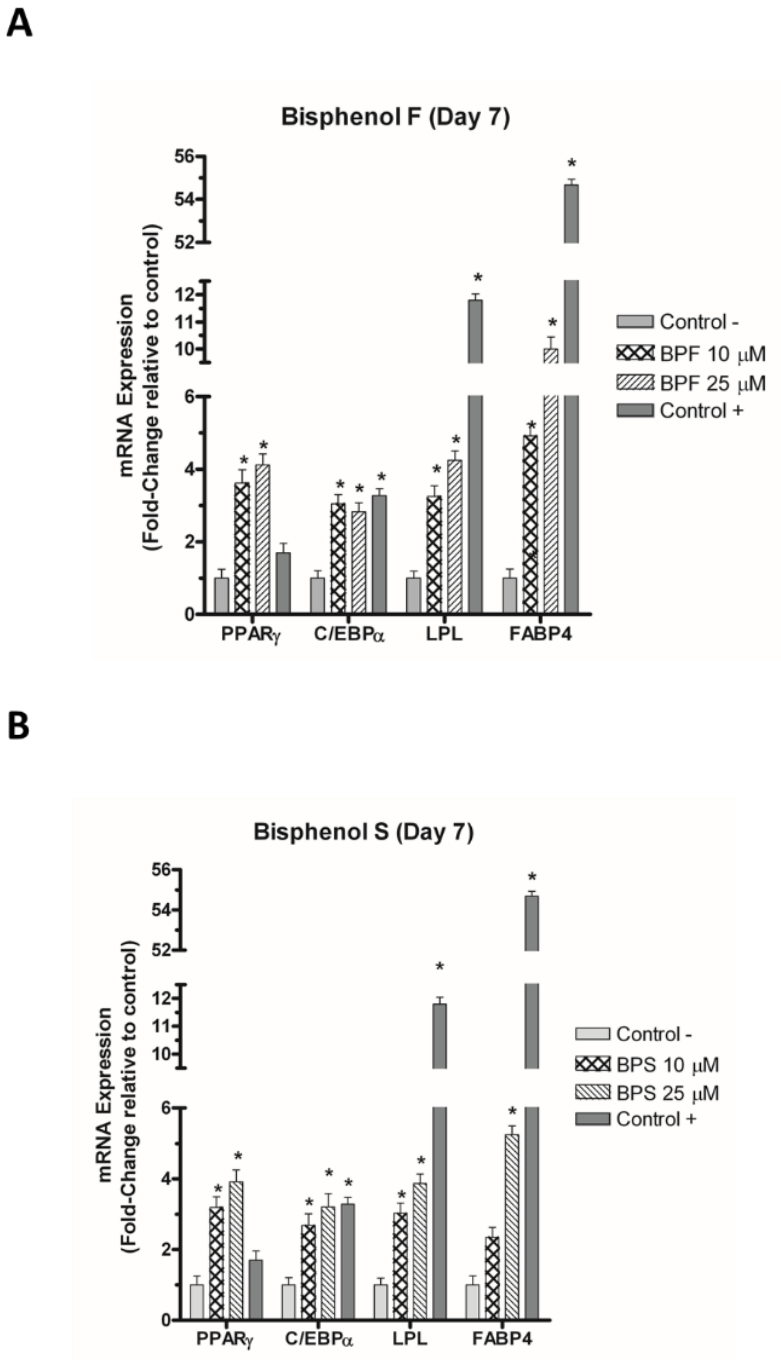


Fig. 2. Effect of BPF and BPS on the expression of adipogenic marker genes at days 7 of adipogenic differentiation. hASCs were differentiated into adipocytes, and the gene expressions of *PPARγ*, *C/EBPα*, *LPL,* and *FABP4* were determined. mRNA levels were

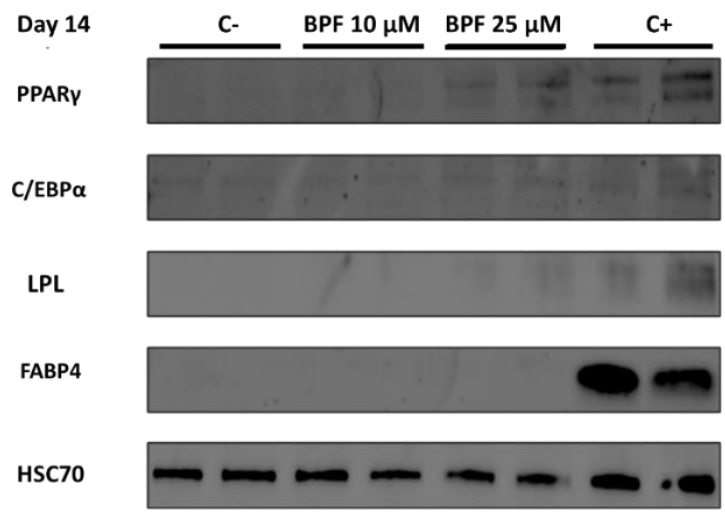
normalized to *HPRT1* and *ACTB* levels and expressed as fold-changes calculated using the 2^-ΔΔCt method. Data were expressed as the mean ± SEM of three independent experiments with multiple replicates for each condition. Significant differences were analyzed using the Mann-Whitney *U* test and were defined as *p < 0.05.

3.2. Effects of BPF and BPS on protein levels of adipogenic markers

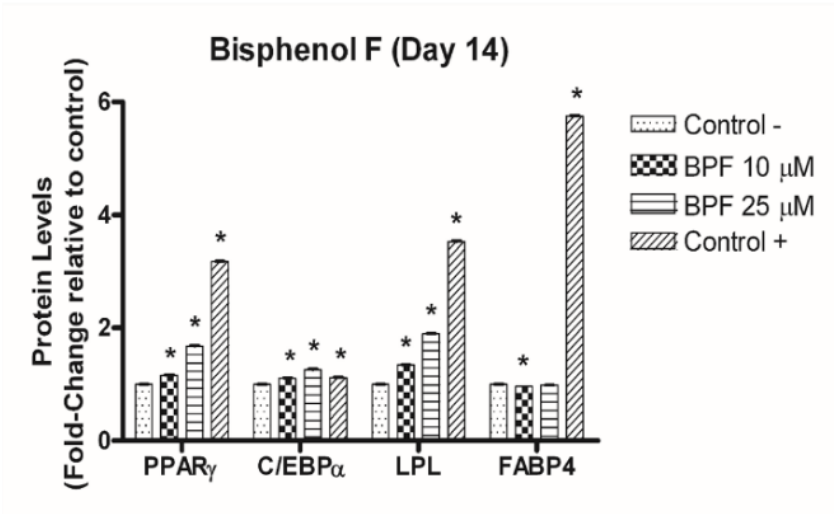
After 14 and 7 days of adipogenic differentiation in different experimental conditions [cell exposure to negative control (0.1% DMSO), positive control (1 μM ROSI), or BPF or BPS at 10 or 25 μM], protein levels of the selected key adipogenic markers were analyzed to determine the possible effect of these bisphenols. Protein expression was quantified by Western Blot analysis and expressed as fold-changes with the negative control as reference.

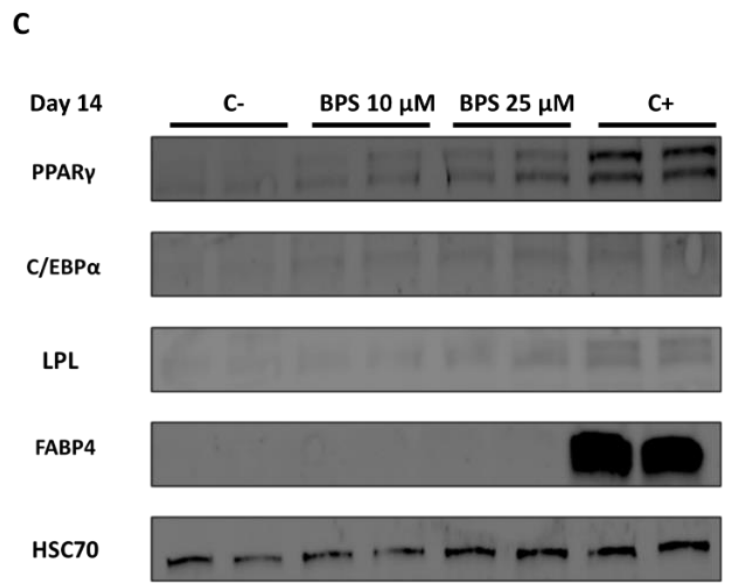
Analysis of the selected adipogenic markers showed alterations in PPARγ, C/EBPα, LPL and FABP4 protein levels after 14 days of adipocyte differentiation in their presence at both bisphenol concentrations (Fig. 3A–D). In the presence of BPF at 10 μM and 25 μM, there were small but significant increases in protein levels of PPARγ (1.17- and 1.68-fold, respectively) and C/EBPα (1.1- and 1.26-fold, respectively) (Fig. 3B). In the presence of 25 μM BPS, there was a small but significant increase in PPARγ protein levels (1.12-fold). BPS treatments at both 10 μM and 25 μM produced a small but significant decrease in C/EBPα protein levels (Fig. 3D). However, no protein levels of PPARγ, C/EBPα, LPL, or FABP4 could be quantified at 7 days of adipocyte cell differentiation in the presence of BPF or BPS at either concentration (data not shown).

A


Day 14
C-
BPF 10 µM
BPF 25 µM
C+
PPARγ
C/EBPα
LPL
FABP4
HSC70

B


Bisphenol F (Day 14)
Protein Levels
(Fold-Change relative to control)
0
2
4
6
PPARγ
C/EBPα
LPL
FABP4
Control -
BPF 10 µM
BPF 25 µM
Control +



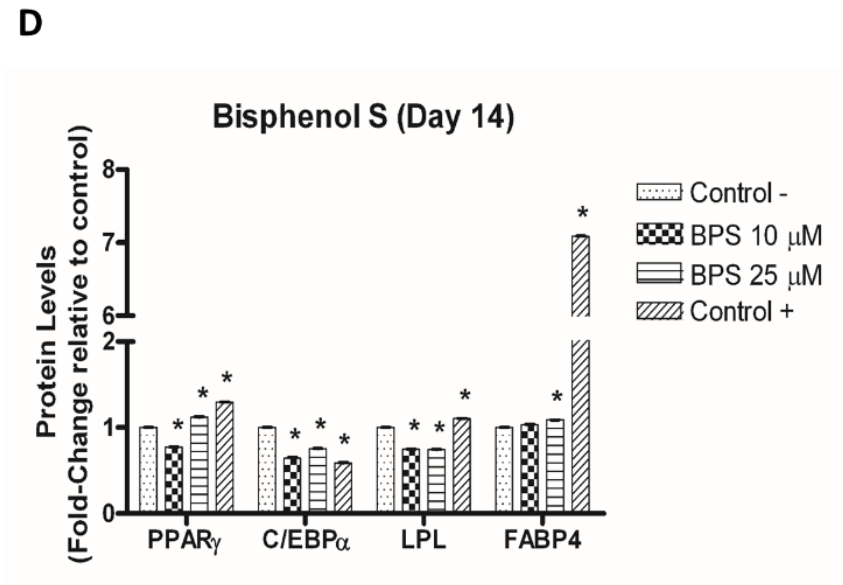


Fig. 3. Effect of BPF and BPS on protein expression of several adipogenic markers. hASCs were differentiated into adipocytes at the aforementioned concentrations, and protein levels were determined by Western blot (A and C) and densitometry (B and D) at day 14 of cell differentiation. Data were expressed as means ± SEM of three independent experiments, performed in duplicate. Significant differences were evaluated using the Mann-Whitney U test and defined as *$p < 0.05$.

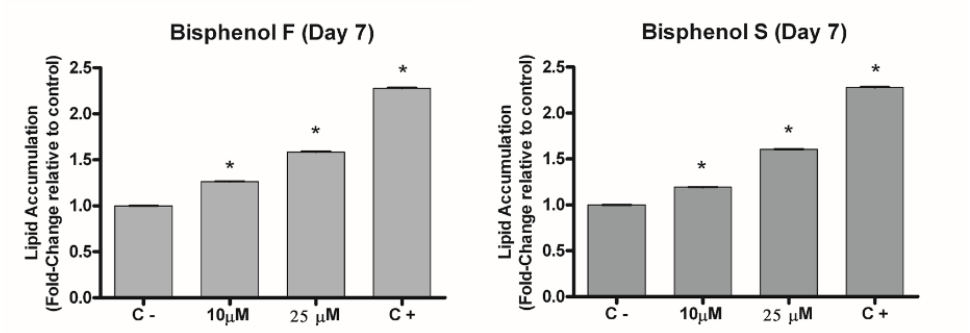
3.3. Effects of BPF and BPS on hASCs viability

The effects of BPF and BPS on cell viability were evaluated during adipogenesis under different experimental [7 days (10 and 25 μM) and 14 days (0.01, 0.1, 1, 10 and 25 μM)] and control (negative and positive) conditions using the trypan-blue assay. Potential toxicity (86% and 71% viability at days 7 and 14, respectively) was only observed in hASCs with BPF at the highest concentration studied (25 μM) (Supplementary Fig. 1A–B).

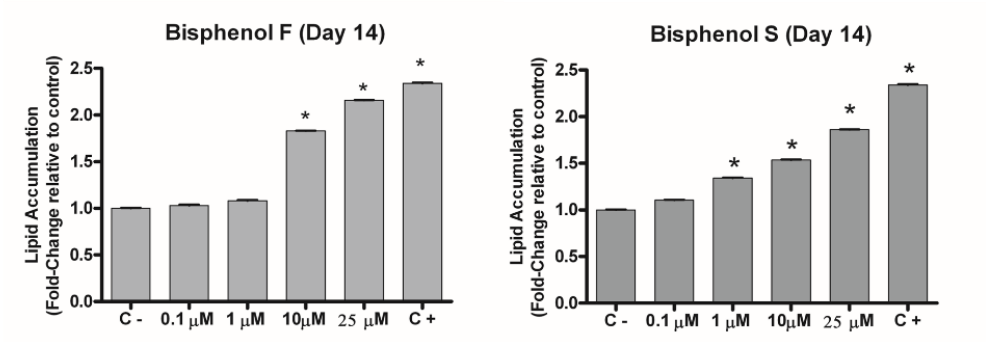
3.4. BPF and BPS promote adipogenesis in hASCs in a dose-dependent manner

The effects of BPF and BPS on adipocyte differentiation and lipid accumulation were evaluated by differentiating hASCs into adipocytes in the presence of 0.1% DMSO (negative control), 1 μM ROSI (positive control), or 0.01, 0.1, 1, 10, or 25 μM BPF or BPS throughout the differentiation process. At 7 or 14 days of culture, adipogenic differentiation was confirmed by the morphological observation of intracellular lipid droplet accumulation, and cells were fixed and stained with ORO (Supplementary Fig. 2). The cells were then treated with isopropanol, and the retained dye was quantified by spectrophotometry at 520 nm. There was a statistically significant increase in the accumulation of intracellular lipids under both experimental conditions (positive control with ROSI and BPF or BPS in a dose-dependent manner) at both 7 and 14 days of the differentiation process (Fig. 4A–B). The maximal response was observed with 25 μM BPF (2.16 ± 0.001) or 25 μM BPS (1.862 ± 0.002) at day 14 (Fig. 4B).

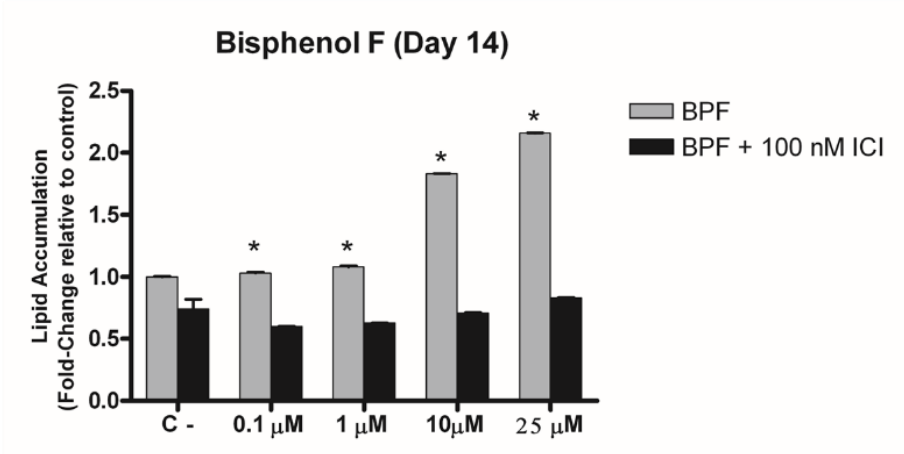
A



B


Bisphenol F (Day 14)
Lipid Accumulation (Fold-Change relative to control)
2.5
2.0
1.5
1.0
0.5
0.0
C -
0.1 μM
1 μM
10μM
25 μM
C +
Bisphenol S (Day 14)
Lipid Accumulation (Fold-Change relative to control)
C -
0.1 μM
1 μM
10μM
25 μM
C +

C


Bisphenol F (Day 14)
Lipid Accumulation (Fold-Change relative to control)
2.5
2.0
1.5
1.0
0.5
0.0
C -
0.1 μM
1 μM
10μM
25 μM
BPF
BPF + 100 nM ICI

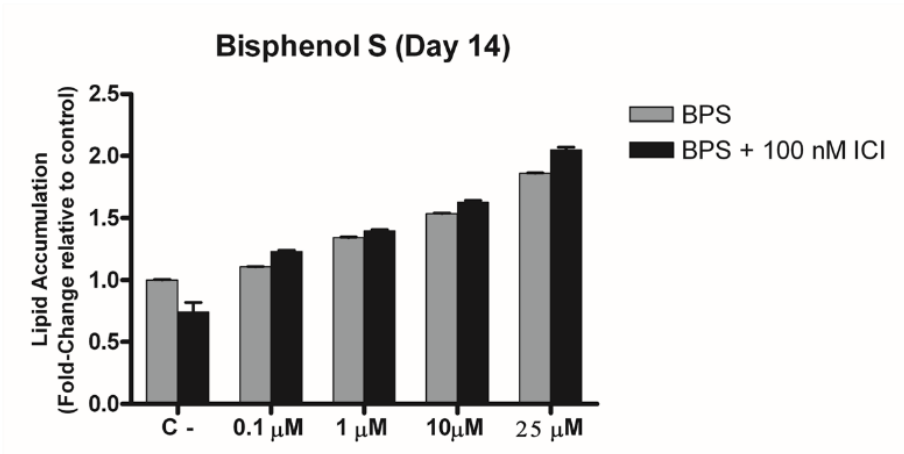

Bisphenol S (Day 14)
Lipid Accumulation (Fold-Change relative to control)
2.5
2.0
1.5
1.0
0.5
0.0
C -
0.1 μM
1 μM
10μM
25 μM
BPS
BPS + 100 nM ICI

Fig. 4. Effect of BPF and BPS on intracellular lipid accumulation in hASCs. Lipid accumulation in hASCs was quantified by ORO staining assay (absorbance at 520 nm) at days 7 (A) and 14 (B and C). Data were expressed as means ± standard error of the mean (SEM) from three experiments with multiple replicates under each experimental condition. Significant differences were analyzed using the Mann-Whitney *U* test and defined as *p < 0.05.

It was also tested whether BPF and BPS promoted adipogenesis *via* an ER-mediated pathway. hASCs were differentiated for 14 days under the previously described culture conditions in the presence of 100 nM ICI (estrogen antagonist). Treatment with ICI alone did not significantly affect lipid accumulation. The addition of 100 nM ICI inhibited adipogenesis at all tested concentrations of BPF (38.4% at 25 μM) but not with BPS treatment, as histochemically demonstrated by ORO quantification of mature adipocytes (Fig. 4C and Supplementary Fig. 1A–B). Treatment of cells with estradiol had no effect on lipid accumulation (data not shown).

4. Discussion

This *in vitro* study examined the impact of BPF and BPS, proposed as industrial alternatives to BPA, on the lipid accumulation and adipogenesis of hASCs. Treatment with BPF for 7 or 14 days at concentrations of 10 or 25 μM interfered with the developmental programming of hASCs, enhancing their capacity to differentiate into adipocytes and accumulate intracellular lipid droplets, as previously observed after treatment with BPA (Ahmed and Atlas, 2016; Boucher et al., 2014; Choi et al., 2020; Héliès-Toussaint et al., 2014; Martínez et al., 2020; Ohlstein et al., 2014; Ramskov Tetzlaff et al., 2020; Wang et al., 2017) and BPS (Ahmed and Atlas, 2016; Boucher et al., 2016a; Choi et al., 2020; Héliès-Toussaint et al., 2014; Martínez et al., 2020; Ramskov Tetzlaff et al., 2020; Wang et al., 2017). These effects were dose-dependent and supported by the upregulation of specific adipogenic genes and related protein levels. To our best knowledge, this is the first report on the obesogenic potential of BPF in human adipocytes.

The potential adverse obesogenic effects of BPS and BPF remain understudied (González et al., 2019). BPF and BPS have mainly been assessed in murine cell lines, especially in 3T3-L1 (Ahmed and Atlas, 2016; Choi et al., 2020; Héliès-Toussaint et al., 2014; Martínez et al., 2020; Masuno et al., 2002; Masuno et al., 2005; Ramskov Tetzlaff et al., 2020). However, the current trend in toxicology is towards human cell

models. A very small number of studies have already investigated BPS-induced adipogenesis in human adipocytes (Boucher et al., 2016a, 2016b; Peshdary et al., 2020; Wang et al., 2017) or in *in vivo* bioassays (Ivry Del Moral et al., 2016); however, no research has been conducted on the potential effects of BPF on human adipogenesis. Thus, recent studies, including our work, are providing new insights using hASCs, suggesting that their utilization may provide a closer view of human adipogenesis. Importantly, while 3T3-L1 cells are already committed preadipocytes, hASCs can differentiate into many cell lines, more accurately reflecting the fate of embryonic stem cells (Linehan et al., 2012; Ohlstein et al., 2014; Wang et al., 2017).

Selection of the range of concentrations for our experiments was based on previous studies of bisphenols in murine and human cell lines and of human exposure to these compounds. Our aim was to compare whether the previously described results could also be replicated in hASCs at the same range of concentrations tested for the selected bisphenols [1 fM to 80 µM] (Ahmed and Atlas, 2016; Liu et al., 2019; Martínez et al., 2020). Thus, our results support the findings of Martínez et al. (2020) on obesogenic activity at 32 µM concentration of BPS and BPF in a 3T3-L1 preadipocyte cell line. We also aimed to compare the effect of BPS and BPF at the same doses as used for BPA in different *in vitro* studies, usually at micromolar concentrations, given that a meaningful comparison can best be achieved by testing similar concentrations (Boucher et al., 2014, 2016a, 2016b; Choi et al., 2020; Ohlstein et al., 2014; Peshdary et al., 2020; Ramskov Tetzlaff et al., 2020; Verbanck et al., 2017; Wang et al., 2017). Interestingly, a recent meta-analysis confirming the obesogenic actions of BPA in rodent models (Wassenaar et al., 2017) suggested that micromolar concentrations of bisphenols tested in cell models can correctly identify obesogenic compounds of concern.

With the aim of identifying the molecular pathways involved in differentiation process, we evaluated genes related to adipogenesis and lipid metabolism on days 7 and 14 of differentiation at different concentrations (0.01, 0.1, 1, 10 and 25 µM) of both bisphenols. Our study focused on *PPARγ,* a central transcriptional regulator of adipogenesis that is commonly described as the only nuclear receptor that is necessary and sufficient for its activation (Ahmed and Atlas, 2016; Kassotis and Stapleton, 2019; Lefterova and Lazar, 2009; Luo and Liu, 2016). We also selected for investigation *C/EBPα*,

an important transcriptional factor considered as marker of the mid-stage of differentiation (Kassotis and Stapleton, 2019; Lefterova and Lazar, 2009; Luo and Liu, 2016), and *LPL* and *FABP4,* standard differentiation gene markers expressed in the mature stage (Bernlohr et al., 2002; Kassotis and Stapleton, 2019; Lefterova and Lazar, 2009; Luo and Liu, 2016).

At day 14 of differentiation, the mRNA expression pattern of these transcription factors was similar for both bisphenol compounds. Specifically, treatments with BPF and BPS produced a significant dose-response increase in PPARγ expression (10, 25 µM and 1, 10 and 25 µM, respectively) and C/EBPα expression (1, 10 and 25 µM). The gene expression of LPL, the late differentiation marker, showed a significant increase with 25 µM BPF and with 1 µM, 10 µM and 25 µM BPS. Both BPF and BPS produced a non-statistically significant increase in FABP4 expression, with the exception of 10 µM BPF. In the same line, Peshdary et al. (2020) reported that 25 µM BPS for 12 days of differentiation significantly increased FABP4 and LPL mRNA expressions (11-fold and 3.2-fold, respectively) in humans preadipocytes. However, Wang et al. (2017) found that the treatment of hASCs with 10 µM BPS did not increase the expression of either adipogenic gene (FABP4 or PPARγ) after 14 days of treatment.

Based on the results on day 14, hASCs were treated during 7 days of the differentiation process with BPF at only 10 µM or 25 µM, finding a significantly upregulated mRNA expression of *PPARγ*, *C/EBPα,* and *LPL* (between 3- and 4-fold) and of *FABP4 (*5- and 10-fold). BPS treatments showed the same pattern for *PPARγ*, *C/EBPα* and *LPL*, but the increase in *FABP4* levels was slightly smaller (2.5- and 5-fold). These results are consistent with previous findings in human progenitor cells (Boucher et al., 2016a; Peshdary et al., 2020) and in 3T3-L1 murine preadipocytes (Ahmed and Atlas, 2016; Ramskov Tetzlaff et al., 2020).

Adipogenesis-related gene expressions observed after 14 days of exposure to the higher concentration of BPF may be related to a decrease in cell viability, which could impact on their mRNA levels. Indeed, this treatment (25 µM for 14 days) was found to be slightly cytotoxic in the cell viability assay. These results are in agreement with the data obtained in murine preadipocytes (Choi et al., 2020; Héliès-Toussaint et al., 2014; Martínez et al., 2020; Ramskov Tetzlaff et al., 2020).

Analysis of protein levels at the end of the hASC differentiation process (day 14) showed a significant dose-dependent increase in the protein expression of PPARγ after BPF treatment (Boucher et al., 2014). By contrast, BPS treatment had no appreciable effect on protein expression levels in the present study. However, a previous study with human preadipocytes exposed to 10 µM or 25 µM BPS showed a significant increase in LPL and FABP4 levels at day 14 of differentiation (Boucher et al., 2016a). Further comparisons are not possible because of the scarcity of *in vitro* human studies relating protein expression to BPS and the absence of such studies on BPF. However, various authors have described the effect of BPF and/or BPS on 3T3-L1 preadipocytes. For instance, Ahmed and Atlas (2016) found a significant dose-dependent increase in FABP4 and LPL protein expression at 6 days of differentiation with BPS at concentrations ranging from 0.01 to 50 µM BPS, and Martínez et al. (2020) reported a significant increase in PPARγ, C/EBPα, and FABP4 protein levels at day 8 of differentiation after treatment with 32 µM BPS. Choi et al. (2020) also reported a significant increase in PPARγ, C/EBPα, and FABP4 protein expression after 10 days of treatment with 20 µM BPF or BPS. However, analysis of these adipogenic markers was unable to identify any protein at day 7 of differentiation. Likewise, no PPARγ was detected by Western blot in human mesenchymal stem cells by Janderová et al. (2003), among others, in contrast to its abundance in murine cell lines (Choi et al., 2020).

Intracellular lipid quantification using ORO as adipogenesis marker is a widely validated procedure, but it cannot elucidate whether an increase in lipid is primarily due to a larger number of hASCs undergoing adipogenesis and/or to a hypertrophic effect in already-differentiated adipocytes (Ramírez-Zacarías et al., 1992). Previous observations are confirmed by the present findings of a dose-dependent increase in lipid accumulation in hASCs exposed to 10 µM or 25 µM of BPF or BPS for 7 or 14 days during the differentiation process. These results suggest that both bisphenols are potential obesogens, promoting obesity by increasing the number and/or size of fat cells (Grün and Blumberg, 2007). Similar results in hASCs were found for BPS by Boucher et al. (2016a) and by Wang et al. (2017), although the latter study did not observe a dose-dependent relationship.

Previous studies indicated that the adipogenic effect of BPA is exerted *via* ER activation because this effect is blocked by the competitive ER antagonist ICI 182,780, representing a potential mode of action underlying the association between BPA and obesity (Ohlstein et al., 2014; Mustieles et al., 2020). We tested here whether the adipogenic effects of BPF and BPS are also mediated in an ER-dependent manner. The obesogenic capacity of BPF in human ASCs was inhibited by treatment with ICI at all concentrations tested; surprisingly, however, this treatment had no such effect on the capacity of BPS under these study conditions. Treatment with ICI alone did not significantly affect lipid accumulation.

Our group previously explored the agonistic and antagonistic activities of BPS and BPF, among other BPA congeners, by using a panel of *in vitro* bioassays to detect different steroid receptor-mediated activities (Molina-Molina et al., 2013). As in the case of BPA, we found that BPF and BPS also bind to both ERα and ERβ, triggering classical nuclear-initiated estrogenic pathways, but differ in their estrogenic potency in the order BPA > BPF > BPS. In addition, whole cell competitive binding assays yielded IC50 values of 401 ± 126 nM (BPA), 1452 ± 261 nM (BPF), and 3452 ± 878 nM (BPS). Unlike BPF, BPS was more active in ERβ *versus* ERα (Molina-Molina et al., 2013). We conclude that the capacity of these compounds to act as estrogen agonists derives not only from their receptor binding but also from their ligand polarity and solvation binding energy, among other physicochemical properties (see Supplemental material), which may explain the differences in activity between BPS and BPF (Marroqui et al., 2021; Molina-Molina et al., 2013).

With regard to potential mechanisms, our results suggest that an estrogenic mode of action may mainly mediate the obesogenic effects of BPF but not those of BPS. It has previously been observed that BPS can exert obesogenic effects *via* PPARγ activation (Ahmed and Atlas, 2016; Boucher et al., 2016). Using a murine adipogenic model, Ahmed and Atlas (2016) found that BPS but not BPA competitively inhibited rosiglitazone-activated PPARγ, indicating that the interaction with PPARγ differs between BPS and BPA. Boucher et al. also reported that the obesogenic effects of BPS were mainly mediated *via* direct activation of the nuclear receptor PPARγ in human hASCs, proposing that GR had a coactivation function in this context (Boucher et al., 2016a). Discrepancies in the action mechanisms described by studies

may be attributable to differences in the composition of differentiation cocktails and in the experimental conditions. Further studies are needed to verify whether the effects of BPF are predominantly mediated *via* an ER-dependent pathway and those of BPS through PPARγ activation. Given the affinity of bisphenols for diverse receptors and enzymes, their *in vitro* and *in vivo* obesogenic effects likely result from a combination of pathways rather than from a single mechanism. Further research is warranted on the manner in which chemical mixtures interfere with adipogenic differentiation.

In summary, BPS and BPF showed a similar transcriptional profile and therefore a relatively similar adipogenic potential, as indicated by other studies (Boucher et al., 2016b; Héliès-Toussaint et al., 2014; Ramskov Tetzlaff et al., 2020; Wang et al., 2017). According to the present study, both compounds also significantly upregulate the mRNA expression of specific adipogenic genes at days 7 and 14 of differentiation. As in the case of BPA, both BPF and BPS increase adipocyte differentiation by promoting the gene expression of PPARγ, C/EBPα, LPL, and FABP4 (Ahmed and Atlas, 2016; Boucher et al., 2014; Masuno et al., 2005; Ohlstein et al., 2014; Wang et al., 2013).

Limitations of our *in vitro* study include the inability to directly extrapolate findings to humans. This is due to: the supra-physiological concentrations of hormones in the hormone cocktail needed for adipogenic differentiation; the lack of discrimination between adipocyte hyperplasia and hypertrophy; and the difficulty of identifying the hormonal mechanisms that underlie the impact of bisphenols on adipocyte biology. Additionally, there is a need to improve comparability among studies by standardizing the protocols used for human adipocyte differentiation (Scott et al., 2011). Results for the adipogenic properties of environmental chemicals can vary among cell lines and experimental protocols (Kassotis et al., 2017), and responses can be influenced by the composition and concentration of hormones in the DM cocktail and by the frequency of its replacement (Boucher et al., 2016a; Ohlstein et al., 2014; Patel et al., 2003; Peshdary et al., 2020; Wang et al., 2013, 2017). Study strengths include the timely evaluation of BPF and BPS; the utilization of a human model of adipogenesis, improving the relevance to biological effects in human populations; the selection of hASCs from the same donor in all experiments, avoiding genetic variability; and the assessment of gene

expression and protein levels at two stages of adipocyte differentiation, providing a broader perspective.

Scant information is available on human exposure to BPS and BPF. BPF and BPS levels in the general population are lower than BPA levels because the utilization of BPA has not been completely restricted. However, the incorporation of these BPA replacements is increasing yearly and will likely reach current levels of BPA in the near future (Lehmler et al., 2018; Liu et al., 2018; Rochester and Bolden, 2015; Ye et al., 2015).

5. Conclusion

These findings indicate that both BPF and BPS promote human adipogenesis by disrupting the developmental programming of adipocytes and increasing the accumulation of intracellular lipids, supporting their obesogenic potential. However, additional research is required to confirm the effects of BPF and to elucidate the mechanisms underlying the actions of BPS and BPF. This information adds to the growing body of evidence suggesting that BPF and BPS may not be safe alternatives to BPA.

CRediT authorship contribution statement

Iris Reina-Pérez: Methodology, Validation, Formal analysis, Investigation, Conceptualization, Writing – original draft. **Alicia Olivas-Martínez:** Methodology, Validation, Formal analysis, Investigation, Conceptualization, Writing – original draft. **Vicente Mustieles:** Investigation, Methodology, Writing – review & editing. **Francisco Javier Ruiz-Ojeda:** Investigation, Methodology, Writing – review & editing. **José Manuel Molina-Molina:** Investigation, Methodology. **Nicolás Olea:** Writing – review & editing. **Mariana F. Fernández:** Global idea and research supervision, Conceptualization, Writing – review & editing, Project administration.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2021.112216.

References

Ahmed, S., Atlas, E., 2016. Bisphenol S- and bisphenol A-induced adipogenesis of murine preadipocytes occurs through direct peroxisome proliferator-activated receptor gamma activation. Int. J. Obes. 40 (10), 1566–1573. https://doi.org/10.1038/ijo.2016.95.

Bernlohr, D.A., Jenkins, A.E., Bennaars, A.A., 2002. Chapter 10 adipose tissue and lipid metabolism. Biochemistry of lipids, lipoproteins and membranes, 4th edition. N. Compr. Biochem. 36, 263–289.

Björnsdotter, M.K., Jonker, W., Legradi, J., Kool, J., Ballesteros-Gómez, A., 2017. Bisphenol A alternatives in thermal paper from The Netherlands, Spain, Sweden and Norway. Screening and potential toxicity. Sci. Total Environ. 601–602, 210–221. https://doi.org/10.1016/j.scitotenv.2017.05.171.

Boucher, J.G., Boudreau, A., Atlas, E., 2014. Bisphenol a induces differentiation of human preadipocytes in the absence of glucocorticoid and is inhibited by an estrogen-receptor antagonist. Nutr. Diabetes 4 (1), 1–8. https://doi.org/10.1038/ nutd.2013.43.

Boucher, J.G., Ahmed, S., Atlas, E., 2016a. Bisphenol S induces adipogenesis in primary human preadipocytes from female donors. Endocrinology 157 (4), 1397–1407. https://doi.org/10.1210/en.2015-1872.

Boucher, J.G., Gagné, R., Rowan-Carroll, A., Boudreau, A., Yauk, C.L., Atlas, E., 2016b. Bisphenol A and bisphenol S induce distinct transcriptional profiles in differentiating human primary preadipocytes. PloS One 11 (9), 1–22. https://doi.org/10.1371/ journal.pone.0163318.

Choi, S. Il, Lee, J.S., Lee, S., Sim, W.S., Kim, Y.C., Lee, O.H., 2020. Potentilla rugulosa nakai extract attenuates bisphenol A-, S- and F-induced ROS production and differentiation of 3T3-L1 preadipocytes in the absence of dexamethasone. Antioxidants 9 (2), 113. https://doi.org/10.3390/antiox9020113.

Darbre, P.D., 2020. Chemical components of plastics as endocrine disruptors: overview and commentary. Birth Defects Res. https://doi.org/10.1002/bdr2.1778.

Drobna, Z., Talarovicova, A., Schrader, H.E., Fennell, T.R., Snyder, R.W., Rissman, E.F., 2019. Bisphenol F has different effects on preadipocytes differentiation and weight gain in adult mice as compared with Bisphenol A and S. Toxicology 420, 66–72. https://doi.org/10.1016/j.tox.2019.03.016.

Eladak, S., Grisin, T., Moison, D., Guerquin, M.J., N'Tumba-Byn, T., Pozzi-Gaudin, S., Benachi, A., Livera, G., Rouiller-Fabre, V., Habert, R., 2015. A new chapter in the bisphenol a story: bisphenol S and bisphenol F are not safe alternatives to this compound. Fertil. Steril. 103 (1), 11–21. https://doi.org/10.1016/j. fertnstert.2014.11.005.

Frank, J., 2016. Origins of the obesity pandemic can be analysed. Nature 532 (7598),149. https://doi.org/10.1038/532149a.

Fraser, J.K., Zhu, M., Wulur, I., Alfonso, Z., 2008. Adipose-derived stem cells. Methods Mol. Biol. 449, 59–67. https://doi.org/10.1007/978-1-60327-169-1_4.

González, N., Cunha, S.C., Monteiro, C., Fernandes, J.O., Marquès, M., Domingo, J.L., Nadal, M., 2019. Quantification of eight bisphenol analogues in blood and urine samples of workers in a hazardous waste incinerator. Environ. Res. 176, 108576. https://doi.org/10.1016/j.envres.2019.108576.

Grün, F., Blumberg, B., 2007. Perturbed nuclear receptor signaling by environmental obesogens as emerging factors in the obesity crisis. Rev. Endocr. Metab. Disord. 8 (2), 161–171. https://doi.org/10.1007/s11154-007-9049-x.

Grün, F., Blumberg, B., 2006. Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. Endocrinology 147 (6), 50–55. https://doi.org/10.1210/en.2005-1129.

Heindel, J.J., Blumberg, B., Cave, M., Machtinger, R., Mantovani, A., Mendez, M.A., Nadal, A., Palanza, P., Panzica, G., Sargis, R., Vandenberg, L.N., Vom Saal, F., 2017. Metabolism disrupting chemicals and metabolic disorders. Reprod. Toxicol. 68, 3–33. https://doi.org/10.1016/j.reprotox.2016.10.001.

Heindel, J.J., 2019. History of the obesogen field: looking back to look forward. Front. Endocrinol. 10, 14. https://doi.org/10.3389/fendo.2019.00014.

Héliès-Toussaint, C., Peyre, L., Costanzo, C., Chagnon, M.C., Rahmani, R., 2014. Is bisphenol S a safe substitute for bisphenol A in terms of metabolic function? An in vitro study. Toxicol. Appl. Pharmacol. 280 (2), 224–235. https://doi.org/10.1016/j.taap.2014.07.025.

Ivry Del Moral, L., Le Corre, L., Hélène, P., Isabelle, N., Caroline, T., Jean-François, M., Patrick, R., Philippe, B., Roger, R., Marie-Christine, C., 2016. Obesogen effects after perinatal exposure of 4,4′-sulfonyldiphenol (Bisphenol S) in C57BL/6 mice. Toxicology 357–358, 11–20. https://doi.org/10.1016/j.tox.2016.05.023.

Janderová, L., McNeil, M., Murrell, A.N., Mynatt, R.L., Smith, S.R., 2003. Human mesenchymal stem cells as an in vitro model for human adipogenesis. Obes. Res. 11 (1), 65–74. https://doi.org/10.1038/oby.2003.11.

Janesick, A., Blumberg, B., 2011. Endocrine disrupting chemicals and the developmental programming of adipogenesis and obesity. Birth Defects Res. Part C Embryo Today - Rev. 93 (1), 34–50. https://doi.org/10.1002/bdrc.20197.

Kassotis, C.D., Masse, L., Kim, S., Schlezinger, J.J., Webster, T.F., Stapleton, H.M., 2017. Characterization of adipogenic chemicals in three different cell culture systems: implications for reproducibility based on cell source and handling. Sci. Rep. 7, 42104. https://doi.org/10.1038/srep42104.

Kassotis, C.D., Stapleton, H.M., 2019. Endocrine-mediated mechanisms of metabolic disruption and new approaches to examine the public health threat. Front. Endocrinol. 10, 39. https://doi.org/10.3389/fendo.2019.00039.

Lefterova, M.I., Lazar, M.A., 2009. New developments in adipogenesis. Trends Endocrinol. Metabol. 20 (3), 107–114. https://doi.org/10.1016/j.tem.2008.11.005.

Lehmler, H.J., Liu, B., Gadogbe, M., Bao, W., 2018. Exposure to bisphenol A, bisphenol F, and bisphenol S in U.S. Adults and children: the national health and nutrition examination survey 2013-2014. ACS Omega 3 (6), 6523–6532. https://doi.org/10.1021/acsomega.8b00824.

Liu, J., Wattar, N., Field, C.J., Dinu, I., Dewey, D., Martin, J.W., 2018. Exposure and dietary sources of bisphenol A (BPA) and BPA-alternatives among mothers in the APrON cohort study. Environ. Int. 119, 319–326. https://doi.org/10.1016/j.envint.2018.07.001.

Luo, L., Liu, M., 2016. Adipose tissue in control of metabolism. J. Endocrinol. 231 (3), 77–99. https://doi.org/10.1530/JOE-16-0211.

Martínez, M., Blanco, J., Rovira, J., Kumar, V., Domingo, J.L., Schuhmacher, M., 2020. Bisphenol A analogues (BPS and BPF) present a greater obesogenic capacity in 3T3- L1 cell line. Food Chem. Toxicol. 140, 111298. https://doi.org/10.1016/j.fct.2020.111298.

Masuno, H., Iwanami, J., Kidani, T., Sakayama, K., Honda, K., 2005. Bisphenol a accelerates terminal differentiation of 3T3-L1 cells into adipocytes through the phosphatidylinositol 3-kinase pathway. Toxicol. Sci. 84 (2), 319–327. https://doi.org/10.1093/toxsci/kfi088.

Marroqui, L., Martinez-Pinna, J., Castellano-Muñoz, M., Dos Santos, R.S., Medina-Gali, R.M., Soriano, S., Quesada, I., Gustafsson, J.A., Encinar, J.A., Nadal, A., 2021. Bisphenol-S and Bisphenol-F alter mouse pancreatic β-cell ion channel expression and activity and insulin release through an estrogen receptor ERβ mediated pathway. Chemosphere 265, 129051. https://doi.org/10.1016/j.chemosphere.2020.129051.

Molina-Molina, J.M., Amaya, E., Grimaldi, M., Sáenz, J.M., Real, M., Fernández, M.F., Balaguer, P., Olea, N., 2013. In vitro study on the agonistic and antagonistic activities of bisphenol-S and other bisphenol-A congeners and derivatives via nuclear receptors. Toxicol. Appl. Pharmacol. 272, 127–136. https://doi.org/10.1016/j. taap.2013.05.015.

Mustieles, V., Pérez-Lobato, R., Olea, N., Fernández, M.F., 2015. Bisphenol A: human exposure and neurobehavior. Neurotoxicology 49, 174–184. https://doi.org/10.1016/j.neuro.2015.06.002.

Mustieles, V., D'Cruz, S.C., Couderq, S., Rodríguez-Carrillo, A., Fini, J.B., Hofer, T., Steffensen, I.L., Dirven, H., Barouki, R., Olea, N., Fernández, M.F., David, A., 2020. Bisphenol A and its

analogues: a comprehensive review to identify and prioritize effect biomarkers for human biomonitoring. Environ. Int. 144, 105811. https://doi. org/10.1016/j.envint.2020.105811.

Ohlstein, J.F., Strong, A.L., McLachlan, J.A., Gimble, J.M., Burow, M.E., Bunnell, B.A., 2014. Bisphenol a enhances adipogenic differentiation of human adipose stromal/ stem cells. J. Mol. Endocrinol. 53 (3), 345–353. https://doi.org/10.1530/JME-14-0052.

Patel, N.G., Holder, J.C., Smith, S.A., Kumar, S., Eggo, M.C., 2003. Differential regulation of lipogenesis and leptin production by independent signaling pathways and rosiglitazone during human adipocyte differentiation. Diabetes 52 (1), 43–50. https://doi.org/10.2337/diabetes.52.1.43.

Peshdary, V., Styles, G., Gagné, R., Yauk, C.L., Sorisky, A., Atlas, E., 2020. Depot-specific analysis of human adipose cells and their responses to Bisphenol S. Endocrinology 161 (6), 44. https://doi.org/10.1210/endocr/bqaa044.

Petrakis, D., Vassilopoulou, L., Mamoulakis, C., Psycharakis, C., Anifantaki, A., Sifakis, S., Docea, A.O., Tsiaoussis, J., Makrigiannakis, A., Tsatsakis, A.M., 2017. Endocrine disruptors leading to obesity and related diseases. Int. J. Environ. Res. Publ. Health 14 (10), 1282. https://doi.org/10.3390/ijerph14101282.

Ramírez-Zacarías, J.L., Castro-Muñozledo, F., Kuri-Harcuch, W., 1992. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with oil red O. Histochemistry 97 (6), 493–497. https://doi.org/10.1007/BF00316069.

Ramskov Tetzlaff, C.N., Svingen, T., Vinggaard, A.M., Rosenmai, A.K., Taxvig, C., 2020. Bisphenols B, E, F, and S and 4-cumylphenol induce lipid accumulation in mouse adipocytes similarly to bisphenol A. Environ. Toxicol. 35 (5), 543–552. https://doi. org/10.1002/tox.22889.

Rochester, J.R., Bolden, A.L., 2015. Bisphenol S and F: a systematic review and comparison of the hormonal activity of bisphenol a substitutes. Environ. Health Perspect. 123 (7), 643–650. https://doi.org/10.1289/ehp.1408989.

Ruiz-Ojeda, F.J., Rupérez, A.I., Gomez-Llorente, C., Gil, A., Aguilera, C.M., 2016. Cell models and their application for studing adipogenic differentiation in relation to obesity: review. Int. J. Mol. Sci. 17 (7), 1040. https://doi.org/10.3390/ ijms17071040.

Russo, G., Barbato, F., Mita, D.G., Grumetto, L., 2019. Occurrence of Bisphenol A and its analogues in some foodstuff marketed in

Europe. Food Chem. Toxicol. 131, 110575. https://doi.org/10.1016/j.fct.2019.110575.
Scott, M.A., Nguyen, V.T., Levi, B., James, A.W., 2011. Current methods of adipogenic differentiation of mesenchymal stem cells. Stem Cell. Dev. 20 (10), 1793–1804. https://doi.org/10.1089/scd.2011.0040.
Strober, W., 2015. Trypan blue exclusion test of cell viability. Curr. Protoc. Im. 111, 1–3. https://doi.org/10.1002/0471142735.ima03bs111.
Usman, A., Ahmad, M., 2016. From BPA to its analogues: is it a safe journey? Chemosphere 158, 131–142. https://doi.org/10.1016/j.chemosphere.2016.05.070.
Vandenberg, L.N., Chahoud, I., Heindel, J.J., Padmanabhan, V., Paumgartten, F.J.R., Schoenfelder, G., 2010. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. Environ. Health Perspect. 118 (8), 1055–1070. https://doi.org/10.1289/ehp.0901716.
Vandenberg, L.N., Hauser, R., Marcus, M., Olea, N., Welshons, W.V., 2007. Human exposure to bisphenol A (BPA). Reprod. Toxicol. 24 (2), 139–177. https://doi.org/10.1016/j.reprotox.2007.07.010.
Verbanck, M., Canouil, M., Leloire, A., Dhennin, V., Coumoul, X., Yengo, L., Froguel, P., Poulain-Godefroy, O., 2017. Low-dose exposure to bisphenols A, F and S of human primary adipocyte impacts coding and non-coding RNA profiles. PloS One 12 (6), e0179583. https://doi.org/10.1371/journal.pone.0179583.
Wang, J., Sun, B., Hou, M., Pan, X., Li, X., 2013. The environmental obesogen bisphenol A promotes adipogenesis by increasing the amount of 11β-hydroxysteroid dehydrogenase type 1 in the adipose tissue of children. Int. J. Obes. 37 (7), 999–1005. https://doi.org/10.1038/ijo.2012.173.
Wang, R.Y., Abbott, R.D., Zieba, A., Borowsky, F.E., Kaplan, D.L., 2017. Development of a three-dimensional adipose tissue model for studying embryonic exposures to obesogenic chemicals. Ann. Biomed. Eng. 45 (7), 1807–1818. https://doi.org/10.1007/s10439-016-1752-x.
Wassenaar, P.N.H., Trasande, L., Legler, J., 2017. Systematic review and meta-analysis of early-life exposure to bisphenol A and obesity-related outcomes in rodents. Environ. Health Perspect. 125 (10), 106001. https://doi.org/10.1289/EHP1233.

Williams, E.P., Mesidor, M., Winters, K., Dubbert, P.M., Wyatt, S.B., 2015. Overweight and obesity: prevalence, consequences, and causes of a growing public health problem. Curr. Obes. Rep. 4 (3), 363–370. https://doi.org/10.1007/s13679-015-0169-4.

Ye, X., Wong, L.Y., Kramer, J., Zhou, X., Jia, T., Calafat, A.M., 2015. Urinary concentrations of Bisphenol A and three other bisphenols in convenience samples of U.S. adults during 2000-2014. Environ. Sci. Technol. 49 (19), 11834–11839. https://doi.org/10.1021/acs.est.5b02135.

Article #2

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The Mixture of Bisphenol-A and Its Substitutes Bisphenol-S and Bisphenol-F Exerts Obesogenic Activity on Human Adipose-Derived Stem Cells

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Abstract

Bisphenol A (BPA) and its substitutes, bisphenol F (BPF) and S (BPS), have previously shown in vitro obesogenic activity. This study was designed to investigate their combined effect on the adipogenic differentiation of human adipose-derived stem cells (hASCs). Cells were exposed for 14 days to an equimolar mixture of bisphenols (MIX) (range 10 nM–10 μM). Oil Red staining was used to measure intracellular lipid accumulation, quantitative real-time polymerase chain reaction (qRT-PCR) to study gene expression of adipogenic markers (PPARγ, C/EBPα, LPL, and FABP4), and Western Blot to determine their corresponding proteins. The MIX promoted intracellular lipid accumulation in a dose-dependent manner with a maximal response at 10 μM. Co-incubation with pure antiestrogen (ICI 182,780) inhibited lipid accumulation, suggesting that the effect was mediated by the estrogen receptor. The MIX also significantly altered the expression of PPARγ, C/EBPα, LPL, and FABP4 markers, observing a non-monotonic (U-shaped) dose-response, with maximal gene expression at 10 nM and 10 μM and lesser expression at 1 μM. This pattern was not observed when bisphenols were tested individually. Exposure to MIX (1–10 μM) also increased all encoded proteins except for FABP4, which showed no changes. Evaluation of the combined effect of relevant chemical mixtures is needed rather than single chemical testing.

Keywords: mixtures, bisphenols, bisphenol A (BPA), bisphenol S (BPS), bisphenol F (BPF), endocrine disruptors, dose addition

1. Introduction

Evidence of the contribution of excessive and/or dysfunctional adipose tissue to the development of obesity-related diseases, including metabolic syndrome and cancer [1], has prompted a rapid increase in research on adipose tissue function. Adipocytes are key regulators of whole-body energy homeostasis, and numerous novel regulators of adipose tissue differentiation and function have been identified [2,3,4,5]. The morphology of adipose tissue is defined by the number and size distribution of its adipocytes. Tissue with numerous small adipocytes is associated with a hyperplastic morphology, and tissue with a small number of large adipocytes is associated with a hypertrophic morphology [2,6]. Increased adipocyte size in both visceral and subcutaneous adipose tissue has been associated with insulin resistance, diabetes, and cardiovascular disease, among other adverse effects [6].

Adipose tissue both stores lipids and acts as an endocrine organ that synthesises and secretes hormones; however, it can also accumulate lipophilic environmental chemical compounds known as endocrine disruptors [2,5,7,8,9]. Endocrine-disrupting chemicals (EDCs) are exogenous substances that interact with endogenous hormones at multiple levels; a) interacting, activating, and antagonising hormonal receptors; b) altering hormone receptor expression and signal transduction in hormone-responsive cells; c) inducing epigenetic modifications in hormone-producing or hormone-responsive cells; and d) altering the synthesis of hormones, their transport across cell membranes, their distribution and/or circulating levels, their metabolism or clearance, and the fate of hormone-producing or hormone-responsive cells [10,11]. There is increasing evidence of an association between exposure to some EDCs, designated as obesogens, and obesity-related diseases. These obesogens include both persistent organic pollutants (POPs) and non-persistent compounds such as bisphenols [12,13,14]. EDCs interact with multiple nuclear hormone receptors, producing an imbalance in the hormonal system [5]. Adipocyte physiology is regulated by nuclear hormone receptors, and several of these have emerged as molecular targets of various EDCs [4].

Bisphenol A (BPA), a well-known EDC, is frequently detected in both the environment and humans [15]. However, its prohibition in multiple products in 2011, when the first European BPA restrictions became effective [16], led manufacturers to start replacing BPA with its analogues [e.g., bisphenol F (BPF), bisphenol S (BPS)] in similar structure applications (thermoplastics, polycarbonate plastics, and epoxy resins), increasing human exposure to these substitute chemicals. For example, a recent European study showed that the average cumulative exposure to unconjugated BPA had decreased from 3.8 ng/kg bw/day to 2.1 ng/kg bw/day before and after the restrictions in 2011 [17]. However, despite the increased detection of BPF and BPS, they have not yet reached the levels of BPA still found in human biological samples because utilisation of the latter has not been totally prohibited, among other reasons [17,18,19,20,21].

Adverse health impacts of environmental chemicals are still assessed substance-by-substance, neglecting, for example, that humans are simultaneously exposed to mixtures of bisphenols. Evaluating their combined exposure effects is difficult but needed, and in vitro methods are potential tools enabling a better understanding of the underlying mechanisms of mixture effect [22].

In vitro and in vivo studies have supported the so-called "something from nothing" effect, i.e., the significant effect of mixtures of chemicals that are individually at a non-detectable or "no-observed effects concentration" (NOEC) [23,24,25,26,27,28]. Most research on mixtures of EDCs has addressed their activity on nuclear receptors (mainly estrogenic and androgenic receptors) and their toxic reproductive and developmental effects using in vivo models [25,29,30,31,32,33]. However, there have been scant in vivo or in vitro studies of the adipogenic activity of ad hoc mixtures of EDCs [34,35,36].

One of the most studied mechanisms of action, and for which a greater number of scientific studies are available, is related to the affinity and agonist (and/or antagonist) activity of bisphenols, BPA (to a greater extent), and its analogues BPF and BPS (to a lesser extent), on the nuclear estrogen receptors ERα and ERβ [12,17,25,37,38,39,40,41,42].

Our group recently demonstrated the effects of individual exposure to BPF and BPS on the adipogenesis and lipid metabolism of human adipose tissue-derived stem cells (hASCs) and on the expression of related genes and proteins [43]. We hypothesised that different

mechanisms of action might cause interactions during combined exposure, which could lead to unpredictable effects of combined exposure [44]. The objective of the present investigation was to use a similar approach to study the ternary mixture of BPA, BPF, and BPS and determine their combined in vitro effect on adipogenic differentiation in hASCs. A secondary objective was to evaluate the role of estrogenic pathways in the action mechanism of the ad hoc bisphenol mixture.

2. Materials and Methods

2.1. Cell Culture

A commercial hASC cell line isolated from a single healthy non-diabetic adult through subcutaneous lipoaspirate, collected during elective surgical liposuction procedures (Poietics™ Normal Human ADSCs, PT-5006, Lot 0F4505, Lonza, Switzerland), was cultured and expanded following the manufacturer's recommendations under previously reported conditions [43,45]. In total, six hASC vials with the same reference and lot number were used to carry out all experiments. In brief, cells were seeded, incubated, and expanded in growth medium (GM) at 37 °C in a humidified atmosphere containing 5% CO_2. The GM comprised Advanced-Dulbecco's Modified Eagle Medium (advanced-DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM GlutaMAX, 100 U mL^{-1} penicillin, and 100 µg/mL streptomycin. The culture medium was replaced every 2–3 days, and cells were passaged up to 6 times. Cells were subcultured using a mixture of 0.25% trypsin-EDTA. All products were supplied by Thermo Fisher Scientific (Gibco, Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Adipogenic Differentiation

Differentiation was induced as previously described by Reina-Pérez et al. [43]. Briefly, hASCs were seeded in 24-well plates at initial concentrations of 40,000 cells per well in GM. When confluence was reached (2 days), the GM was replaced with a differentiation medium (DM) consisting of GM supplemented with 1 µM dexamethasone (DEX), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1.7 µM human insulin, and 0.1% dimethyl sulfoxide (DMSO) (vehicle) as negative control, or by the DM plus 1 µM rosiglitazone (ROSI) as positive control. Individual BPA, BPF, BPS, and the mixture of the 3 compounds (MIX) at a ratio of 1:1:1, were tested. All individual bisphenols dissolved in DMSO were mixed to produce a standard stock solution at different concentrations

(0.01, 0.1, 1, and 10 mM) (Table S1). The final DMSO concentration in both the individual compounds and the mixture never exceeded 0.1% (*v*/*v*) of the culture medium [46]. All experimental conditions were finally diluted in DM and tested at concentrations of 0.01, 0.1, 1, and 10 µM during adipogenic differentiation of hASCs for 14 days [47].

hASCs were also cultured in DM containing individual bisphenols and MIX at 0.01, 0.1, 1, and 10 µM in the presence of the ER antagonist ICI 182,780 (100 nM). The DM was replaced every 2–3 days during the adipogenic process. All differentiation products were supplied by Sigma (Sigma-Aldrich, St. Louis, MO, USA) except for ICI 182,780 (Tocris Bioscience, Bristol, UK).

2.3. Quantitative Oil Red O Staining Assay

At 14 days of adipogenic differentiation, Oil Red O (ORO) staining was performed to quantify the accumulation of intracellular lipids, mainly triglycerides, in mature adipocytes obtained from all controls and under different experimental conditions (0.1, 1, and 10 µM with or without ICI 182,780 at 100 nM). Briefly, cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (Electron Microscopy Science Hartfield, PA, USA) for 1 h at room temperature. After washing with milliQ-water and 60% isopropanol, cells were stained with a filtered ORO solution (0.5%, *w*/*v*) in milliQ-water (60/40, *v*/*v*) for 45 min, followed by washing with 60% isopropanol and again with milliQ-water. Cells were first observed and photographed under a Leica DMi8 microscope (Leica Microsystems, Wetzlar, Germany) with the Leica Application Suite (LAS) X software, and the retained dye was extracted with 100% isopropanol, measuring the optical density at a wavelength of 520 nm with a microplate reader (BioTek HTX, Fisher Scientific, Waltham, MA, USA) [48]. All aforementioned products were purchased from Thermo Fisher Scientific (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) or Sigma Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

2.4. RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

On day 14 of adipogenic differentiation, total RNA was extracted from cells using the RNeasy Mini kit supplied by Qiagen (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNase-Free DNase kit supplied by Qiagen (Qiagen, Hilden, Germany) was used

to eliminate genomic DNA, following the manufacturer's instructions. The final RNA concentration and quality (260/280 ratio) were determined with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to transcribe 1000 ng of total RNA into cDNA, following the manufacturer's instructions.

RT-qPCR was used to measure the expression levels of genes involved in the adipogenic process, including peroxisome proliferator-activated receptor gamma (*PPARγ*), CCAT/enhancer-binding protein (*C/EBPα*), lipoprotein-lipase (*LPL*), and fatty acid-binding protein 4 (*FABP4*). Hypoxanthine-guanine phosphoribosyltransferase-1 (*HPRT1*) and β-actin (*ACTB*) served as control or housekeeping genes for all experiments. Primer pairs for each target gene were *PPARγ* (Assay ID qHsaCED0044425), *C/EBPα* (Assay ID qHsaCED0019045), *LPL* (Assay ID qHsaCED0047106), *FABP4* (Assay ID qHsaCED0057474), *HPRT1* (Assay ID qHsaCID0016375), and *ACTB* (Assay ID qhsaLED0214042), all acquired from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). RT-qPCR was carried out with an ABI Prism 7900HT instrument using SYBR Green PCR (Bio-Rad Laboratories, Hercules, CA, USA). The Qiagen Data Analysis Center (GeneGlobe Data Analysis Center, Qiagen, Hilden, Germany) was used for the quantification. The stability of reference genes was statistically validated for each sample in duplicate, employing the $2^{-\Delta\Delta Ct}$ method to express the results as fold-changes and using the negative control as reference [49].

2.5. Western Blot

At 14 days of adipogenic differentiation, protein levels of selected genes were measured in cells cultured under the different experimental conditions (0.01, 0.1, 1, and 10 µM MIX). Briefly, lysis was performed using cell lysis buffer [10 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 100% glycerol (Sigma-Aldrich, St. Louis, MO, USA)], a protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA), and β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), and samples were then placed on ice for 20 min [43]. After centrifugation for 30 min at 13,000× *g* and 4 °C, the protein content of the supernatant was quantified by DC Protein Assay, using 50 µg of protein sample mixed with 4X Laemmli sample buffer containing 10% β-mercaptoethanol and milliQ-water. Samples were separated with SDS-PAGE using TGX Any kD gel and transferred onto a nitrocellulose membrane, which was incubated in blocking buffer [5% non-fat milk

in 1X Tris-buffered saline (TBS) with 0.5% Tween 20 (TBS-T)] for 1 h at room temperature. Primary antibodies to incubate blotted membrane for PPARγ, C/EBPα, FABP4, LPL, and HSC-70 (B6) were used with appropriate horseradish peroxidase-labelled secondary antibodies (Table S2). Immunoreactive signals were detected using the Clarity Western ECL Substrate Kit, and membranes were digitally imaged with Image Reader LAS-4000 and quantified by densitometry using ImageJ software. Protein levels were represented as the fold-changes in expression relative to the control (HSC-70). All these products were acquired from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA) or Sigma Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

2.6. Cell Viability

The number of viable cells was determined using the trypan blue test described by Strober with slight modifications [50]. Briefly, after 14 days of culture under the aforementioned experimental conditions (0.01, 0.1, 1, and 10 µM of MIX), cells were dissociated with trypsin, resuspended in PBS, mixed with trypan blue dye, visually examined, and counted in a Neubauer chamber to determine the percentage of viability under each experimental condition. An Olympus IX51 inverted microscope with Olympus TH4-200 lamp was used.

2.7. Statistical Analysis

Cell cultures were repeated at least three times for each condition with multiple replicates. Analyses of gene and protein expression were performed in duplicate for each sample. The viability and ORO staining assays were carried out with multiple replicates for each experimental condition. Data were expressed as means ± standard error of the mean (SEM). Significant differences in assay results were evaluated using the non-parametric Mann–Whitney U test. A ≥1.5-fold increase and decrease in gene expression was considered as up-regulation and down-regulation, respectively. SPSS version 23 (IBM SPSS, Armonk, NY, USA) was used for statistical analyses, considering * $p < 0.05$ as significant.

3. Results

3.1. The Bisphenol Mixture Promotes Intracellular Lipid Accumulation in a Dose-Dependent Manner

hASCs were differentiated into mature adipocytes under the following experimental conditions for 14 days: negative control (0.1% DMSO-vehicle), positive control (1 μM ROSI), BPA, BPS, BPF, or bisphenol mixture at concentrations of 0.01, 0.1, 1, or 10 μM. Adipogenic differentiation was confirmed morphologically by the accumulation of intracellular lipid droplets. A dose-dependent increase in intracellular lipid accumulation of mature adipocytes was observed, with a maximum response at 10 μM but with different potencies, in the order BPS (1.422 ± 0.004) > BPF (1.322 ± 0.007) > MIX (1.134 ± 0.005) > BPA (1.126 ± 0.005) (Figure 1 and Figure S1, Table S3).

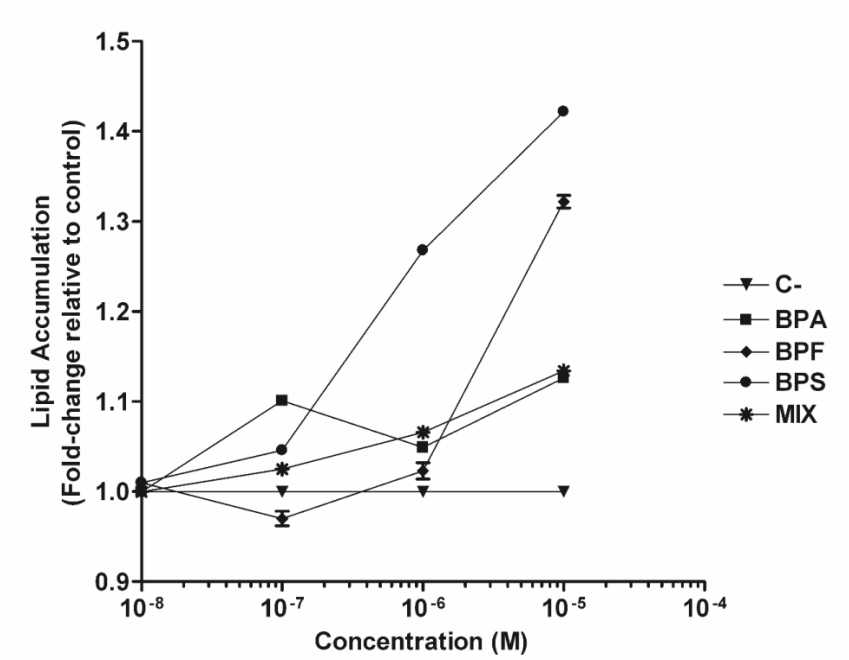


Figure 1. Effect of BPA, BPS, BPF, or a mixture of three bisphenols (BPA, BPS, and BPF) on intracellular lipid accumulation in human adipose-derived stem cells (hASCs). Lipid accumulation was quantified by Oil Red O staining assay (at 520 nm) after 14 days in the presence of 0.01, 0.1, 1, or 10 μM. Lipid content was normalised using the negative control and expressed as fold-changes. Data were expressed as means ± SEM from three independent experiments with multiple replicates for each experimental condition. BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S; MIX (BPA, BPF, and BPS); C–, negative control.

hASCs were also treated under the same experimental conditions in the presence of the antiestrogen ICI 182,780 at a fixed concentration of 100 nM. Lipid accumulation was also studied for both individual bisphenols and the bisphenol mixture. The intracellular lipid

accumulation of mature adipocytes (by ORO staining assay) was significantly reduced at most of all tested concentrations of the bisphenol mixture (15%, 17.3%, 21.2%, and 13% at 0.01, 0.1, 1, and 10 µM, respectively; range: 79–87%) (Figure 2, Table S3), finding lipid accumulation inhibition ranging from 80 to 88%. Similar results were found for BPA, with higher inhibition rates (50 to 60%) with BPF but no inhibition with BPS, as demonstrated by ORO results for mature adipocytes (Figure 2, Table S3). Treatment with ICI alone did not significantly affect lipid accumulation (data not shown).

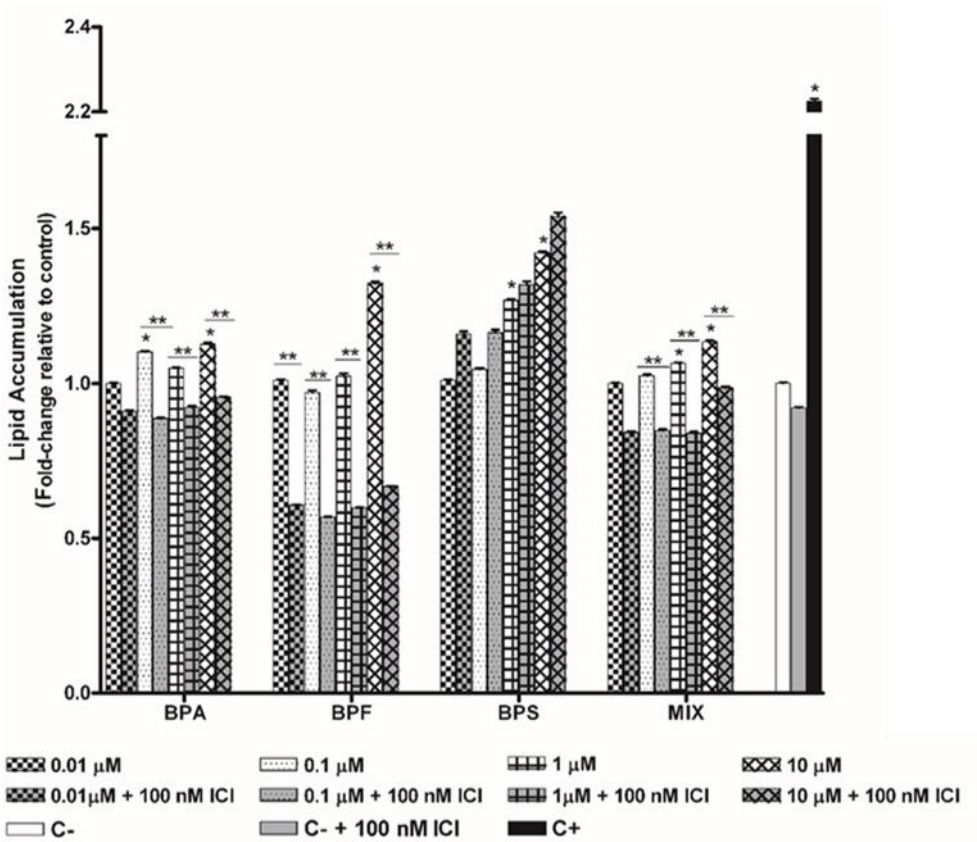
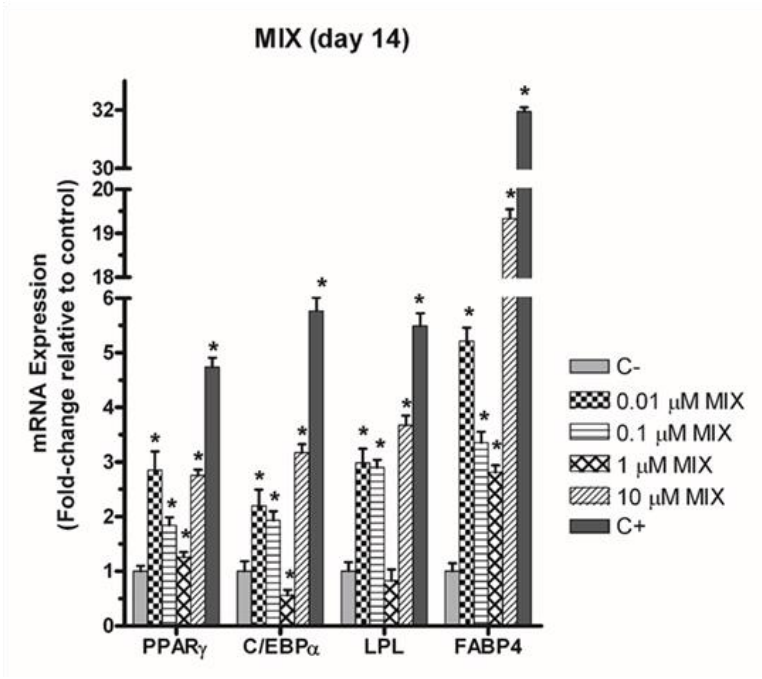


Figure 2. Lipid accumulation in mature adipocytes was quantified by ORO staining assay (absorbance at 520 nm) after 14 days of hASCs differentiation in the presence of BPA, BPS, BPF, or the mixture, with or without 100 nM ICI 182,780. Lipid content was normalised using the negative control and expressed as fold changes. Data were expressed as means ± SEM from three independent experiments with multiple replicates for each experimental condition. Significant differences were analysed using the Mann–Whitney U test and defined as * $p < 0.05$ respect to negative control, and ** $p < 0.05$ respect to the experimental condition without ICI. BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S; MIX (BPA, BPF, BPS); C–, negative control.

3.2. The Bisphenol Mixture Alters the Expression of Adipogenesis-Related Genes in a Non-Monotonic Manner

hASC treatment with the bisphenol MIX (0.01, 0.1, 1, and 10 µM) significantly increased the mRNA expression of *PPARγ*, *C/EBPα*, *LPL*, and *FABP4* genes, observing a non-monotonic dose-response in all cases. In this way, *PPARγ* mRNA expression was increased 2.85-fold at 0.01 µM, 1.26-fold at 1 µM, and 2.75-fold at 10 µM (Figure 3A). *C/EBPα* and *LPL* expressions showed the same pattern (2.2-, 1.93-, 0.56-, and 3.17-fold, and 2.98-, 2.90-, 0.83, and 3.67-fold, respectively, at 0.01, 0.1, 1, and 10 µM MIX) (Figure 3A). The highest increase was in *FABP4* mRNA expression (5.22-, 3.36-, 2.81-, and 19.34-fold, at 0.01, 0.1, 1, and 10 µM of MIX, respectively) (Figure 3A).

A



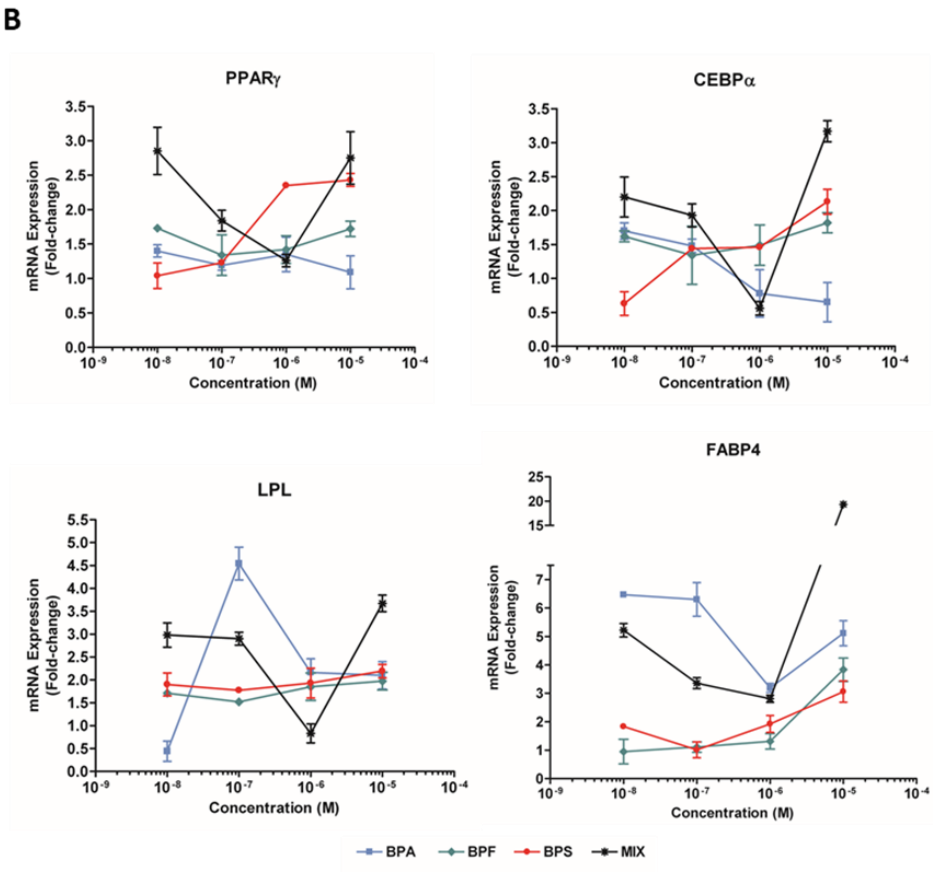


Figure 3. (**A**) Effect of the mixture of three bisphenols (BPA, BPS, and BPF), and (**B**) effect of individual compounds, on the expression of the adipogenic marker genes *PPARγ*, *C/EBPα*, *LPL*, and *FABP4* at 14 days of adipogenic differentiation in hASC cultures. mRNA levels were normalised using the levels of control genes (*HPRT1* and *ACTB*) and expressed as fold changes by the $2^{-\Delta\Delta Ct}$ method. Data were expressed as means ± SEM of three independent experiments with multiple replicates for each condition. Significant differences were analysed using the Mann–Whitney U test and defined as * $p < 0.05$. *ACTB*, β-actin; *C/EBPα*, CCAT/enhancer-binding protein; *FABP4*, fatty acid-binding protein 4; *HPRT1*, hypoxanthine-guanine phosphoribosyltransferase-1; *LPL*, lipoprotein-lipase; *PPARγ*, peroxisome proliferator-activated receptor gamma; MIX (BPA, BPF and BPS); C–, negative control; C+, positive control.

The same genes were evaluated on day 14 in the presence of BPA, BPF, or BPS. Results indicate that the effect of the mixture differs from those of individual components (Figure 3B, Table S4).

3.3. The Bisphenol Mixture Alters Protein Levels of Selected Adipogenic Markers

hASC treatment with the bisphenol MIX (0.01, 0.1, 1, and 10 µM) increased the levels of proteins encoded by PPARγ, C/EBPα, and LPL at the higher concentrations used (1 and 10 µM) (Figure 4A,B). Specifically, a slight but significant increase was observed in LPL protein levels (1.16-fold) at 1 µM MIX (Figure 4B) and in C/EBPα and LPL protein levels (1.22- and 1.44-fold, respectively) at 10 µM MIX (Figure 4B). FABP4 protein levels did not differ from negative control results at any of the tested concentrations of MIX. At the lowest MIX concentrations tested (0.01 and 0.1 µM) the control protein bands (HSC70) were detected for the conditions studied but not for selected adipogenic markers (PPARγ, C/EBPα, LPL, and FABP4) (Figure S2).

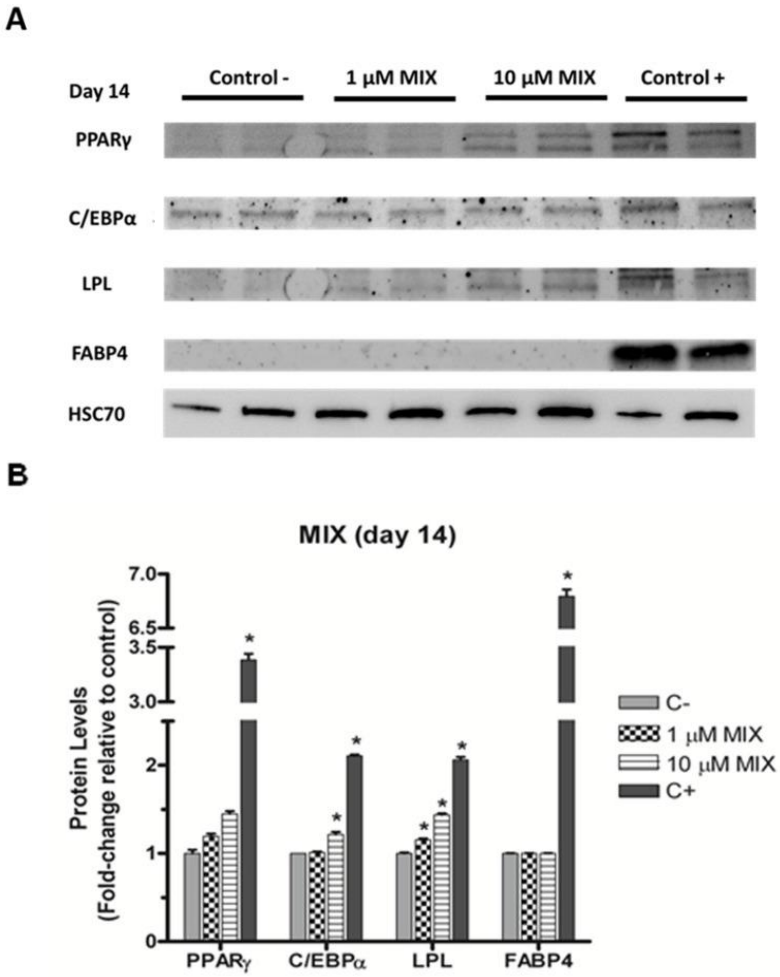


Figure 4. Effect of a mixture of three bisphenols (BPA, BPS, and BPF) on the protein expression of the adipogenic markers PPARγ, C/EBPα, LPL, and FABP4. Protein levels were determined by Western Blot (**A**) and densitometry (**B**) after 14 days of hASCs differentiation in the presence of MIX at 1 and 10 µM. Protein levels were normalised using protein control levels (HSC70) and expressed as fold changes. Data were expressed as means ± SEM of three independent experiments, performed in duplicate for each condition. Significant differences were evaluated using the Mann–Whitney U test and defined as * $p < 0.05$. C–, negative control; C+, positive control.

3.4. Effects of Bisphenols Mixture on hASC Viability

No cell cytotoxicity was observed in hASCs at any concentration (0.01, 0.1, 1, or 10 µM). At least 90% cell viability was obtained under all experimental conditions (Figure S3).

4. Discussion

An equimolar mixture of BPA, BPF, and BPS interfered with the differentiation programming of hASCs into adipocytes and altered their intracellular lipid accumulation, as previously described for each of these bisphenols [43,51,52,53,54]. However, our results indicated that the effect of the mixture differed from that of the individual components. Furthermore, this ternary bisphenol mixture affected the expression of genes involved in the adipogenesis process, but in a non-monotonic dose-response manner, not observed when analysing bisphenols individually. To our best knowledge, this is the first in vitro study to investigate the combined obesogenic activity of a mixture of three known endocrine disruptors (BPA and its analogues BPF and BPS) on the adipogenic differentiation, lipid accumulation, gene expression, and protein synthesis of stem cells derived from human adipose tissue.

The potential obesogenic effect of each individual bisphenol (BPA, BPF, and BPS) has been widely demonstrated in in vivo and in vitro studies [43,51,52,53,54,55,56,57]. In this way, in vitro studies have assessed the effects of these bisphenols on the adipogenic differentiation of murine 3T3-L1 cells, committed to being preadipocytes [58,59,60,61,62] and hASCs, which are of greater relevance to human adipogenesis [43,51,52,53,54,55]. Individually, all three bisphenols interfered with the developmental programming of hASCs, enhancing their differentiation into adipocytes and accumulation of intracellular lipid droplets [43,51,52,53,55,63,64]. Most of the effects shown were dose-dependent and corroborated by the up-regulation of specific adipogenic genes and their corresponding proteins [43,54]. However,

there is still some heterogeneity in the responses of many metabolism-disrupting chemicals in adipogenic differentiation studies. In previous studies, binary bisphenol mixtures (BPA+BPF and BPA+BPS) were found to exert potentially synergistic cytotoxic effects on *Ctenopharyngodon Idella* kidney cells [65]. A synergistic effect was also observed for mixtures of three components of BPA and BADGE analogues [65]. Some in vitro studies revealed additive effects of binary and multicomponent mixtures of bisphenols on estrogen and androgen receptor activity [25,47]. Likewise, an in vitro study on their genotoxic activity found that mixtures of bisphenols increased the gene expression of pivotal metabolic enzymes CYP1A1 and UGT1A1 by HepG2 cells in an additive manner [30]. In this sense, Backhaus and Faust suggested that since cases of more than additive mixtures effects seem to be rare, to consider additivity as precautious first tier, regardless of the mechanisms of action of the mixture components [22].

The present study contributes new evidence on the combined effect of bisphenol mixtures, drawing attention to the potentially hazardous effects of the coexistence of certain bisphenols in the environment [31]. Interestingly, we observed that some of the effects depended on concentrations but in a paradoxical manner. For instance, the adipogenic gene expression was stimulated at both high (10 μM) and low (10 nM) MIX concentrations, but the effect was always lower at intermediate concentrations (1 μM). The concentrations tested in this study were selected because they have been used in in vitro studies of these bisphenols in human cell lines [43,63,66,67] and are considered environmentally relevant [18,19,20,21]. No in vitro data are available on human cell lines treated with similar concentration mixtures for comparisons; however, the non-monotonic U-shaped response observed for some of the genes in the present study was also described by Wang and co-workers [68].

Elucidation of the precise signalling pathways altered by exposure to environmental contaminants will help to identify and understand chemicals that pose a threat to metabolic health. In the present study, we have investigated some of the molecular pathways involved in the differentiation process, evaluating genes related to adipocyte development and lipid metabolism. The selected genes were *PPARγ*, the master regulator gene of adipogenesis [58,69]; *C/EBPα*, a mid-stage marker [69,70]; and *LPL* and *FABP4*, late markers of adipocyte

maturation [69,71]. The mRNA expression pattern of each gene was also assessed. In comparison to the results recently published by our group for the individual bisphenols in the mixture at the same concentration range and under the same culture conditions [43], the gene expression was generally higher (1- to 20-fold for MIX, 1- to 4-fold for BPF, 1- to 3-fold for BPS, and 1- to 8-fold for BPA) (Figure 3A,B).

Statistically significant increases were also observed in protein levels of PPARγ, C/EBPα, and LPL at 14 days of hASCs differentiation in the presence of the mixture at concentrations of 1 and 10 μM (Figure 4). PPARγ, C/EBPα, and LPL protein levels were generally higher when treated at a concentration of 10 μM with the mixture of bisphenols (BPA, BPF, and BPS) than when treated with each individual bisphenol at the same concentration: 1- to 1.5-fold for MIX, 1- to 1.35-fold for BPF, and 1- to 1.1-fold for BPS. However, Western Blot results showed no effect on FABP4 protein levels at these concentrations, as previously reported for individual treatments with BPF and BPS at 10 μM [43], indicating perhaps that higher gene expression would be needed to obtain quantifiable levels of this protein, as shown by its positive control.

Previous studies have shown that the adipogenic effect of BPA is exerted, at least in part, through ER activation, as this effect can be blocked by the ER antagonist ICI 182,780 [15,53], as is confirmed in this work. This mode of action would therefore represent one of the underlying pathways between bisphenols exposure and obesity risk [53]. The obesogenic capacity of BPF was also inhibited by ICI at all concentrations tested but had no such effect on BPS under these study conditions, as reported [43]. Similarly, in their zebrafish reporter gene assay on the in vitro effect of individual bisphenols on ERs (ERα, ERβ1, and ERβ2), Le Fol et al. [72] also found that ICI 182,780 at 1 μM blocks the effect of BPA and BPF but not of BPS. Estrogen receptor pathways do not emerge as the main activation pathway for BPS, which could exert its obesogenic effects via PPARγ activation [51,58]. In this regard, Schaffert et al. recently investigated the mode of action of BPA and four of its substitutes during the differentiation of human preadipocytes [73]. BPA, BPS, and BPF disrupted crucial metabolic functions and insulin signalling at low and environmentally relevant concentrations, and the effects were mediated via the inhibition of PPARγ. Previously, Boucher et al. observed that BPS promotes the adipogenic differentiation of primary human preadipocytes and induces lipid

accumulation via PPARγ pathway [51,52]. Overall, given that adipogenesis involves the timely expression of various key transcription factors (PPARγ, GR, C/EBPα, ER, etc.) and bisphenols have an affinity for several receptors and enzymes, it is highly likely that their obesogenic effects result from a combination of multiple pathways [41,69].

The estrogenic activity of bisphenols mixtures remains underexplored [25,47]. In this work, we investigated whether the adipogenic capacity of the bisphenols mixture could be blocked by the competitive ER antagonist ICI 182,780. According to the present results, the presence of 100 nM ICI for 14 days induced inhibition of intracellular lipid accumulation in hASCs treated with the mixture at all tested concentrations (Figure 2, Table S3). The inhibition was not concentration-dependent and differed from that found with the individual components of the mixture. In our study, the inhibitory action of ICI on the bisphenol mixture could not be predicted from in vitro data on the action of each individual bisphenol. Other authors reported that binary mixtures of BPA, BPF, and/or BPS had similar effects on various ERs [74]. It has been proposed that when individual environmental pollutants act via the same mechanism of action (e.g., ER activation), the effect of their mixture can be predicted according to a concentration addition model [25].

Study limitations include: the difficulty in identifying the molecular mechanisms underlying the impact of bisphenols on adipocyte biology; the lack of discrimination between hyperplasia and hypertrophy in mature adipocytes; and the influence on the results of the characteristics of the selected human mesenchymal stem cell line, given the wide variability according to the sex, ethnicity, and physiological status of donors (in the present case, a single donor). Furthermore, these in vitro results cannot be directly extrapolated to humans, and the proportions of BPA, BPF, and BFS in the ad hoc mixture do not necessarily reflect detectable levels in the environment. On the other hand, all hASCs studied were from the same donor, avoiding genetic variability, and the relevance of findings to humans was enhanced by using hASCs as a model of adipogenesis. Strengths also include the contribution of new evidence on the combined effect of mixtures of bisphenols, highlighting the potentially harmful impact of the co-presence of certain bisphenols and derivatives in the environment.

It is necessary to implement standardised adipogenic differentiation protocols to improve the consistency of results. Kassotis et al. recently published a comprehensive assessment of inter-laboratory reproducibility, analysing factors contributing to the variability in responses to these chemical contaminants [75]. Human mesenchymal stem cell and pre-adipocyte models require further validation to improve the translation of this knowledge to human metabolic health. Scientific knowledge can also be improved by combining traditional in vitro models with new assessment techniques such as three-dimensional and co-culture techniques that allow the analysis of environmentally relevant levels of exposure [76].

5. Conclusions

Humans are simultaneously exposed to complex mixtures of different bisphenols, among numerous environmental EDCs. This study sheds some light on mixtures of bisphenols' obesogenic effects on human adipose-derived stem cells. A ternary mixture of bisphenols altered lipid accumulation and the mRNA expression of genes and proteins of adipogenesis markers at 14 days of differentiation, inducing changes that differed from the individual effects of each bisphenol. Evaluation of the combined effect of relevant EDC mixtures is needed rather than single chemicals testing, and in vitro methods allow a better understanding of the underlying molecular mechanisms. Further studies should be performed to identify the causal mechanism underlying the observed effects.

Acknowledgments

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Abbreviations

ACTB, β-actin; advanced-DMEM, Advance-Dulbecco Modified Eagle Medium; BPA, bisphenol A, 4,4′-isopropylidenediphenol; BPF, bisphenol F, 4,4′-methylenediphenol; BPS, bisphenol S, 4,4′-sulfonyldiphenol; cDNA, complementary deoxyribonucleic acid; C/EBPα, CCAT/enhancer-binding protein; DEX, dexamethasone; DM, differentiation medium; DMSO, dimethyl sulfoxide; EDC, endocrine disrupting chemical; ER, estrogen receptor; FABP4, fatty acid binding protein 4; FBS, fetal bovine serum; GM, growth medium; hASCs, human adipose-derived stem cells; ICI, ER antagonist ICI 182,780; INS, human insulin; HPRT1, hypoxanthine-guanine phosphoribosyltransferase-1;

IBMX, 3-isobutyl-1-methylxanthine; LPL, lipoprotein-lipase; MIX, bisphenols mixture; µg, micrograms; µM, micromolar; mM, millimolar; mL, millilitre; min, minutes; mRNA, messenger ribonucleic acid; ng, nanograms; nm, nanometers; C−, negative control; NOEC, no observed effects concentration; ORO, Oil Red O; PPARγ, peroxisome proliferator-activated receptor gamma; PBS, phosphate buffered saline; POPs, persistent organic pollutants; C+, positive control; qRT-PCR, quantitative real-time polymerase chain reaction; ROSI, rosiglitazone; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, standard error of the mean; TBS, tris-buffered saline; TBS-T, Tris-buffered saline-Tween 20.

Supplementary Materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/toxics10060287/s1, Figure S1: hASCs visualised by Oil Red O staining assay after 14 days of adipogenic differentiation; Figure S2: Western Blot of the control protein (HSC70) at the lowest concentrations of the mixture tested (0.01 and 0.1 µM). Figure S3: Effect of the mixture of three bisphenols (BPA, BPS, and BPF) on the viability of hASCs after 14 days of adipogenic differentiation. Table S1: Standard stock solutions of a mixture of three bisphenols (BPA, BPF, and BPS); Table S2: Brief description of primary and secondary antibodies used in Western Blot; Table S3. Intracellular lipids assessed by Oil Red O assay in hASCs after 14 days; Table S4. Gene expression of the adipogenic markers PPARγ, C/EBPα, LPL, and FABP4, in hASCs after 14 days of adipogenic differentiation.

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Author Contributions

I.R.-P.: Methodology, Validation, Formal analysis, Investigation, Conceptualization, Writing—original draft. A.O.-M.: Methodology, Validation, Formal analysis, Investigation, Conceptualization, Writing—original draft. V.M.: Investigation, Methodology, Writing—review & editing. E.S.-F.: Investigation, Methodology, Writing—review & editing. J.M.M.-M.: Investigation, Methodology. N.O., M.F.F.: Global idea and research supervision, Investigation, Conceptualization, Writing—review & editing, and approval original draft, Project administration. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

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Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the reported results are available on request.

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Footnotes

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References

1. World Cancer Research Fund/American Institute for Cancer Research. Diet, Nutrition, Physical Activity and Cancer: A Global Perspective. Continuous Update Project Expert Report. 2018. Available online: http://dietandcancerreport.org (accessed on 11 November 2021).

2. Janesick, A.S.; Blumberg, B. Obesogens: An emerging threat to public health. *Am. J. Obstet. Gynecol.* 2016;214:559–565. doi: 10.1016/j.ajog.2016.01.182.
3. Lauretta, R.; Sansone, A.; Sansone, M.; Romanelli, F.; Appetecchia, M. Endocrine disrupting chemicals: Effects on endocrine glands. *Front. Endocrinol. (Lausanne)* 2019;10:178. doi: 10.3389/fendo.2019.00178.
4. Regnier, S.M.; Sargis, R.M. Adipocytes under assault: Environmental disruption of adipose physiology. *Biochim. Biophys. Acta.* 2014;1842:520–533. doi: 10.1016/j.bbadis.2013.05.028.
5. Papalou, O.; Kandaraki, E.A.; Papadakis, G.; Diamanti-Kandarakis, E. Endocrine disrupting chemicals: An occult mediator of metabolic disease. *Front. Endocrinol. (Lausanne)* 2019;10:112. doi: 10.3389/fendo.2019.00112.
6. Tandon, P.; Wafer, R.; Minchin, J.E.N. Adipose morphology and metabolic disease. *J. Exp. Biol.* 2018;221:jeb164970. doi: 10.1242/jeb.164970.
7. Artacho-Cordón, F.; Fernández, M.F.; Frederiksen, H.; Iribarne-Durán, L.M.; Jiménez-Díaz, I.; Vela-Soria, F.; Andersson, A.M.; Martin-Olmedo, P.; Peinado, F.M.; Olea, N.; et al. Environmental phenols and parabens in adipose tissue from hospitalized adults in Southern Spain. *Environ. Int.* 2018;119:203–211. doi: 10.1016/j.envint.2018.05.052.
8. Mustieles, V.; Arrebola, J.P. How polluted is your fat? What the study of adipose tissue can contribute to environmental epidemiology. *J. Epidemiol. Community Health.* 2020;74:401–407. doi: 10.1136/jech-2019-213181.
9. Fernandez, M.F.; Arrebola, J.P.; Taoufiki, J.; Navalón, A.; Ballesteros, O.; Pulgar, R.; Vilchez, J.L.; Olea, N. Bisphenol-A and chlorinated derivatives in adipose tissue of women. *Reprod. Toxicol.* 2007;24:259–264. doi: 10.1016/j.reprotox.2007.06.007.
10. La Merrill, M.A.; Vandenberg, L.N.; Smith, M.T.; Goodson, W.; Browne, P.; Patisaul, H.B.; Guyton, K.Z.; Kortenkamp, A.; Cogliano, V.J.; Woodruff, T.J.; et al. Consensus on the key characteristics of endocrine-disrupting chemicals as a basis for hazard identification. *Nat. Rev. Endocrinol.* 2020;16:45–57. doi: 10.1038/s41574-019-0273-8.
11. Thomas Zoeller, R.; Brown, T.R.; Doan, L.L.; Gore, A.C.; Skakkebaek, N.E.; Soto, A.M.; Woodruff, T.J.; Vom Saal, F.S. Endocrine-disrupting chemicals and public health protection: A statement of principles from The Endocrine

Society. *Endocrinology.* 2012;153:4097–4110. doi: 10.1210/en.2012-1422.
12. Gore, A.C.; Chappell, V.A.; Fenton, S.E.; Flaws, J.A.; Nadal, A.; Prins, G.S.; Toppari, J.; Zoeller, R.T. EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. *Endocr. Rev.* 2015;36:E1–E150. doi: 10.1210/er.2015-1010.
13. Heindel, J.J. History of the Obesogen Field: Looking Back to Look Forward. *Front. Endocrinol. (Lausanne)* 2019;10:14. doi: 10.3389/fendo.2019.00014.
14. Heindel, J.J.; Blumberg, B. Environmental Obesogens: Mechanisms and Controversies. *Annu. Rev. Pharmacol. Toxicol.* 2019;59:89–106. doi: 10.1146/annurev-pharmtox-010818-021304.
15. Mustieles, V.; D'Cruz, S.C.; Couderq, S.; Rodríguez-Carrillo, A.; Fini, J.B.; Hofer, T.; Steffensen, I.L.; Dirven, H.; Barouki, R.; Olea, N.; et al. Bisphenol A and its analogues: A comprehensive review to identify and prioritize effect biomarkers for human biomonitoring. *Environ. Int.* 2020;144:105811. doi: 10.1016/j.envint.2020.105811.
16. European Commission. Commission Implementing Regulation (EU) No 321/2011 of 1 April 2011 Amending Regulation (EU) No 10/2011 as Regards the Restriction of Use of Bisphenol A in Plastic Infant Feeding Bottles. Available online: http://data.europa.eu/eli/reg_impl/2011/321/oj (accessed on 11 September 2021).
17. Karrer, C.; De Boer, W.; Delmaar, C.; Cai, Y.; Crépet, A.; Hungerbühler, K.; Von Goetz, N. Linking Probabilistic Exposure and Pharmacokinetic Modeling to Assess the Cumulative Risk from the Bisphenols BPA, BPS, BPF, and BPAF for Europeans. *Environ. Sci. Technol.* 2019;53:9181–9191. doi: 10.1021/acs.est.9b01749.
18. Lehmler, H.J.; Liu, B.; Gadogbe, M.; Bao, W. Exposure to Bisphenol A, Bisphenol F, and Bisphenol S in U.S. Adults and Children: The National Health and Nutrition Examination Survey 2013—2014. *ACS Omega.* 2018;3:6523–6532. doi: 10.1021/acsomega.8b00824.
19. Liu, J.; Wattar, N.; Field, C.J.; Dinu, I.; Dewey, D.; Martin, J.W. Exposure and dietary sources of bisphenol A (BPA) and BPA-alternatives among mothers in the APrON cohort study. *Environ. Int.* 2018;119:319–326. doi: 10.1016/j.envint.2018.07.001.
20. Rochester, J.R.; Bolden, A.L. Bisphenol S and F: A systematic review and comparison of the hormonal activity of bisphenol a

substitutes. *Environ. Health Perspect.* 2015;123:643–650. doi: 10.1289/ehp.1408989.
21. Ye, X.; Wong, L.Y.; Kramer, J.; Zhou, X.; Jia, T.; Calafat, A.M. Urinary Concentrations of Bisphenol A and Three Other Bisphenols in Convenience Samples of U.S. Adults during 2000–2014. *Environ. Sci. Technol.* 2015;49:11834–11839. doi: 10.1021/acs.est.5b02135.
22. Backhaus, T.; Faust, M. Predictive environmental risk assessment of chemical mixtures: A conceptual framework. *Environ. Sci. Technol.* 2012;46:2564–2573. doi: 10.1021/es2034125.
23. Gómez, L.; Niegowska, M.; Navarro, A.; Amendola, L.; Arukwe, A.; Ait-Aissa, S.; Balzamo, S.; Barreca, S.; Belkin, S.; Bittner, M.; et al. Estrogenicity of chemical mixtures revealed by a panel of bioassays. *Sci. Total Environ.* 2021;785:147284. doi: 10.1016/j.scitotenv.2021.147284.
24. Kortenkamp, A.; Faust, M. Regulate to reduce chemical mixture risk. *Science.* 2018;361:224–226. doi: 10.1126/science.aat9219.
25. Skledar, D.G.; Mašič, L.P. In vitro estrogenic activity of binary and multicomponent mixtures with bisphenol A. *Sci. Total Environ.* 2020;707:1352111. doi: 10.1016/j.scitotenv.2019.135211.
26. Thrupp, T.J.; Runnalls, T.J.; Scholze, M.; Kugathas, S.; Kortenkamp, A.; Sumpter, J.P. The consequences of exposure to mixtures of chemicals: Something from "nothing" and "a lot from a little" when fish are exposed to steroid hormones. *Sci. Total Environ.* 2018;619–620:1482–1492. doi: 10.1016/j.scitotenv.2017.11.081.
27. Doan, T.Q.; Cannolly, L.; Igout, A.; Nott, K.; Muller, M.; Scippo, M.I. In vitro profiling of the potential endocrine disrupting activities affecting steroid and aryl hydrocarbon receptors of compounds and mixtures prevalent in human drinking water resources. *Chemosphere.* 2020;258:127332. doi: 10.1016/j.chemosphere.2020.127332.
28. Silva, E.; Rajapakse, N.; Kortenkamp, A. Something from "nothing"—Eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environ. Sci. Technol.* 2002;36:1751–1756. doi: 10.1021/es0101227.
29. Christiansen, S.; Axelstad, M.; Scholze, M.; Johansson, H.K.L.; Hass, U.; Mandrup, K.; Frandsen, H.L.; Frederiksen, H.; Isling, L.K.; Boberg, J. Grouping of endocrine disrupting chemicals for mixture risk assessment—Evidence from a rat study. *Environ. Int.* 2020;142:105870. doi: 10.1016/j.envint.2020.105870.

30. Hercog, K.; Maisanaba, S.; Filipič, M.; Sollner-Dolenc, M.; Kač, L.; Žegura, B. Genotoxic activity of bisphenol A and its analogues bisphenol S, bisphenol F and bisphenol AF and their mixtures in human hepatocellular carcinoma (HepG2) cells. *Sci. Total Environ.* 2019;687:267–276. doi: 10.1016/j.scitotenv.2019.05.486.
31. Jatkowska, N.; Kudłak, B.; Lewandowska, P.; Liu, W.; Williams, M.J.; Schiöth, H.B. Identification of synergistic and antagonistic actions of environmental pollutants: Bisphenols A, S and F in the presence of DEP, DBP, BADGE and BADGE·2HCl in three component mixtures. *Sci. Total Environ.* 2021;767:144286. doi: 10.1016/j.scitotenv.2020.144286.
32. Repouskou, A.; Papadopoulou, A.K.; Panagiotidou, E.; Trichas, P.; Lindh, C.; Bergman, Å.; Gennings, C.; Bornehag, C.G.; Rüegg, J.; Kitraki, E.; et al. Long term transcriptional and behavioral effects in mice developmentally exposed to a mixture of endocrine disruptors associated with delayed human neurodevelopment. *Sci. Rep.* 2020;10:9367. doi: 10.1038/s41598-020-66379-x.
33. Yu, H.; Caldwell, D.J.; Suri, R.P. In vitro estrogenic activity of representative endocrine disrupting chemicals mixtures at environmentally relevant concentrations. *Chemosphere.* 2019;215:396–403. doi: 10.1016/j.chemosphere.2018.10.067.
34. Kassotis, C.D.; Hoffman, K.; Phillips, A.L.; Zhang, S.; Cooper, E.M.; Webster, T.F.; Stapleton, H.M. Characterization of adipogenic, PPARγ, and TRβ activities in house dust extracts and their associations with organic contaminants. *Sci. Total Environ.* 2021;758:143707. doi: 10.1016/j.scitotenv.2020.143707.
35. Biemann, R.; Fischer, B.; Navarrete Santos, A. Adipogenic effects of a combination of the endocrine-disrupting compounds bisphenol A, diethylhexylphthalate, and tributyltin. *Obes. Facts.* 2014;7:48–56. doi: 10.1159/000358913.
36. Mentor, A.; Brunström, B.; Mattsson, A.; Jönsson, M. Developmental exposure to a human relevant mixture of endocrine disruptors alters metabolism and adipogenesis in zebrafish (Danio rerio). *Chemosphere.* 2020;238:124584. doi: 10.1016/j.chemosphere.2019.124584.
37. Gore, A.C.; Chappell, V.A.; Fenton, S.E.; Flaws, J.A.; Nadal, A.; Prins, G.S.; Toppari, J.; Zoeller, R.T. Executive Summary to EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-

Disrupting Chemicals. *Endocr. Rev.* 2015;36:593–602. doi: 10.1210/er.2015-1093.
38. Grimaldi, M.; Boulahtouf, A.; Toporova, L.; Balaguer, P. Functional profiling of bisphenols for nuclear receptors. *Toxicology.* 2019;420:39–45. doi: 10.1016/j.tox.2019.04.003.
39. Riu, A.; le Maire, A.; Grimaldi, M.; Audebert, M.; Hillenweck, A.; Bourguet, W.; Balaguer, P.; Zalko, D. Characterization of Novel Ligands of ERα, Erβ, and PPARγ: The Case of Halogenated Bisphenol A and Their Conjugated Metabolites. *Toxicol. Sci.* 2011;122:372–382. doi: 10.1093/toxsci/kfr132.
40. Riu, A.; Grimaldi, M.; le Maire, A.; Bey, G.; Phillips, K.; Boulahtouf, A.; Perdu, E.; Zalko, D.; Bourguet, W.; Balaguer, P. Peroxisome proliferator-activated receptor γ is a target for halogenated analogs of bisphenol A. *Environ. Health Perspect.* 2011;119:1227–1232. doi: 10.1289/ehp.1003328.
41. Molina-Molina, J.M.; Amaya, E.; Grimaldi, M.; Sáenz, J.M.; Real, M.; Fernández, M.F.; Balaguer, P.; Olea, N. In vitro study on the agonistic and antagonistic activities of bisphenol-S and other bisphenol-A congeners and derivatives via nuclear receptors. *Toxicol. Appl. Pharmacol.* 2013;272:127–136. doi: 10.1016/j.taap.2013.05.015.
42. Delfosse, V.; Grimaldi, M.; Pons, J.L.; Boulahtouf, A.; Le Maire, A.; Cavailles, V.; Labesse, G.; Bourguet, W.; Balaguer, P. Structural and mechanistic insights into bisphenols action provide guidelines for risk assessment and discovery of bisphenol A substitutes. *Proc. Natl. Acad. Sci. USA.* 2012;109:14930–14935. doi: 10.1073/pnas.1203574109.
43. Reina-Pérez, I.; Olivas-Martínez, A.; Mustieles, V.; Ruiz-Ojeda, F.J.; Molina-Molina, J.M.; Olea, N.; Fernández, M.F. Bisphenol F and bisphenol S promote lipid accumulation and adipogenesis in human adipose-derived stem cells. *Food Chem. Toxicol.* 2021;152:112216. doi: 10.1016/j.fct.2021.112216.
44. Zhou, R.; Xia, M.; Zhang, L.; Cheng, W.; Yan, J.; Sun, Y.; Wang, Y.; Jiang, H. Individual and combined effects of BPA, BPS and BPAF on the cardiomyocyte differentiation of embryonic stem cells. *Ecotoxicol. Environ. Saf.* 2021;220:112366. doi: 10.1016/j.ecoenv.2021.112366.
45. Ruiz-Ojeda, F.J.; Rupérez, A.I.; Gomez-Llorente, C.; Gil, A.; Aguilera, C.M. Cell Models and Their Application for Studying Adipogenic Differentiation in Relation to Obesity: A Review. *Int. J. Mol. Sci.* 2016;17:1040. doi: 10.3390/ijms17071040.

46. Pinto, C.; Grimaldi, M.; Boulahtouf, A.; Pakdel, F.; Brion, F.; Aït-Aïssa, S.; Cavaillès, V.; Bourguet, W.; Gustafsson, J.A.; Bondesson, M.; et al. Selectivity of natural, synthetic and environmental estrogens for zebrafish estrogen receptors. *Toxicol. Appl. Pharmacol.* 2014;280:60–69. doi: 10.1016/j.taap.2014.07.020.
47. Park, C.; Song, H.; Choi, J.; Sim, S.; Kojima, H.; Park, J.; Iida, M.; Lee, Y.J. The mixture effects of bisphenol derivatives on estrogen receptor and androgen receptor. *Environ. Pollut.* 2020;260:114036. doi: 10.1016/j.envpol.2020.114036.
48. Ramírez-Zacarías, J.L.; Castro-Muñozledo, F.; Kuri-Harcuch, W. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with oil red O. *Histochemistry.* 1992;97:493–497. doi: 10.1007/BF00316069.
49. Schmittgen, T.D.; Livak, K.J. Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 2008;3:1101–1108. doi: 10.1038/nprot.2008.73.
50. Strober, W. Trypan Blue Exclusion Test of Cell Viability. *Curr. Protoc. Immunol.* 2015;11:A3.B.1–A3.B.3. doi: 10.1002/0471142735.ima03bs111.
51. Boucher, J.G.; Ahmed, S.; Atlas, E. Bisphenol S induces adipogenesis in primary human preadipocytes from female donors. *Endocrinology.* 2016;157:1397–1407. doi: 10.1210/en.2015-1872.
52. Boucher, J.G.; Gagné, R.; Rowan-Carroll, A.; Boudreau, A.; Yauk, C.L.; Atlas, E. Bisphenol A and bisphenol S induce distinct transcriptional profiles in differentiating human primary preadipocytes. *PLoS ONE.* 2016;11:e0163318. doi: 10.1371/journal.pone.0163318.
53. Ohlstein, J.F.; Strong, A.L.; McLachlan, J.A.; Gimble, J.M.; Burow, M.E.; Bunnell, B.A. Bisphenol a enhances adipogenic differentiation of human adipose stromal/stem cells. *J. Mol. Endocrinol.* 2014;53:345–353. doi: 10.1530/JME-14-0052.
54. Wang, R.Y.; Abbott, R.D.; Zieba, A.; Borowsky, F.E.; Kaplan, D.L. Development of a Three-Dimensional Adipose Tissue Model for Studying Embryonic Exposures to Obesogenic Chemicals. *Ann. Biomed. Eng.* 2017;45:1807–1818. doi: 10.1007/s10439-016-1752-x.
55. Boucher, J.G.; Boudreau, A.; Atlas, E. Bisphenol A induces differentiation of human preadipocytes in the absence of glucocorticoid and is inhibited by an estrogen-receptor antagonist. *Nutr. Diabetes.* 2014;4:e102. doi: 10.1038/nutd.2013.43.

56. Gao, P.; Wang, L.; Yang, N.; Wen, J.; Zhao, M.; Su, G.; Zhang, J.; Weng, D. Peroxisome proliferator-activated receptor gamma (PPARγ) activation and metabolism disturbance induced by bisphenol A and its replacement analog bisphenol S using in vitro macrophages and in vivo mouse models. *Environ. Int.* 2020;134:1005328. doi: 10.1016/j.envint.2019.105328.
57. Desai, M.; Ferrini, M.G.; Jellyman, J.K.; Han, G.; Ross, M.G. In vivo and in vitro bisphenol A exposure effects on adiposity. *J. Dev. Orig. Health Dis.* 2018;9:678–687. doi: 10.1017/S2040174418000600.
58. Ahmed, S.; Atlas, E. Bisphenol S- and bisphenol A-induced adipogenesis of murine preadipocytes occurs through direct peroxisome proliferator-activated receptor gamma activation. *Int. J. Obes. (Lond)* 2016;40:1566–1573. doi: 10.1038/ijo.2016.95.
59. Choi, S.I.; Lee, J.S.; Lee, S.; Sim, W.S.; Kim, Y.C.; Lee, O.H. Potentilla rugulosa nakai extract attenuates bisphenol A-, S-and F-induced ROS production and differentiation of 3T3-L1 preadipocytes in the absence of dexamethasone. *Antioxidants (Basel).* 2020;9:113. doi: 10.3390/antiox9020113.
60. Héliès-Toussaint, C.; Peyre, L.; Costanzo, C.; Chagnon, M.C.; Rahmani, R. Is bisphenol S a safe substitute for bisphenol A in terms of metabolic function? An in vitro study. *Toxicol. Appl. Pharmacol.* 2014;280:224–235. doi: 10.1016/j.taap.2014.07.025.
61. Martínez, M.; Blanco, J.; Rovira, J.; Kumar, V.; Domingo, J.L.; Schuhmacher, M. Bisphenol A analogues (BPS and BPF) present a greater obesogenic capacity in 3T3-L1 cell line. *Food Chem. Toxicol.* 2020;140:111298. doi: 10.1016/j.fct.2020.111298.
62. Ramskov Tetzlaff, C.N.; Svingen, T.; Vinggaard, A.M.; Rosenmai, A.K.; Taxvig, C. Bisphenols B, E, F, and S and 4-cumylphenol induce lipid accumulation in mouse adipocytes similarly to bisphenol A. *Environ. Toxicol.* 2020;35:543–552. doi: 10.1002/tox.22889.
63. Dong, H.; Yao, X.; Liu, S.; Yin, N.; Faiola, F. Non-cytotoxic nanomolar concentrations of bisphenol A induce human mesenchymal stem cell adipogenesis and osteogenesis. *Ecotoxicol. Environ. Saf.* 2018;164:448–454. doi: 10.1016/j.ecoenv.2018.08.052.
64. Cohen, I.C.; Cohenour, E.R.; Harnett, K.G.; Schuh, S.M. BPA, BPAF and TMBPF Alter Adipogenesis and Fat Accumulation in Human Mesenchymal Stem Cells, with Implications for Obesity. *J. Mol. Sci.* 2021;22:5363. doi: 10.3390/ijms22105363.

65. Zhu, X.; Wu, G.; Xing, Y.; Wang, C.; Yuan, X.; Li, B. Evaluation of single and combined toxicity of bisphenol A and its analogues using a highly-sensitive micro-biosensor. *J. Hazard. Mater.* 2020;381:120908. doi: 10.1016/j.jhazmat.2019.120908.
66. Harnett, K.G.; Chin, A.; Schuh, S.M. Cytotoxic and apoptotic data of BPA and BPA alternatives TMBPF, BPAF, and BPS in female adult rat and human stem cells. *Data Brief.* 2021;37:107183. doi: 10.1016/j.dib.2021.107183.
67. Harnett, K.G.; Chin, A.; Schuh, S.M. BPA and BPA alternatives BPS, BPAF, and TMBPF, induce cytotoxicity and apoptosis in rat and human stem cells. *Ecotoxicol. Environ. Saf.* 2021;216:112210. doi: 10.1016/j.ecoenv.2021.112210.
68. Wang, J.; Sun, B.; Hou, M.; Pan, X.; Li, X. The environmental obesogen bisphenol A promotes adipogenesis by increasing the amount of 11β-hydroxysteroid dehydrogenase type 1 in the adipose tissue of children. *Int. J. Obes. (Lond)* 2013;37:999–1005. doi: 10.1038/ijo.2012.173.
69. Kassotis, C.D.; Stapleton, H.M. Endocrine-mediated mechanisms of metabolic disruption and new approaches to examine the public health threat. *Front. Endocrinol. (Lausanne)* 2019;10:39. doi: 10.3389/fendo.2019.00039.
70. Luo, L.; Liu, M. Adipose tissue in control of metabolism. *J. Endocrinol.* 2016;231:R77–R99. doi: 10.1530/JOE-16-0211.
71. Lefterova, M.I.; Lazar, M.A. New developments in adipogenesis. *Trends Endocrinol. Metab.* 2009;20:107–114. doi: 10.1016/j.tem.2008.11.005.
72. Le Fol, V.; Aït-Aïssa, S.; Sonavane, M.; Porcher, J.M.; Balaguer, P.; Cravedi, J.P.; Zalko, D.; Brion, F. In vitro and in vivo estrogenic activity of BPA, BPF and BPS in zebrafish-specific assays. *Ecotoxicol. Environ. Saf.* 2017;142:150–156. doi: 10.1016/j.ecoenv.2017.04.009.
73. Schaffert, A.; Krieg, L.; Weiner, J.; Schlichting, R.; Ueberham, E.; Karkossa, I.; Bauer, M.; Landgraf, K.; Junge, K.M.; Wabitsch, M.; et al. Alternatives for the worse: Molecular insights into adverse effects of bisphenol a and substitutes during human adipocyte differentiation. *Environ. Int.* 2021;156:106730. doi: 10.1016/j.envint.2021.106730.
74. Kudłak, B.; Wieczerzak, M.; Namieśnik, J. Bisphenols (A, S, and F) affect the basic hormonal activity determined for pharmaceuticals—Study of Saccharomyces cerevisiae. *Environ. Pollut.* 2019;246:914–920. doi: 10.1016/j.envpol.2018.12.052.
75. Kassotis, C.D.; Hoffman, K.; Völker, J.; Pu, Y.; Veiga-Lopez, A.; Kim, S.M.; Schlezinger, J.J.; Bovolin, P.; Cottone, E.; Saraceni, A.; et al.

Reproducibility of adipogenic responses to metabolism disrupting chemicals in the 3T3-L1 pre-adipocyte model system: An interlaboratory study. *Toxicology.* 2021;461:152900. doi: 10.1016/j.tox.2021.152900.

76. Kassotis, C.D.; Vom Saal, F.S.; Babin, P.J.; Lagadic-Gossmann, D.; Le Mentec, H.; Blumberg, B.; Mohajer, N.; Legrand, A.; Munic Kos, V.; Martin-Chouly, C.; et al. Obesity III: Obesogen assays: Limitations, strengths, and new directions. *Biochem. Pharmacol.* 2022;199:115014. doi: 10.1016/j.bcp.2022.115014.

Article #3

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Cross-sectional associations of Persistent Organic Pollutants measured in adipose tissue and Metabolic Syndrome in clinically diagnosed middle-aged adults

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Highlights

• HCB and γ-HCH fat levels were associated with metabolic syndrome and its components.

• HCB and γ-HCH were positively associated with blood pressure and glucose levels.

• HCB, *o,p'*-DDT, *p,p'*-DDE and γ-HCH were correlated with increased waist circumference.

• Higher fat HCB levels were inversely associated with HDL cholesterol levels.

Abstract

Introduction: Although often overlooked in clinical settings, accumulation of persistent organic pollutants (POPs) in visceral adipose tissue (VAT) is thought to be a relevant risk factor for metabolic syndrome (MetS).

Methods: One hundred and seventeen patients undergoing non-oncological surgery were randomly recruited and classified as MetS+ if presented 3 out of the 5 MetS components: waist circumference (WC), systolic and diastolic blood pressure (SBP and DBP, respectively), serum glucose, insulin, triglycerides (TG) and high-density lipoprotein (HDL) cholesterol levels, according International Diabetes Federation (IDF) criteria. Seventeen organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) were measured in adipose tissue samples. Linear, logistic and weighted quantile sum regression (WQS) models regression models, adjusted for age and sex, were performed.

Results: One third of the participants were males (36.8%) with a median age of 44 years, showing clinical evidences of MetS (35.0%). Adjusted linear regression models showed that WC correlated positively with all OCP concentrations. Higher fasting serum glucose levels were related to higher HCB and γ-HCH concentrations. The remaining OCPs and PCBs were not associated with this MetS component. HCB was inversely associated with HDL cholesterol levels, while PCB-180 was positively associated. HCB and γ-HCH concentrations were positively correlated with DBP and SBP levels. PCB-138 was also positively associated with SBP. Adjusted logistic models revealed that exposure to HCB and γ-HCH were associated with increased odds of MetS [ORs (95% CI) = 1.53 (1.22-1.92) and 1.39

(1.10-1.76) respectively; p< 0.01]. No associations were observed for the remaining POPs. WQS models showed a positive and significant mixture effect of POPs on the odds of MetS (exp[beta] = 2.34; p<0.001), with γ-HCH (52.9%), *o,p'*-DDT (26.9%) and HCB (19.7%) driving the association.

Conclusions: Our findings support that POPs in VAT, specifically HCB and γ-HCH, are associated with both isolated components and clinically-diagnosed MetS.

Keywords

Adipose tissue, metabolic syndrome (MetS), endocrine disruptors, persistent organic pollutants (POPs), hexachlorobenzene (HCB), hexachlorohexane (HCH)

Abbreviations

Adipose tissue (AT), Adult Treatment Panel III (ATPIII), American Association of Clinical Endocrinologists (AACE), body mass index (BMI), diastolic blood pressure (DBP), dichlorodiphenyltrichloroethane (DDT), gas chromatography and mass spectrometry (GC-MS/MS), hexachlorobenzene (HCB), hexachlorohexane (HCH), high-density lipoprotein (HDL), high-performance liquid chromatography (HPLC), International Diabetes Federation (IDF), limit of detection (LOD), metabolic syndrome (MetS), metabolism-disrupting chemicals (MDCs), organochlorine pesticides (OCPs), *o,p'*-dichlorodiphenyltrichloroethane (*o,p'*-DDT), *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE), persistent organic pollutants (POPs), polychlorinated biphenyls (PCBs), standard deviation (SD), systolic blood pressure (SBP), triglycerides (TG), type-2 diabetes (T2D), visceral adipose tissue (VAT), waist circumference (WC), weight/height squared (kg/m^2), World Health Organization (WHO)

1. Introduction

Metabolic syndrome (MetS) describes a complex cluster of adverse health conditions (also named 'MetS components') that substantially increases the risk of other chronic diseases such as heart failure, type-2 diabetes (T2D) or non-alcoholic fatty liver disease (Mustieles and Arrebola, 2020). In spite of several subsets of criteria proposed by different international organizations [Cholesterol Education

Program's Adult Treatment Panel III (ATPIII), World Health Organization (WHO), and American Association of Clinical Endocrinologists (AACE)], the working definition for MetS classification used worldwide was proposed in 2009 by the International Diabetes Federation (IDF). It included 5 MetS components: fasting glucose, triglycerides (TG) and high-density lipoprotein (HDL) cholesterol, as well as blood pressure and waist circumference (WC), considering that the presence of any 3 out 5 MetS components constitutes a clinical diagnosis of MetS (Alberti et al., 2009). Although the trends of elevated TG and blood pressure have decreased in the last decade, mainly due to increased prescription of anti-hypertensives and lipid modifying agents (Shin et al., 2018), some reports that have assessed the prevalence of MetS, for example in the United States, have found an increase over the years (from 25.3% in 1988–1994 to 36.9% in 2015–2016) (Hirode and Wong, 2020; Moore et al., 2017). This is particularly relevant given the enormous economic costs derived from MetS patients as well as the healthcare expenditure associated to POP exposure (Pérez-Carrascosa et al., 2022, 2021).

It remains unclear which factors trigger the development of MetS, although all indicates that MetS has a multifactorial origin (Fahed et al., 2022; Lam and LeRoith., 2019). In this regard, it has been suggested that, in addition to unhealthy lifestyle factors (excessive calorie intake and sedentary lifestyle), body fat distribution, birth weight, smoking habits during pregnancy and maternal weight would be important determinants (Fahed et al., 2022; González-Jiménez et al., 2015; Müller et al., 2012). Also, human exposure to some environmental chemicals has been linked to the development of some metabolic disorders such as obesity and T2D, those classified as 'metabolism-disrupting chemicals' (MDCs) (Heindel et al., 2015; Nadal et al., 2017; Sun et al., 2022). These MDCs include persistent organic pollutants (POPs), a wide group of synthetic chemicals [including organochlorine pesticides (OCPs) such as dichlorodiphenyltrichloroethane (DDT) and its metabolite *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE), hexachlorobenzene (HCB), hexachlorohexane (HCH), and polychlorinated biphenyls (PCBs)], with a tendency to bioaccumulate in fat compartments, given their lipophilicity and high environmental persistence (Bokobza et al., 2021; La Merrill et al., 2013). Adipose tissue (AT) is therefore regarded as the main reservoir of these compounds, accounting for all routes and sources of exposure and representing a stable and long-term reservoir of these chemicals

(Artacho-Cordón et al., 2015b; Bokobza et al., 2021; Jackson et al., 2017; Kohlmeier and Kohlmeier, 1995; La Merrill et al., 2013). Despite the fact that the production, handling and disposal of POPs was banned or severely restricted in most countries starting in the early 1970s, levels of POPs are currently still detected in biological samples of most humans, worldwide (Artacho-Cordón et al., 2015a; Bergonzi et al., 2011; Bjerregaard-Olesen et al., 2017; Björvang et al., 2021; Centers for Disease Control and Prevention, 2017; Fernández-Rodríguez et al., 2015).

Contrary to the traditional consideration of AT as a simple energy store depot, its relevant immunological and endocrine roles have been acknowledged during the last decades (Kershaw and Flier, 2004). In fact, AT dysfunction has been demonstrated to be the main precursor of MetS (Fahed et al., 2022, van Greevenbroek et al., 2016). Although the exact mechanisms of action for the development of MetS are not fully understood, hormonal imbalance (Banos et al., 2011), chronic low-grade inflammation (Esser et al., 2014), altered redox status (Höhn et al., 2016; Seillier et al., 2015; Spanidis et al., 2016) and mitochondrial dysfunction in AT (Fahed et al., 2022; Grundy, 2016; Kim and Lee, 2014), may play a crucial role. In this regards, exposure to POPs might interfere with the endocrine system (Grünfeld and Bonefeld-Jorgensen, 2004) and it has been also related to inflammatory responses (Pandolfi et al., 2016; Rolle-Kampczyk et al., 2020), macromolecule oxidation (Artacho-Cordón et al., 2016), and mitochondrial dysfunction (Kim et al., 2019). Although mounting research suggests a relevant role of POP exposure in the development of MetS, the relationship between POPs exposure and MetS has not been fully elucidated. Many of the epidemiological studies that assessed POPs exposure did so in serum samples (Lee et al., 2007, 2011, 2014; Tomar et al., 2013), which may be influenced by point exposures or altered by AT dynamics (Mustieles and Arrebola, 2020). Furthermore, most of the investigations that have observed relationships between AT POP concentrations and risk of MetS components (Arrebola et al., 2015, 2014; Mustieles et al., 2017; Pestana et al., 2014; Tawar et al., 2022; Valvi et al., 2020), often did not include clinical diagnoses of MetS as an outcome (Mustieles et al., 2017). The recent systematic review by Lamat et al. (2022) indicates that the occurrence of MetS would be linked to visceral adiposity, supporting that the influence of adiposity may be a

very important factor for the influence of POP exposure on MetS (Mustieles et al., 2017).

Therefore, given the relevance of visceral adiposity disruption of AT physiology in the development of MetS, the aim of this study was to evaluate the relationship between cumulative POP concentrations in AT and the presence of individual MetS components, as well as to assess the risk of clinically diagnosis of MetS in a hospitalized adult population from Southern Spain.

2. Materials and methods

2.1. Study population and sample collection

The population of this cross-sectional clinical study consisted of 117 middle-aged adults selected from among patients undergoing non-oncologic surgery (mostly hiatal hernia, umbilical hernia, inguinal hernia, para-oesophageal hernia, achalasia and cholecystectomy), in both the Endocrinology and Nutrition Unit and the Surgery Unit at the Virgen de la Victoria University Hospital (Málaga, Spain).

The recruitment took place between 2013 and 2015. Exclusion criteria included patients receiving blood glucose-lowering treatment, major cardiovascular disease in the last 6 months before recruitment, evidence of acute/chronic inflammatory disease (such us chronic inflammatory bowel diseases, ulcerative colitis and Crohn's disease), cancer patients and infectious diseases, as well as those who did not signed the informed consent to participate in this study. Sociodemographic data was recorded during recruitment. The height and weight of the participants were recorded, and body mass index (BMI) as weight/height squared (kg/m^2), was calculated. All participant data were extracted from medical records.

Approximately 300 mg of visceral adipose tissue (VAT) and 10 mL of blood were collected under fasting conditions during surgery. Blood samples were immediately centrifuged for 5 min at 2500 rpm and 4 °C to separate the serum. Both serum and AT samples were immediately coded and stored at −80 °C until chemical analysis. Blood samples were used to determine clinical biomarkers of MetS.

The study was approved by the Ethics Committee of Virgen de la Victoria Hospital before all participants were informed of the objective and characteristics of the study and gave their signed consent. The

study was conducted in accordance with the Declaration of Helsinki, and all the information collected was anonymized. Once all the fat samples had been collected, they were sent to the chemistry laboratories of the ibs. GRANADA/University of Granada (Spain) for analysis.

2.2. Adipose tissue sample extraction and chemical analyses

Concentrations of 17 POPs were determined in 150 mg of AT, as previously described (Martínez Vidal et al., 2002; Moreno Frías et al., 2004). AT samples were spiked with p-chlorobenzophenone as internal standard, and extracted following a two-step methodology, consisting on a mechanical homogenization in n-hexane followed by a filtration through a glass column with 200 mg of alumina. Extracts were dried under a continuous nitrogen stream and fractionated in duplicate by high-performance liquid chromatography (HPLC). Residues of *o,p'*-DDT, *p,p'*-DDE, HCB, α-HCH, β-HCH γ-HCH, aldrin, isodrin, endrin, dieldrin, heptachlor, vinclozolin, endosulfan I and II and PCBs (congeners −138, −153, and −180) were quantified by gas chromatography and mass spectrometry (GC-MS/MS) with an Agilent 7890 A system and MS Agilent 7000 GC/MS (triple quadrupole) mass spectrometer (Agilent Tehcnologies, Santa Clara, CA) after reconstitution in 200 µL *n*-hexane (Arrebola et al., 2014; Moreno Frías et al., 2004). Procedural laboratory blanks with solvents alone were tested and always yielded a negative result. Laboratory fortified matrix samples at different concentrations were used for quality control procedures. Inter- and intra-day variabilities were calculated by analyzing fortified samples within the same day (repeatability) and on different days (intermediate precision), always yielding values < 20%, respectively. The limit of detection (LOD) was determined as the smallest amount of the analyte that gave a signal-to-noise ratio ≥3 and was set at 4 ng/mL for *o,p'*-DDT and *p,p'*-DDE, and 2 ng/mL for the rest of POPs. Concentrations below the LOD were assigned half of the LOD.

2.3. Assessment of MetS components and clinical diagnosis of MetS

Waist circumference (WC) was measured at the nearest 0.1 cm using an inelastic tape in a standing position and after a gentle exhalation by trained clinicians using standardized protocols. Fasting glucose, TG and HDL cholesterol levels were quantified in serum samples by using a Dimension autoanalyzer, Dade Behring Inc. (Deerfield, IL) in the analytical unit of Virgen de la Victoria Hospital. Diastolic and systolic

blood pressures (DBP and SBP, respectively) were also measured. Blood pressure was measured twice in a sitting position after a 5-min rest at 5-min intervals, and the mean of both measurements used.

Following the IDF working group criteria, the following 5 MetS components were considered and their respective cut-off points: fasting glucose (≥100 mg/dL), TG (≥150 mg/dL), HDL cholesterol (<40 mg/dL in males and <50 mg/dL in females), blood pressure [SBP ≥130 mmHg and/or DBP ≥85 mmHg], and WC (≥94 cm for males and ≥80 cm for females from European population). In addition, participants were classified as MetS+ when the simultaneous presence of 3 of the above 5 MetS components was detected. Those participants with less than 3 MetS components were included in the MetS- group (Alberti et al., 2009).

2.4. Statistical analyses

Description of continuous variables was performed using arithmetic mean ± standard deviation (SD), median and 25th-75th percentiles, while categorical variables were expressed as percentages.

Individual MetS components were considered as continuous, but also as categorical variables considering the cut-off points established in the IDF working definition for the clinical diagnosis of MetS (MetS +/−) (Alberti et al., 2009). POP concentrations were natural-log transformed to reduce the skewness of the distributions and the influence of extreme values, especially in the context of a modest sample size. Spearman's correlation test was used to assess relationships between POPs concentrations (Fig. 1). Continuous outcomes were also log-transformed. The linearity of the relationship between POP exposure and MetS components was examined using generalized additive models (GAMs), supporting the modelling of POP concentrations as continuous variables (Fig. S1). Thus, associations between concentrations of selected POPs (HCB, *o,p'*-DDT, *p,p'*-DDE, γ-HCH, PCB-138, PCB-153 and PCB-180) and MetS components were examined using both multivariable linear and logistic regression analyses when outcomes were coded as continuous or categorical variables, respectively. Linear regression models were also performed using dichotomized exposure variables (exposed *vs*. not exposed) for those POPs with ~50% below LOD (HCB, *o,p'*-DDT, γ-HCH) in the adipose samples. Furthermore, logistic regression analyses were performed to explore the influence of AT levels of selected POPs on the risk of

clinically-diagnosed MetS. All models were adjusted for age (years) and sex (male/female) as potential confounders. In addition, a sensitivity analysis was performed further adjusting models by BMI.

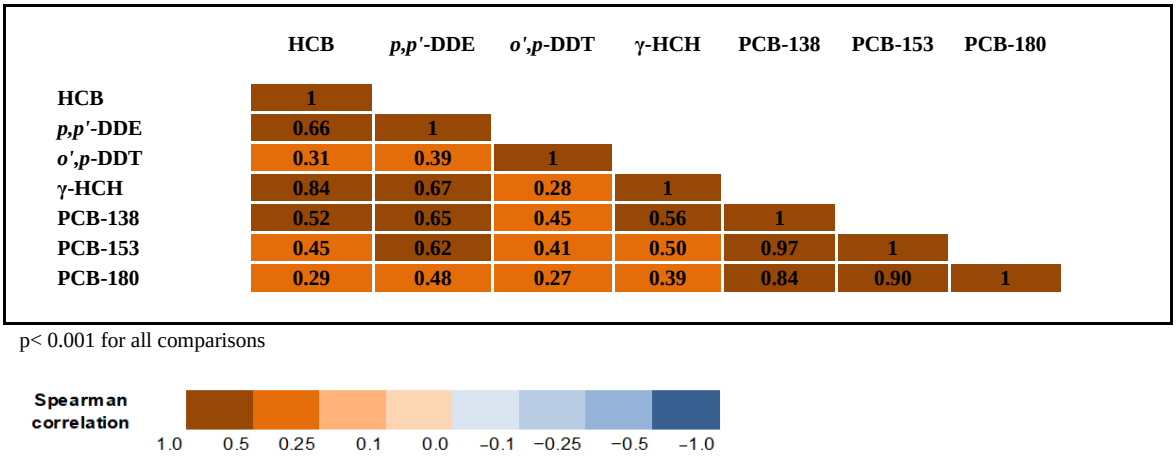

Fig. 1. Correlation heatmap for POP concentrations in adipose tissue samples.

The potential POP mixture effect on MetS was assessed using Weighted Quantile Sum Regression (WQS) (Carrico et al., 2015), which combines the individual associations into a weighted index, and estimates the specific weight of each chemical on the mixture. Associations between each WQS index and the outcome were further studied by using multivariable logistic regression adjusting for age, sex and BMI. The WQS analyses were performed with log-transformed continuous pollutant concentrations, using a training set defined as a 40% random sample of the dataset, being the remaining 60% used for model validation. The final weights were calculated using a total of 1000 bootstrap steps. On the basis of our hypothesis and the associations found in the models for individual chemicals, the WQS index was calculated for a priori assumed positive associations.

The results were interpreted taking into their internal validity, consistency and coherence, the existing epidemiological and toxicological evidence, and not exclusively considering the statistical significance (Amrhein et al., 2019). Given the hypothesis investigated in this work, and the moderate number of comparisons tested, we did not perform a post-hoc correction for multiple comparisons to avoid a

disproportionate increase in the frequency of type II errors (Rothman, 2014). Diagnosis of the models was performed in order to ensure the goodness of fit and the fulfilment of implementation conditions. The significance level was set at 0.05 (two-sided), and data were stored and processed using SPSS 20.0 (IBM, Chicago, IL).

3. Results

3.1. Characteristics of the study population and adipose tissue concentrations of selected POPs

Table 1 summarizes the characteristics of the study population. A total of 41 participants (35.0%) had a clinical diagnosis of MetS (i.e., showed at least 3 components of MetS established by the IDF classification). More than one third of the sample population was male (36.8%) and the median age across the entire study population was 44 years, with older individuals in the MetS+ group relative to the MetS- group (50 *vs.* 42 years, $p = 0.04$). As expected, compared with the MetS- group, participants in the MetS+ group showed higher BMI (34.2 kg/m^2 *vs.* 30.5 kg/m^2, respectively; $p < 0.01$) and WC (121.0 cm *vs.* 95 cm, respectively; $p < 0.01$). Median TG and HDL cholesterol concentrations were 160.0 mg/dL and 44.0 mg/dL in the MetS+ group, respectively, whereas lower median concentrations of 92.5 mg/dL and 53.0 mg/dL for TG and HDL cholesterol, respectively, were detected in the MetS- group ($p < 0.01$ in both cases). As for blood pressure, median DBP and SBP were 85.0 mmHg and 140.0 mmHg in the MetS+ group and 76.0 mmHg and 119.0 mmHg in the MetS- group, respectively ($p < 0.01$ in both cases). Considering the IDF cut-off points, 84 out of the 117 participants (68.3%) showed elevated WC, 56 (45.5%) elevated blood pressure, 37 (30.1%) reduced HDL cholesterol, 45 (36.6%) elevated serum TG levels and 17 (13.8%) had elevated fasting blood glucose (data not shown in the tables).

Detection levels and concentrations of OCPs and PCBs in VAT are also shown in Table 1. Median concentrations of HCB and γ-HCH were 4.5 ng/g tissue and 11.4 ng/g tissue, respectively, showing significantly higher concentrations in the MetS+ group (28.6 ng/g tissue and 34.4 ng/g tissue, respectively) than in the MetS- group (median concentrations of both chemicals were below the LOD). Median concentration of *o,p'*-DDT and *p,p'*-DDE were 6.6 ng/g tissue and 49.9 ng/g tissue, showing non-significant differences between MetS groups. Finally, levels of PCBs were 21.2 ng/g tissue, 26.8 ng/g

tissue and 10.0 ng/g tissue for PCB-138, PCB-153 and PCB-180, respectively, with similar median levels in both groups.

3.2. Associations between adipose tissue levels of POPs and MetS components

Table S2 and Table 2 shows unadjusted and adjusted (sex and age) linear regression analyses assessing the influence of POP burden on each individual MetS component (considered as continuous variables), respectively. WC was positively correlated with concentrations of all OCPs, either in the unadjusted analysis or in the multivariate models adjusted. None of the PCB congeners were associated with WC. Higher fasting blood glucose levels were associated with higher HCB and γ-HCH concentrations, while the rest of OCPs and PCBs were not associated with this MetS component. Exposure to HCB was inversely associated with HDL cholesterol levels after adjusting, while PCB-180 and HDL cholesterol were positively associated. Concentrations of HCB and γ-HCH in VAT were positively correlated with TG in the unadjusted analyses and borderline associated in the adjusted models [exp(β) (95%CI) = 1.04 (1.00–1.09), p = 0.07 for HCB and 1.04 (0.99–1.10) p = 0.09 for γ-HCH]. DBP and SBP levels were positively associated with exposure to HCB and γ-HCH, both in the unadjusted and in the multivariate analysis. PCB-138 was also positively associated with SBP after adjusting. Similar results were found for HCB, *o,p'*-DDT, and γ-HCH (POPs around 50% detection) when the assessment was performed between exposed *vs.* not exposed subjects (Table S3). GAMs analyses confirmed the presence of linear relationships for POPs and MetS components (Figure S1).

Additionally, unadjusted (Table S4) and adjusted logistic regression analyses were performed to explore the role of the cumulative POP concentrations in the VAT on the odds of each individual MetS component (considered as categorical variables) (Table 3). Exposure to HCB and γ-HCH consistently showed a positive association for elevated WC and blood pressure, while the association with elevated TG was only detected in the unadjusted analysis. HCB exposure was also associated with higher odds of reduced HDL and borderline associated with elevated blood glucose [OR (95%CI) = 1.22 (0.98–1.53), p = 0.08]. Intriguingly, an inverse relationship was found between exposure to *p,p'*-DDE and the odds of elevated TG in the adjusted models.

Table 1. Characteristics of the study population (N=117)

	Total (N=117)					MetS + (N=41)					MetS - (N=76)					
	DF (%)	Mean	SD	Percentiles			Mean	SD	Percentiles			Mean	SD	Percentiles			p-value
				25	50	75			25	50	75			25	50	75	
Age (yr)		47.14	14.37	38.00	44.00	58.75	50.83	15.22	38.00	50.00	65.00	45.12	13.56	36.00	42.00	53.00	***0.040***
Sex*																	0.217
Male		43 (36.8%)	-	-	-	-	12 (29.3%)	-	-	-	-	31 (40.8%)	-	-	-	-	
Female		74 (63.2%)	-	-	-	-	29 (70.7%)	-	-	-	-	45 (59.2%)	-	-	-	-	
BMI (kg/m^2)		35.25	12.96	24.11	32.05	44.27	39.76	11.56	31.14	34.22	50.81	32.82	13.09	23.15	30.45	38.73	***0.005***
Fasting blood glucose (mg/dL)		100.76	24.09	89.00	94.00	102.00	114.98	32.24	92.50	104.00	133.00	92.88	12.68	87.00	92.00	96.25	***<0.001***
Triglycerides (mg/dL)		130.75	65.04	83.00	115.00	156.00	180.52	59.45	132.90	160.00	214.50	103.18	50.06	71.25	92.50	126.25	***<0.001***
HDL cholesterol (mg/dL)		50.44	12.57	41.00	50.00	58.25	45.44	11.13	37.50	44.00	52.50	53.25	12.52	45.00	53.00	61.00	***0.001***
Diastolic blood pressure (mmHg)		79.59	11.50	70.00	80.00	87.00	85.33	10.58	80.00	85.00	90.00	76.25	10.73	70.00	76.00	83.00	***<0.001***
Systolic blood pressure (mmHg)		128.70	21.50	112.75	125.00	146.00	140.97	21.01	125.00	140.00	153.00	121.55	18.44	110.00	119.00	134.00	***<0.001***
Waist circumference (cm)		106.60	25.73	88.00	102.00	123.00	127.60	23.64	109.00	121.00	151.00	100.28	24.98	82.25	95.00	109.00	***<0.001***
Adipose tissue concentration of POPs (ng/g tissue)																	
Organochlorine pesticides (OCPs)																	
HCB	49.6	36.52	65.31	<LOD	4.47	46.05	65.94	87.14	2.55	28.55	85.85	20.64	42.66	<LOD	<LOD	17.84	***<0.001***
p,p'-DDE	62.6	228.93	672.67	<LOD	49.89	211.39	337.25	1085.22	<LOD	54.83	257.13	170.50	248.72	<LOD	49.66	235.82	0.202
o,p'-DDT	56.1	15.88	36.46	<LOD	6.59	12.25	21.81	49.56	<LOD	7.33	16.05	12.67	26.76	<LOD	6.42	11.44	0.197
γ-HCH	54.5	41.62	80.50	<LOD	11.44	40.94	68.99	113.24	5.83	34.42	70.29	26.85	50.53	<LOD	<LOD	31.46	***0.006***

Polychlorinated biphenyls (PCBs)																
PCB-138	78.0	50.77	137.73	5.77	21.18	50.29	79.07	222.82	9.92	28.63	58.97	35.51	46.75	4.42	18.86	46.24	0.103
PCB-153	79.7	61.17	161.72	6.02	26.79	59.09	92.29	260.59	10.24	31.45	84.88	44.38	58.46	5.56	22.94	59.06	0.127
PCB-180	62.60	35.98	107.15	<LOD	9.95	40.93	52.57	173.03	<LOD	12.05	45.07	27.03	39.47	<LOD	7.99	40.04	0.220

** N (%); BMI: body mass index; DF: Detection frequency; HDL: high density lipoprotein; mean: arithmetic mean; SD: standard deviation*

Table 2. Multivariate linear regression analyses between MetS components and log-transformed adipose tissue POP concentration (ng/g tissue)

	Adjusted Modela					Adjusted Modelb				
	β	**exp(β)**	**CI95%**		**p-value**	**β**	**exp(β)**	**CI95%**		**p-value**
Waist circumference (cm)										
HCB	**0.08**	**1.08**	**1.06**	**1.10**	**<0.001**	0.06	1.06	1.00	1.02	0.236
p,p'-DDE	**0.03**	**1.03**	**1.01**	**1.05**	**0.003**	-0.07	0.94	0.99	1.00	0.112
o,p'-DDT	**0.06**	**1.06**	**1.02**	**1.10**	**0.002**	-0.02	0.98	0.98	1.01	0.635
γ-HCH	**0.07**	**1.07**	**1.05**	**1.10**	**<0.001**	0.07	1.07	1.00	1.02	0.172
PCB-138	0.03	1.03	1.00	1.06	0.074	-0.06	0.94	0.98	1.00	0.143
PCB-153	0.01	1.01	0.98	1.04	0.609	-0.07	0.93	0.98	1.00	0.077
PCB-180	-0.02	0.98	0.96	1.01	0.233	-0.05	0.95	0.98	1.00	0.213
Fasting blood glucose (mg/dL)										
HCB	**0.02**	**1.02**	**1.00**	**1.04**	**0.020**	0.06	1.06	0.98	1.03	0.611
p,p'-DDE	0.00	1.00	0.99	1.02	0.660	-0.06	0.94	0.98	1.01	0.523
o,p'-DDT	0.02	1.02	0.99	1.05	0.308	0.01	1.01	0.97	1.03	0.888
γ-HCH	**0.02**	**1.02**	**1.00**	**1.04**	**0.028**	0.08	1.08	0.99	1.03	0.487
PCB-138	0.01	1.01	0.99	1.04	0.418	0.03	1.03	0.98	1.03	0.772
PCB-153	0.01	1.01	0.98	1.03	0.556	0.05	1.05	0.98	1.03	0.640
PCB-180	0.01	1.01	0.98	1.03	0.573	0.12	1.12	0.99	1.04	0.261
Triglycerides (mg/dL)										
HCB	0.04	1.04	1.00	1.09	0.072	0.05	1.05	0.96	1.07	0.641
p,p'-DDE	-0.03	0.97	0.94	1.02	0.223	-0.22	0.80	0.92	1.00	0.030
o,p'-DDT	-0.02	0.98	0.91	1.05	0.545	-0.13	0.88	0.88	1.02	0.166
γ-HCH	0.04	1.04	0.99	1.10	0.089	0.07	1.07	0.96	1.08	0.556
PCB-138	-0.04	0.96	0.90	1.02	0.189	-0.19	0.83	0.89	1.00	0.067
PCB-153	-0.05	0.95	0.89	1.01	0.080	**-0.20**	**0.82**	**0.89**	**1.00**	**0.049**
PCB-180	-0.05	0.96	0.90	1.01	0.112	-0.14	0.87	0.91	1.02	0.194
HDL cholesterol (mg/dL)										
HCB	**-0.03**	**0.97**	**0.95**	**0.99**	**0.012**	-0.05	0.96	0.97	1.02	0.689

p,p'-DDE	0.00	1.00	0.98	1.02	0.986	0.14	1.16	0.99	1.04	0.149
o,p'-DDT	0.00	1.00	0.96	1.04	0.842	0.10	1.10	0.98	1.06	0.289
γ-HCH	-0.02	0.98	0.96	1.01	0.142	0.05	1.05	0.98	1.03	0.663
PCB-138	0.02	1.02	0.99	1.06	0.133	**0.25**	**1.29**	**1.01**	**1.07**	**0.011**
PCB-153	0.03	1.03	1.00	1.06	0.087	**0.22**	**1.25**	**1.00**	**1.06**	**0.027**
PCB-180	**0.03**	**1.03**	**1.00**	**1.06**	**0.046**	0.17	1.19	1.00	1.05	0.096
Diastolic Blood Pressure (mmHg)										
HCB	**0.02**	**1.02**	**1.00**	**1.03**	**0.028**	0.05	1.05	0.99	1.02	0.677
p,p'-DDE	0.00	1.00	0.98	1.01	0.881	-0.11	0.90	0.98	1.01	0.297
o,p'-DDT	0.01	1.01	0.99	1.04	0.277	0.03	1.03	0.98	1.03	0.772
γ-HCH	**0.02**	**1.02**	**1.00**	**1.04**	**0.033**	0.10	1.10	0.99	1.03	0.421
PCB-138	0.02	1.02	1.00	1.04	0.077	0.15	1.16	0.99	1.03	0.157
PCB-153	0.02	1.02	1.00	1.04	0.099	0.19	1.21	1.00	1.04	0.077
PCB-180	0.01	1.01	0.99	1.03	0.469	0.19	1.21	1.00	1.04	0.089
Systolic Blood Pressure (mmHg)										
HCB	**0.02**	**1.02**	**1.00**	**1.03**	**0.044**	-0.05	0.95	0.98	1.01	0.668
p,p'-DDE	0.00	1.00	0.99	1.02	0.575	-0.05	0.95	0.98	1.01	0.604
o,p'-DDT	0.02	1.02	1.00	1.05	0.077	0.07	1.08	0.99	1.04	0.409
γ-HCH	**0.02**	**1.02**	**1.01**	**1.04**	**0.014**	0.09	1.09	0.99	1.03	0.440
PCB-138	**0.02**	**1.02**	**1.00**	**1.05**	**0.032**	0.17	1.19	1.00	1.04	0.078
PCB-153	0.02	1.02	0.99	1.04	0.172	0.15	1.16	1.00	1.04	0.123
PCB-180	0.00	1.00	0.98	1.02	0.731	0.17	1.18	1.00	1.03	0.112

aAdjusted for age (yr) and sex (male/female); bAdjusted for age (yr) and sex (male/female) and BMI (body mass index); CI: confidence interval

Table 3. ORs (95%CI) of the risk of MetS components according to adipose tissue POP concentration (ng/g tissue)

	Adjusted Modela				Adjusted Modelb			
	OR	**CI95%**		**p-value**	**OR**	**CI95%**		**p-value**
Elevated waist circumference (≥ 94 cm men or ≥ 80 cm women)								
HCB	**1.92**	**1.35**	**2.73**	**<0.001**	0.63	0.31	1.29	0.207
p,p'-DDE	1.06	0.85	1.31	0.625	**0.42**	**0.21**	**0.83**	**0.012**
o,p'-DDT	1.45	0.94	2.22	0.092	0.74	0.33	1.66	0.461
γ-HCH	**1.78**	**1.28**	**2.48**	**0.001**	0.90	0.50	1.64	0.730
PCB-138	1.17	0.86	1.59	0.322	0.81	0.46	1.43	0.473
PCB-153	1.02	0.75	1.39	0.896	0.82	0.46	1.46	0.506
PCB-180	0.85	0.63	1.15	0.294	0.97	0.58	1.64	0.912
Elevated fasting blood glucose (≥100 mg/dL)								
HCB	1.22	0.98	1.53	0.081	1.05	0.81	1.37	0.701
p,p'-DDE	0.99	0.81	1.21	0.921	0.90	0.72	1.12	0.332
o,p'-DDT	1.01	0.70	1.47	0.947	0.87	0.59	1.30	0.500
γ-HCH	1.20	0.93	1.53	0.159	1.03	0.78	1.36	0.829
PCB-138	0.93	0.68	1.27	0.637	0.84	0.61	1.17	0.315
PCB-153	0.92	0.68	1.25	0.584	0.88	0.64	1.22	0.450
PCB-180	0.96	0.72	1.28	0.784	1.01	0.75	1.36	0.951
Elevated triglycerides (≥ 150 mg/dL)								
HCB	1.17	0.95	1.45	0.137	1.22	0.94	1.58	0.135
p,p'-DDE	**0.77**	**0.62**	**0.95**	**0.014**	**0.73**	**0.58**	**0.92**	**0.007**

o,p'-DDT	0.76	0.52	1.10	0.145	0.72	0.49	1.07	0.105
γ-HCH	1.19	0.95	1.51	0.136	1.22	0.93	1.60	0.145
PCB-138	0.77	0.57	1.04	0.086	0.75	0.56	1.02	0.069
PCB-153	0.77	0.57	1.03	0.082	0.77	0.57	1.03	0.076
PCB-180	0.87	0.66	1.15	0.336	0.88	0.67	1.16	0.358
Reduced HDL cholesterol (<40 mg/dL in men or <50 mg/dL in women)								
HCB	**1.28**	**1.04**	**1.57**	**0.022**	1.06	0.83	1.37	0.629
p,p'-DDE	1.07	0.89	1.28	0.497	0.95	0.77	1.16	0.617
o,p'-DDT	1.24	0.89	1.72	0.197	1.06	0.74	1.52	0.760
γ-HCH	1.15	0.92	1.43	0.221	0.94	0.73	1.22	0.636
PCB-138	0.86	0.65	1.12	0.264	**0.73**	**0.54**	**1.00**	**0.049**
PCB-153	0.87	0.67	1.13	0.301	0.81	0.61	1.08	0.154
PCB-180	0.81	0.63	1.05	0.112	0.83	0.63	1.10	0.196
Elevated blood pressure (SBP ≥130mmHg and/or DBP ≥85mmHg)								
HCB	**1.27**	**1.02**	**1.59**	**0.034**	0.95	0.71	1.27	0.726
p,p'-DDE	1.01	0.84	1.22	0.924	0.89	0.71	1.10	0.284
o,p'-DDT	1.20	0.85	1.70	0.295	0.99	0.66	1.48	0.959
γ-HCH	**1.39**	**1.09**	**1.79**	**0.009**	1.16	0.87	1.54	0.315
PCB-138	1.24	0.94	1.64	0.128	1.19	0.87	1.62	0.268
PCB-153	1.11	0.85	1.45	0.450	1.15	0.85	1.55	0.374
PCB-180	1.03	0.80	1.33	0.826	1.24	0.92	1.66	0.152

[a]Adjusted for age (yr) and sex (male/female); [b]Adjusted for age (yr) and sex (male/female) and BMI (body mass index); CI: confidence interval

3.3. Associations between chronic adipose tissue POPs concentrations and MetS risk

The risk for MetS according to AT concentrations of each POP is shown in Table 4. Both unadjusted (Table S5) and adjusted logistic models for age and sex revealed that the exposure to HCB and γ-HCH was associated with increased odds of MetS [ORs (95%CI) = 1.53 (1.22–1.92) and 1.39 (1.10–1.76), respectively; $p < 0.01$]. No associations with MetS were observed for the remaining contaminants analyzed in AT samples. When BMI was included as an additional variable, the previously found associations were attenuated in all models (Table 2, Table 3, Table 4), probably because BMI is in the causal pathway between POPs and MetS.

Table 4. ORs (95%CI) of the risk of MetS according to adipose tissue POP concentration (ng/g tissue)

	Adjusted Modela				Adjusted Modelb			
	OR	**IC95%**		**p-value**	**OR**	**IC95%**		**p-value**
HCB	**1.53**	**1.22**	**1.92**	**<0.001**	**1.36**	**1.05**	**1.76**	**0.021**
p,p'-DDE	0.98	0.82	1.18	0.849	0.86	0.70	1.06	0.163
o,p'-DDT	1.25	0.89	1.75	0.199	1.07	0.74	1.53	0.736
γ-HCH	**1.39**	**1.10**	**1.76**	**0.006**	1.20	0.92	1.56	0.174
PCB-138	1.15	0.87	1.52	0.316	1.06	0.78	1.43	0.716
PCB-153	1.15	0.87	1.51	0.323	1.12	0.84	1.50	0.449
PCB-180	1.06	0.82	1.37	0.676	1.13	0.86	1.50	0.377

[a]Adjusted for age (yr) and sex (male/female); bAdjusted for age (yr) and sex (male/female) and BMI; CI: confidence interval

3.4. Mixture associations between chronic adipose tissue POPs concentrations and MetS risk

The combined effect of POPs on MetS, assessed using WQS, showed that WQS index for the seven POPs was positively and significantly associated with MetS, in both the adjusted model for age and sex [(exp [beta] = 2.34; $p < 0.001$)], as well as in the model adjusted for age, sex and BMI [(exp [beta] = 2.44; $p = 0.016$)] (Table 5). Concentrations of γ-HCH (52.9%), *o,p'*-DDT (26.9%) and HCB (19.7%) drove the mixture effect in the model adjusted for age and sex; while γ-HCH (47.6%), PCB-180 (26.2%), *o,p'*-DDT (21.7%) and PCB-153 (4.5%) drove the mixture effect in the model adjusted for age, sex and BMI.

Table 5. Estimation of the mixture effect of POPs on the risk of MetS using WQS

Adjusted for age (yr) and sex (male/female)			Adjusted for age (yr), sex (male/female) and BMI		
exp(Beta)	Standard Error	p-value	exp(Beta)	Standard Error	p-value
2.34	0.25	<0.001	2.44	0.37	0.016
	%			%	
γ-HCH	52.9		γ-HCH	47.6	
o,p'-DDT	26.9		PCB-180	26.2	
HCB	19.7		*o,p'*-DDT	21.7	
p,p'-DDE	0.33		PCB-153	4.53	

% shows individual contributions to the WQS index; BMI: body mass index; MetS: metabolic syndrome; POPs: persistent organic pollutants; WQS: Weighted Quantile Sum Regression

4. Discussion

This cross-sectional epidemiological study shows that VAT concentrations of HCB and γ-HCH, are associated with a greater risk of clinically-diagnosed MetS in an adult hospital population. Participants with higher VAT concentrations of these two chemical compounds also showed higher risk for several MetS components when considered individually. Hence, considering IDF cut-off points, HCB and γ-HCH AT concentrations were associated with elevated WC and blood pressure. Moreover, HCB concentrations were also related to lower HDL cholesterol levels. Consistently, linear positive associations were further detected between both HCB and γ-HCH levels in VAT and WC, fasting blood glucose, DBP, SBP and TG, while HCB was inversely associated with HDL cholesterol levels. PCBs in VAT were not associated with greater risk of MetS or any MetS component in our study, except for PCB-138 whose concentration was positively associated with SBP levels. WQS models further showed a positive and significant mixture effect of POPs on MetS, with γ-HCH (52.9%), and HCB (19.7%) being two of the main drivers of the association, in line which what was reported in the systematic review and meta-analysis of Lamat et al. (2022).

Our data show that VAT levels of both HCB and γ-HCH were positively associated with the prevalence of MetS. These results confirm previous articles published by our team in a prospective study in the Granada province (Southern Spain). For example, Mustieles et al.

(2017) investigated AT concentrations of OCPs and PCBs in 387 hospitalized adults in relation to the prevalence of being "metabolically compromised" (defined as having ≥1 diagnosis of the following diagnosis: T2D, hypertension, hypertriglyceridemia or low HDL cholesterol), identifying HCB and β-HCH) as the main contributors in cross-sectional analyses. Interestingly, those participants without any MetS component at baseline were followed during 10 years on average, and again AT HCB and β-HCH levels at baseline were found to be longitudinally associated with the risk of being metabolically compromised (Mustieles et al., 2017). Our current study, performed in a similar population in the neighbouring province of Málaga, complements these results showing that VAT concentrations of HCB and γ-HCH are not only associated with single MetS components, but with the risk of clinically-diagnosed MetS, strengthening the weight of evidence. In other words, this study reinforces the clinical relevance of POP-metabolism associations found in population studies. In line with our results, it has been interestingly reported that living in close proximity to environmental sources of POPs is associated with a statistically significant 39.2% increase in MetS-related hospitalizations in a population-based semi-ecological study based on zip codes of residence (Sergeev and Carpenter, 2011).

Few previous investigations have investigated the relationship between POP AT concentrations and risk of MetS components using either cross-sectional, longitudinal (Arrebola et al., 2015, 2014; Dirinck et al., 2015, 2016; Lee et al., 2012, 2014; Mustieles et al., 2017; Penell et al., 2014), or combined designs (Dirinck et al., 2014; Mustieles et al., 2017). Thus, some studies using cross-sectional design reported increased risk for MetS related to serum POP exposure (Aminov and Carpenter, 2020; Gasull et al., 2018; Lee et al., 2014; Park et al., 2010; Rosenbaum et al., 2017), yielding similar conclusions. Tomar et al. (2013) and Kim et al. (2018) detected increased serum levels of β-HCH among those MetS patients. Lee et al. (2007) also revealed that patients with detectable serum levels of β-HCH had higher risk for MetS compared with patients with β-HCH levels below LOD. However, Pestana et al. (2014) found no statistically significant differences between AT γ-HCH levels and MetS prevalence, although they did detect a positive association when POP exposure was considered as the sum of single chemicals. Regarding PCBs, we observed non-significant higher concentrations of PCBs in patients with MetS. Similarly, Dirinck et al. (2016) found that MetS+ patients

had significantly higher serum concentrations of PCB-153, -138 and −180, and the sum of these PCBs (ΣPCBs) than MetS- patients.

We also explored the influence of POP exposure in the prevalence of individual MetS components. It was observed that HCB and γ-HCH levels in VAT was related to higher blood pressure and/or hypertension risk, in line with previous findings (Arrebola et al., 2015; Lee et al., 2007; Park et al., 2010). Similarly, other authors have also found positive associations between any POP and hypertension. For instance, Henríquez-Hernández et al. (2014) found positive associations between serum *p,p'*-DDE and hypertension, and between *p,p'*-DDE and blood pressure (systolic and diastolic) in subjects not taking anti-hypertensive drugs. However, other studies have reported inverse associations between hypertension and exposure to HCH congeners (Pestana et al., 2014; Valera et al., 2013). Regarding HDL cholesterol, we also detected an inverse relationship between HCB concentrations and HDL cholesterol, either when HDL cholesterol was considered as a continuous variable and after using IDF cut-off point. Similarly, another study found inverse associations between HDL cholesterol levels and concentrations of HCB, in both Caucasian and African American residents of Alabama (Aminov et al., 2014), although a study carried out in northern Europe reported no association between HCB and changes in HDL cholesterol over a 5-year follow-up (Penell et al., 2014). Inversely, significant quadratic association between HDL cholesterol and HCB was found by Arrebola et al. (2014). Concerning the elevated serum TG, other MetS component, we found no statistically significant associations between the studied POPs and TG, unlike Arrebola et al. (2014) that found quadratic associations between TG and PCB-138 and -180. Increased WC comprises another MetS component with different cut-off point depending on the sex and population/country. Following this definition, we observed elevated WC among those participants with higher HCB and γ-HCH levels. Consistently, positive associations were found for WC and HCB and γ-HCH levels, as well as *p,p'*-DDE and *o,p'*-DDT, which is in line with previous findings (Lee et al., 2012, 2007; Tawar et al., 2022). We did not observe statistically significant risk with the exposure to POPs for increased fasting plasma glucose, another MetS component. Nevertheless, other groups have reported positive associations between fasting plasma glucose and some POPs. For instance, Tawar et al. (2022) found the VAT levels of δ-HCH, *p,p'*-DDT, heptachlor and endrin, were positively and

significantly correlated with fasting plasma glucose. In this line, other groups also observed positive associations between fasting plasma glucose and serum levels of different PCB congeners [PCB-153, -138 and −180, and the sum of PCBs (ΣPCBs = PCB-153 + PCB-138 + PCB-180)] (Dirinck et al., 2014; Mehta et al., 2021), as well for DDE, HCB and various PCBs (Aminov and Carpenter, 2020). Overall, while the associations between different POPs and specific single MetS components are rather heterogenous in the scientific literature, the associations between these chemical pollutants and the risk of MetS are more consistent. This may be explained by the fact that POPs seem to show a complex interplay with different metabolic organs including AT, the liver, the pancreas and other critical organs (Heindel et al., 2015). Thus, clinically-diagnosed MetS may better reflect the final expected outcome of POPs exposure compared to isolated components.

POPs may contribute to MetS by: 1) promoting obesity through different mechanisms including increased fat accumulation, decreased thermogenesis and disruption of satiety signals; and 2) by altering metabolic signalling in AT and other crucial metabolic organs (e.g., liver, pancreas) and not necessarily mediated through an increase in fat increase (Aaseth et al., 2022; Mustieles and Arrebola, 2020). At a molecular level, POPs may interact with different nuclear receptors involved in adipogenesis and metabolism such as peroxisome-proliferator activated receptors (PPARs) and estrogenic receptors (ERs) among others (Heindel et al., 2015; Mrema et al., 2013; Pereira-Fernandes et al., 2014), as well as with the protein and gene expressions of endoplasmatic stress and pro-inflammatory markers (Tawar et al., 2022). The best-known mechanism of action of some obesogenic POPs is the alteration of PPAR-γ, considered the main regulator of adipogenesis and lipid homeostasis (Gore et al., 2015). Zhang et al. (2015) also reported a POP-induced modification of gut microbiota-host metabolic homeostasis via activation of the AhR.

The mechanisms of action linking POP exposure to MetS are not fully elucidated, but probably involve endocrine disruption, oxidative stress/inflammation and epigenetic mechanisms, which can lead to insulin resistance, as well as the secretion of adipokines and inflammatory cytokines (Artacho-Cordón et al., 2016; Desai et al., 2015; Grünfeld and Bonefeld-Jorgensen, 2004; Mrema et al., 2013; Mustieles et al., 2021; Pandolfi et al., 2016), recently reviewed by Aaseth et al. (2022). In the last decade, additional pathways have

been identified, which might also lead to MetS thorough mitochondrial dysfunction (Kim and Lee, 2014), among other mechanisms. All of them contribute to a greater or lesser extent to dysfunctional AT due to POPs exposure. In addition to these typical changes, exposure to POPs has been linked to altered lipid metabolism (lipolytic and lipogenic processes) and altered adipokines involved in energy balance (e.g., leptin and adiponectin) (Bays et al., 2013; Cano-Sancho et al., 2017). Recently, Valvi et al. (2020) analyzed the levels of 18 POPs in abdominal AT from 11 obese adolescents, in addition to potentially altered biological pathways, by untargeted plasma metabolomics assessment. The results found showed high correlations between POPs and metabolic alterations in the pathways of amino acid, lipid and fatty acid metabolism, and carbohydrate metabolism (Valvi et al., 2020). A recent meta-analysis by Lamat et al. (2022) has reported that HCH would be involved in oxidative dysregulation, altering cytochrome P450, glutathione-S-transferase and glucose-6-phosphate dehydrogenase activities, increasing the risk of MetS compared to other POPs. Previous studies of our team also support the involvement of γ-HCH in the oxidative microenvironment of AT (Artacho-Cordón et al., 2016; Mustieles et al., 2021). Future studies should explore potential modes of action linking POP exposure to MetS, especially using multidisciplinary approaches including combined *in vitro, in vivo* and observational designs.

Several limitations should be acknowledged in this study. Firstly, the sample size was limited, although it was sufficient to yield statistically significant associations. Moreover, our hospital-based population might not be entirely representative of the general population. It is also important to note the cross-sectional design of this study, which only allow us to highlight potential associations, and reverse causality cannot be ruled out, since it was not possible to explore the temporal link between exposure and outcome. Another shortcoming of this study was the limited adjustment for potential confounders (mainly age and sex). Other known potential confounders, such as food intake, physical activity, smoking or alcohol, were not available in the clinical database, nor was the fat content of biopsies determined. Furthermore, mechanisms of action were not addressed in this study. Nevertheless, several mechanistic studies conducted in animals support our findings (Hong et al., 2015; Howell and Mangum, 2011; Mulligan et al., 2017; Ruzzin et al., 2010). Among the strengths of this study was the examination of the associations between exposure and clinically

diagnosed MetS. Additionally, we evaluated the influence of POPs on each related MetS components, considering them as continuous and clinical diagnostic components (according to IDF cut-off points). Although the continuous outcomes allowed to increase the statistical power to detect associations, the binary results facilitated the clinical interpretation of the magnitude of the associations found. In addition, the coherence between both types of modelling (continuous *vs*. binary) could be interpreted as a greater degree of confidence in the associations described.

In contrast to previous studies addressing the influence of circulating POP levels in MetS, we have analyzed the contribution of visceral adipose POP levels on the development of MetS. Ample research points to AT being a central factor in POP toxicity (Barrett, 2013; La Merrill et al., 2013), and the recent systematic review of Lamat et al. (2022) supports that the onset of MetS would be linked to visceral adiposity. Moreover, by using AT samples, we were able to partially counteract the possibility of reverse causality, more plausible by measuring POPs in serum samples, due to the so-called disease progression bias (Porta, 2014). AT is also the preferred reservoir for POP accumulation, and concentrations in this matrix can be considered indicative of long-term exposure (Crinnion WJ, 2009; Kohlmeier and Kohlmeier, 1995; Mustieles and Arrebola, 2020).

Although the results found in this study may only indicate an association between POP exposure and MetS risk, and not causation, it is of interest to highlight that a previous study by our team also identified HCB and γ-HCH levels in AT as predictors of MetS components, both in the cross-sectional and longitudinal designs of that study (Mustieles et al., 2017). Moreover, even using different approaches (clinical *vs.* population-based population), different analytical laboratories, and different study areas [the province of Málaga in the current study *vs*. the province of Granada in Mustieles et al. (2017)], similar conclusions have been obtained, supporting the epidemiological literature with this hypothesis.

Our findings shed light on the potential of POPs accumulated in the AT to trigger the risk of MetS, as well as most of the MetS components. It is necessary to recognize the increasing prevalence of the MetS in the world, and therefore, the need to identify preventable risk factors and establish intervention measures to halt and potentially reverse the progression of this syndrome (Saklayen, 2018).

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Ethics

This paper includes human samples for the investigation. The study was approved by the Ethics Committee of Virgen de la Victoria Hospital and conducted in accordance with the Declaration of Helsinki. All participants gave their signed consent after being fully informed of the goal and characteristics of the study at recruitment.

Declaration of competing interest

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

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Appendix A. Supplementary data

The following is the Supplementary data to this article.

References

Aaseth, J., Javorac, D., Djordjevic, A.B., Bulat, Z., Skalny, A. V., Zaitseva, I.P., Aschner, M., Tinkov, A.A., 2022. The Role of Persistent Organic Pollutants in Obesity: A Review of Laboratory and Epidemiological Studies. Toxics 10. https://doi.org/10.3390/TOXICS10020065

Alberti, K.G.M.M., Eckel, R.H., Grundy, S.M., Zimmet, P.Z., Cleeman, J.I., Donato, K.A., Fruchart, J.C., James, W.P.T., Loria, C.M., Smith, S.C., 2009. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation 120, 1640–1645. https://doi.org/10.1161/CIRCULATIONAHA.109.192644

Aminov, Z., Carpenter, D.O., 2020. Serum concentrations of persistent organic pollutants and the metabolic syndrome in Akwesasne

Mohawks, a Native American community. Environ. Pollut. 260. https://doi.org/10.1016/J.ENVPOL.2020.114004

Aminov, Z., Haase, R., Olson, J.R., Pavuk, M., Carpenter, D.O., Bartell, S., Olson, J., Foushee, R., Percy, A., Carpenter, D.O., Cash, J., Frumkin, H., Rosenbaum, P., Silverstone, A., Weinstock, R., 2014. Racial differences in levels of serum lipids and effects of exposure to persistent organic pollutants on lipid levels in residents of Anniston, Alabama. Environ. Int. 73, 216–223. https://doi.org/10.1016/J.ENVINT.2014.07.022

Amrhein, V., Greenland, S., McShane, B., 2019. Scientists rise up against statistical significance. Nature 567, 305–307. https://doi.org/10.1038/D41586-019-00857-9

Arrebola, J.P., Fernández, M.F., Martin-Olmedo, P., Bonde, J.P., Martín-Rodriguez, J.L., Expósito, J., Rubio-Domínguez, A., Olea, N., 2015. Historical exposure to persistent organic pollutants and risk of incident hypertension. Environ. Res. 138, 217–223. https://doi.org/10.1016/J.ENVRES.2015.02.018

Arrebola, J.P., Ocaña-Riola, R., Arrebola-Moreno, A.L., Fernández-Rodríguez, M., Martin-Olmedo, P., Fernández, M.F., Olea, N., 2014. Associations of accumulated exposure to persistent organic pollutants with serum lipids and obesity in an adult cohort from Southern Spain. Environ. Pollut. 195, 9–15. https://doi.org/10.1016/J.ENVPOL.2014.08.003

Artacho-Cordón, F., Belhassen, H., Arrebola, J.P., Ghali, R., Amira, D., Jiménez-Díaz, I., Pérez-Lobato, R., Boussen, H., Hedili, A., Olea, N., 2015a. Serum levels of persistent organic pollutants and predictors of exposure in Tunisian women. Sci. Total Environ. 511, 530–534. https://doi.org/10.1016/J.SCITOTENV.2014.12.093

Artacho-Cordón, F., Fernández-Rodríguez, M., Garde, C., Salamanca, E., Iribarne-Durán, L.M., Torné, P., Expósito, J., Papay-Ramírez, L., Fernández, M.F., Olea, N., Arrebola, J.P., 2015b. Serum and adipose tissue as matrices for assessment of exposure to persistent organic pollutants in breast cancer patients. Environ. Res. 142, 633–643. https://doi.org/10.1016/J.ENVRES.2015.08.020

Artacho-Cordón, F., León, J., Saénz, J.M., Fernndez, M.F., Piedad, M.O., Olea, N., Arrebola, J.P., 2016. Contribution of Persistent Organic Pollutant Exposure to the Adipose Tissue Oxidative Microenvironment in an Adult Cohort: A Multipollutant

Approach. Environ. Sci. Technol. 50, 13529–13538. https://doi.org/10.1021/ACS.EST.6B03783

Banos, G., Guarner, V., El Hafidi, M., Perez-Torres, I., 2011. Sex hormones, metabolic syndrome and kidney. Curr. Top. Med. Chem. 11, 1694–1705. https://doi.org/10.2174/156802611796117577

Barrett, J.R., 2013. POPs vs. fat: persistent organic pollutant toxicity targets and is modulated by adipose tissue. Environ. Health Perspect. 121. https://doi.org/10.1289/EHP.121-A61

Bays, H.E., Toth, P.P., Kris-Etherton, P.M., Abate, N., Aronne, L.J., Brown, W.V., Gonzalez-Campoy, J.M., Jones, S.R., Kumar, R., La Forge, R., Samuel, V.T., 2013. Obesity, adiposity, and dyslipidemia: a consensus statement from the National Lipid Association. J. Clin. Lipidol. 7, 304–383. https://doi.org/10.1016/J.JACL.2013.04.001

Bergonzi, R., De Palma, G., Specchia, C., Dinolfo, M., Tomasi, C., Frusca, T., Apostoli, P., 2011. Persistent organochlorine compounds in fetal and maternal tissues: evaluation of their potential influence on several indicators of fetal growth and health. Sci. Total Environ. 409, 2888–2893. https://doi.org/10.1016/J.SCITOTENV.2011.04.031

Bjerregaard-Olesen, C., Long, M., Ghisari, M., Bech, B.H., Nohr, E.A., Uldbjerg, N., Henriksen, T.B., Olsen, J., Bonefeld-Jørgensen, E.C., 2017. Temporal trends of lipophilic persistent organic pollutants in serum from Danish nulliparous pregnant women 2011-2013. Environ. Sci. Pollut. Res. Int. 24, 16592–16603. https://doi.org/10.1007/S11356-017-8992-7

Björvang, R.D., Vinnars, M.T., Papadogiannakis, N., Gidlöf, S., Mamsen, L.S., Mucs, D., Kiviranta, H., Rantakokko, P., Ruokojärvi, P., Lindh, C.H., Andersen, C.Y., Damdimopoulou, P., 2021. Mixtures of persistent organic pollutants are found in vital organs of late gestation human fetuses. Chemosphere 283. https://doi.org/10.1016/J.CHEMOSPHERE.2021.131125

Bokobza, E., Hinault, C., Tiroille, V., Clavel, S., Bost, F., Chevalier, N., 2021. The Adipose Tissue at the Crosstalk Between EDCs and Cancer Development. Front. Endocrinol. (Lausanne). https://doi.org/10.3389/fendo.2021.691658

Cano-Sancho, G., Salmon, A.G., La Merrill, M.A., 2017. Association between Exposure to p,p′-DDT and Its Metabolite p,p′-DDE with Obesity: Integrated Systematic Review and Meta-

Analysis. Environ. Health Perspect. 125. https://doi.org/10.1289/EHP527

Carrico, C., Gennings, C., Wheeler, D.C., Factor-Litvak, P., 2015. Characterization of Weighted Quantile Sum Regression for Highly Correlated Data in a Risk Analysis Setting. J. Agric. Biol. Environ. Stat. 20, 100–120. https://doi.org/10.1007/S13253-014-0180-3

Centers for Disease Control and Prevention., 2017. Fourth National Report on Human Exposure to Environmental Chemicals, Updated Tables, January 2017. Dep. Heal. Hum. Serv. Centers Dis. Control Prev. Atlanta, GA U.S. 1.

Crinnion WJ, 2009. Chlorinated pesticides: threats to health and importance of detection. Altern Med Rev. 14, 347–59.

Desai, M., Jellyman, J.K., Ross, M.G., 2015. Epigenomics, gestational programming and risk of metabolic syndrome. Int. J. Obes. (Lond). 39, 633–641. https://doi.org/10.1038/IJO.2015.13

Dirinck, E., Dirtu, A.C., Jorens, P.G., Malarvannan, G., Covaci, A., Van Gaal, L.F., 2015. Pivotal Role for the Visceral Fat Compartment in the Release of Persistent Organic Pollutants During Weight Loss. J. Clin. Endocrinol. Metab. 100, 4463–4471. https://doi.org/10.1210/JC.2015-2571

Dirinck, E.L., Dirtu, A.C., Govindan, M., Covaci, A., Jorens, P.G., Van Gaal, L.F., 2016. Endocrine-disrupting polychlorinated biphenyls in metabolically healthy and unhealthy obese subjects before and after weight loss: difference at the start but not at the finish. Am. J. Clin. Nutr. 103, 989–998. https://doi.org/10.3945/AJCN.115.119081

Dirinck, E.L., Dirtu, A.C., Govindan, M., Covaci, A., Van Gaal, L.F., Jorens, P.G., 2014. Exposure to persistent organic pollutants: relationship with abnormal glucose metabolism and visceral adiposity. Diabetes Care 37, 1951–1958. https://doi.org/10.2337/DC13-2329

Esser, N., Legrand-Poels, S., Piette, J., Scheen, A.J., Paquot, N., 2014. Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. Diabetes Res. Clin. Pract. 105, 141–150. https://doi.org/10.1016/J.DIABRES.2014.04.006

Fahed, G., Aoun, L., Zerdan, Morgan Bou, Allam, S., Zerdan, Maroun Bou, Bouferraa, Y., Assi, H.I., 2022. Metabolic Syndrome: Updates on Pathophysiology and Management in 2021. Int. J. Mol. Sci. 23. https://doi.org/10.3390/IJMS23020786

Fernández-Rodríguez, M., Arrebola, J.P., Artacho-Cordón, F., Amaya, E., Aragones, N., Llorca, J., Perez-Gomez, B., Ardanaz, E., Kogevinas, M., Castano-Vinyals, G., Pollan, M., Olea, N., 2015. Levels and predictors of persistent organic pollutants in an adult population from four Spanish regions. Sci. Total Environ. 538, 152–161. https://doi.org/10.1016/J.SCITOTENV.2015.07.162

Gasull, M., Castell, C., Pallarès, N., Miret, C., Pumarega, J., Téllez-Plaza, M., López, T., Salas-Salvadó, J., Lee, D.H., Goday, A., Porta, M., 2018. Blood Concentrations of Persistent Organic Pollutants and Unhealthy Metabolic Phenotypes in Normal-Weight, Overweight, and Obese Individuals. Am. J. Epidemiol. 187, 494–506. https://doi.org/10.1093/AJE/KWX267

González-Jiménez, E., Montero-Alonso, M.A., Schmidt-RioValle, J., García-García, C.J., Padez, C., 2015. Metabolic syndrome in Spanish adolescents and its association with birth weight, breastfeeding duration, maternal smoking, and maternal obesity: a cross-sectional study. Eur. J. Nutr. 54, 589–597. https://doi.org/10.1007/S00394-014-0740-X

Gore, A.C., Chappell, V.A., Fenton, S.E., Flaws, J.A., Nadal, A., Prins, G.S., Toppari, J., Zoeller, R.T., 2015. EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. Endocr. Rev. https://doi.org/10.1210/er.2015-1010

Grundy, S.M., 2016. Metabolic syndrome update. Trends Cardiovasc. Med. 26, 364–373. https://doi.org/10.1016/J.TCM.2015.10.004

Grünfeld, H.T., Bonefeld-Jorgensen, E.C., 2004. Effect of in vitro estrogenic pesticides on human oestrogen receptor alpha and beta mRNA levels. Toxicol. Lett. 151, 467–480. https://doi.org/10.1016/J.TOXLET.2004.03.021

Heindel, J.J., Vom Saal, F.S., Blumberg, B., Bovolin, P., Calamandrei, G., Ceresini, G., Cohn, B.A., Fabbri, E., Gioiosa, L., Kassotis, C., Legler, J., La Merrill, M., Rizzir, L., Machtinger, R., Mantovani, A., Mendez, M.A., Montanini, L., Molteni, L., Nagel, S.C., Parmigiani, S., Panzica, G., Paterlini, S., Pomatto, V., Ruzzin, J., Sartor, G., Schug, T.T., Street, M.E., Suvorov, A., Volpi, R., Zoeller, R.T., Palanza, P., 2015. Parma consensus statement on metabolic disruptors. Environ. Health 14. https://doi.org/10.1186/S12940-015-0042-7

Henríquez-Hernández, L.A., Luzardo, O.P., Zumbado, M., Camacho, M., Serra-Majem, L., Álvarez-León, E.E., Boada, L.D., 2014. Blood pressure in relation to contamination by polychlorobiphenyls and organochlorine pesticides: Results from a population-based study in the Canary Islands (Spain). Environ. Res. 135, 48–54. https://doi.org/10.1016/J.ENVRES.2014.05.036

Hirode, G., Wong, R.J., 2020. Trends in the Prevalence of Metabolic Syndrome in the United States, 2011-2016. JAMA 323, 2526–2528. https://doi.org/10.1001/JAMA.2020.4501

Höhn, A., König, J., Jung, T., 2016. Metabolic Syndrome, Redox State, and the Proteasomal System. Antioxid. Redox Signal. 25, 902–917. https://doi.org/10.1089/ARS.2016.6815

Hong, M.Y., Lumibao, J., Mistry, P., Saleh, R., Hoh, E., 2015. Fish Oil Contaminated with Persistent Organic Pollutants Reduces Antioxidant Capacity and Induces Oxidative Stress without Affecting Its Capacity to Lower Lipid Concentrations and Systemic Inflammation in Rats. J. Nutr. 145, 939–944. https://doi.org/10.3945/JN.114.206607

Howell, G., Mangum, L., 2011. Exposure to bioaccumulative organochlorine compounds alters adipogenesis, fatty acid uptake, and adipokine production in NIH3T3-L1 cells. Toxicol. In Vitro 25, 394–402. https://doi.org/10.1016/J.TIV.2010.10.015

Jackson, E., Shoemaker, R., Larian, N., Cassis, L., 2017. Adipose Tissue as a Site of Toxin Accumulation. Compr. Physiol. 7, 1085–1135. https://doi.org/10.1002/CPHY.C160038

Kershaw, E.E., Flier, J.S., 2004. Adipose tissue as an endocrine organ. J. Clin. Endocrinol. Metab. 89, 2548–2556. https://doi.org/10.1210/JC.2004-0395

Kim, J.T., Kang, J.H., Chang, Y.S., Lee, D.H., Choi, S.D., 2018. Determinants of serum organochlorine pesticide and polychlorinated biphenyl levels in middle-aged Korean adults. Environ. Sci. Pollut. Res. Int. 25, 249–259. https://doi.org/10.1007/S11356-017-0382-7

Kim, J.T., Lee, H.K., 2014. Metabolic syndrome and the environmental pollutants from mitochondrial perspectives. Rev. Endocr. Metab. Disord. 15, 253–262. https://doi.org/10.1007/S11154-014-9297-5

Kim, Y.A., Park, J.B., Woo, M.S., Lee, S.Y., Kim, H.Y., Yoo, Y.H., 2019. Persistent Organic Pollutant-Mediated Insulin Resistance. Int. J. Environ. Res. Public Health 16. https://doi.org/10.3390/IJERPH16030448

Kohlmeier, L., Kohlmeier, M., 1995. Adipose tissue as a medium for epidemiologic exposure assessment. Environ. Health Perspect. 103 Suppl 3, 99–106. https://doi.org/10.1289/EHP.95103S399

La Merrill, M., Emond, C., Kim, M.J., Antignac, J.P., Le Bizec, B., Clément, K., Birnbaum, L.S., Barouki, R., 2013. Toxicological function of adipose tissue: focus on persistent organic pollutants. Environ. Health Perspect. 121, 162–169. https://doi.org/10.1289/EHP.1205485

Lam D.W. and LeRoith D., 2019. Metabolic Syndrome - PubMed [WWW Document]. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000–. https://doi.org/PMID: 25905173.

Lamat, H., Sauvant-Rochat, M.P., Tauveron, I., Bagheri, R., Ugbolue, U.C., Maqdasi, S., Navel, V., Dutheil, F., 2022. Metabolic syndrome and pesticides: A systematic review and meta-analysis. Environ. Pollut. 305. https://doi.org/10.1016/J.ENVPOL.2022.119288

Lee, D.H., Lee, I.K., Porta, M., Steffes, M., Jacobs, D.R., 2007. Relationship between serum concentrations of persistent organic pollutants and the prevalence of metabolic syndrome among non-diabetic adults: results from the National Health and Nutrition Examination Survey 1999-2002. Diabetologia 50, 1841–1851. https://doi.org/10.1007/S00125-007-0755-4

Lee, D.H., Lind, L., Jacobs, D.R., Salihovic, S., van Bavel, B., Lind, P.M., 2012. Associations of persistent organic pollutants with abdominal obesity in the elderly: The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study. Environ. Int. 40, 170–178. https://doi.org/10.1016/J.ENVINT.2011.07.010

Lee, D.H., Steffes, M.W., Sjödin, A., Jones, R.S., Needham, L.L., Jacobs, D.R., 2011. Low dose organochlorine pesticides and polychlorinated biphenyls predict obesity, dyslipidemia, and insulin resistance among people free of diabetes. PLoS One 6. https://doi.org/10.1371/JOURNAL.PONE.0015977

Lee, Y.M., Kim, K.S., Kim, S.A., Hong, N.S., Lee, S.J., Lee, D.H., 2014. Prospective associations between persistent organic

pollutants and metabolic syndrome: a nested case-control study. Sci. Total Environ. 496, 219–225. https://doi.org/10.1016/J.SCITOTENV.2014.07.039

Martínez Vidal, J.L., Moreno Frías, M., Garrido Frenich, A., Olea-Serrano, F., Olea, N., 2002. Determination of endocrine-disrupting pesticides and polychlorinated biphenyls in human serum by GC-ECD and GC-MS-MS and evaluation of contributions to the uncertainty of the results. Anal. Bioanal. Chem. 372, 766–775. https://doi.org/10.1007/S00216-002-1272-4

Mehta, S.S., James-Todd, T., Applebaum, K.M., Bellavia, A., Coleman-Phox, K., Adler, N., Laraia, B., Epel, E., Parry, E., Wang, M., Park, J.S., Zota, A.R., 2021. Persistent organic pollutants and maternal glycemic outcomes in a diverse pregnancy cohort of overweight women. Environ. Res. 193. https://doi.org/10.1016/J.ENVRES.2020.110551

Moore, J.X., Chaudhary, N., Akinyemiju, T., 2017. Metabolic Syndrome Prevalence by Race/Ethnicity and Sex in the United States, National Health and Nutrition Examination Survey, 1988-2012. Prev. Chronic Dis. 14. https://doi.org/10.5888/PCD14.160287

Moreno Frías, M., Jiménez Torres, M., Garrido Frenich, A., Martínez Vidal, J.L., Olea-Serrano, F., Olea, N., 2004. Determination of organochlorine compounds in human biological samples by GC-MS/MS. Biomed. Chromatogr. 18, 102–111. https://doi.org/10.1002/BMC.300

Mrema, E.J., Rubino, F.M., Brambilla, G., Moretto, A., Tsatsakis, A.M., Colosio, C., 2013. Persistent organochlorinated pesticides and mechanisms of their toxicity. Toxicology 307, 74–88. https://doi.org/10.1016/J.TOX.2012.11.015

Müller, M.J., Lagerpusch, M., Enderle, J., Schautz, B., Heller, M., Bosy-Westphal, A., 2012. Beyond the body mass index: tracking body composition in the pathogenesis of obesity and the metabolic syndrome. Obes. Rev. 13 Suppl 2, 6–13. https://doi.org/10.1111/J.1467-789X.2012.01033.X

Mulligan, C., Kondakala, S., Yang, E.J., Stokes, J. V., Stewart, J.A., Kaplan, B.L.F., Howell, G.E., 2017. Exposure to an environmentally relevant mixture of organochlorine compounds and polychlorinated biphenyls Promotes hepatic steatosis in male Ob/Ob mice. Environ. Toxicol. 32, 1399–1411. https://doi.org/10.1002/TOX.22334

Mustieles, V., Arrebola, J.P., 2020. How polluted is your fat? What the study of adipose tissue can contribute to environmental epidemiology. J. Epidemiol. Community Health 74, 401–407. https://doi.org/10.1136/JECH-2019-213181

Mustieles, V., Fernández, M.F., Martin-Olmedo, P., González-Alzaga, B., Fontalba-Navas, A., Hauser, R., Olea, N., Arrebola, J.P., 2017. Human adipose tissue levels of persistent organic pollutants and metabolic syndrome components: Combining a cross-sectional with a 10-year longitudinal study using a multi-pollutant approach. Environ. Int. 104, 48–57. https://doi.org/10.1016/J.ENVINT.2017.04.002

Mustieles, V., Pérez-Carrascosa, F.M., León, J., Lange, T., Bonde, J.P., Gómez-Peña, C., Artacho-Cordón, F., Barrios-Rodríguez, R., Olmedo-Requena, R., Expósito, J., Jiménez-Moleón, J.J., Arrebola, J.P., 2021. Adipose Tissue Redox Microenvironment as a Potential Link between Persistent Organic Pollutants and the 16-Year Incidence of Non-hormone-Dependent Cancer. Environ. Sci. Technol. 55, 9926–9937. https://doi.org/10.1021/ACS.EST.0C08180

Nadal, A., Quesada, I., Tudurí, E., Nogueiras, R., Alonso-Magdalena, P., 2017. Endocrine-disrupting chemicals and the regulation of energy balance. Nat. Rev. Endocrinol. 13, 536–546. https://doi.org/10.1038/NRENDO.2017.51

Pandolfi, J.B., Ferraro, A.A., Sananez, I., Gancedo, M.C., Baz, P., Billordo, L.A., Fainboim, L., Arruvito, L., 2016. ATP-Induced Inflammation Drives Tissue-Resident Th17 Cells in Metabolically Unhealthy Obesity. J. Immunol. 196, 3287–3296. https://doi.org/10.4049/JIMMUNOL.1502506

Park, S.K., Son, H.K., Lee, S.K., Kang, J.H., Chang, Y.S., Jacobs, D.R., Lee, D.H., 2010. Relationship between serum concentrations of organochlorine pesticides and metabolic syndrome among non-diabetic adults. J. Prev. Med. Public Health 43, 1–18. https://doi.org/10.3961/JPMPH.2010.43.1.1

Penell, J., Lind, L., Salihovic, S., van Bavel, B., Lind, P.M., 2014. Persistent organic pollutants are related to the change in circulating lipid levels during a 5 year follow-up. Environ. Res. 134, 190–197. https://doi.org/10.1016/J.ENVRES.2014.08.005

Pereira-Fernandes, A., Dirinck, E., Dirtu, A.C., Malarvannan, G., Covaci, A., Van Gaal, L., Vanparys, C., Jorens, P.G., Blust, R., 2014.

Expression of obesity markers and Persistent Organic Pollutants levels in adipose tissue of obese patients: reinforcing the obesogen hypothesis? PLoS One 9. https://doi.org/10.1371/JOURNAL.PONE.0084816

Pérez-Carrascosa, F.M., Barrios-Rodríguez, R., Gómez-Peña, C., Salcedo-Bellido, I., Velasco-García, M.E., Jiménez- Moleón, J.J., García-Ruiz, A., Navarro-Espigares, J.L., Requena, P., Muñoz-Sánchez, C., Arrebola, J.P., 2022. Public healthcare costs associated with long-term exposure to mixtures of persistent organic pollutants in two areas of Southern Spain: A longitudinal analysis. Environ. Res. 213, 113609. https://doi.org/10.1016/J.ENVRES.2022.113609

Pérez-Carrascosa, F.M., Gómez-Peña, C., Echeverría, R., Jiménez-Moleón, J.J., Melchor, J.M., García-Ruiz, A., Navarro-Espigares, J.L., Cabeza-Barrera, J., Martin-Olmedo, P., Ortigosa-García, J.C., Arrebola, J.P., 2021. Historical exposure to persistent organic pollutants and cardiovascular disease: A 15-year longitudinal analysis focused on pharmaceutical consumption in primary care. Environ. Int. 156. https://doi.org/10.1016/J.ENVINT.2021.106734

Pestana, D., Faria, G., Sá, C., Fernandes, V.C., Teixeira, D., Norberto, S., Faria, A., Meireles, M., Marques, C., Correia-Sá, L., Cunha, A., Guimarães, J.T., Taveira-Gomes, A., Santos, A.C., Domingues, V.F., Delerue-Matos, C., Monteiro, R., Calhau, C., 2014. Persistent organic pollutant levels in human visceral and subcutaneous adipose tissue in obese individuals--depot differences and dysmetabolism implications. Environ. Res. 133, 170–177. https://doi.org/10.1016/J.ENVRES.2014.05.026

Porta, M., 2014. A dictionary of epidemiology., 6th ed. ed. Oxford University Press/International Epidemiological Association.

Rolle-Kampczyk, U., Gebauer, S., Haange, S.B., Schubert, K., Kern, M., Moulla, Y., Dietrich, A., Schön, M.R., Klöting, N., von Bergen, M., Blüher, M., 2020. Accumulation of distinct persistent organic pollutants is associated with adipose tissue inflammation. Sci. Total Environ. 748. https://doi.org/10.1016/J.SCITOTENV.2020.142458

Rosenbaum, P.F., Weinstock, R.S., Silverstone, A.E., Sjödin, A., Pavuk, M., 2017. Metabolic syndrome is associated with exposure to organochlorine pesticides in Anniston, AL, United States. Environ. Int. 108, 11–21. https://doi.org/10.1016/J.ENVINT.2017.07.017

Rothman, K.J., 2014. Six persistent research misconceptions. J. Gen. Intern. Med. 29, 1060–1064. https://doi.org/10.1007/S11606-013-2755-Z

Ruzzin, J., Petersen, R., Meugnier, E., Madsen, L., Lock, E.J., Lillefosse, H., Ma, T., Pesenti, S., Sonne, S.B., Marstrand, T.T., Malde, M.K., Du, Z.Y., Chavey, C., Fajas, L., Lundebye, A.K., Brand, C.L., Vidal, H., Kristiansen, K., Frøyland, L., 2010. Persistent organic pollutant exposure leads to insulin resistance syndrome. Environ. Health Perspect. 118, 465–471. https://doi.org/10.1289/EHP.0901321

Saklayen, M.G., 2018. The Global Epidemic of the Metabolic Syndrome. Curr. Hypertens. Rep. 20. https://doi.org/10.1007/S11906-018-0812-Z

Seillier, M., Pouyet, L., N'Guessan, P., Nollet, M., Capo, F., Guillaumond, F., Peyta, L., Dumas, J., Varrault, A., Bertrand, G., Bonnafous, S., Tran, A., Meur, G., Marchetti, P., Ravier, M.A., Dalle, S., Gual, P., Muller, D., Rutter, G.A., Servais, S., Iovanna, J.L., Carrier, A., 2015. Defects in mitophagy promote redox-driven metabolic syndrome in the absence of TP53INP1. EMBO Mol. Med. 7, 802–818. https://doi.org/10.15252/EMMM.201404318

Sergeev, A. V., Carpenter, D.O., 2011. Increase in metabolic syndrome-related hospitalizations in relation to environmental sources of persistent organic pollutants. Int. J. Environ. Res. Public Health 8, 762–776. https://doi.org/10.3390/IJERPH8030762

Shin, D., Kongpakpaisarn, K., Bohra, C., 2018. Trends in the prevalence of metabolic syndrome and its components in the United States 2007-2014. Int. J. Cardiol. 259, 216–219. https://doi.org/10.1016/J.IJCARD.2018.01.139

Spanidis, Y., Mpesios, A., Stagos, D., Goutzourelas, N., Bar-Or, D., Karapetsa, M., Zakynthinos, E., Spandidos, D.A., Tsatsakis, A.M., Leon, G., Kouretas, D., 2016. Assessment of the redox status in patients with metabolic syndrome and type 2 diabetes reveals great variations. Exp. Ther. Med. 11, 895–903. https://doi.org/10.3892/ETM.2016.2968

Sun, J., Fang, R., Wang, H., Xu, D.X., Yang, J., Huang, X., Cozzolino, D., Fang, M., Huang, Y., 2022. A review of environmental metabolism disrupting chemicals and effect biomarkers associating disease risks: Where exposomics meets metabolomics. Environ. Int. 158. https://doi.org/10.1016/J.ENVINT.2021.106941

Tawar, N., Banerjee, B.D., Madhu, S.V., Agrawal, V., Gupta, S., 2022. Association of Organochlorine Pesticides With Genetic Markers of Endoplasmic Reticulum Stress in Type 2 Diabetes Mellitus: A Case-Control Study Among the North-Indian Population. Front. Endocrinol. (Lausanne). 13. https://doi.org/10.3389/FENDO.2022.841463

Tomar, L., Agarwal, M., Avasthi, R., Tyagi, V., Mustafa, M., Banerjee, B., 2013. Serum organochlorine pesticide levels in patients with metabolic syndrome. Indian J. Endocrinol. Metab. 17, 342. https://doi.org/10.4103/2230-8210.119612

Valera, B., Ayotte, P., Poirier, P., Dewailly, É., 2013. Associations between plasma persistent organic pollutant levels and blood pressure in Inuit adults from Nunavik. Environ. Int. 59, 282–289. https://doi.org/10.1016/J.ENVINT.2013.06.019

Valvi, D., Walker, D.I., Inge, T., Bartell, S.M., Jenkins, T., Helmrath, M., Ziegler, T.R., La Merrill, M.A., Eckel, S.P., Conti, D., Liang, Y., Jones, D.P., McConnell, R., Chatzi, L., 2020. Environmental chemical burden in metabolic tissues and systemic biological pathways in adolescent bariatric surgery patients: A pilot untargeted metabolomic approach. Environ. Int. 143. https://doi.org/10.1016/J.ENVINT.2020.105957

van Greevenbroek, M.M.J., Schalkwijk, C.G., Stehouwer, C.D.A., 2016. Dysfunctional adipose tissue and low-grade inflammation in the management of the metabolic syndrome: Current practices and future advances. F1000Research 5. https://doi.org/10.12688/F1000RESEARCH.8971.1/DOI

Zhang, L., Nichols, R.G., Correll, J., Murray, I.A., Tanaka, N., Smith, P.B., Hubbard, T.D., Sebastian, A., Albert, I., Hatzakis, E., Gonzalez, F.J., Perdew, G.H., Patterson, A.D., 2015. Persistent Organic Pollutants Modify Gut Microbiota-Host Metabolic Homeostasis in Mice Through Aryl Hydrocarbon Receptor Activation. Environ. Health Perspect. 123, 679–688. https://doi.org/10.1289/EHP.1409055

Discusión

Efecto obesogénico de bisfenoles presentes en la población

La Iniciativa Europea de Biomonitorización Humana (HBM4EU), en la que nuestro grupo de investigación ha participado, confirma que la mayoría de los europeos tienen en su organismo concentraciones cuantificables de algunos contaminantes ambientales-obesógenos, como por ejemplo BPA, prohibido o restringido en ciertas aplicaciones en la Unión Europea desde 2011 (Bousoumah et al., 2021; Colorado-Yohar et al., 2021; Meslin et al., 2022; Ougier et al., 2021). Los datos de HBM4EU indican, además, que algunos europeos tienen concentraciones altas de algunos de estos contaminantes, y que al menos la mitad de los europeos investigados están expuestos, no sólo a BPA, sino también a otros bisfenoles sustitutos de BPA, cada vez más usados, como por ejemplo BPS y BPF (Bousoumah et al., 2021; Colorado-Yohar et al., 2021; Meslin et al., 2022; Ougier et al., 2021).

Dada la evidencia, cada vez mayor, del riesgo para la salud humana que implica la exposición a contaminantes ambientales-obesógenos, como, BPA, en esta Tesis Doctoral Internacional nos planteamos investigar como hipótesis de trabajo si la exposición a EDCs-obesógenos contribuye al desarrollo de la obesidad, además de otras enfermedades metabólicas asociadas a ella, como el MetS.

En el momento de proponer el plan de investigación de esta Tesis Doctoral Internacional ya era conocido el efecto obesogénico *in vitro* de BPA (Ahmed and Atlas, 2016; Boucher et al., 2016b, 2014; Desai et al., 2018; Masuno et al., 2005, 2002; Ohlstein et al., 2014; Ramskov Tetzlaff et al., 2020), pero eran pocos los trabajos que habían investigado el efecto obesogénico de algunos de sus sustitutos mayoritarios, como BPF (Martínez et al., 2020; Ramskov Tetzlaff et al., 2020) y BPS (Ahmed and Atlas, 2016; Boucher et al., 2016a, 2016b;

Martínez et al., 2020; Peshdary et al., 2020; Ramskov Tetzlaff et al., 2020), y aún menos los que habían profundizado en el desarrollo de metodologías que permitieran estudiar el efecto combinado de la exposición conjunta a estos contaminantes, analizando, por ejemplo, el efecto de mezclas reales de obesógenos presentes en matrices humanas.

Para abordar el objetivo de esta Tesis Doctoral Internacional, investigamos en primer lugar el efecto de la exposición individual de los sustitutos de BPA seleccionados, BPF y BPS, sobre la diferenciación de células madre mesenquimales derivadas del tejido adiposo humano (hASCs). Nuestro propósito era profundizar en el conocimiento de los mecanismos mediante los cuales los EDCs-obesogénicos alteran la regulación del proceso de la adipogénesis, promoviendo el desarrollo de obesidad, y, por ende, el MetS.

Para ello, células madre mesenquimales humanas hASCs se expusieron a estos dos sustitutos (BPF y BPS) en un rango de concentraciones que incluían las detectadas en muchas matrices biológicas humanas como orina, suero y leche humana (10 nM-25 μM) (Dualde et al., 2019; Gao et al., 2021; Jin et al., 2020; Kang et al., 2020; Karrer et al., 2019; Lehmler et al., 2018; Liu et al., 2018; Niu et al., 2021, 2017; Rochester and Bolden, 2015; Song et al., 2019; Usman and Ahmad, 2016; Ye et al., 2015). Se planearon dos diseños experimentales, uno primero exponiendo a las células a estos contaminantes de manera individual durante 7 días, lo que correspondería a la etapa temprana de la diferenciación adipocitaria; y un segundo diseño de exposición más prolongada, durante 14 días, para con ello analizar la etapa tardía o madura de la diferenciación adipocitaria (Ambele et al., 2016). Las células hASCs también se expusieron a una mezcla ternaria compuesta

por la combinación equimolar (1:1:1) de los tres bisfenoles, BPA, BPF y BPS, como posible ejemplo "real" de exposición humana a esta clase de compuestos. En este caso, la exposición a la mezcla también se realizó durante 14 días, con objeto de investigar el efecto combinado de la mezcla sobre los mismos objetivos: alteración de la adipogénesis y efecto sobre la acumulación de lípidos en los adipocitos.

Los resultados obtenidos revelaron que la exposición de las células hASCs, tanto a BPF como a BPS, de forma individual, a las mayores concentraciones testadas (10 μM y 25 μM), interfería con la programación de la diferenciación celular, de manera que las células hASCs acumulaban más lípidos intracelulares, aumentando la diferenciación de las células, y, por tanto, se obtenía un mayor número de adipocitos. Los adipocitos obtenidos presentaban, además, gotas lipídicas de mayor tamaño (**artículo #1**, Reina-Pérez et al., 2021).

Concretamente, el tratamiento con BPF a concentraciones 10 μM y 25 μM, durante 7 y 14 días, aumentó la capacidad de las células hASCs para diferenciarse en adipocitos y acumular gotas de lípidos intracelulares, como ya se había observado previamente tras el tratamiento de las mismas células con BPA (Dong et al., 2018; Ohlstein et al., 2014). Los efectos obtenidos sobre la adipogénesis fueron dependientes de la dosis y se corroboraron por la regulación al alza de los marcadores adipogénicos seleccionados (**artículo #1**, Reina-Pérez et al., 2021). Así, una mayor diferenciación de las células madre hacia adipocitos se correspondió con alteraciones en los niveles proteicos y en la expresión génica de los marcadores adipogénicos PPARγ, C/EBPα, LPL y FABP4. Específicamente, tras 14 días de diferenciación y en presencia de BPF a 10 μM y 25 μM, se observaron incrementos moderados pero significativos en los niveles proteicos de PPARγ (1,17- y 1,68- veces) y

de C/EBPα (1,1- y 1,26- veces), respectivamente. Tras 14 días de diferenciación y en presencia de BPS a 25 μM, se observó también un aumento similar y significativo de los niveles de proteína PPARγ (1,12 veces). El tratamiento con BPS, tanto a 10 μM como a 25 μM, indujo, sin embargo, una reducción pequeña pero significativa, de los niveles de proteína C/EBPα (**artículo #1,** Reina-Pérez et al., 2021). Hay que tener en cuenta que cuando consideramos la evolución temporal, al acelerar el desarrollo de los adipocitos estos presentan más PPARγ y a la vez menos C/EBPα.

No fue posible cuantificar los niveles proteicos de PPARγ, C/EBPα, LPL o FABP4, en el diseño experimental de 7 días de diferenciación, en ninguna de las concentraciones testadas, probablemente porque este marco temporal era excesivamente corto para detectar niveles proteicos de los marcadores seleccionados, algunos de ellos marcadores tardíos de diferenciación. Así, por ejemplo, mientras que C/EBPα, es un factor de transcripción implicado en la etapa intermedia de la diferenciación del adipocito (Kassotis and Stapleton, 2019; Lefterova and Lazar, 2009; Luo and Liu, 2016); FABP4 y LPL son marcadores adipogénicos en la etapa madura (tardía) de la diferenciación del adipocito (adipogénesis) (Bernlohr et al., 2002; Kassotis and Stapleton, 2019; Lefterova and Lazar, 2009; Luo and Liu, 2016). Hubiéramos esperado, sin embargo, cuantificar al menos los niveles proteicos de PPARγ, considerado como único y fundamental receptor nuclear, necesario y suficiente para iniciar la adipogénesis (Ahmed and Atlas, 2016; Kassotis and Stapleton, 2019; Lefterova and Lazar, 2009; Luo and Liu, 2016). Pero, este hallazgo (la no detección de expresión proteica con el Western Blot en el día 7) fue también encontrado por Janderová *et al.* (2003) con células madre mesenquimales humanas y la proteína PPARγ.

Otros grupos de investigación habían confirmado con anterioridad lo ya observado en esta Tesis Doctoral Internacional, principalmente para BPA y unos pocos para BPS, pero la mayoría de los trabajos lo habían hecho con diseños experimentales en adipocitos murinos, principalmente con células 3T3-L1 (Ahmed and Atlas, 2016; Choi et al., 2020; Drobna et al., 2019; Héliès-Toussaint et al., 2014; Martínez et al., 2020; Masuno et al., 2005, 2002; Ramskov Tetzlaff et al., 2020); en algunos ocasiones en adipocitos humanos (Boucher et al., 2016a, 2016b, 2014; Ohlstein et al., 2014; Peshdary et al., 2020; Ramskov Tetzlaff et al., 2020; Wang et al., 2017), e incluso mediante estudios *in vivo* (Drobna et al., 2019; Gao et al., 2020; Ivry Del Moral et al., 2016; Wang et al., 2017). Sin embargo, ningún trabajo con anterioridad había estudiado el efecto obesogénico de BPF. Por tanto, hasta donde sabemos, este es el primer trabajo que evidencia el potencial obesogénico *in vitro* de BPF sobre la diferenciación de adipocitos a partir de células madre humanas (**artículo #1**, Reina-Pérez et al., 2021).

Además de investigar el efecto de la exposición individual de los sustitutos de BPA seleccionados (BPF y BPS), sobre la diferenciación de células (hASCs), queríamos saber qué sucedía si las células se exponían a una mezcla de bisfenoels fabricada *ad hoc* ya que, hasta ese momento, no se habían publicado estudios *in vitro* con mezclas de contaminantes químicos-obesógenos. Por ello, células mesenquimales humanas hASCs se expusieron, siguiendo las mismas condiciones experimentales a los trabajos de exposición individual, a una mezcla ternaria equimolar de BPA, BPF y BPS (**artículo #2**, Reina-Pérez et al., 2022). En estas condiciones, se encontraron diferencias en los resultados de la acumulación lipídica y la expresión génica y proteica al comparar la exposición individual a dos de los componentes de la

mezcla y la exposición combinada de los tres (BPA+BPF+BPS). Así, por ejemplo, se observó una mayor diferenciación de las células, esto es, un mayor número de células madre evolucionaron a adipocitos, y éstos presentaban un mayor contenido lipídico, con gotas de mayor tamaño. Además, cuando se analizó la expresión génica y proteica de los marcadores seleccionados, la respuesta observada no fue monotónica como la obtenida en los ensayos individuales, sino con cambios de fase adoptando forma de U (**artículo #2**, Reina-Pérez et al., 2022).

El rango de concentraciones seleccionado en los estudios *in vitro* llevados a cabo en este trabajo, tanto de exposición individual como combinada, se decidió tras consultar estudios previos con líneas celulares, tanto murinas como humanas, además de estudios epidemiológicos descriptivos sobre exposición humana y ambiental a estos disruptores metabólicos (Ahmed and Atlas, 2016; Boucher et al., 2016b, 2016a, 2014; Choi et al., 2020; Dong et al., 2018; Harnett et al., 2021a, 2021b; Lehmler et al., 2018; Liu et al., 2018; Martínez et al., 2020; Ohlstein et al., 2014; Peshdary et al., 2020; Ramskov Tetzlaff et al., 2020; Rochester and Bolden, 2015; Verbanck et al., 2017; Wang et al., 2017; Ye et al., 2015). La intención era que pudiéramos comparar los resultados obtenidos con los hallados en otros trabajos, fundamentalmente con aquellos realizados en células hASCs (Ahmed and Atlas, 2016; Boucher et al., 2014, 2016a, 2016b; Choi et al., 2020; Martínez et al., 2020; Ohlstein et al., 2014; Peshdary et al., 2020; Ramskov Tetzlaff et al., 2020; Verbanck et al., 2017; Wang et al., 2017).

Para cuantificar la acumulación lipídica, esto es, las gotas de lípidos intracelulares contenidas en los adipocitos, se utilizó la tinción ORO, técnica validada y utilizada como marcador de adipogénesis. Sin embargo, esta técnica no está exenta de algunas limitaciones ya que no

permite conocer si el aumento lipídico es debido a una mayor hipertrofia y/o por el contrario es consecuencia de un aumento del número de adipocitos (Ramírez-Zacarías et al., 1992). Los resultados obtenidos en esta Tesis Doctoral Internacional indican que, tras la exposición de las células a los compuestos individuales ensayados (BPS y BPF), se obtenía un aumento significativo, dosis-dependiente de la acumulación lipídica, tanto tras la exposición de las hASCs durante 7 días, como tras la exposición durante 14 días de diferenciación. También la exposición a la mezcla de bisfenoles supuso un incremento de la acumulación lipídica dependiente de la dosis (0,01, 0,1, 1, o 10 μM), y significativo a 1 y 10 μM, fundamentalmente tras 14 días de exposición. Estos resultados confirman el potencial obesogénico de los bisfenoles individuales analizados, y de la mezcla *ad hoc* preparada, propuesto por Grün and Blumberg (2009) como criterio de clasificación de un EDC-obesogénico. No obstante, algunos de nuestros resultados difieren respecto a otros trabajos previos publicados, concretamente en lo que se refiere a la dependencia o no de las dosis testadas (Boucher et al., 2016a; Wang et al., 2017).

Como siguiente etapa de este trabajo de Tesis, quisimos profundizar en los mecanismos de acción mediante los cuales los EDCs-obesogénicos ejercen su acción. En este sentido, el receptor de estrógenos (ER) ha sido propuesto como responsable principal del efecto que ejercen algunos de estos contaminantes sobre la adipogénesis, como por ejemplo BPA. Así, la evidencia científica indica que la co-exposición de células madre mesenquimales humanas a BPA junto con el antiestrogéno ICI 182,780 (ICI, *fulvestrant*) inhibe la diferenciación de las células a adipocitos (Ohlstein et al., 2014). Este compuesto es un potente antagonista del ER. Cuando en el protocolo *in vitro* seguido en esta Tesis se añadió ICI, es decir, cuando las células se expusieron a los

bisfenoles de manera individual o combinada junto con ICI, encontramos que la co-exposición con el antiestrógeno inhibía el efecto obesogénico de BPA y BPF contrarrestando la diferenciación, mientras que no limitaba el efecto de BPS. Estos resultados indicarían que la unión al ER sería uno de los eventos clave responsables del efecto adipogénico total o parcial de BPA, BPF y de la mezcla, pero no de BPS, que actuaría mediante otros mecanismos de acción, posiblemente a través del receptor PPARγ (figura 4C, **artículo #1**, Reina-Pérez et al., 2021; figura 2, **artículo #2**, Reina-Pérez et al., 2022). Estos mismos hallazgos han sido corroborados también mediante otros diseños experimentales, por ejemplo, utilizando genes *reporters* de peces cebra (*zebrafish reporter gene assays*) (Le Fol et al., 2017).

Las diferencias encontradas respecto a la capacidad de los bisfenoles analizados para actuar como agonistas completos o parciales del ER podrían estar en parte justificadas por sus diferentes características físico-químicas, respecto a polaridad, y energía de unión de solvatación (Marroqui et al., 2021), como ya fue previamente evidenciado por nuestro grupo de investigación (Molina-Molina et al., 2013).

También se ensayó, como trabajo de Tesis, la actividad de la mezcla de bisfenoles en presencia del antiestrógeno ICI. En este caso la inhibición de la adipogénesis fue distinta a la encontrada con los compuestos individuales, y los resultados obtenidos no fueron posibles de predecir a partir de los resultados *in vitro* obtenidos con la exposición a los compuestos individuales que componen la mezcla (figura 2**, artículo #2**, Reina-Pérez et al., 2022). El efecto tampoco fue dosis dependiente. No fue posible comparar los resultados obtenidos con datos de estudios previos, ya que son muy pocos los trabajos que habían

investigado hasta el momento el efecto de mezclas de EDCs sobre esta vía de acción (Park et al., 2020; Skledar and Mašič, 2020).

Sin embargo, son muchos los trabajos que se han centrado en el estudio de la afinidad y actividad (agonista y/o antagonista) de unión de los bisfenoles sobre los ERs (Delfosse et al., 2012; Gore et al., 2015a, 2015b; Grimaldi et al., 2019; Karrer et al., 2019; Molina-Molina et al., 2013; Riu et al., 2011a, 2011b; Skledar and Mašič, 2020). No obstante, a pesar de tratarse de un mecanismo frecuentemente estudiado de acción estrogénica de BPA, y algunos de sus análogos y sustitutos, aún no ha sido dilucidado los mecanismos más precisos responsables del efecto de estos obesógenos sobre la adipogénesis. El proceso de adipogénisis debe involucrar, por tanto, otras vías, como la desencadenada por BPS, cuyo mecanismo principal propuesto sería a través de PPARγ (Ahmed and Atlas, 2016; Boucher et al., 2016a, 2016b), aunque probablemente intervengan otras dianas moleculares (Schaffert et al., 2021). De hecho, la opción más probable es que los compuestos individuales y sobre todo las mezclas de EDCs-obesógenos actúen a través de varios mecanismos de acción, principalmente relacionados con PPARγ, ER y GR, sin excluir otros mecanismos alternativos (Kassotis and Stapleton, 2019; Molina-Molina et al., 2013).

Es por ello que en este trabajo de Tesis Doctoral Internacional se investigaron algunas otras dianas moleculares que podrían estar implicadas en este proceso, seleccionando para ello algunos genes, que según la literatura científica tienen un papel clave en la adipogénesis, como el *PPARγ*, *C/EBPα*, *FABP4* y *LPL*. Por ejemplo, PPARγ está considerado el único y fundamental receptor nuclear, necesario y suficiente para iniciar la adipogénesis (Ahmed and Atlas, 2016;

Kassotis and Stapleton, 2019; Lefterova and Lazar, 2009; Luo and Liu, 2016).

La expresión de los marcadores lipogénicos seleccionados en las células en cultivo, difirió según la exposición individual o combinada (MIX). Así, la expresión génica, en general, fue mayor cuando las células se expusieron a la mezcla, alcanzándose valores de hasta veinte veces superiores a los basales, significativamente mayores que cuando se procedió a la exposición a compuestos individuales, que resultaron ser de hasta 4 veces más para BPF, hasta 3 veces más para BPS y hasta 8 veces más para BPA (figuras 3A y 3B, **artículo #2**, Reina-Pérez et al., 2022). En concreto, en el diseño de exposición más largo, 14 días, el incremento significativo en la expresión de los genes estudiados fue similar que tras la exposición individual de BPF y BPS (figuras 1A y 1B, **artículo #1**, Reina-Pérez et al., 2021) en línea con los resultados publicados por Peshdary *et al.* (2020), pero en dirección opuesta a los resultados del trabajo de Wang *et al.* (2017) que indican que no hubo cambios significativos en la expresión génica (*PPARγ* y *FABP4*). En el diseño de exposición más corto, 7 días, en general también observamos un incremento significativo de la expresión de los genes seleccionados, tanto tras la exposición de BPF como BPS a 10 y 25 μM, excepto para la expresión de *FABP4* cuando las células fueron expuestas a BPS 10 μM (figuras 2A y 2B, **artículo #1**, Reina-Pérez et al., 2021). Los resultados obtenidos (BPS a día 7) están en la misma línea que los publicados por otros grupos de investigación que han trabajado con preadipocitos murinos 3T3-L1 (Ahmed and Atlas, 2016; Ramskov Tetzlaff et al., 2020).

Un aspecto importante a mencionar es que, cuando las células se expusieron a BPF (25 μM) durante 14 días, encontramos cierta

toxicidad (71%) en el ensayo de viabilidad celular, lo que pudo influir en la expresión génica. Otros trabajos también han referido toxicidades en preadipocitos murinos expuestos a BPA (Héliès-Toussaint et al., 2014; Martínez et al., 2020). No se evidenció citotoxicidad en los ensayos de viabilidad celular que se hicieron para el resto de las condiciones experimentales llevadas a cabo en esta Tesis Doctoral Internacional (BPA, BPF, BPS y MIX).

En cuanto a la expresión génica en el estudio de mezcla de bisfenoles, encontramos una mayor expresión de marcadores lipogénicos tanto a la concentración más baja (10 nM) como a la más alta (10 μM), así como una menor expresión a la concentración intermedia (1 μM). Es decir, los niveles de expresión génica se relacionaron de forma no monotónica (no lineal) con la dosis, concretamente en forma de U (figura 3, **artículo #2**, Reina-Pérez et al., 2022). A pesar de que no hay estudios *in vitro* disponibles que hayan investigado el efecto de mezclas de bisfenoles a concentraciones similares en líneas celulares humanas para poder realizar comparaciones, la respuesta no monotónica en forma de U encontrada en nuestro trabajo ha sido previamente descrita para la expresión de algunos de los genes estudiados; concretamente para *PPARγ* y *LPL* en un trabajo llevado a cabo por Wang *et al.* (2013) con preadipocitos viscerales humanos expuestos a BPA (10 nM, 1 y 80 μM durante 14 días).

También se analizaron en este trabajo de Tesis los niveles de proteína de los genes seleccionados, tanto a los 7 como a los 14 días de diferenciación adipocitaria. Tras 14 días de ensayo, observamos que los bisfenoles a nivel individual causaron un incremento en los niveles proteicos de los marcadores lipogénicos. Así, se alcanzaron incrementos estadísticamente significativos y dosis dependiente para

PPARγ, C/EBPα y LPL, tras la exposición a BPF y BPS a 10 y 25 µM. En el estudio de mezclas, también se observó un incremento para LPL tras la exposición a la mezcla a 1 y 10 µM. También fue significativo el incremento en los niveles de las proteínas FABP4 tras la exposición a BPF 10 µM y a BPS 25 µM, así como los niveles de C/EBPα tras la exposición al MIX a 10 µM (figura 3, **artículo #1**, Reina-Pérez et al., 2021; figura 4, **artículo #2**, Reina-Pérez et al., 2022). No son muchos los trabajos que hayan publicado los niveles de proteínas de los genes seleccionados, pero los resultados obtenidos están en línea con los pocos encontrados tanto en líneas celulares murinas como en humanas (PPARγ, Boucher et al., 2014; PPARγ, C/EBPα y FABP4, Martínez et al., 2020; FABP4 y LPL, Ahmed and Atlas, 2016). No coinciden, sin embargo, con otros trabajos (LPL y FABP4, Boucher et al., 2016a).

Como decíamos anteriormente, no fue posible cuantificar los niveles proteicos de PPARγ, C/EBPα, LPL o FABP4, en el diseño experimental de 7 días de diferenciación, en ninguna de las concentraciones testadas. Igualmente, Janderová *et al.* (2003) tampoco detectaron proteína PPARγ en el día 7 en células madre mesenquimales humanas.

Entre las limitaciones de los estudios *in vitro* realizados en esta Tesis Doctoral Internacional cabe destarcar en primer lugar, la imposibilidad de extrapolar los resultados a lo que ocurre en la población debido fundamentalmente a que para favorecer la diferenciación adipogénica de las hASCs se emplea un cóctel hormonal suprafisiológico, totalmente diferente a lo que ocurre en el individuo. Además, aunque las concentraciones de los ensayos individuales se seleccionaron teniendo en cuenta los datos de exposición humana y ambiental, las concentraciones empleadas para preparar la mezcla *ad hoc* no necesariamente representan estos niveles. Otras de las limitaciones

sería la no determinación de hiperplasia e hipertrofia de los adipocitos en los ensayos realizados, y, por último, la dificultad de identificar los mecanismos hormonales y moleculares que subyacen en la exposición y efecto tanto de los compuestos individuales como de la mezcla estudiados. Hay que tener en cuenta, asimismo, la variabilidad en las respuestas adipogénicas según el tipo de compuesto químico estudiado y según la línea celular utilizada (Kassotis et al., 2017), así como la concentración hormonal y la composición del cóctel de diferenciación adipogénica, además de la frecuencia de remplazo del cóctel durante el proceso de diferenciación (Boucher et al., 2016a; Ohlstein et al., 2014; Patel et al., 2003; Peshdary et al., 2020; Wang et al., 2013, 2017).

En este sentido en los últimos años se está haciendo un esfuerzo por estandarizar los protocolos de diferenciación adipogénica entre distintos laboratorios para posibilitar una mejor comparación de los estudios que evalúan el efecto adipogénico de sustancias químicas (Kassotis et al., 2021).

Entre las fortalezas de los estudios *in vitro* en esta Tesis Doctoral Internacional cabe destacar, en primer lugar, que el estudio del efecto sobre la adipogénesis se ha planteado tanto tras de la exposición de compuestos individuales como a mezclas complejas, y que se han programado exposiciones durante un periodo de tiempo idóneo que cubre desde la etapa temprana hasta la madura de la diferenciación adipogénica. En segundo lugar, es importante resaltar la utilización de una línea celular humana (hASCs) en un intento de entender mejor lo que puede estar ocurriendo en la clínica humana. En este sentido, es necesario indicar que la selección de las células hASCs utilizadas en todos los experimentos realizados en esta Tesis Doctoral Internacional,

procedían de un único donante para evitar la variabilidad genética. Los resultados de diferenciación, se completaron, además, con el análisis de la expresión génica y proteica de marcadores específicos de diferenciación en un intento de dilucidar algunos de los mecanismos implicados en este proceso. El estudio de mezclas realizado aporta nuevas pruebas sobre el efecto combinado de mezclas de compuestos químicos, utilizando los bisfenoles a modo de ejemplo, evidenciando la dificultad a la hora de predecir el efecto de las mezclas a partir de actividad biológicas individuales.

En resumen, los resultados de esta Tesis Doctoral Internacional indican que los bisfenoles BPF y el BPS presentan un perfil transcripcional y un potencial adipogénico similares a los ya mostrados por BPA en otros estudios. La mezcla de bisfenoles ensayada (BPA+BPF+BPS) mostró igualmente un perfil transcripcional y un potencial adipogénico, aunque diferente al de sus componentes individuales. Por lo tanto, BPA, BPS, BPF o su mezcla poseen capacidad para aumentar la diferenciación adipocitaria de células mesenquimales humanas al incrementar la expresión génica de marcadores específicos (PPARγ, C/EBPα, LPL y FABP4).

Efecto obesogénico y disruptor metabólico de compuestos persistentes en un estudio clínico

Como segundo bloque de objetivos de esta Tesis Doctoral nos planteamos investigar cuál era la exposición real de la población a EDCs persistentes empleando para ello la matriz biológica por la que tienen más apetencia, el tejido adiposo; de forma particular se seleccionó el tejido adiposo visceral, por ser éste el tejido asociado con un mayor riesgo de enfermedades metabólicas (Jackson et al., 2017; Kim et al., 2019; Lamat et al., 2022).

En su día nuestro grupo de investigación ya describió la presencia de BPA en tejido adiposo (Fernandez et al., 2007), hecho del mayor interés ya que se trata de un compuesto químico considerado no persistente desde el punto de vista ambiental (*non-persistent environmental pollutants*, npEPs). Actualmente, aunque haya consenso en que BPA y otros npEPs son metabolizados y excretados en orina en horas/días, nuestro grupo ha mostrado, en no pocos casos, que también están presentes en aquellas matrices biológicas que informan de la exposición a contaminantes a medio y/o largo plazo, como es el tejido adiposo, lo que podría reflejar una cierta acumulación transitoria en tejidos específicos (Artacho-Cordón et al., 2017, 2018, 2019; Fernandez et al., 2007).

Las características fisicoquímicas de los POPs, alta lipofilidad y persistencia, favorecen su acumulación en los compartimientos grasos de los organismos. Es por ello que el tejido adiposo es considerado como una matriz biológica que representa la carga corporal a estas sustancias químicas a lo largo de la vida, actuando como principal reservorio de los mismos (Artacho-Cordón et al., 2015; Bokobza et al., 2021; Crinnion, 2009; Jackson et al., 2017; Kohlmeier and Kohlmeier, 1995; La Merrill et al., 2013; Mustieles and Arrebola, 2020).

En este trabajo de Tesis Doctoral investigamos la presencia y concentración de varios EDCs-persistentes. Además de describir la exposición, nos propusimos también evaluar las posibles asociaciones entre POPs y el diagnóstico clínico de MetS, utilizando tanto la información de biomarcadores de exposición como de los biomarcadores de efecto clínicos disponibles para la población de estudio (**artículo #3**, Reina-Pérez et al., 2023).

Planteamos para ello un estudio transversal con 117 adultos de mediana edad, seleccionados entre pacientes sometidos a cirugía no oncológica en el Hospital Universitario Virgen de la Victoria (Málaga, España). El reclutamiento se realizó entre 2013-2015. Como biomarcadores de exposición seleccionamos los 17 *compuestos* persistentes *o,p'*-DDT, *p,p'*-DDE, aldrina, isodrina, endrina, dieldrina, heptacloro, vinclozolina, endosulfán I y II, α-HCH, β-HCH, γ-HCH, HCB, y tres PCBs, congéneres -138, -153 y -180, que se midieron en tejido adiposo visceral. Como biomarcadores clínicos de efecto se seleccionaron todos aquellos considerados en el diagnóstico del MetS, es decir, niveles de glucosa en ayunas, TG y colesterol HDL medidos en muestras de sangre recogidas en ayunas antes de la intervención quirúrgica, así como medidas de tensión arterial, y antropométricas (perímetro abdominal, IMC, etc.), obtenidas de la historia clínica. Los participantes se clasificaron en base a la presencia o ausencia de síndrome metabólico según presentaran o no de forma simultánea, 3 de los 5 componentes anteriormente comentados (Alberti et al., 2009).

Los resultados del estudio transversal volvieron a mostrar (en comparación con poblaciones reclutadas en décadas anteriores), que la presencia de POPs en el tejido adiposo visceral de una población del sur de España, sigue siendo frecuente, y que la exposición ocurre a más de un compuesto, a pesar de que en nuestro país, al igual que en otros muchos, estos compuestos químicos están prohibidos o se ha limitado enormemente su uso desde hace ya más de tres décadas (Artacho-Cordón et al., 2015a; Bergonzi et al., 2011; Bjerregaard-Olesen et al., 2017; Björvang et al., 2021; Centers for Disease Control and Prevention, 2017; Fernández-Rodríguez et al., 2015). Los resultados obtenidos coinciden, además, con los descritos por muchos otros trabajos de la literatura científica realizados en otras poblaciones y

muestras biológicas (Artacho-Cordón et al., 2015a; Bergonzi et al., 2011; Bjerregaard-Olesen et al., 2017; Björvang et al., 2021; Centers for Disease Control and Prevention., 2017; Fernández-Rodríguez et al., 2015).

Las concentraciones encontradas en las muestras de tejido adiposo analizadas indicaron que los niveles de OCPs y PCBs eran significativamente mayores en el grupo de pacientes MetS+ (28,6 ng/g de tejido y 34,4 ng/g de tejido, respectivamente) respecto al grupo MetS-, donde todas las sustancias químicas se encontraron por debajo del LOD. También se pudieron evidenciar asociaciones positivas entre ciertos POPs y el diagnóstico clínico de MetS o algunos de sus componentes individuales (**artículo #3**, Reina-Pérez et al., 2023). Específicamente, los modelos de regresión lineal ajustados por edad y sexo mostraron que todos los OCPs medidos se asociaban positivamente con el perímetro de cintura. Además, niveles más altos de HCB y γ-HCH se relacionaron con concentraciones más altas de glucosa en ayunas, con una presión arterial más elevada y con un mayor riesgo de MetS. Los niveles de HCB se relacionaron además inversamente con los niveles de colesterol HDL, mientras que la concentración de PCB-180 se asoció positivamente. PCB-138 también se asoció positivamente con la presión arterial.

Los modelos logísticos ajustados por edad y sexo indicaron igualmente la asociación entre los niveles de HCB y al γ-HCH y una mayor probabilidad de padecer MetS [OR (IC95%): 1,53 (1,22-1,92) y 1,39 (1,10-1,76) respectivamente; $p< 0,01$]. No se observaron asociaciones entre MetS y el resto de OCPs. Se encontraron asociaciones similares, cuando la exposición a los contaminantes se categorizó por frecuencia de detección (detectados vs. no detectados), ajustando por edad y sexo.

Una de las fortalezas de este trabajo es que además se comprobó que no sólo es importante investigar el efecto de la exposición a contaminantes individuales, sino que el efecto combinado de todos ellos puede aportar igualmente información del mayor interés. Así, los modelos WQS, considerando los siete OCPs, mostraron que la exposición de la mezcla encontrada en el tejido adiposo se asociaba de manera positiva y significativa con el riesgo de MetS, tanto en el modelo ajustado por edad y sexo [(exp [beta] = 2,34; p< 0,001)], como en el modelo ajustado por edad, sexo e IMC [(exp [beta] = 2,44; p= 0,016)]. Las concentraciones de γ-HCH, *o,p'*-DDT y HCB eran responsables del efecto de la mezcla en un 52,9%, 26,9% y 19,7%, respectivamente, en el modelo ajustado por edad y sexo; mientras que γ-HCH (47,6%), PCB-180 (26,2%), *o,p'*-DDT (21,7%) y PCB-153 (4,5%) lideraban el modelo ajustado por edad, sexo y e IMC.

Los resultados hallados en este estudio transversal son coincidentes con otros estudios transversales y longitudinales previos que evaluaron la exposición a POPs, empleando habitualmente muestras de suero, y el riesgo de MetS o de alguno de sus componentes (Aminov et al., 2014; Aminov and Carpenter, 2020; Dirinck et al., 2016; Gasull et al., 2018; Henríquez-Hernández et al., 2014; Kim et al., 2018; Lee et al., 2007, 2014; Park et al., 2010; Rosenbaum et al., 2017; Tomar et al., 2013; Valera et al., 2013). Muchos de estos trabajos se han incluido en la reciente revisión sistemática y meta-análisis de Lamat *et al.* (2022), realizada con el objetivo de establecer la relación entre la exposición a plaguicidas y MetS. El meta-análisis muestra que la exposición global a plaguicidas aumenta el riesgo de MetS en un 30% (IC95: 22%-37%), correspondiendo a los OCPs un 23% del riesgo de MetS (14-32%). Al igual que los resultados de nuestro estudio transversal, en el meta-análisis de Lamat *et al.* (2022), HCH se relaciona con un aumento del

53% del riesgo de MetS (28-78%) y la exposición a HCB con un 40% (0,01-80%) (Lamat et al., 2022).

Los resultados obtenidos en el estudio transversal también están en consonancia con estudios previos llevados a cabo por nuestro grupo de investigación en otras áreas geográficas de España, como por ejemplo en la provincia de Granada (Arrebola et al., 2015a, 2014; Mustieles et al., 2017). Así, por ejemplo, Mustieles *et al.* (2017) investigaron las concentraciones en tejido adiposo de OCPs y PCBs en 387 adultos hospitalizados, en relación con la prevalencia de estar "metabólicamente comprometido", definido como tener ≥1 diagnóstico de algunos de los componentes del MetS, y los siguieron en el tiempo una media de diez años. Curiosamente encontraron que aquellos participantes sin ningún componente de MetS al inicio del estudio, pero con mayores niveles de HCB y β-HCH en su tejido adiposo, tenían un mayor riesgo de estar metabólicamente comprometidos a lo largo de los diez años siguientes (Mustieles et al., 2017). Nuestro estudio actual, realizado en una población similar de la vecina provincia de Málaga, complementa estos resultados mostrando que las concentraciones de HCB y γ-HCH no sólo se asocian con componentes individuales del MetS, sino con el riesgo de MetS diagnosticado clínicamente, reforzando el peso de la evidencia y la relevancia clínica de estas exposiciones ambientales.

Estudios previos que utilizaron diseños transversales también encontraron un mayor riesgo de MetS asociado a la exposición a POPs medidos en muestras de suero (Aminov and Carpenter, 2020; Gasull et al., 2018; Lee et al., 2014; Park et al., 2010; Rosenbaum et al., 2017), con conclusiones similares a las encontradas en nuestro trabajo. Así, por ejemplo, Tomar *et al.* (2013) y Kim *et al.* (2018) detectaron un

aumento de los niveles séricos de β-HCH entre aquellos pacientes con MetS (Kim et al., 2018; Tomar et al., 2013). Por su parte, Lee *et al.* (2007) indicaron que los pacientes con niveles séricos detectables de β-HCH tenían un mayor riesgo de padecer MetS, en comparación con aquellos con niveles de β-HCH inferiores al LOD (Lee et al., 2007). Sin embargo, Pestana *et al.* (2014) no encontraron diferencias estadísticamente significativas entre los niveles en tejido adiposo de γ-HCH y la prevalencia de MetS, aunque sí encontraron una asociación positiva cuando la exposición a POPs se consideró como la suma de sustancias químicas individuales cuantificadas en las muestras de suero de los participantes (Pestana et al., 2014).

Respecto a los niveles de PCBs, en nuestro estudio transversal no observamos concentraciones significativamente más altas en los pacientes con MetS+, al contrario de lo descrito por Dirinck *et al.* (2016) que indicaron que los pacientes con MetS+ tenían niveles séricos significativamente más altos de PCB-153 (Dirinck et al., 2016).

También exploramos en esta Tesis Doctoral la influencia de la exposición a POPs en la prevalencia de componentes individuales del MetS. Como se ha comentado antes, observamos que los niveles de HCB y γ-HCH estaban relacionados con un mayor riesgo de hipertensión, en consonancia con hallazgos previos (Arrebola et al., 2015a; Lee et al., 2007; Park et al., 2010). En la misma línea, otros autores también han encontrado asociaciones positivas entre POPs e hipertensión, como por ejemplo, Henríquez-Hernández *et al.* (2014), que describieron asociaciones positivas entre niveles séricos de *p,p′*-DDE y presión arterial (e hipertensión) en sujetos sin medicación con antihipertensivos. Sin embargo, otros estudios describieron

asociaciones inversas entre hipertensión y exposición a HCH (Pestana et al., 2014; Valera et al., 2013).

En nuestro trabajo detectamos una relación inversa entre las concentraciones de HCB y colesterol HDL (tanto cuando la variable se consideró de forma continua como dicotómica). De forma similar, Aminov et al. (2014), encontraron asociaciones inversas entre los niveles de colesterol HDL y las concentraciones de HCB, tanto en residentes caucásicos como afroamericanos de Alabama (Aminov et al., 2014). Sin embargo, otros estudios no encontraron asociaciones entre HCB y cambios en los niveles de colesterol HDL en una cohorte de seguimiento (Penell et al., 2014). Con respecto a los TG séricos elevados, el trabajo de esta Tesis no encontró asociaciones estadísticamente significativas entre este componente MetS y los contaminantes seleccionados, a diferencia del estudio de Arrebola *et al.* (2014) que sí evidenciaron asociaciones con PCB-138 y PCB-180. Si observamos, por el contrario, un aumento del perímetro de la cintura entre los participantes con niveles más elevados de HCB y γ-HCH. Análogamente, también se hallaron asociaciones positivas entre el perímetro de la cintura y los niveles de HCB y γ-HCH, lo que concuerda con hallazgos anteriores (Lee et al., 2012, 2007; Tawar et al., 2022). Otros grupos han informado también de asociaciones positivas entre los niveles de glucosa en ayunas, y OCPs; como por ejemplo, Tawar *et al.* (2022) que encontraron correlaciones positivas con las concentraciones de *p,p'*-DDT, δ-HCH, heptacloro y endrina, al igual que otros estudios (Aminov and Carpenter, 2020; Dirinck et al., 2014; Mehta et al., 2021).

En resumen, mientras que las asociaciones entre los distintos POPs y los componentes individuales del MetS son bastante heterogéneas en

la bibliografía científica, las asociaciones entre estos contaminantes químicos y el riesgo de MetS son más consistentes (Heindel et al., 2015b).

La heterogeneidad en los resultados entre estudios podría deberse a diferentes razones, como por ejemplo el tipo de matriz biológica donde se analizan los contaminantes, la diversidad en las poblaciones estudiadas, así como el diseño de estudio, o el diagnóstico de la enfermedad estudiada. Las diferencias en las asociaciones descritas también podrían explicarse por el hecho de que los POPs parecen mostrar una compleja interacción con diferentes órganos metabólicos tales como el tejido adiposo, hígado y páncreas, además de otros órganos críticos implicados en el metabolismo (Heindel et al., 2015b).

Son varios los mecanismos de acción mediante los cuales los POPs pueden contribuir al riesgo de MetS. En primer lugar, los POPs promueven la obesidad desde los mismos adipocitos, como por ejemplo, el aumento de la diferenciación adipogénica y la acumulación lipídica, descrito en los primeros trabajos de esta Tesis Doctoral Internacional, la disminución de la termogénesis y la variación de las señales de saciedad, o alterando la señalización metabólica en el tejido adiposo u otros órganos (Aaseth et al., 2022; Mustieles and Arrebola, 2020). Pueden actuar también a nivel molecular uniéndose a algunos receptores nucleares implicados en la adipogénesis y metabolismo (Gore et al., 2015a; Heindel et al., 2015b, 2015a; Jackson et al., 2017; Mrema et al., 2013; Pereira-Fernandes et al., 2014; Zhang et al., 2015), modificando la expresión génica y proteica de marcadores específicos (Tawar et al., 2022), como también indican los resultados de esta Tesis. Más aún, los POPs también interfieren con el desarrollo de los

adipocitos a través de modificaciones epigenéticas (van den Dungen et al., 2017).

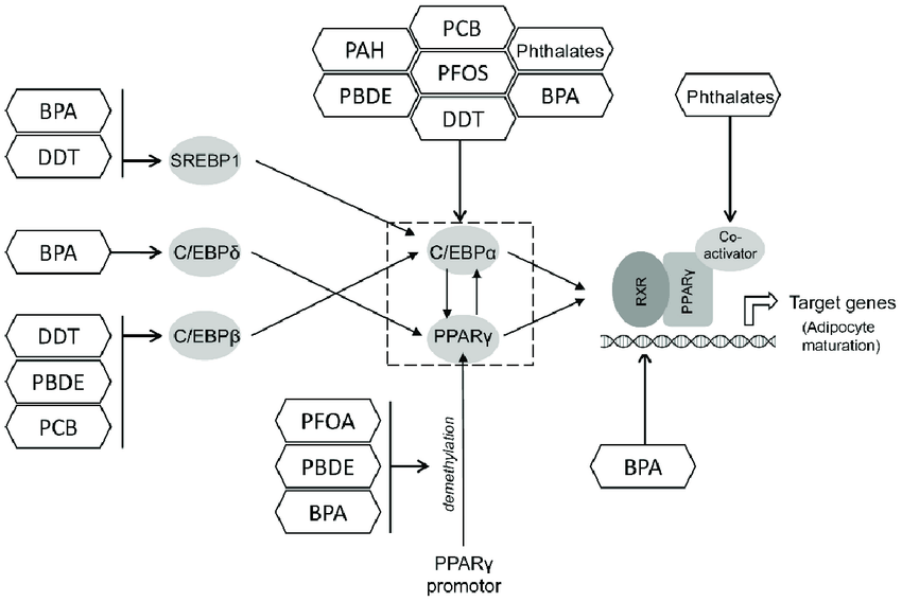


Figura 1. Posibles mecanismos subyacentes a los efectos adipogénicos de los POPs en adipocitos (Aaseth et al., 2022).

Otros de los modos de acción y eventos claves propuestos en la literatura científica ligando la disfunción del tejido adiposo al MetS (Fahed et al., 2022; van Greevenbroek et al., 2016), son: el desequilibrio hormonal (Banos et al., 2011), la inflamación crónica de bajo grado (Esser et al., 2014), el estado redox alterado (Höhn et al., 2016; Seillier et al., 2015; Spanidis et al., 2016) y/o la disfunción mitocondrial en el tejido adiposo (Fahed et al., 2022; Grundy, 2016; Kim and Lee, 2014). De esta forma los POPs afectarían al sistema endocrino (Grünfeld and Bonefeld-Jorgensen, 2004), a las respuestas inflamatorias (Pandolfi et al., 2016; Rolle-Kampczyk et al., 2020), a la oxidación de macromoléculas (Artacho-Cordón et al., 2016) e incluso a la disfunción mitocondrial (Kim et al., 2019).

Los mecanismos de acción responsable de entre la asociación exposición a POPs y MetS han sido recientemente revisados por Aaseth *et al.* (2022). Los autores de la revisión indican que es el conjunto de las alteraciones endocrinas, estrés oxidativo, inflamación junto con mecanismos epigenéticos, lo que puede dar lugar a resistencia a la insulina, además de provocar la secreción de adipoquinas y citocinas inflamatorias específicas, y que todos estos posibles mecanismos participan, en mayor o menor medida, en la disfunción del tejido adiposo producida por estos contaminantes (Artacho-Cordón et al., 2016; Desai et al., 2015; Grünfeld and Bonefeld-Jorgensen, 2004; Mrema et al., 2013; Mustieles et al., 2021; Pandolfi et al., 2016). Algunos autores añaden otras posibilidades como son los cambiossobre la lipólisis y lipogénesis, así como en el metabolismo de los aminoácidos y de los hidratos de carbono, además de en las adipocinas que intervienen en el balance energético (Bays et al., 2013; Cano-Sancho et al., 2017; Valvi et al., 2020).

La revisión sistemática y meta-análisis de Lamat *et al.* (2022) sugiere a su vez, que, aunque los mecanismos de los POPs no han sido dilucidados por completo, parece que concretamente el HCH interviene en la desregulación oxidativa al alterar las actividades del citocromo P450, la glutatión-S-transferasa y la glucosa-6-fosfato deshidrogenasa (Lamat et al., 2022). Estudios previos de nuestro equipo también apoyan la implicación del γ-HCH en el microambiente del tejido adiposo (Artacho-Cordón et al., 2016; Mustieles et al., 2021).

La mayoría de los trabajos publicados en la literatura científica se han centrado en la medida de POPs, en muestras séricas, que han relacionado con MetS, limitando la comparación con los hallazgos encontrados en este estudio transversal. Es por ello, que son necesarias

más investigaciones que exploren el papel de estos contaminantes ambientales-obesógenos en el desarrollo de MetS y cada uno de sus componentes, especialmente mediante estudios de cohortes prospectivas que utilicen el tejido adiposo como matriz biológica de referencia para evaluar la exposición. El punto clave es que los POPs séricos pueden variar debido a exposiciones dietéticas puntuales, a cambios de peso recientes, e incluso aumentar el flujo desde el tejido adiposo a sangre circulante debido a la progresión de la enfermedad metabólica. La medida de POPs en tejido adiposo, por el contrario, puede contrarrestar estos posibles sesgos (Mustieles and Arrebola, 2020). Son necesarios, además estudios que incluyan diseños combinados (*in vitro*, *in vivo* y observacionales), que permitan investigar los modos de acción implicados en la relación entre POPs y MetS, y de este modo aumentar la plausibilidad biológica de las asociaciones observadas.

Es necesario comentar, por último, las limitaciones que presenta el estudio transversal realizado. En primer lugar, su tamaño de muestra limitado, así como el reducido ajuste por variables de confusión, que no impidieron, sin embargo, encontrar algunas asociaciones estadísticamente significativas y coherentes con los estudios toxicológicos y epidemiológicos previos. En segundo lugar, la población de estudio particular seleccionada, ya que se trata de una población hospitalaria no representativa de la población general. Tercero, por su diseño transversal que sólo permite determinar asociaciones potenciales, no permitiendo descartar la causalidad inversa. No se pudo además tener en cuenta otros factores de confusión de interés, como la ingesta de alimentos, la actividad física, el tabaquismo o la ingesta de alcohol, al no estar disponibles en la base de datos clínica, ni tampoco fue posible determinar del contenido graso de

las muestras de tejido adiposo. Tampoco se investigaron los mecanismos de acción implicados en las asociaciones encontradas, sin embargo, la estancia internacional desarrollada por la doctoranda en el grupo de investigación del profesor Patrick Balaguer, ha permitido profundizar en el estudio de la especificidad (actividad agonista, antagonista y/o parcial) de diversos EDCs, entre ellos, BPA, BPF y BPS por los receptores nucleares PPARα, PPARβ/δ y PPARγ, utilizando líneas celulares "reporteras" (*reporter cell lines*).

Entre las fortalezas del estudio se encuentran las asociaciones similares descritas entre exposición, ya sea individual y combinada, a POPs y diagnóstico clínico MetS (según los criterios de la IDF), lo que aporta coherencia y consistencia a los resultados encontrados. Pudimos evaluar, además, la influencia de varios POPs en cada uno de los componentes del MetS. Nuestro trabajo refuerza además la idea de considerar al tejido adiposo como matriz óptima como biomarcador de exposición (Crinnion, 2009; Kohlmeier and Kohlmeier, 1995; Mustieles and Arrebola, 2020), al aparecer como un factor principal de toxicidad ejercida por los POPs (Barrett, 2013; La Merrill et al., 2013).

Como último objetivo de esta Tesis Doctoral Internacional nos planteamos evaluar la carga total obesogénica en muestras de tejido adiposo de pacientes adultos, con peso normal y sobrepeso/obesidad, e investigar su posible asociación con trastornos metabólicos, mediante el biomarcador *in vitro* "O-Screen". Es decir, consistiría en realizar extracciones cromatográficas de las muestras de tejido adiposo, aislando mezclas reales de POPs y otros compuestos, testando su efecto obesogénico en el modelo *in vitro* desarrollado. Aunque la herramienta *in vitro* necesaria para este ensayo ha sido desarrollada y validada [**artículos #1** y **#2**, (Reina-Pérez et al., 2022, 2021)], sin

embargo, el periodo de tiempo cubierto por el contrato FPU no ha sido suficiente para poder finalizar este objetivo, dejando esta labor como perspectiva de futuro para el grupo que apoya esta Tesis Doctoral Internacional.

Esta Tesis Doctoral ha abordado el estudio, mediante ensayos *in vitro,* de algunos EDCs-obesógenos específicos, como biomarcadores de exposición, sobre la adipogénesis, permitiendo identificar la capacidad de actuar como obesógeno de uno de los principales sustitutos de BPA, esto es BPF, al igual que ya se había establecido para otros sustitutos como BPS (Heindel et al., 2022). También ha puesto de nuevo de manifiesto la necesidad de no limitarse al análisis del efecto biológico de los compuestos químicos tomados uno a uno, sino de hacerlo en forma de mezclas extraídas de tejidos y fluidos biológicos o creadas *ad hoc*, como por ejemplo la ensayada en esta Tesis. Nuestro grupo de trabajo tiene una experiencia muy particular en la generación de información sobre el efecto hormonal a partir de los datos obtenidos de la medida de los niveles de exposición en muestras biológicas, como es el caso del tejido adiposo de individuos con muy diferente patologías (Artacho-Cordón et al., 2019, 2018; Echeverría et al., 2021; Mustieles et al., 2017). El efecto combinado de mezclas de compuestos químicos, tan complejo de llevar a cabo, y por ello, tan poco estudiado, ha sido abordado, con éxito a nuestro entender en esta Tesis, y alienta a seguir realizando estudios *in vitro* de combinaciones de EDCs como vía para contribuir al escaso conocimiento sobre los efectos en salud humana producidos por la exposición real a compuestos químicos ambientales.

Se ha profundizado, además en los mecanismos subyacentes al efecto generado por la exposición a contaminantes ambientales, mediante estudios *in vitro*, analizando la expresión de algunos genes

adipogénicos (*PPARγ*, *C/EBPα*, *LPL* y *FABP4*) y estrogénicos (*ERα*) junto con los niveles de sus proteínas, como posibles biomarcadores de efecto tempranos de esta exposición. La implementación de biomarcadores de efecto, como los considerados en esta Tesis Doctoral, considerados como una actividad biológica que puede medirse en ensayos *in vitro*, permitiría añadir nueva información en la detección temprana y/o en el diagnóstico clínico de algunas enfermedades (Vinggaard et al., 2021). Los ensayos *in vitro* permiten además medir el efecto completo al tener en cuenta los efectos combinados de la mezcla, permitiendo la identificación de sustancias químicas responsables del efecto, tanto conocidas como desconocidas (*chemicals of emerging concern*, CECs) (Vinggaard et al., 2021).

Esta Tesis Doctoral ha completado la información experimental con un estudio epidemiológico en el que se han establecido asociaciones entre algunos EDCs-persistentes (fundamentalmente HCB y γ-HCH) con el diagnóstico del MetS y/o sus componentes individuales. Sin embargo, hay que tener en cuenta que los EDCs investigados en los ensayos *in vitro* no han sido los mismos que los analizados en el estudio epidemiológico, y hubiera sido ideal haber complementado la información *in vitro* con la epidemiológica. Este trabajo se está actualmente realizando dentro de nuestro grupo de investigación. Es necesario, por tanto, continuar investigando y realizando más trabajos que nos permitan establecer conclusiones más sólidas sobre el efecto de la exposición a compuestos químicos ambientales (EDCs) en la salud humana.

Para finalizar es interesante proponer algunas perspectivas de futuro a tener en cuenta. Los resultados del programa de biomonitorización europeo HBM4EU indican que la exposición humana a algunos

contaminantes persistentes como los PFAS, y no persistentes, va en aumento (Richterová et al., 2023; Rosolen et al., 2022). Por ello, es necesario seguir profundizando mediante estudios *in vitro*, en el efecto individual y combinado de diferentes EDCs-obesogénicos, mediante protocolos estandarizados de diferenciación adipogénica, con líneas celulares de adipocitos humana, que permitan obtener comparabilidad y fiabilidad en los resultados entre unos trabajos y otros de similares características. Así se ha indicado recientemente en el trabajo de (Kassotis et al., 2021) en el que intervinieron varios laboratorios, donde se resalta la importancia que tiene la reproducibilidad entre laboratorios, así como la estandarización de protocolos de diferenciación adipogénica, cuando se lleva a cabo una evaluación detallada de la reproducibilidad y se analizan los factores que intervienen en la variabilidad de las respuestas a la exposición de compuestos químicos disruptores del metabolismo (MDCs, *metabolism disrupting chemicals*) para incrementar la fiabilidad en los resultados hallados (Kassotis et al., 2021). Estos modelos resultan muy prometedores para extrapolar los conocimientos sobre la salud metabólica de la población.

El conocimiento científico mejorará sin duda combinando los modelos tradicionales *in vitro* con nuevas técnicas de evaluación como por ejemplo los ensayos en 3D y de co-cultivo para evaluar la exposición a mezclas de compuestos adipogénicos y, por tanto, con potencial obesogénico (Kassotis et al., 2022). Es necesario, además, reconocer la creciente prevalencia del MetS en el mundo y, por lo tanto, la necesidad de identificar los factores de riesgo prevenibles, así como establecer medidas de intervención para detener y revertir la progresión de este síndrome (Saklayen, 2018). La participación de los profesionales

clínicos en esta área de investigación será fundamental para acometer los desafíos de salud ambiental a los que hacemos y haremos frente.

Conclusiones

Conclusión #1

La exposición de células hASCs a bisfenol-F (BPF) y bisfenol-S (BPS) promueve la adipogénesis como se comprueba al alterar la programación del desarrollo de los adipocitos y aumentar la acumulación de lípidos intracelulares. De forma particular, la exposición celular con BPF, a concentraciones de 10 o 25 μM durante 7 o 14 días, potencia la diferenciación de las células madre en adipocitos de manera dosis-dependiente, lo que se confirma al comprobar un incremento de la expresión de los genes adipogénicos seleccionados (*PPARγ*, *C/EBPα*, *LPL* y *FABP4*), así como de sus proteínas correspondientes. Nuestros resultados muestran por primera vez el potencial obesogénico de BPF sobre la diferenciación de adipocitos humanos, corroborando datos previos obtenidos en preadipocitos murinos.

Por su parte, como ya habían indicado estudios previos, nuestros propios resultados demuestran que la exposición de células hASCs a BPS resulta en un perfil transcripcional y un potencial adipogénico similar a BPF, tanto en los ensayos de diferenciación de 7 como de 14 días. De esta forma, la exposición celular a concentraciones de 10 o 25 μM de BPS aumenta la diferenciación de las células madre en adipocitos al promover, igualmente, la expresión de los genes *PPARγ*, *C/EBPα*, *LPL* y *FABP4*, y de los niveles proteicos correspondientes.

Los hallazgos encontrados en los estudios *in vitro* indican, por tanto, que el potencial obesogénico, evidenciado tanto para BPF como para BPS, los hacen inviables como alternativas seguras a la toxicidad del bisfenol-A (BPA).

Conclusión #2

La mezcla ternaria equimolar de los tres bisfenoles [bisfenol-F (BPF), bisfenol-S (BPS) y bisfenol-A (BPA), interfiere en la programación de las células hASCs, aumentando su capacidad para diferenciarse en adipocitos. No obstante, el efecto obesogénico de la mezcla difirió del de los compuestos individuales, mostrando niveles de expresión génica mayores a los obtenidos para los componentes individuales de la misma. La exposición de las células resultó en un efecto dosis-dependiente en la acumulación lipídica, mientras que la expresión de los genes mostró una respuesta no monotónica, adoptando forma de U. Este trabajo es uno de pocos estudios *in vitro* que han investigado la actividad obesogénica combinada de mezclas de EDCs-obesógenos sobre la diferenciación adipogénica, la acumulación de lípidos, la expresión génica y la síntesis proteica, en un intento de emular situaciones de exposición real en población humana.

Conclusión #3

Los resultados descritos en esta Tesis Doctoral Internacional indican que el potencial obesogénico de BPF y de la mezcla de obesógenos ensayada, está mediado por la unión al receptor de estrógenos, que se presenta como un evento clave responsable del efecto adipogénico parcial, o total, obtenido. Interesantemente, el efecto obesogénico de BPS estaría parcialmente mediado por otros mecanismos de acción, posiblemente a través del receptor PPARγ. No obstante, la explicación más plausible es que los obesógenos actúen a través de una combinación de mecanismos de acción, principalmente relacionados con los receptores PPARγ, ER y GR, entre otras posibles vías de señalización.

Cuando se estudiaron algunas de las dianas moleculares implicadas en el proceso de adipogénesis, como son PPARγ, C/EBPα, FABP4 y LPL, se comprobó que la expresión *in vitro* de estos marcadores lipogénicos difiere según se trate de exposición individual o combinada de contaminantes. En general, la expresión génica fue mayor cuando las células se expusieron a la mezcla de bisfenoles, alcanzándose valores de hasta veinte veces superiores a los basales. Este estímulo fue significativamente mayor que el observado cuando se analizó la exposición a compuestos individuales, que resultaron ser de hasta 4 veces más para BPF, hasta 3 veces más para BPS y hasta 8 veces más para BPA, respecto a los valores basales. Los niveles de expresión génica se relacionaron, además, de forma no-monotónica con la dosis, adoptando las curvas dosis-respuesta una forma de U.

Conclusión #4

La frecuencia de los POPs detectados en las muestras de tejido adiposo visceral obtenidos en el estudio clínico transversal, indica que la población adulta española sigue expuesta a estos compuestos ambientales persistentes, a pesar de haber sido prohibidos desde hace varias décadas. Las concentraciones encontradas en las muestras indicaron además que los niveles de OCPs y PCBs son significativamente mayores en el grupo de pacientes con síndrome metabólico (MetS) (concentraciones medianas: 28,6 ng/g de tejido y 34,4 ng/g de tejido, respectivamente) que en los individuos sin este síndrome (con concentraciones por debajo del límite de cuantificación).

Se han puesto de manifiesto asociaciones positivas entre ciertos POPs ya sea con el diagnóstico clínico de MetS o algunos de sus componentes. De forma particular, los modelos de regresión lineal ajustados por edad

y sexo muestran que todos los OCPs medidos se asocian positivamente con el perímetro de cintura. Además, niveles más altos de HCB y γ-HCH se relacionan con concentraciones más altas de glucosa en ayunas, con una presión arterial más elevada y con un mayor riesgo de MetS. Los niveles de HCB se relacionan inversamente con los niveles de colesterol HDL, mientras que la concentración de PCB-180 se asoció positivamente. Los modelos logísticos ajustados por edad y sexo indican igualmente la asociación entre los niveles de HCB y al γ-HCH con una mayor probabilidad de padecer MetS.

Nuestros propios resultados sugieren que no sólo es importante investigar el efecto de la exposición a contaminantes individuales, sino que el efecto combinado de todos ellos puede aportar información del mayor interés clínico. Así, los modelos de mezclas WQS mostraron que la exposición de la mezcla de los 7 POPs encontrada en el tejido adiposo se asocia de manera positiva y significativa con el riesgo de MetS, tanto en el modelo ajustado por edad y sexo, como en el modelo ajustado por edad, sexo e IMC. Las concentraciones de γ-HCH (53%), *o,p'*-DDT (27%) y HCB (20%) eran responsables del efecto de la mezcla en el modelo ajustado por edad y sexo; mientras que γ-HCH (48%), PCB-180 (26%), *o,p'*-DDT (28%) and PCB-153 (5%) lideraban el modelo ajustado por edad, sexo y e IMC.

La creciente prevalencia del MetS en el mundo, hace necesaria la identificación de factores de riesgo prevenibles, así como el establecimiento de medidas de intervención para detener y revertir la progresión de este síndrome. Dentro de este contexto se enmarca la exposición a contaminantes ambientales-obesogénicos, que una vez identificados y entendido su papel en la génesis de la enfermedad, deberían ser regulados en sus usos y aplicaciones de forma más

estricta. La participación de los profesionales clínicos en esta área de investigación será fundamental para acometer los desafíos de salud ambiental a los que hacemos y haremos frente con medidas preventivas.

Conclusions

Conclusion #1

Exposure of hASCs to bisphenol-F (BPF) and bisphenol-S (BPS) promotes adipogenesis by altering adipocyte developmental programming and increasing intracellular lipid accumulation. In particular, cell exposure with BPF, at concentrations of 10 or 25 μM for 7 or 14 days, potentiates stem cell differentiation into adipocytes in a dose-dependent manner, confirmed by an increase in the expression of selected adipogenic genes (*PPARγ*, *C/EBPα*, *LPL* and *FABP4*), as well as their corresponding proteins. Our results show for the first time the obesogenic potential of BPF on human adipocyte differentiation, corroborating previous data obtained in murine preadipocytes.

On the other hand, as previous studies had already indicated, our own results demonstrate that exposure of hASCs to BPS results in a transcriptional profile and adipogenic potential similar to BPF, both in the 7- and 14-day differentiation assays. Thus, cell exposure to concentrations of 10 or 25 μM of BPS enhances stem cell differentiation into adipocytes by promoting, likewise, the expression of *PPARγ*, *C/EBPα*, *LPL* and *FABP4* genes and corresponding protein levels.

The findings found in the *in vitro* studies indicate, therefore, that the obesogenic potential, evidenced for both BPF and BPS, make them unfeasible as safe alternatives to bisphenol-A (BPA) toxicity.

Conclusion #2

The equimolar ternary mixture of the three bisphenols [bisphenol-F (BPF), bisphenol-S (BPS) and bisphenol-A (BPA), interferes with the programming of hASCs, increasing their ability to differentiate into adipocytes. However, the obesogenic effect of the mixture differed from that of the individual compounds, showing higher gene expression levels than those obtained for the individual components of

the mixture. Exposure of the cells resulted in a dose-dependent effect on lipid accumulation, while gene expression showed a non-monotonic response, adopting a U-shape. This work is one of the few *in vitro* studies that have investigated the combined obesogenic activity of EDCs-obesogen mixtures on adipogenic differentiation, lipid accumulation, gene expression and protein synthesis, in an attempt to emulate real exposure situations occurring in human populations.

Conclusion #3

The results described in this International Doctoral Thesis indicate that the obesogenic potential of BPF and the mixture of obesogens tested are mediated by binding to the estrogen receptor, which is presented as a key event responsible for the partial or total adipogenic effect obtained. Interestingly, the obesogenic effect of BPS would be partially mediated by other mechanisms of action, possibly through the PPARγ receptor. However, the most plausible explanation is that these obesogens act through a combination of mechanisms, mainly related to PPARγ, ER and GR receptors, among other possible action modes.

When some of the molecular targets involved in the process of adipogenesis, such as PPARγ, C/EBPα, FABP4 and LPL, were studied, it was found that the *in vitro* expression of these lipogenic markers differed according to individual or combined exposure to pollutants. In general, gene expression was higher when cells were exposed to the bisphenol mixture, reaching values up to twenty times higher than basal. This stimulus is significantly greater than that observed when exposure to individual compounds was analyzed, which were up to 4-fold higher for BPF, up to 3-fold higher for BPS and up to 8-fold higher for BPA, compared to baseline values. The gene expression levels were

also non-monotonically related to dose, with the dose-response curves adopting a U-shape.

Conclusion #4

The frequency of POPs detected in the visceral adipose tissue samples obtained in the cross-sectional clinical study indicates that the Spanish adult population is still exposed to these persistent environmental compounds, despite the fact that they were banned for several decades. The concentrations found in the samples further indicate that the levels of OCPs and PCBs are significantly higher in the group of patients with metabolic syndrome (MetS) (median concentrations: 28.6 ng/g tissue and 34.4 ng/g tissue, respectively) than in individuals without this syndrome (with concentrations below the limit of quantification).

Positive associations between certain POPs and either the clinical diagnosis of MetS or some of its components have been shown. Notably, linear regression models adjusted for age and sex show that all measured POPs are positively associated with waist circumference. In addition, higher levels of HCB and γ-HCH are related to higher fasting glucose levels, higher blood pressure and higher risk of MetS. HCB levels were inversely related to HDL cholesterol levels, whereas PCB-180 concentration was positively associated. Logistic models adjusted for age and sex likewise identified associations between HCB and γ-HCH levels and an increased likelihood of MetS.

Our own results suggest that it is not only important to investigate the effect of exposure to individual contaminants, but that the combined effect of all of them may provide information of major clinical interest. Thus, WQS mixture models showed that exposure to the mixture of the 7 POPs found in adipose tissue is positively and significantly associated with the risk of MetS, both in the model adjusted for age and sex, and

in the model adjusted for age, sex and BMI. Concentrations of γ-HCH (53%), *o,p'*-DDT (27%) and HCB (20%) drove the mixture in the age- and sex-adjusted model; whereas γ-HCH (48%), PCB-180 (26%), *o,p'*-DDT (28%) and PCB-153 (5%) drove the age-, sex- and BMI-adjusted model.

The increasing prevalence of MetS in the world makes necessary the identification of preventable risk factors, besides stablishing intervention measures to stop and reverse the progression of this syndrome. Within this context, once the role of exposure to environmental-obesogenic pollutants in metabolic disease has been identified, more strictly regulations in their uses and applications should be implemented. The participation of clinical professionals in this area of research will be essential to deal with the environmental health challenges that we are facing and will face through precautionary measures.

References

Aaseth, J., Javorac, D., Djordjevic, A.B., Bulat, Z., Skalny, A. V., Zaitseva, I.P., Aschner, M., Tinkov, A.A., 2022. The Role of Persistent Organic Pollutants in Obesity: A Review of Laboratory and Epidemiological Studies. Toxics 10. https://doi.org/10.3390/TOXICS10020065

ACOG Committee Opinion, N. 575., 2013. Exposure to toxic environmental agents. Fertil. Steril. 100, 931–934. https://doi.org/10.1016/J.FERTNSTERT.2013.08.043

Ahmed, S., Atlas, E., 2016. Bisphenol S- and bisphenol A-induced adipogenesis of murine preadipocytes occurs through direct peroxisome proliferator-activated receptor gamma activation. Int. J. Obes. https://doi.org/10.1038/ijo.2016.95

Alberti, K.G.M.M., Eckel, R.H., Grundy, S.M., Zimmet, P.Z., Cleeman, J.I., Donato, K.A., Fruchart, J.C., James, W.P.T., Loria, C.M., Smith, S.C., 2009. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation 120, 1640–1645. https://doi.org/10.1161/CIRCULATIONAHA.109.192644

Ambele, M.A., Dessels, C., Durandt, C., Pepper, M.S., 2016. Genome-wide analysis of gene expression during adipogenesis in human adipose-derived stromal cells reveals novel patterns of gene expression during adipocyte differentiation. Stem Cell Res. 16, 725–734. https://doi.org/10.1016/J.SCR.2016.04.011

Aminov, Z., Carpenter, D.O., 2020. Serum concentrations of persistent

organic pollutants and the metabolic syndrome in Akwesasne Mohawks, a Native American community. Environ. Pollut. 260. https://doi.org/10.1016/J.ENVPOL.2020.114004

Aminov, Z., Haase, R., Olson, J.R., Pavuk, M., Carpenter, D.O., Bartell, S., Olson, J., Foushee, R., Percy, A., Carpenter, D.O., Cash, J., Frumkin, H., Rosenbaum, P., Silverstone, A., Weinstock, R., 2014. Racial differences in levels of serum lipids and effects of exposure to persistent organic pollutants on lipid levels in residents of Anniston, Alabama. Environ. Int. 73, 216–223. https://doi.org/10.1016/J.ENVINT.2014.07.022

Amir, S., Shah, S.T.A., Mamoulakis, C., Docea, A.O., Kalantzi, O.I., Zachariou, A., Calina, D., Carvalho, F., Sofikitis, N., Makrigiannakis, A., Tsatsakis, A., 2021. Endocrine Disruptors Acting on Estrogen and Androgen Pathways Cause Reproductive Disorders through Multiple Mechanisms: A Review. Int. J. Environ. Res. Public Health 18, 1–20. https://doi.org/10.3390/IJERPH18041464

Amrhein, V., Greenland, S., McShane, B., 2019. Scientists rise up against statistical significance. Nature 567, 305–307. https://doi.org/10.1038/D41586-019-00857-9

Antonio-Villa, N.E., Fernández-Chirino, L., Vargas-Vázquez, A., Fermín-Martínez, C.A., Aguilar-Salinas, C.A., Bello-Chavolla, O.Y., 2022. Prevalence Trends of Diabetes Subgroups in the United States: A Data-driven Analysis Spanning Three Decades From NHANES (1988-2018). J. Clin. Endocrinol. Metab. 107, 735–742. https://doi.org/10.1210/CLINEM/DGAB762

Arrebola, J.P., Fernández, M.F., Martin-Olmedo, P., Bonde, J.P., Martín-Rodriguez, J.L., Expósito, J., Rubio-Domínguez, A., Olea, N., 2015a.

Historical exposure to persistent organic pollutants and risk of incident hypertension. Environ. Res. 138, 217–223. https://doi.org/10.1016/J.ENVRES.2015.02.018

Arrebola, J.P., Molina-Molina, J.M., Fernández, M.F., Sáenz, J.M., Amaya, E., Indiveri, P., Hill, E.M., Scholze, M., Orton, F., Kortenkamp, A., Olea, N., 2015b. A novel biomarker for anti-androgenic activity in placenta reveals risks of urogenital malformations. Reproduction 149, 605–613. https://doi.org/10.1530/REP-14-0525

Arrebola, J.P., Ocaña-Riola, R., Arrebola-Moreno, A.L., Fernández-Rodríguez, M., Martin-Olmedo, P., Fernández, M.F., Olea, N., 2014. Associations of accumulated exposure to persistent organic pollutants with serum lipids and obesity in an adult cohort from Southern Spain. Environ. Pollut. 195, 9–15. https://doi.org/10.1016/J.ENVPOL.2014.08.003

Artacho-Cordón, F., Arrebola, J.P., Nielsen, O., Hernández, P., Skakkebaek, N.E., Fernández, M.F., Andersson, A.M., Olea, N., Frederiksen, H., 2017. Assumed non-persistent environmental chemicals in human adipose tissue; matrix stability and correlation with levels measured in urine and serum. Environ. Res. 156, 120–127. https://doi.org/10.1016/J.ENVRES.2017.03.030

Artacho-Cordón, F., Belhassen, H., Arrebola, J.P., Ghali, R., Amira, D., Jiménez-Díaz, I., Pérez-Lobato, R., Boussen, H., Hedili, A., Olea, N., 2015a. Serum levels of persistent organic pollutants and predictors of exposure in Tunisian women. Sci. Total Environ. 511, 530–534. https://doi.org/10.1016/J.SCITOTENV.2014.12.093

Artacho-Cordón, F., Fernández-Rodríguez, M., Garde, C., Salamanca, E., Iribarne-Durán, L.M., Torné, P., Expósito, J., Papay-Ramírez, L., Fernández, M.F., Olea, N., Arrebola, J.P., 2015b. Serum and adipose tissue as matrices for assessment of exposure to persistent organic pollutants in breast cancer patients. Environ. Res. 142, 633–643. https://doi.org/10.1016/J.ENVRES.2015.08.020

Artacho-Cordón, F., Fernández, M.F., Frederiksen, H., Iribarne-Durán, L.M., Jiménez-Díaz, I., Vela-Soria, F., Andersson, A.M., Martin-Olmedo, P., Peinado, F.M., Olea, N., Arrebola, J.P., 2018. Environmental phenols and parabens in adipose tissue from hospitalized adults in Southern Spain. Environ. Int. 119, 203–211. https://doi.org/10.1016/J.ENVINT.2018.05.052

Artacho-Cordón, F., León, J., Saénz, J.M., Fernndez, M.F., Piedad, M.O., Olea, N., Arrebola, J.P., 2016. Contribution of Persistent Organic Pollutant Exposure to the Adipose Tissue Oxidative Microenvironment in an Adult Cohort: A Multipollutant Approach. Environ. Sci. Technol. 50, 13529–13538. https://doi.org/10.1021/ACS.EST.6B03783

Artacho-Cordón, F., Ríos-Arrabal, S., León, J., Frederiksen, H., Sáenz, J.M., Martín-Olmedo, P., Fernández, M.F., Olea, N., Arrebola, J.P., 2019. Adipose tissue concentrations of non-persistent environmental phenols and local redox balance in adults from Southern Spain. Environ. Int. 133. https://doi.org/10.1016/J.ENVINT.2019.105118

Badimon, L., Oñate, B., Vilahur, G., 2015. Adipose-derived Mesenchymal Stem Cells and Their Reparative Potential in Ischemic Heart Disease. Rev. Esp. Cardiol. (Engl. Ed). 68, 599–611.

https://doi.org/10.1016/J.REC.2015.02.025

Baillie-Hamilton, P.F., 2002. Chemical toxins: a hypothesis to explain the global obesity epidemic. J. Altern. Complement. Med. 8, 185–192. https://doi.org/10.1089/107555302317371479

Banos, G., Guarner, V., El Hafidi, M., Perez-Torres, I., 2011. Sex hormones, metabolic syndrome and kidney. Curr. Top. Med. Chem. 11, 1694–1705. https://doi.org/10.2174/156802611796117577

Barker, D.J.P., 1995. Intrauterine programming of adult disease. Mol. Med. Today 1, 418–423. https://doi.org/10.1016/S1357-4310(95)90793-9

Barrett, J.R., 2013. POPs vs. fat: persistent organic pollutant toxicity targets and is modulated by adipose tissue. Environ. Health Perspect. 121. https://doi.org/10.1289/EHP.121-A61

Bays, H.E., Toth, P.P., Kris-Etherton, P.M., Abate, N., Aronne, L.J., Brown, W.V., Gonzalez-Campoy, J.M., Jones, S.R., Kumar, R., La Forge, R., Samuel, V.T., 2013. Obesity, adiposity, and dyslipidemia: a consensus statement from the National Lipid Association. J. Clin. Lipidol. 7, 304–383. https://doi.org/10.1016/J.JACL.2013.04.001

Bergman, A., Heindel, J., Jobling, S., Kidd, K., Zoeller, R., 2013. United Nations Environment Programme (UNEP) and World Health Organisation (WHO). State of the science of endocrine disrupting chemicals – 2012.

Bergonzi, R., De Palma, G., Specchia, C., Dinolfo, M., Tomasi, C., Frusca, T., Apostoli, P., 2011. Persistent organochlorine compounds in fetal and maternal tissues: evaluation of their potential influence

on several indicators of fetal growth and health. Sci. Total Environ. 409, 2888–2893. https://doi.org/10.1016/J.SCITOTENV.2011.04.031

Bernlohr, D.A., Jenkins, A.E., Bennaars, A.A., 2002. Chapter 10 Adipose tissue and lipid metabolism.

Bjerregaard-Olesen, C., Long, M., Ghisari, M., Bech, B.H., Nohr, E.A., Uldbjerg, N., Henriksen, T.B., Olsen, J., Bonefeld-Jørgensen, E.C., 2017. Temporal trends of lipophilic persistent organic pollutants in serum from Danish nulliparous pregnant women 2011-2013. Environ. Sci. Pollut. Res. Int. 24, 16592–16603. https://doi.org/10.1007/S11356-017-8992-7

Björvang, R.D., Vinnars, M.T., Papadogiannakis, N., Gidlöf, S., Mamsen, L.S., Mucs, D., Kiviranta, H., Rantakokko, P., Ruokojärvi, P., Lindh, C.H., Andersen, C.Y., Damdimopoulou, P., 2021. Mixtures of persistent organic pollutants are found in vital organs of late gestation human fetuses. Chemosphere 283. https://doi.org/10.1016/J.CHEMOSPHERE.2021.131125

Bokobza, E., Hinault, C., Tiroille, V., Clavel, S., Bost, F., Chevalier, N., 2021. The Adipose Tissue at the Crosstalk Between EDCs and Cancer Development. Front. Endocrinol. (Lausanne). https://doi.org/10.3389/fendo.2021.691658

Boucher, J.G., Ahmed, S., Atlas, E., 2016a. Bisphenol S induces adipogenesis in primary human preadipocytes from female donors. Endocrinology. https://doi.org/10.1210/en.2015-1872

Boucher, J.G., Boudreau, A., Atlas, E., 2014. Bisphenol A induces differentiation of human preadipocytes in the absence of glucocorticoid and is inhibited by an estrogen-receptor

antagonist. Nutr. Diabetes 4. https://doi.org/10.1038/NUTD.2013.43

Boucher, J.G., Gagné, R., Rowan-Carroll, A., Boudreau, A., Yauk, C.L., Atlas, E., 2016b. Bisphenol A and bisphenol S induce distinct transcriptional profiles in differentiating human primary preadipocytes. PLoS One 11, 1–22. https://doi.org/10.1371/journal.pone.0163318

Bousoumah, R., Leso, V., Iavicoli, I., Huuskonen, P., Viegas, S., Porras, S.P., Santonen, T., Frery, N., Robert, A., Ndaw, S., 2021. Biomonitoring of occupational exposure to bisphenol A, bisphenol S and bisphenol F: A systematic review. Sci. Total Environ. 783, 146905. https://doi.org/10.1016/j.scitotenv.2021.146905

Boutari, C., Mantzoros, C.S., 2022. A 2022 update on the epidemiology of obesity and a call to action: as its twin COVID-19 pandemic appears to be receding, the obesity and dysmetabolism pandemic continues to rage on. Metabolism. 133. https://doi.org/10.1016/J.METABOL.2022.155217

Cano-Sancho, G., Salmon, A.G., La Merrill, M.A., 2017. Association between Exposure to p,p'-DDT and Its Metabolite p,p'-DDE with Obesity: Integrated Systematic Review and Meta-Analysis. Environ. Health Perspect. 125. https://doi.org/10.1289/EHP527

Carrico, C., Gennings, C., Wheeler, D.C., Factor-Litvak, P., 2015. Characterization of Weighted Quantile Sum Regression for Highly Correlated Data in a Risk Analysis Setting. J. Agric. Biol. Environ. Stat. 20, 100–120. https://doi.org/10.1007/S13253-014-0180-3

Carvaillo, J.C., Barouki, R., Coumoul, X., Audouze, K., 2019. Linking

Bisphenol S to Adverse Outcome Pathways Using a Combined Text Mining and Systems Biology Approach. Environ. Health Perspect. 127, 47005. https://doi.org/10.1289/EHP4200

Catalán, V., Avilés-Olmos, I., Rodríguez, A., Becerril, S., Fernández-Formoso, J.A., Kiortsis, D., Portincasa, P., Gómez-Ambrosi, J., Frühbeck, G., 2022. Time to Consider the "Exposome Hypothesis" in the Development of the Obesity Pandemic. Nutrients 14. https://doi.org/10.3390/NU14081597

Centers for Disease Control and Prevention., 2017. Fourth National Report on Human Exposure to Environmental Chemicals, Updated Tables, January 2017. Dep. Heal. Hum. Serv. Centers Dis. Control Prev. Atlanta, GA U.S. 1.

Centers for Disease Control and Prevention, C., 2019. National Report on Human Exposure to Environmental Chemicals. Fourth National Report on Human Exposure to Environmental Chemicals, Updated Tables. January 2019.

Choe, S.S., Huh, J.Y., Hwang, I.J., Kim, J.I., Kim, J.B., 2016. Adipose Tissue Remodeling: Its Role in Energy Metabolism and Metabolic Disorders. Front. Endocrinol. (Lausanne). 7. https://doi.org/10.3389/FENDO.2016.00030

Choi, S. Il, Lee, J.S., Lee, S., Sim, W.S., Kim, Y.C., Lee, O.H., 2020. Potentilla rugulosa nakai extract attenuates bisphenol A-, S-and F-induced ROS production and differentiation of 3T3-L1 preadipocytes in the absence of dexamethasone. Antioxidants 9, 1–14. https://doi.org/10.3390/antiox9020113

Christiansen, S., Axelstad, M., Scholze, M., Johansson, H.K.L., Hass, U., Mandrup, K., Frandsen, H.L., Frederiksen, H., Isling, L.K., Boberg,

J., 2020. Grouping of endocrine disrupting chemicals for mixture risk assessment - Evidence from a rat study. Environ. Int. 142. https://doi.org/10.1016/J.ENVINT.2020.105870

Colborn, T., Clement, C., 1992. Chemically-induced alterations in sexual and functional development : the wildlife/human connection.

Colborn, T., Vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspect. 101, 378–384. https://doi.org/10.1289/EHP.93101378

Colorado-Yohar, S.M., Castillo-González, A.C., Sánchez-Meca, J., Rubio-Aparicio, M., Sánchez-Rodríguez, D., Salamanca-Fernández, E., Ardanaz, E., Amiano, P., Fernández, M.F., Mendiola, J., Navarro-Mateu, F., Chirlaque, M.D., 2021. Concentrations of bisphenol-A in adults from the general population: A systematic review and meta-analysis. Sci. Total Environ. 775. https://doi.org/10.1016/j.scitotenv.2021.145755

Commission, E., Agency, E.E., Health, W.E.C. for E. and, Organisation for Economic Co-operation and Development, G.B., Department of the Environment, G., Umweltbundesamt, K. (Sweden), Britain)., M.R.C. (Great, Institute for Environment and Health, E.C. for E. and T. of C., 1997. European Workshop on the Impact of Endocrine Disrupters on Human Health and Wildlife: 2-4 December 1996, Weybridge UK, Report of Proceedings. Environment and Climate Research Programme of DG XII, European Commission.

Crinnion WJ, 2009. Chlorinated pesticides: threats to health and importance of detection. Altern Med Rev. 14, 347–59.

Darbre, P.D., 2019. The history of endocrine-disrupting chemicals.

Curr. Opin. Endocr. Metab. Res. 7, 26–33. https://doi.org/10.1016/J.COEMR.2019.06.007

Delfosse, V., Grimaldi, M., Pons, J.L., Boulahtouf, A., Le Maire, A., Cavailles, V., Labesse, G., Bourguet, W., Balaguer, P., 2012. Structural and mechanistic insights into bisphenols action provide guidelines for risk assessment and discovery of bisphenol A substitutes. Proc. Natl. Acad. Sci. U. S. A. 109, 14930–14935. https://doi.org/10.1073/PNAS.1203574109

Desai, M., Ferrini, M.G., Jellyman, J.K., Han, G., Ross, M.G., 2018. In vivo and in vitro bisphenol A exposure effects on adiposity. J. Dev. Orig. Health Dis. 9, 678–687. https://doi.org/10.1017/S2040174418000600

Desai, M., Jellyman, J.K., Ross, M.G., 2015. Epigenomics, gestational programming and risk of metabolic syndrome. Int. J. Obes. (Lond). 39, 633–641. https://doi.org/10.1038/IJO.2015.13

Diamanti-Kandarakis, E., Bourguignon, J.P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T., Gore, A.C., 2009. Endocrine-disrupting chemicals: An Endocrine Society scientific statement. Endocr. Rev. https://doi.org/10.1210/er.2009-0002

Dirinck, E.L., Dirtu, A.C., Govindan, M., Covaci, A., Jorens, P.G., Van Gaal, L.F., 2016. Endocrine-disrupting polychlorinated biphenyls in metabolically healthy and unhealthy obese subjects before and after weight loss: difference at the start but not at the finish. Am. J. Clin. Nutr. 103, 989–998. https://doi.org/10.3945/AJCN.115.119081

Dirinck, E.L., Dirtu, A.C., Govindan, M., Covaci, A., Van Gaal, L.F., Jorens, P.G., 2014. Exposure to persistent organic pollutants: relationship

with abnormal glucose metabolism and visceral adiposity. Diabetes Care 37, 1951–1958. https://doi.org/10.2337/DC13-2329

Dong, H., Yao, X., Liu, S., Yin, N., Faiola, F., 2018. Non-cytotoxic nanomolar concentrations of bisphenol A induce human mesenchymal stem cell adipogenesis and osteogenesis. Ecotoxicol. Environ. Saf. 164, 448–454. https://doi.org/10.1016/J.ECOENV.2018.08.052

Drobna, Z., Talarovicova, A., Schrader, H.E., Fennell, T.R., Snyder, R.W., Rissman, E.F., 2019. Bisphenol F has different effects on preadipocytes differentiation and weight gain in adult mice as compared with Bisphenol A and S. Toxicology. https://doi.org/10.1016/j.tox.2019.03.016

Dualde, P., Pardo, O., Corpas-Burgos, F., Kuligowski, J., Gormaz, M., Vento, M., Pastor, A., Yusà, V., 2019. Biomonitoring of bisphenols A, F, S in human milk and probabilistic risk assessment for breastfed infants. Sci. Total Environ. 668, 797–805. https://doi.org/10.1016/J.SCITOTENV.2019.03.024

ECHA, E.C.A., 2017. MSC unanimously agrees that Bisphenol A is an endocrine disruptor. ECHA/PR/17/12 [WWW Document]. URL https://echa.europa.eu/es/-/msc-unanimously-agrees-that-bisphenol-a-is-an-endocrine-disruptor (accessed 1.22.23).

Echeverría, R., Vrhovnik, P., Salcedo-Bellido, I., Pérez-Carrascosa, F.M., Gómez-Peña, C., Fiket, Ž., Martin-Olmedo, P., Olea, N., Fernández, M.F., Arrebola, J.P., 2021. Associations of residential and occupational history with the distribution of persistent pollutant mixtures in adipose tissue samples. Environ. Res. 194.

https://doi.org/10.1016/J.ENVRES.2020.110687

Esser, N., Legrand-Poels, S., Piette, J., Scheen, A.J., Paquot, N., 2014. Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. Diabetes Res. Clin. Pract. 105, 141–150. https://doi.org/10.1016/J.DIABRES.2014.04.006

European Commission, 2018. Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions: Towards a comprehensive European Union framework on endocrine disruptors. 2018. [WWW Document]. COM/2018/734. URL https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52018DC0734 (accessed 1.20.23).

Fahed, G., Aoun, L., Zerdan, Morgan Bou, Allam, S., Zerdan, Maroun Bou, Bouferraa, Y., Assi, H.I., 2022. Metabolic Syndrome: Updates on Pathophysiology and Management in 2021. Int. J. Mol. Sci. 23. https://doi.org/10.3390/IJMS23020786

Fernández-Rodríguez, M., Arrebola, J.P., Artacho-Cordón, F., Amaya, E., Aragones, N., Llorca, J., Perez-Gomez, B., Ardanaz, E., Kogevinas, M., Castano-Vinyals, G., Pollan, M., Olea, N., 2015. Levels and predictors of persistent organic pollutants in an adult population from four Spanish regions. Sci. Total Environ. 538, 152–161. https://doi.org/10.1016/J.SCITOTENV.2015.07.162

Fernandez, M.F., Arrebola, J.P., Taoufiki, J., Navalón, A., Ballesteros, O., Pulgar, R., Vilchez, J.L., Olea, N., 2007. Bisphenol-A and chlorinated derivatives in adipose tissue of women. Reprod. Toxicol. 24, 259–264. https://doi.org/10.1016/J.REPROTOX.2007.06.007

Fernández, M.F., Olea, N., 2014. [Endocrine disruptors. Is there sufficient evidence to act?]. Gac. Sanit. 28, 93–95. https://doi.org/10.1016/J.GACETA.2013.11.005

Flynn, K., 2011. Dietary exposure to endocrine-active pesticides: Conflicting opinions in a European workshop. Environ. Int. 37, 980–990. https://doi.org/10.1016/J.ENVINT.2011.03.019

Foulds, C.E., Treviño, L.S., York, B., Walker, C.L., 2017. Endocrine-disrupting chemicals and fatty liver disease. Nat. Rev. Endocrinol. 13, 445–457. https://doi.org/10.1038/NRENDO.2017.42

Frühbeck, G., 2008. Overview of adipose tissue and its role in obesity and metabolic disorders. Methods Mol. Biol. 456, 1–22. https://doi.org/10.1007/978-1-59745-245-8_1

Ganzleben, C., Antignac, J.P., Barouki, R., Castaño, A., Fiddicke, U., Klánová, J., Lebret, E., Olea, N., Sarigiannis, D., Schoeters, G.R., Sepai, O., Tolonen, H., Kolossa-Gehring, M., 2017. Human biomonitoring as a tool to support chemicals regulation in the European Union. Int. J. Hyg. Environ. Health 220, 94–97. https://doi.org/10.1016/J.IJHEH.2017.01.007

Gao, C., He, H., Qiu, W., Zheng, Y., Chen, Y., Hu, S., Zhao, X., 2021. Oxidative Stress, Endocrine Disturbance, and Immune Interference in Humans Showed Relationships to Serum Bisphenol Concentrations in a Dense Industrial Area. Environ. Sci. Technol. 55, 1953–1963. https://doi.org/10.1021/ACS.EST.0C07587

Gao, P., Snyder, M., 2021. Exposome-wide Association Study for Metabolic Syndrome. Front. Genet. 12. https://doi.org/10.3389/FGENE.2021.783930

Gao, P., Wang, L., Yang, N., Wen, J., Zhao, M., Su, G., Zhang, J., Weng, D., 2020. Peroxisome proliferator-activated receptor gamma (PPARγ) activation and metabolism disturbance induced by bisphenol A and its replacement analog bisphenol S using in vitro macrophages and in vivo mouse models. Environ. Int. 134. https://doi.org/10.1016/J.ENVINT.2019.105328

Gasull, M., Castell, C., Pallarès, N., Miret, C., Pumarega, J., Téllez-Plaza, M., López, T., Salas-Salvadó, J., Lee, D.H., Goday, A., Porta, M., 2018. Blood Concentrations of Persistent Organic Pollutants and Unhealthy Metabolic Phenotypes in Normal-Weight, Overweight, and Obese Individuals. Am. J. Epidemiol. 187, 494–506. https://doi.org/10.1093/AJE/KWX267

Gore, A.C., Chappell, V.A., Fenton, S.E., Flaws, J.A., Nadal, A., Prins, G.S., Toppari, J., Zoeller, R.T., 2015a. EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. Endocr. Rev. 36, 1–150. https://doi.org/10.1210/ER.2015-1010

Gore, A.C., Chappell, V.A., Fenton, S.E., Flaws, J.A., Nadal, A., Prins, G.S., Toppari, J., Zoeller, R.T., 2015b. Executive Summary to EDC-2: The Endocrine Society's second Scientific Statement on endocrine-disrupting chemicals. Endocr. Rev. https://doi.org/10.1210/er.2015-1093

Govarts, E., Nieuwenhuijsen, M., Schoeters, G., Ballester, F., Bloemen, K., de Boer, M., Chevrier, C., EggesbØ, M., Guxens, M., Krämer, U., Legler, J., Martínez, D., Palkovicova, L., Patelarou, E., Ranft, U., Rautio, A., Petersen, M.S., Slama, R., Stigum, H., Toft, G., Trnovec, T., Vandentorren, S., Weihe, P., Kuperus, N.W., Wilhelm, M., Wittsiepe, J., Bonde, J.P., 2012. Birth weight and prenatal exposure

to polychlorinated biphenyls (PCBs) and dichlorodiphenyldichloroethylene (DDE): a meta-analysis within 12 European Birth Cohorts. Environ. Health Perspect. 120, 162–170. https://doi.org/10.1289/EHP.1103767

Grimaldi, M., Boulahtouf, A., Toporova, L., Balaguer, P., 2019. Functional profiling of bisphenols for nuclear receptors. Toxicology 420, 39–45. https://doi.org/10.1016/J.TOX.2019.04.003

Grün, F., 2010. Obesogens. Curr. Opin. Endocrinol. Diabetes. Obes. 17, 453–459. https://doi.org/10.1097/MED.0B013E32833DDEA0

Grün, F., Blumberg, B., 2009. Endocrine disrupters as obesogens. Mol. Cell. Endocrinol. 304, 19–29. https://doi.org/10.1016/J.MCE.2009.02.018

Grün, F., Blumberg, B., 2006. Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. Endocrinology 147. https://doi.org/10.1210/EN.2005-1129

Grundy, S.M., 2016. Metabolic syndrome update. Trends Cardiovasc. Med. 26, 364–373. https://doi.org/10.1016/J.TCM.2015.10.004

Grünfeld, H.T., Bonefeld-Jorgensen, E.C., 2004. Effect of in vitro estrogenic pesticides on human oestrogen receptor alpha and beta mRNA levels. Toxicol. Lett. 151, 467–480. https://doi.org/10.1016/J.TOXLET.2004.03.021

Gupta, R., Kumar, P., Fahmi, N., Garg, B., Dutta, S., Sachar, S., Matharu, A.S., Vimaleswaran, K.S., 2020. Endocrine disruption and obesity: A current review on environmental obesogens. Curr. Res. Green Sustain. Chem. 3, 100009. https://doi.org/10.1016/J.CRGSC.2020.06.002

Harnett, K.G., Chin, A., Schuh, S.M., 2021a. Cytotoxic and apoptotic data of BPA and BPA alternatives TMBPF, BPAF, and BPS in female adult rat and human stem cells. Data Br. 37. https://doi.org/10.1016/J.DIB.2021.107183

Harnett, K.G., Chin, A., Schuh, S.M., 2021b. BPA and BPA alternatives BPS, BPAF, and TMBPF, induce cytotoxicity and apoptosis in rat and human stem cells. Ecotoxicol. Environ. Saf. 216. https://doi.org/10.1016/J.ECOENV.2021.112210

Hatch, E.E., Nelson, J.W., Stahlhut, R.W., Webster, T.F., 2010. Association of endocrine disruptors and obesity: perspectives from epidemiological studies. Int. J. Androl. 33, 324–332. https://doi.org/10.1111/J.1365-2605.2009.01035.X

Heindel, J.J., Blumberg, B., Cave, M., Machtinger, R., Mantovani, A., Mendez, M.A., Nadal, A., Palanza, P., Panzica, G., Sargis, R., Vandenberg, L.N., vom Saal, F., 2017. Metabolism disrupting chemicals and metabolic disorders. Reprod. Toxicol. 68, 3–33. https://doi.org/10.1016/J.REPROTOX.2016.10.001

Heindel, J.J., Howard, S., Agay-Shay, K., Arrebola, J.P., Audouze, K., Babin, P.J., Barouki, R., Bansal, A., Blanc, E., Cave, M.C., Chatterjee, S., Chevalier, N., Choudhury, M., Collier, D., Connolly, L., Coumoul, X., Garruti, G., Gilbertson, M., Hoepner, L.A., Holloway, A.C., Howell, G., Kassotis, C.D., Kay, M.K., Ji Kim, M., Lagadic-Gossmann, D., Langouet, S., Legrand, A., Li, Z., Le Mentec, H., Lind, L., Monica Lind, P., Lustig, R.H., Martin-Chouly, C., Munic Kos, V., Podechard, N., Roepke, T.A., Sargis, R.M., Starling, A., Tomlinson, C.R., Touma, C., Vondracek, J., vom Saal, F., Blumberg, B., 2022. Obesity II: Establishing causal links between chemical exposures and

obesity. Biochem. Pharmacol. 199. https://doi.org/10.1016/J.BCP.2022.115015

Heindel, J.J., Newbold, R., Schug, T.T., 2015a. Endocrine disruptors and obesity. Nat. Rev. Endocrinol. 11, 653–661. https://doi.org/10.1038/NRENDO.2015.163

Heindel, J.J., Vom Saal, F.S., Blumberg, B., Bovolin, P., Calamandrei, G., Ceresini, G., Cohn, B.A., Fabbri, E., Gioiosa, L., Kassotis, C., Legler, J., La Merrill, M., Rizzir, L., Machtinger, R., Mantovani, A., Mendez, M.A., Montanini, L., Molteni, L., Nagel, S.C., Parmigiani, S., Panzica, G., Paterlini, S., Pomatto, V., Ruzzin, J., Sartor, G., Schug, T.T., Street, M.E., Suvorov, A., Volpi, R., Zoeller, R.T., Palanza, P., 2015b. Parma consensus statement on metabolic disruptors. Environ. Health 14. https://doi.org/10.1186/S12940-015-0042-7

Héliès-Toussaint, C., Peyre, L., Costanzo, C., Chagnon, M.C., Rahmani, R., 2014. Is bisphenol S a safe substitute for bisphenol A in terms of metabolic function? An in vitro study. Toxicol. Appl. Pharmacol. 280, 224–235. https://doi.org/10.1016/j.taap.2014.07.025

Henríquez-Hernández, L.A., Luzardo, O.P., Zumbado, M., Camacho, M., Serra-Majem, L., Álvarez-León, E.E., Boada, L.D., 2014. Blood pressure in relation to contamination by polychlorobiphenyls and organochlorine pesticides: Results from a population-based study in the Canary Islands (Spain). Environ. Res. 135, 48–54. https://doi.org/10.1016/J.ENVRES.2014.05.036

High Level Political Forum of United Nations on Sustainable Development., 2022. Stockholm Convention on Persistent Organic Pollutants [WWW Document]. URL https://hlpf.un.org/inputs/stockholm-convention-on-

persistent-organic-pollutants-0 (accessed 1.20.23).

Hirode, G., Wong, R.J., 2020. Trends in the Prevalence of Metabolic Syndrome in the United States, 2011-2016. JAMA 323, 2526–2528. https://doi.org/10.1001/JAMA.2020.4501

Höhn, A., König, J., Jung, T., 2016. Metabolic Syndrome, Redox State, and the Proteasomal System. Antioxid. Redox Signal. 25, 902–917. https://doi.org/10.1089/ARS.2016.6815

Ibarluzea, J.M., Fernández, M.F., Santa-Marina, L., Olea-Serrano, M.F., Rivas, A.M., Aurrekoetxea, J.J., Expósito, J., Lorenzo, M., Torné, P., Villalobos, M., Pedraza, V., Sasco, A.J., Olea, N., 2004. Breast cancer risk and the combined effect of environmental estrogens. Cancer Causes Control 15, 591–600. https://doi.org/10.1023/B:CACO.0000036167.51236.86

Ivry Del Moral, L., Le Corre, L., Hélène, P., Isabelle, N., Caroline, T., Jean-François, M., Patrick, R., Philippe, B., Roger, R., Marie-Christine, C., 2016. Obesogen effects after perinatal exposure of 4,4′-sulfonyldiphenol (Bisphenol S) in C57BL/6 mice. Toxicology. https://doi.org/10.1016/j.tox.2016.05.023

Jackson, E., Shoemaker, R., Larian, N., Cassis, L., 2017. Adipose Tissue as a Site of Toxin Accumulation. Compr. Physiol. 7, 1085–1135. https://doi.org/10.1002/CPHY.C160038

Janderová, L., McNeil, M., Murrell, A.N., Mynatt, R.L., Smith, S.R., 2003. Human mesenchymal stem cells as an in vitro model for human adipogenesis. Obes. Res. 11, 65–74. https://doi.org/10.1038/OBY.2003.11

Janesick, A., Blumberg, B., 2012. Obesogens, Stem Cells and the

Developmental Programming of Obesity. Int. J. Androl. 35, 437. https://doi.org/10.1111/J.1365-2605.2012.01247.X

Janesick, A.S., Blumberg, B., 2016. Obesogens: an emerging threat to public health. Am. J. Obstet. Gynecol. 214, 559–565. https://doi.org/10.1016/J.AJOG.2016.01.182

Jin, H., Xie, J., Mao, L., Zhao, M., Bai, X., Wen, J., Shen, T., Wu, P., 2020. Bisphenol analogue concentrations in human breast milk and their associations with postnatal infant growth. Environ. Pollut. 259. https://doi.org/10.1016/J.ENVPOL.2019.113779

Kang, S., Shin, B.H., Kwon, J.A., Lee, C.W., Park, E.K., Park, E.Y., Kim, B., 2020. Urinary bisphenol A and its analogues and haemato-biochemical alterations of pregnant women in Korea. Environ. Res. 182. https://doi.org/10.1016/J.ENVRES.2019.109104

Karmaus, W., Osuch, J.R., Eneli, I., Mudd, L.M., Zhang, J., Mikucki, D., Haan, P., Davis, S., 2009. Maternal levels of dichlorodiphenyl-dichloroethylene (DDE) may increase weight and body mass index in adult female offspring. Occup. Environ. Med. 66, 143–149. https://doi.org/10.1136/OEM.2008.041921

Karrer, C., De Boer, W., Delmaar, C., Cai, Y., Crépet, A., Hungerbühler, K., Von Goetz, N., 2019. Linking Probabilistic Exposure and Pharmacokinetic Modeling To Assess the Cumulative Risk from the Bisphenols BPA, BPS, BPF, and BPAF for Europeans. Environ. Sci. Technol. 53, 9181–9191. https://doi.org/10.1021/ACS.EST.9B01749

Kassotis, C.D., Hoffman, K., Völker, J., Pu, Y., Veiga-Lopez, A., Kim, S.M., Schlezinger, J.J., Bovolin, P., Cottone, E., Saraceni, A., Scandiffio, R., Atlas, E., Leingartner, K., Krager, S., Tischkau, S.A., Ermler, S.,

Legler, J., Chappell, V.A., Fenton, S.E., Mesmar, F., Bondesson, M., Fernández, M.F., Stapleton, H.M., 2021. Reproducibility of adipogenic responses to metabolism disrupting chemicals in the 3T3-L1 pre-adipocyte model system: An interlaboratory study. Toxicology 461. https://doi.org/10.1016/J.TOX.2021.152900

Kassotis, C.D., Masse, L., Kim, S., Schlezinger, J.J., Webster, T.F., Stapleton, H.M., 2017. Characterization of Adipogenic Chemicals in Three Different Cell Culture Systems: Implications for Reproducibility Based on Cell Source and Handling. Sci. Rep. 7, 1–17. https://doi.org/10.1038/srep42104

Kassotis, C.D., Stapleton, H.M., 2019. Endocrine-mediated mechanisms of metabolic disruption and new approaches to examine the public health threat. Front. Endocrinol. (Lausanne). 10, 1–22. https://doi.org/10.3389/fendo.2019.00039

Kassotis, C.D., vom Saal, F.S., Babin, P.J., Lagadic-Gossmann, D., Le Mentec, H., Blumberg, B., Mohajer, N., Legrand, A., Munic Kos, V., Martin-Chouly, C., Podechard, N., Langouët, S., Touma, C., Barouki, R., Ji Kim, M., Audouze, K., Choudhury, M., Shree, N., Bansal, A., Howard, S., Heindel, J.J., 2022. Obesity III: Obesogen assays: Limitations, strengths, and new directions. Biochem. Pharmacol. 199. https://doi.org/10.1016/J.BCP.2022.115014

Kershaw, E.E., Flier, J.S., 2004. Adipose tissue as an endocrine organ. J. Clin. Endocrinol. Metab. 89, 2548–2556. https://doi.org/10.1210/JC.2004-0395

Kim, J.T., Kang, J.H., Chang, Y.S., Lee, D.H., Choi, S.D., 2018. Determinants of serum organochlorine pesticide and polychlorinated biphenyl levels in middle-aged Korean adults. Environ. Sci. Pollut. Res. Int.

25, 249–259. https://doi.org/10.1007/S11356-017-0382-7

Kim, J.T., Lee, H.K., 2014. Metabolic syndrome and the environmental pollutants from mitochondrial perspectives. Rev. Endocr. Metab. Disord. 15, 253–262. https://doi.org/10.1007/S11154-014-9297-5

Kim, Y.A., Park, J.B., Woo, M.S., Lee, S.Y., Kim, H.Y., Yoo, Y.H., 2019. Persistent Organic Pollutant-Mediated Insulin Resistance. Int. J. Environ. Res. Public Health 16. https://doi.org/10.3390/IJERPH16030448

Kohlmeier, L., Kohlmeier, M., 1995. Adipose tissue as a medium for epidemiologic exposure assessment. Environ. Health Perspect. 103 Suppl 3, 99–106. https://doi.org/10.1289/EHP.95103S399

Kojima, H., Takeuchi, S., Sanoh, S., Okuda, K., Kitamura, S., Uramaru, N., Sugihara, K., Yoshinari, K., 2019. Profiling of bisphenol A and eight its analogues on transcriptional activity via human nuclear receptors. Toxicology 413, 48–55. https://doi.org/10.1016/J.TOX.2018.12.001

Kortenkamp, A., 2014. Low dose mixture effects of endocrine disrupters and their implications for regulatory thresholds in chemical risk assessment. Curr. Opin. Pharmacol. 19, 105–111. https://doi.org/10.1016/J.COPH.2014.08.006

Kortenkamp, A., 2008. Low dose mixture effects of endocrine disrupters: implications for risk assessment and epidemiology. Int. J. Androl. 31, 233–240. https://doi.org/10.1111/J.1365-2605.2007.00862.X

Kortenkamp, A., 2007. Ten years of mixing cocktails: a review of

combination effects of endocrine-disrupting chemicals. Environ. Health Perspect. 115 Suppl 1, 98–105. https://doi.org/10.1289/EHP.9357

Kudłak, B., Wieczerzak, M., Namieśnik, J., 2019. Bisphenols (A, S, and F) affect the basic hormonal activity determined for pharmaceuticals – Study of Saccharomyces cerevisiae. Environ. Pollut. https://doi.org/10.1016/j.envpol.2018.12.052

Kurşunoğlu, N.E., Sarer Yurekli, B.P., 2022. Endocrine disruptor chemicals as obesogen and diabetogen: Clinical and mechanistic evidence. World J. Clin. cases 10, 11226–11239. https://doi.org/10.12998/WJCC.V10.I31.11226

La Merrill, M., Emond, C., Kim, M.J., Antignac, J.P., Le Bizec, B., Clément, K., Birnbaum, L.S., Barouki, R., 2013. Toxicological function of adipose tissue: focus on persistent organic pollutants. Environ. Health Perspect. 121, 162–169. https://doi.org/10.1289/EHP.1205485

La Merrill, M.A., Vandenberg, L.N., Smith, M.T., Goodson, W., Browne, P., Patisaul, H.B., Guyton, K.Z., Kortenkamp, A., Cogliano, V.J., Woodruff, T.J., Rieswijk, L., Sone, H., Korach, K.S., Gore, A.C., Zeise, L., Zoeller, R.T., 2020. Consensus on the key characteristics of endocrine-disrupting chemicals as a basis for hazard identification. Nat. Rev. Endocrinol. 16, 45–57. https://doi.org/10.1038/S41574-019-0273-8

Lam D.W. and LeRoith D., 2019. Metabolic Syndrome - PubMed [WWW Document]. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000–. https://doi.org/PMID: 25905173.

Lamat, H., Sauvant-Rochat, M.P., Tauveron, I., Bagheri, R., Ugbolue, U.C.,

Maqdasi, S., Navel, V., Dutheil, F., 2022. Metabolic syndrome and pesticides: A systematic review and meta-analysis. Environ. Pollut. 305. https://doi.org/10.1016/J.ENVPOL.2022.119288

Lambert, S.A., Jolma, A., Campitelli, L.F., Das, P.K., Yin, Y., Albu, M., Chen, X., Taipale, J., Hughes, T.R., Weirauch, M.T., 2018. The Human Transcription Factors. Cell 172, 650–665. https://doi.org/10.1016/J.CELL.2018.01.029

Latchman, D.S., 1993. Transcription factors: an overview. Int. J. Exp. Pathol. 74, 417422.

Le Fol, V., Aït-Aïssa, S., Sonavane, M., Porcher, J.M., Balaguer, P., Cravedi, J.P., Zalko, D., Brion, F., 2017. In vitro and in vivo estrogenic activity of BPA, BPF and BPS in zebrafish-specific assays. Ecotoxicol. Environ. Saf. 142, 150–156. https://doi.org/10.1016/J.ECOENV.2017.04.009

Lee, D.H., Lee, I.K., Porta, M., Steffes, M., Jacobs, D.R., 2007. Relationship between serum concentrations of persistent organic pollutants and the prevalence of metabolic syndrome among non-diabetic adults: results from the National Health and Nutrition Examination Survey 1999-2002. Diabetologia 50, 1841–1851. https://doi.org/10.1007/S00125-007-0755-4

Lee, D.H., Lind, L., Jacobs, D.R., Salihovic, S., van Bavel, B., Lind, P.M., 2012. Associations of persistent organic pollutants with abdominal obesity in the elderly: The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study. Environ. Int. 40, 170–178. https://doi.org/10.1016/J.ENVINT.2011.07.010

Lee, M.J., Wu, Y., Fried, S.K., 2013. Adipose tissue heterogeneity: implication of depot differences in adipose tissue for obesity

complications. Mol. Aspects Med. 34, 1–11. https://doi.org/10.1016/J.MAM.2012.10.001

Lee, Y.M., Kim, K.S., Jacobs, D.R., Lee, D.H., 2017. Persistent organic pollutants in adipose tissue should be considered in obesity research. Obes. Rev. 18, 129–139. https://doi.org/10.1111/OBR.12481

Lee, Y.M., Kim, K.S., Kim, S.A., Hong, N.S., Lee, S.J., Lee, D.H., 2014. Prospective associations between persistent organic pollutants and metabolic syndrome: a nested case-control study. Sci. Total Environ. 496, 219–225. https://doi.org/10.1016/J.SCITOTENV.2014.07.039

Lefterova, M.I., Lazar, M.A., 2009. New developments in adipogenesis. Trends Endocrinol. Metab. https://doi.org/10.1016/j.tem.2008.11.005

Legler, J., Zalko, D., Jourdan, F., Jacobs, M., Fromenty, B., Balaguer, P., Bourguet, W., Kos, V.M., Nadal, A., Beausoleil, C., Cristobal, S., Remy, S., Ermler, S., Margiotta-Casaluci, L., Griffin, J.L., Blumberg, B., Chesné, C., Hoffmann, S., Andersson, P.L., Kamstra, J.H., 2020. The GOLIATH Project: Towards an Internationally Harmonised Approach for Testing Metabolism Disrupting Compounds. Int. J. Mol. Sci. 21. https://doi.org/10.3390/IJMS21103480

Lehmler, H.J., Liu, B., Gadogbe, M., Bao, W., 2018. Exposure to Bisphenol A, Bisphenol F, and Bisphenol S in U.S. Adults and Children: The National Health and Nutrition Examination Survey 2013-2014. ACS omega 3, 6523–6532. https://doi.org/10.1021/ACSOMEGA.8B00824

Lim, M.H., Ong, W.K., Sugii, S., 2014. The current landscape of adipose-

derived stem cells in clinical applications. Expert Rev. Mol. Med. 16. https://doi.org/10.1017/ERM.2014.8

Liu, J., Wattar, N., Field, C.J., Dinu, I., Dewey, D., Martin, J.W., 2018. Exposure and dietary sources of bisphenol A (BPA) and BPA-alternatives among mothers in the APrON cohort study. Environ. Int. 119, 319–326. https://doi.org/10.1016/J.ENVINT.2018.07.001

Liu, J., Zhang, Y., Lavie, C.J., Moran, A.E., 2022. Trends in Metabolic Phenotypes According to Body Mass Index Among US Adults, 1999-2018. Mayo Clin. Proc. 97. https://doi.org/10.1016/J.MAYOCP.2022.02.013

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408. https://doi.org/10.1006/METH.2001.1262

Longo, M., Zatterale, F., Naderi, J., Parrillo, L., Formisano, P., Raciti, G.A., Beguinot, F., Miele, C., 2019. Adipose Tissue Dysfunction as Determinant of Obesity-Associated Metabolic Complications. Int. J. Mol. Sci. 20. https://doi.org/10.3390/IJMS20092358

Lonza, n.d. Human Adipose-Derived Stem Cells (hADSC), PT-5006 | Lonza [WWW Document]. URL https://bioscience.lonza.com/lonza_bs/ES/en/Primary-and-Stem-Cells/p/000000000000186731/hADSC-Human-Adipose-Derived-Stem-Cells# (accessed 1.31.23).

Luo, L., Liu, M., 2016. Adipose tissue in control of metabolism. J. Endocrinol. 231, R77–R99. https://doi.org/10.1530/JOE-16-0211

Lustig, R.H., Collier, D., Kassotis, C., Roepke, T.A., Ji Kim, M., Blanc, E., Barouki, R., Bansal, A., Cave, M.C., Chatterjee, S., Choudhury, M., Gilbertson, M., Lagadic-Gossmann, D., Howard, S., Lind, L., Tomlinson, C.R., Vondracek, J., Heindel, J.J., 2022. Obesity I: Overview and molecular and biochemical mechanisms. Biochem. Pharmacol. 199. https://doi.org/10.1016/J.BCP.2022.115012

Marroqui, L., Martinez-Pinna, J., Castellano-Muñoz, M., dos Santos, R.S., Medina-Gali, R.M., Soriano, S., Quesada, I., Gustafsson, J.A., Encinar, J.A., Nadal, A., 2021. Bisphenol-S and Bisphenol-F alter mouse pancreatic β-cell ion channel expression and activity and insulin release through an estrogen receptor ERβ mediated pathway. Chemosphere 265. https://doi.org/10.1016/J.CHEMOSPHERE.2020.129051

Martínez, M., Blanco, J., Rovira, J., Kumar, V., Domingo, J.L., Schuhmacher, M., 2020. Bisphenol A analogues (BPS and BPF) present a greater obesogenic capacity in 3T3-L1 cell line. Food Chem. Toxicol. 140. https://doi.org/10.1016/J.FCT.2020.111298

Martínez Vidal, J.L., Moreno Frías, M., Garrido Frenich, A., Olea-Serrano, F., Olea, N., 2002. Determination of endocrine-disrupting pesticides and polychlorinated biphenyls in human serum by GC-ECD and GC-MS-MS and evaluation of contributions to the uncertainty of the results. Anal. Bioanal. Chem. 372, 766–775. https://doi.org/10.1007/S00216-002-1272-4

Masuno, H., Iwanami, J., Kidani, T., Sakayama, K., Honda, K., 2005. Bisphenol a accelerates terminal differentiation of 3T3-L1 cells into adipocytes through the phosphatidylinositol 3-kinase pathway. Toxicol. Sci. 84, 319–27.

https://doi.org/10.1093/toxsci/kfi088

Masuno, H., Kidani, T., Sekiya, K., Sakayama, K., Shiosaka, T., Yamamoto, H., Honda, K., 2002. Bisphenol A in combination with insulin can accelerate the conversion of 3T3-L1 fibroblasts to adipocytes. J. Lipid Res. 43, 676–684. https://doi.org/10.1016/S0022-2275(20)30108-5

McEwan, I.J., 2016. The Nuclear Receptor Superfamily at Thirty. Methods Mol. Biol. 1443, 3–9. https://doi.org/10.1007/978-1-4939-3724-0_1

Mehta, S.S., James-Todd, T., Applebaum, K.M., Bellavia, A., Coleman-Phox, K., Adler, N., Laraia, B., Epel, E., Parry, E., Wang, M., Park, J.S., Zota, A.R., 2021. Persistent organic pollutants and maternal glycemic outcomes in a diverse pregnancy cohort of overweight women. Environ. Res. 193. https://doi.org/10.1016/J.ENVRES.2020.110551

Mejhert, N., Rydén, M., 2020. Novel aspects on the role of white adipose tissue in type 2 diabetes. Curr. Opin. Pharmacol. 55, 47–52. https://doi.org/10.1016/J.COPH.2020.09.008

Merrheim, J., Villegas, J., Van Wassenhove, J., Khansa, R., Berrih-Aknin, S., le Panse, R., Dragin, N., 2020. Estrogen, estrogen-like molecules and autoimmune diseases. Autoimmun. Rev. 19. https://doi.org/10.1016/J.AUTREV.2020.102468

Meslin, M., Beausoleil, C., Zeman, F.A., Antignac, J., Kolossa-gehring, M., Rousselle, C., Apel, P., 2022. Human Biomonitoring Guidance Values (HBM-GVs) for Bisphenol S and Assessment of the Risk Due to the Exposure to Bisphenols A and S, in Europe.

Molina-Molina, J.M., Amaya, E., Grimaldi, M., Sáenz, J.M., Real, M., Fernández, M.F., Balaguer, P., Olea, N., 2013. In vitro study on the agonistic and antagonistic activities of bisphenol-S and other bisphenol-A congeners and derivatives via nuclear receptors. Toxicol. Appl. Pharmacol. 272, 127–136. https://doi.org/10.1016/J.TAAP.2013.05.015

Moore, J.X., Chaudhary, N., Akinyemiju, T., 2017. Metabolic Syndrome Prevalence by Race/Ethnicity and Sex in the United States, National Health and Nutrition Examination Survey, 1988-2012. Prev. Chronic Dis. 14. https://doi.org/10.5888/PCD14.160287

Moreno Frías, M., Jiménez Torres, M., Garrido Frenich, A., Martínez Vidal, J.L., Olea-Serrano, F., Olea, N., 2004. Determination of organochlorine compounds in human biological samples by GC-MS/MS. Biomed. Chromatogr. 18, 102–111. https://doi.org/10.1002/BMC.300

Mrema, E.J., Rubino, F.M., Brambilla, G., Moretto, A., Tsatsakis, A.M., Colosio, C., 2013. Persistent organochlorinated pesticides and mechanisms of their toxicity. Toxicology 307, 74–88. https://doi.org/10.1016/J.TOX.2012.11.015

Muntner, P., Hardy, S.T., Fine, L.J., Jaeger, B.C., Wozniak, G., Levitan, E.B., Colantonio, L.D., 2020. Trends in Blood Pressure Control Among US Adults With Hypertension, 1999-2000 to 2017-2018. JAMA 324, 1190–1200. https://doi.org/10.1001/JAMA.2020.14545

Mustieles, V., Arrebola, J.P., 2020. How polluted is your fat? What the study of adipose tissue can contribute to environmental epidemiology. J. Epidemiol. Community Health 74, 401–407. https://doi.org/10.1136/JECH-2019-213181

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

Mustieles, V., Fernández, M.F., Martin-Olmedo, P., González-Alzaga, B., Fontalba-Navas, A., Hauser, R., Olea, N., Arrebola, J.P., 2017. Human adipose tissue levels of persistent organic pollutants and metabolic syndrome components: Combining a cross-sectional with a 10-year longitudinal study using a multi-pollutant approach. Environ. Int. 104, 48–57. https://doi.org/10.1016/J.ENVINT.2017.04.002

Mustieles, V., Pérez-Carrascosa, F.M., León, J., Lange, T., Bonde, J.P., Gómez-Peña, C., Artacho-Cordón, F., Barrios-Rodríguez, R., Olmedo-Requena, R., Expósito, J., Jiménez-Moleón, J.J., Arrebola, J.P., 2021. Adipose Tissue Redox Microenvironment as a Potential Link between Persistent Organic Pollutants and the 16-Year Incidence of Non-hormone-Dependent Cancer. Environ. Sci. Technol. 55, 9926–9937. https://doi.org/10.1021/ACS.EST.0C08180

Nadal, A., Quesada, I., Tudurí, E., Nogueiras, R., Alonso-Magdalena, P., 2017. Endocrine-disrupting chemicals and the regulation of energy balance. Nat. Rev. Endocrinol. 13, 536–546. https://doi.org/10.1038/NRENDO.2017.51

National Research Council (U.S.)., Committee on Human Biomonitoring for Environmental Toxicants., Toxicology., N.R.C. (U. S.)., And, B.

on E.S., 2006. Human biomonitoring for environmental chemicals 291. https://doi.org/10.17226/11700

Newbold, R.R., Padilla-Banks, E., Jefferson, W.N., 2009. Environmental estrogens and obesity. Mol. Cell. Endocrinol. 304, 84–89. https://doi.org/10.1016/J.MCE.2009.02.024

Newbold, R.R., Padilla-Banks, E., Snyder, R.J., Jefferson, W.N., 2005. Developmental exposure to estrogenic compounds and obesity. Birth Defects Res. A. Clin. Mol. Teratol. 73, 478–480. https://doi.org/10.1002/BDRA.20147

Niu, Y., Wang, B., Yang, R., Wu, Y., Zhao, Y., Li, C., Zhang, J., Xing, Y., Shao, B., 2021. Bisphenol Analogues and Their Chlorinated Derivatives in Breast Milk in China: Occurrence and Exposure Assessment. J. Agric. Food Chem. 69, 1391–1397. https://doi.org/10.1021/ACS.JAFC.0C06938

Niu, Y., Wang, B., Zhao, Y., Zhang, J., Shao, B., 2017. Highly Sensitive and High-Throughput Method for the Analysis of Bisphenol Analogues and Their Halogenated Derivatives in Breast Milk. J. Agric. Food Chem. 65, 10452–10463. https://doi.org/10.1021/acs.jafc.7b04394

Noubiap, J.J., Nansseu, J.R., Lontchi-Yimagou, E., Nkeck, J.R., Nyaga, U.F., Ngouo, A.T., Tounouga, D.N., Tianyi, F.L., Foka, A.J., Ndoadoumgue, A.L., Bigna, J.J., 2022a. Geographic distribution of metabolic syndrome and its components in the general adult population: A meta-analysis of global data from 28 million individuals. Diabetes Res. Clin. Pract. 188. https://doi.org/10.1016/J.DIABRES.2022.109924

Noubiap, J.J., Nansseu, J.R., Lontchi-Yimagou, E., Nkeck, J.R., Nyaga, U.F.,

Ngouo, A.T., Tounouga, D.N., Tianyi, F.L., Foka, A.J., Ndoadoumgue, A.L., Bigna, J.J., 2022b. Global, regional, and country estimates of metabolic syndrome burden in children and adolescents in 2020: a systematic review and modelling analysis. Lancet. Child Adolesc. Heal. 6, 158–170. https://doi.org/10.1016/S2352-4642(21)00374-6

Ohlstein, J.F., Strong, A.L., McLachlan, J.A., Gimble, J.M., Burow, M.E., Bunnell, B.A., 2014. Bisphenol a enhances adipogenic differentiation of human adipose stromal/stem cells. J. Mol. Endocrinol. 53, 345–353. https://doi.org/10.1530/JME-14-0052

Ougier, E., Zeman, F., Antignac, J.P., Rousselle, C., Lange, R., Kolossa-Gehring, M., Apel, P., 2021. Human biomonitoring initiative (HBM4EU): Human biomonitoring guidance values (HBM-GVs) derived for bisphenol A. Environ. Int. 154, 106563. https://doi.org/10.1016/j.envint.2021.106563

Palmer, M.K., Toth, P.P., 2019. Trends in Lipids, Obesity, Metabolic Syndrome, and Diabetes Mellitus in the United States: An NHANES Analysis (2003-2004 to 2013-2014). Obesity (Silver Spring). 27, 309–314. https://doi.org/10.1002/OBY.22370

Pandolfi, J.B., Ferraro, A.A., Sananez, I., Gancedo, M.C., Baz, P., Billordo, L.A., Fainboim, L., Arruvito, L., 2016. ATP-Induced Inflammation Drives Tissue-Resident Th17 Cells in Metabolically Unhealthy Obesity. J. Immunol. 196, 3287–3296. https://doi.org/10.4049/JIMMUNOL.1502506

Papalou, O., Kandaraki, E.A., Papadakis, G., Diamanti-Kandarakis, E., 2019. Endocrine disrupting chemicals: An occult mediator of metabolic disease. Front. Endocrinol. (Lausanne).

https://doi.org/10.3389/fendo.2019.00112

Park, C., Song, H., Choi, J., Sim, S., Kojima, H., Park, J., Iida, M., Lee, Y.J., 2020. The mixture effects of bisphenol derivatives on estrogen receptor and androgen receptor. Environ. Pollut. 260. https://doi.org/10.1016/J.ENVPOL.2020.114036

Park, S.K., Son, H.K., Lee, S.K., Kang, J.H., Chang, Y.S., Jacobs, D.R., Lee, D.H., 2010. Relationship between serum concentrations of organochlorine pesticides and metabolic syndrome among non-diabetic adults. J. Prev. Med. Public Health 43, 1–18. https://doi.org/10.3961/JPMPH.2010.43.1.1

Pastor-Barriuso, R., Fernández, M.F., Castaño-Vinyals, G., Whelan, D., Pérez-Gómez, B., Llorca, J., Villanueva, C.M., Guevara, M., Molina-Molina, J.M., Artacho-Cordón, F., Barriuso-Lapresa, L., Tusquets, I., Dierssen-Sotos, T., Aragonés, N., Olea, N., Kogevinas, M., Pollán, M., 2016. Total Effective Xenoestrogen Burden in Serum Samples and Risk for Breast Cancer in a Population-Based Multicase-Control Study in Spain. Environ. Health Perspect. 124, 1575–1582. https://doi.org/10.1289/EHP157

Patel, N.G., Holder, J.C., Smith, S.A., Kumar, S., Eggo, M.C., 2003. Differential regulation of lipogenesis and leptin production by independent signaling pathways and rosiglitazone during human adipocyte differentiation. Diabetes 52, 43–50. https://doi.org/10.2337/DIABETES.52.1.43

Penell, J., Lind, L., Salihovic, S., van Bavel, B., Lind, P.M., 2014. Persistent organic pollutants are related to the change in circulating lipid levels during a 5 year follow-up. Environ. Res. 134, 190–197. https://doi.org/10.1016/J.ENVRES.2014.08.005

Pereira-Fernandes, A., Dirinck, E., Dirtu, A.C., Malarvannan, G., Covaci, A., Van Gaal, L., Vanparys, C., Jorens, P.G., Blust, R., 2014. Expression of obesity markers and Persistent Organic Pollutants levels in adipose tissue of obese patients: reinforcing the obesogen hypothesis? PLoS One 9. https://doi.org/10.1371/JOURNAL.PONE.0084816

Perrini, S., Cignarelli, A., Ficarella, R., Laviola, L., Giorgino, F., 2009. Human adipose tissue precursor cells: a new factor linking regulation of fat mass to obesity and type 2 diabetes? Arch. Physiol. Biochem. 115, 218–226. https://doi.org/10.1080/13813450903260864

Peshdary, V., Styles, G., Gagné, R., Yauk, C.L., Sorisky, A., Atlas, E., 2020. Depot-Specific Analysis of Human Adipose Cells and Their Responses to Bisphenol S. Endocrinology 161. https://doi.org/10.1210/ENDOCR/BQAA044

Pestana, D., Faria, G., Sá, C., Fernandes, V.C., Teixeira, D., Norberto, S., Faria, A., Meireles, M., Marques, C., Correia-Sá, L., Cunha, A., Guimarães, J.T., Taveira-Gomes, A., Santos, A.C., Domingues, V.F., Delerue-Matos, C., Monteiro, R., Calhau, C., 2014. Persistent organic pollutant levels in human visceral and subcutaneous adipose tissue in obese individuals--depot differences and dysmetabolism implications. Environ. Res. 133, 170–177. https://doi.org/10.1016/J.ENVRES.2014.05.026

Peters, S.A.E., Muntner, P., Woodward, M., 2019. Sex Differences in the Prevalence of, and Trends in, Cardiovascular Risk Factors, Treatment, and Control in the United States, 2001 to 2016. Circulation 139, 1025–1035.

https://doi.org/10.1161/CIRCULATIONAHA.118.035550

Ramírez-Zacarías, J.L., Castro-Muñozledo, F., Kuri-Harcuch, W., 1992. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. Histochemistry 97, 493–497. https://doi.org/10.1007/BF00316069

Ramskov Tetzlaff, C.N., Svingen, T., Vinggaard, A.M., Rosenmai, A.K., Taxvig, C., 2020. Bisphenols B, E, F, and S and 4-cumylphenol induce lipid accumulation in mouse adipocytes similarly to bisphenol A. Environ. Toxicol. 35, 543–552. https://doi.org/10.1002/tox.22889

Reina-Pérez, I., Artacho-Cordón, F., Mustieles, V., Castellano-Castillo, D., Cardona, F., Jiménez-Díaz, I., López-Medina, J.A., Alcaide, J., Ocaña-Wilhelmi, L., Iribarne-Durán, L.M., Arrebola, J.P., Olea, N., Tinahones, F.J., Fernández, M.F., 2023. Cross-sectional associations of persistent organic pollutants measured in adipose tissue and metabolic syndrome in clinically diagnosed middle-aged adults. Environ. Res. 115350. https://doi.org/10.1016/J.ENVRES.2023.115350

Reina-Pérez, I., Olivas-Martínez, A., Mustieles, V., Ruiz-Ojeda, F.J., Molina-Molina, J.M., Olea, N., Fernández, M.F., 2021. Bisphenol F and bisphenol S promote lipid accumulation and adipogenesis in human adipose-derived stem cells. Food Chem. Toxicol. https://doi.org/10.1016/j.fct.2021.112216

Reina-Pérez, I., Olivas-Martínez, A., Mustieles, V., Salamanca-Fernández, E., Molina-Molina, J.M., Olea, N., Fernández, M.F., 2022. The Mixture of Bisphenol-A and Its Substitutes Bisphenol-S and Bisphenol-F Exerts Obesogenic Activity on Human Adipose-

Derived Stem Cells. Toxics 10. https://doi.org/10.3390/TOXICS10060287

Repouskou, A., Papadopoulou, A.K., Panagiotidou, E., Trichas, P., Lindh, C., Bergman, Å., Gennings, C., Bornehag, C.G., Rüegg, J., Kitraki, E., Stamatakis, A., 2020. Long term transcriptional and behavioral effects in mice developmentally exposed to a mixture of endocrine disruptors associated with delayed human neurodevelopment. Sci. Rep. https://doi.org/10.1038/s41598-020-66379-x

Richterová, D., Govarts, E., Fábelová, L., Rausová, K., Rodriguez Martin, L., Gilles, L., Remy, S., Colles, A., Rambaud, L., Riou, M., Gabriel, C., Sarigiannis, D., Pedraza-Diaz, S., Ramos, J.J., Kosjek, T., Snoj Tratnik, J., Lignell, S., Gyllenhammar, I., Thomsen, C., Haug, L.S., Kolossa-Gehring, M., Vogel, N., Franken, C., Vanlarebeke, N., Bruckers, L., Stewart, L., Sepai, O., Schoeters, G., Uhl, M., Castaño, A., Esteban López, M., Göen, T., Palkovičová Murínová, 2023. PFAS levels and determinants of variability in exposure in European teenagers - Results from the HBM4EU aligned studies (2014-2021). Int. J. Hyg. Environ. Health 247. https://doi.org/10.1016/J.IJHEH.2022.114057

Riu, A., Grimaldi, M., le Maire, A., Bey, G., Phillips, K., Boulahtouf, A., Perdu, E., Zalko, D., Bourguet, W., Balaguer, P., 2011a. Peroxisome proliferator-activated receptor γ is a target for halogenated analogs of bisphenol A. Environ. Health Perspect. 119, 1227–1232. https://doi.org/10.1289/EHP.1003328

Riu, A., le Maire, A., Grimaldi, M., Audebert, M., Hillenweck, A., Bourguet, W., Balaguer, P., Zalko, D., 2011b. Characterization of novel ligands of ERα, Erβ, and PPARγ: the case of halogenated bisphenol

A and their conjugated metabolites. Toxicol. Sci. 122, 372–382. https://doi.org/10.1093/TOXSCI/KFR132

Rochester, J.R., Bolden, A.L., 2015. Bisphenol S and F: A Systematic Review and Comparison of the Hormonal Activity of Bisphenol A Substitutes. Environ. Health Perspect. 123, 643–650. https://doi.org/10.1289/EHP.1408989

Rolle-Kampczyk, U., Gebauer, S., Haange, S.B., Schubert, K., Kern, M., Moulla, Y., Dietrich, A., Schön, M.R., Klöting, N., von Bergen, M., Blüher, M., 2020. Accumulation of distinct persistent organic pollutants is associated with adipose tissue inflammation. Sci. Total Environ. 748. https://doi.org/10.1016/J.SCITOTENV.2020.142458

Rosenbaum, P.F., Weinstock, R.S., Silverstone, A.E., Sjödin, A., Pavuk, M., 2017. Metabolic syndrome is associated with exposure to organochlorine pesticides in Anniston, AL, United States. Environ. Int. 108, 11–21. https://doi.org/10.1016/J.ENVINT.2017.07.017

Rosolen, V., Giordani, E., Mariuz, M., Parpinel, M., Ronfani, L., Vecchi Brumatti, L., Bin, M., Calamandrei, G., Mustieles, V., Gilles, L., Govarts, E., Baken, K., Rodriguez Martin, L., Schoeters, G., Sepai, O., Sovcikova, E., Fabelova, L., Šidlovská, M., Kolena, B., Kold Jensen, T., Frederiksen, H., Kolossa-Gehring, M., Lange, R., Apel, P., Castano, A., Esteban López, M., Jacobs, G., Voorspoels, S., Jurdáková, H., Górová, R., Barbone, F., 2022. Concurrent Assessment of Phthalates/HEXAMOLL® DINCH Exposure and Wechsler Intelligence Scale for Children Performance in Three European Cohorts of the HBM4EU Aligned Studies. Toxics 10. https://doi.org/10.3390/TOXICS10090538

Rothman, K.J., 2014. Six persistent research misconceptions. J. Gen. Intern. Med. 29, 1060–1064. https://doi.org/10.1007/S11606-013-2755-Z

Ruiz-Ojeda, F.J., Rupérez, A.I., Gomez-Llorente, C., Gil, A., Aguilera, C.M., 2016. Cell Models and Their Application for Studying Adipogenic Differentiation in Relation to Obesity: A Review. Int. J. Mol. Sci. 17. https://doi.org/10.3390/IJMS17071040

Sakkiah, S., Wang, T., Zou, W., Wang, Y., Pan, B., Tong, W., Hong, H., 2017. Endocrine Disrupting Chemicals Mediated through Binding Androgen Receptor Are Associated with Diabetes Mellitus. Int. J. Environ. Res. Public Health 15. https://doi.org/10.3390/IJERPH15010025

Saklayen, M.G., 2018. The Global Epidemic of the Metabolic Syndrome. Curr. Hypertens. Rep. 20. https://doi.org/10.1007/S11906-018-0812-Z

Sallout, B., Walker, M., 2003. The fetal origin of adult diseases. J. Obstet. Gynaecol. 23, 555–560. https://doi.org/10.1080/0144361031000156483

Schaffert, A., Krieg, L., Weiner, J., Schlichting, R., Ueberham, E., Karkossa, I., Bauer, M., Landgraf, K., Junge, K.M., Wabitsch, M., Lehmann, J., Escher, B.I., Zenclussen, A.C., Körner, A., Blüher, M., Heiker, J.T., von Bergen, M., Schubert, K., 2021. Alternatives for the worse: Molecular insights into adverse effects of bisphenol a and substitutes during human adipocyte differentiation. Environ. Int. 156, 106730. https://doi.org/10.1016/J.ENVINT.2021.106730

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 3, 1101–1108.

https://doi.org/10.1038/NPROT.2008.73

Schneider, H.J., Friedrich, N., Klotsche, J., Pieper, L., Nauck, M., John, U., Dörr, M., Felix, S., Lehnert, H., Pittrow, D., Silber, S., Völzke, H., Stalla, G.K., Wallaschofski, H., Wittchen, H.U., 2010. The predictive value of different measures of obesity for incident cardiovascular events and mortality. J. Clin. Endocrinol. Metab. 95, 1777–1785. https://doi.org/10.1210/JC.2009-1584

Seillier, M., Pouyet, L., N'Guessan, P., Nollet, M., Capo, F., Guillaumond, F., Peyta, L., Dumas, J., Varrault, A., Bertrand, G., Bonnafous, S., Tran, A., Meur, G., Marchetti, P., Ravier, M.A., Dalle, S., Gual, P., Muller, D., Rutter, G.A., Servais, S., Iovanna, J.L., Carrier, A., 2015. Defects in mitophagy promote redox-driven metabolic syndrome in the absence of TP53INP1. EMBO Mol. Med. 7, 802–818. https://doi.org/10.15252/EMMM.201404318

Sexton, K., Needham, L.L., Pirkle, J.L., 2004. Human biomonitoring of environmental chemicals: measuring chemicals in human tissues is the 'Gold Standard' for assessing people's exposure to pollution. Am. Sci. 92, 38–46.

Silva, E., Rajapakse, N., Kortenkamp, A., 2002. Something from "nothing"--eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environ. Sci. Technol. 36, 1751–1756. https://doi.org/10.1021/ES0101227

Skledar, D.G., Mašič, L.P., 2020. In vitro estrogenic activity of binary and multicomponent mixtures with bisphenol A. Sci. Total Environ. 707. https://doi.org/10.1016/J.SCITOTENV.2019.135211

Smink, A., Ribas-Fito, N., Garcia, R., Torrent, M., Mendez, M.A., Grimalt,

J.O., Sunyer, J., 2008. Exposure to hexachlorobenzene during pregnancy increases the risk of overweight in children aged 6 years. Acta Paediatr. 97, 1465–1469. https://doi.org/10.1111/J.1651-2227.2008.00937.X

Søeborg, T., Frederiksen, H., Andersson, A.M., 2014. Considerations for estimating daily intake values of nonpersistent environmental endocrine disruptors based on urinary biomonitoring data. Reproduction 147, 455–463. https://doi.org/10.1530/REP-13-0458

Song, S., Duan, Y., Zhang, T., Zhang, B., Zhao, Z., Bai, X., Xie, L., He, Y., Ouyang, J. ping, Huang, X., Sun, H., 2019. Serum concentrations of bisphenol A and its alternatives in elderly population living around e-waste recycling facilities in China: Associations with fasting blood glucose. Ecotoxicol. Environ. Saf. 169, 822–828. https://doi.org/10.1016/J.ECOENV.2018.11.101

Spanidis, Y., Mpesios, A., Stagos, D., Goutzourelas, N., Bar-Or, D., Karapetsa, M., Zakynthinos, E., Spandidos, D.A., Tsatsakis, A.M., Leon, G., Kouretas, D., 2016. Assessment of the redox status in patients with metabolic syndrome and type 2 diabetes reveals great variations. Exp. Ther. Med. 11, 895–903. https://doi.org/10.3892/ETM.2016.2968

Strober, W., 2015. Trypan Blue Exclusion Test of Cell Viability. Curr. Protoc. Immunol. 111, A3.B.1-A3.B.3. https://doi.org/10.1002/0471142735.IMA03BS111

Sun, H., Saeedi, P., Karuranga, S., Pinkepank, M., Ogurtsova, K., Duncan, B.B., Stein, C., Basit, A., Chan, J.C.N., Mbanya, J.C., Pavkov, M.E., Ramachandaran, A., Wild, S.H., James, S., Herman, W.H., Zhang, P.,

Bommer, C., Kuo, S., Boyko, E.J., Magliano, D.J., 2022. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. Diabetes Res. Clin. Pract. 183. https://doi.org/10.1016/J.DIABRES.2021.109119

Swan, S.H., 2000. Intrauterine exposure to diethylstilbestrol: long-term effects in humans. APMIS 108, 793–804. https://doi.org/10.1111/J.1600-0463.2000.TB00001.X

Tawar, N., Banerjee, B.D., Madhu, S.V., Agrawal, V., Gupta, S., 2022. Association of Organochlorine Pesticides With Genetic Markers of Endoplasmic Reticulum Stress in Type 2 Diabetes Mellitus: A Case-Control Study Among the North-Indian Population. Front. Endocrinol. (Lausanne). 13. https://doi.org/10.3389/FENDO.2022.841463

Tomar, L., Agarwal, M., Avasthi, R., Tyagi, V., Mustafa, M., Banerjee, B., 2013. Serum organochlorine pesticide levels in patients with metabolic syndrome. Indian J. Endocrinol. Metab. 17, 342. https://doi.org/10.4103/2230-8210.119612

Usman, A., Ahmad, M., 2016. From BPA to its analogues: Is it a safe journey? Chemosphere 158, 131–142. https://doi.org/10.1016/j.chemosphere.2016.05.070

Valera, B., Ayotte, P., Poirier, P., Dewailly, É., 2013. Associations between plasma persistent organic pollutant levels and blood pressure in Inuit adults from Nunavik. Environ. Int. 59, 282–289. https://doi.org/10.1016/J.ENVINT.2013.06.019

Valvi, D., Walker, D.I., Inge, T., Bartell, S.M., Jenkins, T., Helmrath, M., Ziegler, T.R., La Merrill, M.A., Eckel, S.P., Conti, D., Liang, Y., Jones, D.P., McConnell, R., Chatzi, L., 2020. Environmental chemical

burden in metabolic tissues and systemic biological pathways in adolescent bariatric surgery patients: A pilot untargeted metabolomic approach. Environ. Int. 143. https://doi.org/10.1016/J.ENVINT.2020.105957

van den Dungen, M.W., Murk, A.J., Kok, D.E., Steegenga, W.T., 2017. Persistent organic pollutants alter DNA methylation during human adipocyte differentiation. Toxicol. In Vitro 40, 79–87. https://doi.org/10.1016/J.TIV.2016.12.011

van Greevenbroek, M.M.J., Schalkwijk, C.G., Stehouwer, C.D.A., 2016. Dysfunctional adipose tissue and low-grade inflammation in the management of the metabolic syndrome: Current practices and future advances. F1000Research 5. https://doi.org/10.12688/F1000RESEARCH.8971.1/DOI

Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs, D.R., Lee, D.H., Shioda, T., Soto, A.M., vom Saal, F.S., Welshons, W. V., Zoeller, R.T., Myers, J.P., 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33, 378–455. https://doi.org/10.1210/ER.2011-1050

Vandenberg, L.N., Hauser, R., Marcus, M., Olea, N., Welshons, W. V., 2007. Human exposure to bisphenol A (BPA). Reprod. Toxicol. 24, 139–177. https://doi.org/10.1016/J.REPROTOX.2007.07.010

Veiga-Lopez, A., Pu, Y., Gingrich, J., Padmanabhan, V., 2018. Obesogenic Endocrine Disrupting Chemicals: Identifying Knowledge Gaps. Trends Endocrinol. Metab. 29, 607–625. https://doi.org/10.1016/J.TEM.2018.06.003

Verbanck, M., Canouil, M., Leloire, A., Dhennin, V., Coumoul, X., Yengo,

L., Froguel, P., Poulain-Godefroy, O., 2017. Low-dose exposure to bisphenols A, F and S of human primary adipocyte impacts coding and non-coding RNA profiles. PLoS One 12, 1–20. https://doi.org/10.1371/journal.pone.0179583

Vermeulen, R., Schymanski, E.L., Barabási, A.L., Miller, G.W., 2020. The exposome and health: Where chemistry meets biology. Science 367, 392–396. https://doi.org/10.1126/SCIENCE.AAY3164

Vilahur, N., Molina-Molina, J.M., Bustamante, M., Murcia, M., Arrebola, J.P., Ballester, F., Mendez, M.A., Garcia-Esteban, R., Guxens, M., Marina, L.S., Tardón, A., Sunyer, J., Olea, N., Fernandez, M.F., 2013. Male specific association between xenoestrogen levels in placenta and birthweight. Environ. Int. 51, 174–181. https://doi.org/10.1016/J.ENVINT.2012.10.004

Vinggaard, A.M., Bonefeld-Jørgensen, E.C., Jensen, T.K., Fernandez, M.F., Rosenmai, A.K., Taxvig, C., Rodriguez-Carrillo, A., Wielsøe, M., Long, M., Olea, N., Antignac, J.P., Hamers, T., Lamoree, M., 2021. Receptor-based in vitro activities to assess human exposure to chemical mixtures and related health impacts. Environ. Int. 146. https://doi.org/10.1016/J.ENVINT.2020.106191

Völker, J., Ashcroft, F., Vedøy, Å., Zimmermann, L., Wagner, M., 2022. Adipogenic Activity of Chemicals Used in Plastic Consumer Products. Environ. Sci. Technol. 56, 2487–2496. https://doi.org/10.1021/ACS.EST.1C06316

Wajchenberg, B.L., 2000. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. Endocr. Rev. 21, 697–738. https://doi.org/10.1210/EDRV.21.6.0415

Wang, J., Sun, B., Hou, M., Pan, X., Li, X., 2013. The environmental

obesogen bisphenol A promotes adipogenesis by increasing the amount of 11β-hydroxysteroid dehydrogenase type 1 in the adipose tissue of children. Int. J. Obes. 37, 999–1005. https://doi.org/10.1038/ijo.2012.173

Wang, L., Li, X., Wang, Z., Bancks, M.P., Carnethon, M.R., Greenland, P., Feng, Y.Q., Wang, H., Zhong, V.W., 2021. Trends in Prevalence of Diabetes and Control of Risk Factors in Diabetes Among US Adults, 1999-2018. JAMA 326, 704–716. https://doi.org/10.1001/JAMA.2021.9883

Wang, R.Y., Abbott, R.D., Zieba, A., Borowsky, F.E., Kaplan, D.L., 2017. Development of a Three-Dimensional Adipose Tissue Model for Studying Embryonic Exposures to Obesogenic Chemicals. Ann. Biomed. Eng. 45, 1807–1818. https://doi.org/10.1007/s10439-016-1752-x

Ward, Z.J., Bleich, S.N., Cradock, A.L., Barrett, J.L., Giles, C.M., Flax, C., Long, M.W., Gortmaker, S.L., 2019. Projected U.S. State-Level Prevalence of Adult Obesity and Severe Obesity. N. Engl. J. Med. 381, 2440–2450. https://doi.org/10.1056/NEJMSA1909301

Wild, C.P., 2005. Complementing the genome with an "exposome": the outstanding challenge of environmental exposure measurement in molecular epidemiology. Cancer Epidemiol. Biomarkers Prev. 14, 1847–1850. https://doi.org/10.1158/1055-9965.EPI-05-0456

World Health Organization, 2022. Noncommunicable diseases [WWW Document]. URL https://www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases (accessed 1.27.23).

World Health Organization, 2021. Obesity and overweight [WWW

Document]. URL https://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight (accessed 1.27.23).

World Health Organization, 2012. State of the science of endocrine disrupting chemicals 2012 [WWW Document]. URL https://www.who.int/publications/i/item/9789241505031 (accessed 1.20.23).

Ye, X., Wong, L.Y., Kramer, J., Zhou, X., Jia, T., Calafat, A.M., 2015. Urinary Concentrations of Bisphenol A and Three Other Bisphenols in Convenience Samples of U.S. Adults during 2000-2014. Environ. Sci. Technol. 49, 11834–11839. https://doi.org/10.1021/acs.est.5b02135

Yu, H., Caldwell, D.J., Suri, R.P., 2019. In vitro estrogenic activity of representative endocrine disrupting chemicals mixtures at environmentally relevant concentrations. Chemosphere. https://doi.org/10.1016/j.chemosphere.2018.10.067

Zare Jeddi, M., Hopf, N.B., Viegas, S., Price, A.B., Paini, A., van Thriel, C., Benfenati, E., Ndaw, S., Bessems, J., Behnisch, P.A., Leng, G., Duca, R.C., Verhagen, H., Cubadda, F., Brennan, L., Ali, I., David, A., Mustieles, V., Fernandez, M.F., Louro, H., Pasanen-Kase, R., 2021. Towards a systematic use of effect biomarkers in population and occupational biomonitoring. Environ. Int. 146. https://doi.org/10.1016/J.ENVINT.2020.106257

Zhang, L., Nichols, R.G., Correll, J., Murray, I.A., Tanaka, N., Smith, P.B., Hubbard, T.D., Sebastian, A., Albert, I., Hatzakis, E., Gonzalez, F.J., Perdew, G.H., Patterson, A.D., 2015. Persistent Organic Pollutants Modify Gut Microbiota-Host Metabolic Homeostasis in Mice Through Aryl Hydrocarbon Receptor Activation. Environ. Health

Perspect. 123, 679–688. https://doi.org/10.1289/EHP.1409055

Zhou, B., Bentham, J., Di Cesare, M., Bixby, H., Danaei, G., Cowan, M.J., Paciorek, C.J., Singh, G., Hajifathalian, K., Bennett, J.E., Taddei, C., Bilano, V., Carrillo-Larco, R.M., Djalalinia, S., Khatibzadeh, S., Lugero, C., Peykari, N., Zhang, W.Z., Lu, Y., Stevens, G.A., Riley, L.M., Bovet, P., Elliott, P., Gu, D., Ikeda, N., Jackson, R.T., Joffres, M., Kengne, A.P., Laatikainen, T., Lam, T.H., Laxmaiah, A., Liu, J., Miranda, J.J., Mondo, C.K., Neuhauser, H.K., Sundström, J., Smeeth, L., Sorić, M., Woodward, M., Ezzati, M., Abarca-Gómez, L., Abdeen, Z.A., Rahim, H.A., Abu-Rmeileh, N.M., Acosta-Cazares, B., Adams, R., Aekplakorn, W., Afsana, K., Aguilar-Salinas, C.A., Agyemang, C., Ahmadvand, A., Ahrens, W., Al Raddadi, R., Al Woyatan, R., Ali, M.M., Alkerwi, A., Aly, E., Amouyel, P., Amuzu, A., Andersen, L.B., Anderssen, S.A., Ängquist, L., Anjana, R.M., Ansong, D., Aounallah-Skhiri, H., Araújo, J., Ariansen, I., Aris, T., Arlappa, N., Aryal, K., Arveiler, D., Assah, F.K., Assunção, M.C.F., Avdicová, M., Azevedo, A., Azizi, F., Babu, B. V., Bahijri, S., Balakrishna, N., Bandosz, P., Banegas, J.R., Barbagallo, C.M., Barceló, A., Barkat, A., Barros, A.J.D., Barros, M. V., Bata, I., Batieha, A.M., Baur, L.A., Beaglehole, R., Romdhane, H. Ben, Benet, M., Benson, L.S., Bernabe-Ortiz, A., Bernotiene, G., Bettiol, H., Bhagyalaxmi, A., Bharadwaj, S., Bhargava, S.K., Bi, Y., Bikbov, M., Bjerregaard, P., Bjertness, E., Björkelund, C., Blokstra, A., Bo, S., Bobak, M., Boeing, H., Boggia, J.G., Boissonnet, C.P., Bongard, V., Braeckman, L., Brajkovich, I., Branca, F., Breckenkamp, J., Brenner, H., Brewster, L.M., Bruno, G., Bueno-de-Mesquita, H.B., Bugge, A., Burns, C., Bursztyn, M., de León, A.C., Cacciottolo, J., Cameron, C., Can, G., Cândido, A.P.C., Capuano, V., Cardoso, V.C., Carlsson, A.C., Carvalho, M.J., Casanueva, F.F., Casas, J.P., Caserta, C.A., Chamukuttan, S., Chan,

A.W., Chan, Q., Chaturvedi, H.K., Chaturvedi, N., Chen, C.J., Chen, F., Chen, H., Chen, S., Chen, Z., Cheng, C.Y., Dekkaki, I.C., Chetrit, A., Chiolero, A., Chiou, S.T., Chirita-Emandi, A., Cho, B., Cho, Y., Chudek, J., Cifkova, R., Claessens, F., Clays, E., Concin, H., Cooper, C., Cooper, R., Coppinger, T.C., Costanzo, S., Cottel, D., Cowell, C., Craig, C.L., Crujeiras, A.B., Cruz, J.J., D'Arrigo, G., d'Orsi, E., Dallongeville, J., Damasceno, A., Dankner, R., Dantoft, T.M., Dauchet, L., De Backer, G., De Bacquer, D., de Gaetano, G., De Henauw, S., De Smedt, D., Deepa, M., Dehghan, A., Delisle, H., Deschamps, V., Dhana, K., Di Castelnuovo, A.F., Dias-da-Costa, J.S., Diaz, A., Dickerson, T.T., Do, H.T.P., Donfrancesco, C., Dobson, A.J., Donoso, S.P., Döring, A., Doua, K., Drygas, W., Dulskiene, V., Džakula, A., Dzerve, V., Dziankowska-Zaborszczyk, E., Ekelund, U., El Ati, J., Ellert, U., Elosua, R., Erasmus, R.T., Erem, C., Eriksen, L., Escobedo-de la Peña, J., Evans, A., Faeh, D., Fall, C.H., Farzadfar, F., Felix-Redondo, F.J., Ferguson, T.S., Fernández-Bergés, D., Ferrante, D., Ferrari, M., Ferreccio, C., Ferrieres, J., Finn, J.D., Fischer, K., Föger, B., Foo, L.H., Forslund, A.S., Forsner, M., Fortmann, S.P., Fouad, H.M., Francis, D.K., do Carmo Franco, M., Franco, O.H., Frontera, G., Fuchs, F.D., Fuchs, S.C., Fujita, Y., Furusawa, T., Gaciong, Z., Gareta, D., Garnett, S.P., Gaspoz, J.M., Gasull, M., Gates, L., Gavrila, D., Geleijnse, J.M., Ghasemian, A., Ghimire, A., Giampaoli, S., Gianfagna, F., Giovannelli, J., Goldsmith, R.A., Gonçalves, H., González-Gross, M.M., González Rivas, J.P., Gottrand, F., Graff-Iversen, S., Grafnetter, D., Grajda, A., Gregor, R.D., Grodzicki, T., Grøntved, A., Gruden, G., Grujic, V., Guan, O.P., Gudnason, V., Guerrero, R., Guessous, I., Guimaraes, A.L., Gulliford, M.C., Gunnlaugsdottir, J., Gunter, M., Gupta, P.C., Gureje, O., Gurzkowska, B., Gutierrez, L., Gutzwiller, F., Hadaegh, F., Halkjær,

J., Hambleton, I.R., Hardy, R., Harikumar, R., Hata, J., Hayes, A.J., He, J., Hendriks, M.E., Henriques, A., Cadena, L.H., Herqutanto, Herrala, S., Heshmat, R., Hihtaniemi, I.T., Ho, S.Y., Ho, S.C., Hobbs, M., Hofman, A., Dinc, G.H., Hormiga, C.M., Horta, B.L., Houti, L., Howitt, C., Htay, T.T., Htet, A.S., Hu, Y., Huerta, J.M., Husseini, A.S., Huybrechts, I., Hwalla, N., Iacoviello, L., Iannone, A.G., Ibrahim, M.M., Ikram, M.A., Irazola, V.E., Islam, M., Ivkovic, V., Iwasaki, M., Jacobs, J.M., Jafar, T., Jamrozik, K., Janszky, I., Jasienska, G., Jelakovic, B., Jiang, C.Q., Johansson, M., Jonas, J.B., Jørgensen, T., Joshi, P., Juolevi, A., Jurak, G., Jureša, V., Kaaks, R., Kafatos, A., Kalter-Leibovici, O., Kamaruddin, N.A., Kasaeian, A., Katz, J., Kauhanen, J., Kaur, P., Kavousi, M., Kazakbaeva, G., Keil, U., Boker, L.K., Keinänen-Kiukaanniemi, S., Kelishadi, R., Kemper, H.C.G., Kersting, M., Key, T., Khader, Y.S., Khalili, D., Khang, Y.H., Khaw, K.T., Kiechl, S., Killewo, J., Kim, J., Klumbiene, J., Kolle, E., Kolsteren, P., Korrovits, P., Koskinen, S., Kouda, K., Koziel, S., Kristensen, P.L., Krokstad, S., Kromhout, D., Kruger, H.S., Kubinova, R., Kuciene, R., Kuh, D., Kujala, U.M., Kula, K., Kulaga, Z., Krishna Kumar, R., Kurjata, P., Kusuma, Y.S., Kuulasmaa, K., Kyobutungi, C., Lachat, C., Landrove, O., Lanska, V., Lappas, G., Larijani, B., Laugsand, L.E., Le Nguyen Bao, K., Le, T.D., Leclercq, C., Lee, Jeannette, Lee, Jeonghee, Lehtimäki, T., Rampal, L., León-Muñoz, L.M., Levitt, N.S., Li, Y., Lilly, C.L., Lim, W.Y., Lima-Costa, M.F., Lin, H.H., Lin, X., Linneberg, A., Lissner, L., Litwin, M., Lorbeer, R., Lotufo, P.A., Lozano, J.E., Luksiene, D., Lundqvist, A., Lunet, N., Lytsy, P., Ma, G., Ma, J., Machado-Coelho, G.L.L., Machi, S., Maggi, S., Magliano, D.J., Majer, M., Makdisse, M., Malekzadeh, R., Malhotra, R., Rao, K.M., Malyutina, S., Manios, Y., Mann, J.I., Manzato, E., Margozzini, P., Marques-Vidal, P., Marrugat, J., Martorell, R., Mathiesen, E.B.,

Matijasevich, A., Matsha, T.E., Mbanya, J.C.N., McDonald Posso, A.J., McFarlane, S.R., McGarvey, S.T., McLachlan, S., McLean, R.M., McNulty, B.A., MdKhir, A.S., Mediene-Benchekor, S., Medzioniene, J., Meirhaeghe, A., Meisinger, C., Menezes, A.M.B., Menon, G.R., Meshram, I.I., Metspalu, A., Mi, J., Mikkel, K., Miller, J.C., Miquel, J.F., Mišigoj-Durakovic, M., Mohamed, M.K., Mohammad, K., Mohammadifard, N., Mohan, V., Mohd Yusoff, M.F., Møller, N.C., Molnár, D., Momenan, A., Monyeki, K.D.K., Moreira, L.B., Morejon, A., Moreno, L.A., Morgan, K., Moschonis, G., Mossakowska, M., Mota, J., Mostafa, A., Motlagh, M.E., Motta, J., Muiesan, M.L., Müller-Nurasyid, M., Murphy, N., Mursu, J., Musil, V., Nagel, G., Naidu, B.M., Nakamura, H., Námešná, J., Nang, E.E.K., Nangia, V.B., Narake, S., Navarrete-Muñoz, E.M., Ndiaye, N.C., Neal, W.A., Nenko, I., Nervi, F., Nguyen, N.D., Nguyen, Q.N., Nieto-Martínez, R.E., Niiranen, T.J., Ning, G., Ninomiya, T., Nishtar, S., Noale, M., Noboa, O.A., Noorbala, A.A., Norat, T., Noto, D., Al Nsour, M., O'Reilly, D., Oh, K., Olinto, M.T.A., Oliveira, I.O., Omar, M.A., Onat, A., Ordunez, P., Osmond, C., Ostojic, S.M., Otero, J.A., Overvad, K., Owusu-Dabo, E., Paccaud, F.M., Padez, C., Pahomova, E., Pajak, A., Palli, D., Palmieri, L., Panda-Jonas, S., Panza, F., Papandreou, D., Parnell, W.R., Parsaeian, M., Pecin, I., Pednekar, M.S., Peer, N., Peeters, P.H., Peixoto, S.V., Pelletier, C., Peltonen, M., Pereira, A.C., Pérez, R.M., Peters, A., Petkeviciene, J., Pham, S.T., Pigeot, I., Pikhart, H., Pilav, A., Pilotto, L., Pitakaka, F., Plans-Rubió, P., Polakowska, M., Polašek, O., Porta, M., Portegies, M.L.P., Pourshams, A., Pradeepa, R., Prashant, M., Price, J.F., Puiu, M., Punab, M., Qasrawi, R.F., Qorbani, M., Radic, I., Radisauskas, R., Rahman, M., Raitakari, O., Raj, M., Rao, S.R., Ramachandran, A., Ramos, E., Rampal, S., Rangel Reina, D.A., Rasmussen, F., Redon, J., Reganit, P.F.M., Ribeiro, R., Riboli, E.,

Rigo, F., Rinke de Wit, T.F., Ritti-Dias, R.M., Robinson, S.M., Robitaille, C., Rodríguez-Artalejo, F., del Cristo Rodriguez-Perez, M., Rodríguez-Villamizar, L.A., Rojas-Martinez, R., Rosengren, A., Rubinstein, A., Rui, O., Ruiz-Betancourt, B.S., Russo Horimoto, A.R.V., Rutkowski, M., Sabanayagam, C., Sachdev, H.S., Saidi, O., Sakarya, S., Salanave, B., Martinez, E.S., Salmerón, D., Salomaa, V., Salonen, J.T., Salvetti, M., Sánchez-Abanto, J., Sans, S., Santos, D., Santos, I.S., dos Santos, R.N., Santos, R., Saramies, J.L., Sardinha, L.B., Margolis, G.S., Sarrafzadegan, N., Saum, K.U., Savva, S.C., Scazufca, M., Schargrodsky, H., Schneider, I.J., Schultsz, C., Schutte, A.E., Sen, A., Senbanjo, I.O., Sepanlou, S.G., Sharma, S.K., Shaw, J.E., Shibuya, K., Shin, D.W., Shin, Y., Siantar, R., Sibai, A.M., Santos Silva, D.A., Simon, M., Simons, J., Simons, L.A., Sjöström, M., Skovbjerg, S., Slowikowska-Hilczer, J., Slusarczyk, P., Smith, M.C., Snijder, M.B., So, H.K., Sobngwi, E., Söderberg, S., Solfrizzi, V., Sonestedt, E., Song, Y., Sørensen, T.I.A., Jérome, C.S., Soumare, A., Staessen, J.A., Starc, G., Stathopoulou, M.G., Stavreski, B., Steene-Johannessen, J., Stehle, P., Stein, A.D., Stergiou, G.S., Stessman, J., Stieber, J., Stöckl, D., Stocks, T., Stokwiszewski, J., Stronks, K., Strufaldi, M.W., Sun, C.A., Sung, Y.T., Suriyawongpaisal, P., Sy, R.G., Tai, E.S., Tammesoo, M.L., Tamosiunas, A., Tang, L., Tang, X., Tao, Y., Tanser, F., Tarawneh, M.R., Tarqui-Mamani, C.B., Taylor, A., Theobald, H., Thijs, L., Thuesen, B.H., Tjonneland, A., Tolonen, H.K., Tolstrup, J.S., Topbas, M., Topór-Madry, R., Tormo, M.J., Torrent, M., Traissac, P., Trichopoulos, D., Trichopoulou, A., Trinh, O.T.H., Trivedi, A., Tshepo, L., Tulloch-Reid, M.K., Tuomainen, T.P., Tuomilehto, J., Turley, M.L., Tynelius, P., Tzourio, C., Ueda, P., Ugel, E., Ulmer, H., Uusitalo, H.M.T., Valdivia, G., Valvi, D., van der Schouw, Y.T., Van Herck, K., van Rossem, L., van Valkengoed, I.G.M., Vanderschueren,

D., Vanuzzo, D., Vatten, L., Vega, T., Velasquez-Melendez, G., Veronesi, G., Verschuren, W.M.M., Verstraeten, R., Victora, C.G., Viet, L., Viikari-Juntura, E., Vineis, P., Vioque, J., Virtanen, J.K., Visvikis-Siest, S., Viswanathan, B., Vollenweider, P., Voutilainen, S., Vrdoljak, A., Vrijheid, M., Wade, A.N., Wagner, A., Walton, J., Wan Mohamud, W.N., Wang, M.D., Wang, Q., Wang, Y.X., Wannamethee, S.G., Wareham, N., Wederkopp, N., Weerasekera, D., Whincup, P.H., Widhalm, K., Widyahening, I.S., Wijga, A.H., Wiecek, A., Wilks, R.J., Willeit, J., Willeit, P., Williams, E.A., Wilsgaard, T., Wojtyniak, B., Wong, T.Y., Wong-McClure, R.A., Woo, J., Wu, A.G., Wu, F.C., Wu, S.L., Xu, H., Yan, W., Yang, X., Ye, X., Yiallouros, P.K., Yoshihara, A., Younger-Coleman, N.O., Yusoff, A.F., Yusoff, M.F.M., Zambon, S., Zdrojewski, T., Zeng, Y., Zhao, D., Zhao, W., Zheng, Y., Zimmermann, E., Cisneros, J.Z., Zhu, D., Eggertsen, R., 2017. Worldwide trends in blood pressure from 1975 to 2015: a pooled analysis of 1479 population-based measurement studies with 19·1 million participants. Lancet (London, England) 389, 37–55. https://doi.org/10.1016/S0140-6736(16)31919-5

Zhou, B., Carrillo-Larco, R.M., Danaei, G., Riley, L.M., Paciorek, C.J., Stevens, G.A., Gregg, E.W., Bennett, J.E., Solomon, B., Singleton, R.K., Sophiea, M.K., Iurilli, M.L.C., Lhoste, V.P.F., Cowan, M.J., Savin, S., Woodward, M., Balanova, Y., Cifkova, R., Damasceno, A., Elliott, P., Farzadfar, F., He, J., Ikeda, N., Kengne, A.P., Khang, Y.H., Kim, H.C., Laxmaiah, A., Lin, H.H., Maira, P.M., Miranda, J.J., Neuhauser, H., Sundström, J., Varghese, C., Widyahening, I.S., Zdrojewski, T., Ezzati, M., Abarca-Gómez, L., Abdeen, Z.A., Abdul Rahim, H.F., Abu-Rmeileh, N.M., Acosta-Cazares, B., Adams, R.J., Aekplakorn, W., Afsana, K., Afzal, S., Agdeppa, I.A., Aghazadeh-Attari, J., Aguilar-Salinas, C.A., Agyemang, C., Ahmad, N.A., Ahmadi, A., Ahmadi,

Naser, Ahmadi, Nastaran, Ahmadizar, F., Ahmed, S.H., Ahrens, W., Ajlouni, K., Al-Raddadi, R., Alarouj, M., AlBuhairan, F., AlDhukair, S., Ali, M.M., Alkandari, A., Alkerwi, A., Allin, K., Aly, E., Amarapurkar, D.N., Amougou, N., Amouyel, P., Andersen, L.B., Anderssen, S.A., Anjana, R.M., Ansari-Moghaddam, A., Ansong, D., Aounallah-Skhiri, H., Araújo, J., Ariansen, I., Aris, T., Arku, R.E., Arlappa, N., Aryal, K.K., Aspelund, T., Assah, F.K., Assunção, M.C.F., Auvinen, J., Avdićová, M., Azevedo, A., Azimi-Nezhad, M., Azizi, F., Azmin, M., Babu, B. V., Bahijri, S., Balakrishna, N., Bamoshmoosh, M., Banach, M., Banadinović, M., Bandosz, P., Banegas, J.R., Baran, J., Barbagallo, C.M., Barceló, A., Barkat, A., Barreto, M., Barros, A.J.D., Barros, M.V.G., Bartosiewicz, A., Basit, A., Bastos, J.L.D., Bata, I., Batieha, A.M., Batyrbek, A., Baur, L.A., Beaglehole, R., Belavendra, A., Romdhane, H. Ben, Benet, M., Benson, L.S., Berkinbayev, S., Bernabe-Ortiz, A., Bernotiene, G., Bettiol, H., Bezerra, J., Bhagyalaxmi, A., Bhargava, S.K., Bia, D., Biasch, K., Lele, E.C.B., Bikbov, M.M., Bista, B., Bjerregaard, P., Bjertness, E., Bjertness, M.B., Björkelund, C., Bloch, K. V., Blokstra, A., Bo, S., Bobak, M., Boeing, H., Boggia, J.G., Boissonnet, C.P., Bojesen, S.E., Bongard, V., Bonilla-Vargas, A., Braeckman, L., Bruno, G., Bueno-De-Mesquita, H.B., Burns, C., Cardoso, V.C., Cervantes-Loaiza, M., Chadjigeorgiou, C.A., Chamukuttan, S., Chan, A.W., Chan, Q., Chaturvedi, H.K., Chaturvedi, N., Chee, M.L., Chen, C.J., Chen, F., Chen, H., Chen, S., Chen, Z., Cheng, C.Y., Cheraghian, B., Dekkaki, I.C., Chetrit, A., Chien, K.L., Chiolero, A., Chiou, S.T., Chirita-Emandi, A., Chirlaque, M.D., Cho, B., Christensen, K., Christofaro, D.G., Chudek, J., Cinteza, E., Claessens, F., Clarke, J., Clays, E., Cohen, E., Concin, H., Cooper, C., Coppinger, T.C., Costanzo, S., Cottel, D., Cowell, C., Craig, C.L., Crampin, A.C., Crujeiras, A.B., Cruz, J.J., Csilla,

S., Cui, L., Cureau, F. V., Cuschieri, S., D'Arrigo, G., d'Orsi, E., Dallongeville, J., Dankner, R., Dantoft, T.M., Dauchet, L., Davletov, K., De Backer, G., De Bacquer, D., De Curtis, A., de Gaetano, G., De Henauw, S., de Oliveira, P.D., De Ridder, D., De Smedt, D., Deepa, M., Deev, A.D., DeGennaro, V., Delisle, H., Demarest, S., Dennison, E., Deschamps, V., Dhimal, M., Di Castelnuovo, A.F., Dias-Da-Costa, J.S., Diaz, A., Dickerson, T.T., Dika, Z., Djalalinia, S., Do, H.T.P., Dobson, A.J., Donfrancesco, C., Donoso, S.P., Döring, A., Dorobantu, M., Dörr, M., Doua, K., Dragano, N., Drygas, W., Duante, C.A., Duboz, P., Duda, R.B., Dulskiene, V., Dushpanova, A., Džakula, A., Dzerve, V., Dziankowska-Zaborszczyk, E., Eddie, R., Eftekhar, E., Eggertsen, R., Eghtesad, S., Eiben, G., Ekelund, U., El-Khateeb, M., El Ati, J., Eldemire-Shearer, D., Eliasen, M., Elosua, R., Erasmus, R.T., Erbel, R., Erem, C., Eriksen, L., Eriksson, J.G., Escobedo-De la Peña, J., Eslami, S., Esmaeili, A., Evans, A., Faeh, D., Fakhretdinova, A.A., Fall, C.H., Faramarzi, E., Farjam, M., Fattahi, M.R., Fawwad, A., Felix-Redondo, F.J., Felix, S.B., Ferguson, T.S., Fernandes, R.A., Fernández-Bergés, D., Ferrante, D., Ferrao, T., Ferrari, M., Ferrario, M.M., Ferreccio, C., Ferreira, H.S., Ferrer, E., Ferrieres, J., Figueiró, T.H., Fink, G., Fischer, K., Foo, L.H., Forsner, M., Fouad, H.M., Francis, D.K., do Carmo Franco, M., Frikke-Schmidt, R., Frontera, G., Fuchs, F.D., Fuchs, S.C., Fujita, Y., Fumihiko, M., Furdela, V., Furer, A., Furusawa, T., Gaciong, Z., Galbarczyk, A., Galenkamp, H., Galvano, F., Gao, J., Gao, P., Garcia-De-la-Hera, M., Garcia, P., Gareta, D., Garnett, S.P., Gaspoz, J.M., Gasull, M., Gazzinelli, A., Gehring, U., Geleijnse, J.M., George, R., Ghanbari, A., Ghasemi, E., Gheorghe-Fronea, O.F., Ghimire, A., Gialluisi, A., Giampaoli, S., Gieger, C., Gill, T.K., Giovannelli, J., Gironella, G., Giwercman, A., Gkiouras, K., Goldberg, M., Goldsmith, R.A., Gomez, L.F., Gomula, A., Gonçalves

Cordeiro da Silva, B., Gonçalves, H., Gonçalves, M., Gonzalez-Chica, D.A., Gonzalez-Gross, M., González-Rivas, J.P., González-Villalpando, C., González-Villalpando, M.E., Gonzalez, A.R., Gorbea, M.B., Gottrand, F., Graff-Iversen, S., Grafnetter, D., Grajda, A., Grammatikopoulou, M.G., Gregor, R.D., Grodzicki, T., Grosso, G., Gruden, G., Gu, D., Guan, O.P., Gudmundsson, E.F., Gudnason, V., Guerrero, R., Guessous, I., Guimaraes, A.L., Gulliford, M.C., Gunnlaugsdottir, J., Gunter, M.J., Gupta, P.C., Gupta, R., Gureje, O., Gurzkowska, B., Gutierrez, L., Gutzwiller, F., Ha, S., Hadaegh, F., Haghshenas, R., Hakimi, H., Halkjær, J., Hambleton, I.R., Hamzeh, B., Hange, D., Hanif, A.A.M., Hantunen, S., Hao, J., Hardman, C.M., Hari Kumar, R., Hashemi-Shahri, S.M., Hata, J., Haugsgjerd, T., Hayes, A.J., He, Y., Heier, M., Hendriks, M.E., Henrique, R.D.S., Henriques, A., Hernandez Cadena, L., Herqutanto, Herrala, S., Heshmat, R., Hill, A.G., Ho, S.Y., Ho, S.C., Hobbs, M., Holdsworth, M., Homayounfar, R., Horasan Dinc, G., Horimoto, A.R.V.R., Hormiga, C.M., Horta, B.L., Houti, L., Howitt, C., Htay, T.T., Htet, A.S., Htike, M.M.T., Hu, Y., Huerta, J.M., Huhtaniemi, I.T., Huiart, L., Huisman, M., Husseini, A.S., Huybrechts, I., Hwalla, N., Iacoviello, L., Iannone, A.G., Ibrahim, M.M., Ibrahim Wong, N., Ikram, M.A., Iotova, V., Irazola, V.E., Ishida, T., Isiguzo, G.C., Islam, M., Islam, S.M.S., Iwasaki, M., Jackson, R.T., Jacobs, J.M., Jaddou, H.Y., Jafar, T., James, K., Jamrozik, K., Janszky, I., Janus, E., Jarvelin, M.R., Jasienska, G., Jelaković, A., Jelaković, B., Jennings, G., Jha, A.K., Jiang, C.Q., Jimenez, R.O., Jöckel, K.H., Joffres, M., Johansson, M., Jokelainen, J.J., Jonas, J.B., Jørgensen, T., Joshi, P., Joukar, F., Jóźwiak, J., Juolevi, A., Jurak, G., Jureša, V., Kaaks, R., Kafatos, A., Kajantie, E.O., Kalmatayeva, Z., Kalpourtzi, N., Kalter-Leibovici, O., Kampmann, F.B., Kannan, S., Karaglani, E., Kårhus, L.L., Karki, K.B., Katibeh, M.,

Katz, J., Kauhanen, J., Kaur, P., Kavousi, M., Kazakbaeva, G.M., Keil, U., Boker, L.K., Keinänen-Kiukaanniemi, S., Kelishadi, R., Kemper, H.C.G., Keramati, M., Kerimkulova, A., Kersting, M., Key, T., Khader, Y.S., Khalili, D., Khaw, K.T., Kheiri, B., Kheradmand, M., Khosravi, A., Kiechl-Kohlendorfer, U., Kiechl, S., Killewo, J., Kim, D.W., Kim, J., Klakk, H., Klimek, M., Klumbiene, J., Knoflach, M., Kolle, E., Kolsteren, P., Kontto, J.P., Korpelainen, R., Korrovits, P., Kos, J., Koskinen, S., Kouda, K., Kowlessur, S., Koziel, S., Kratenova, J., Kriaucioniene, V., Kristensen, P.L., Krokstad, S., Kromhout, D., Kruger, H.S., Kubinova, R., Kuciene, R., Kujala, U.M., Kulaga, Z., Krishna Kumar, R., Kurjata, P., Kusuma, Y.S., Kutsenko, V., Kuulasmaa, K., Kyobutungi, C., Laatikainen, T., Lachat, C., Laid, Y., Lam, T.H., Landrove, O., Lanska, V., Lappas, G., Larijani, B., Latt, T.S., Le Coroller, G., Le Nguyen Bao, K., Le, T.D., Lee, Jeannette, Lee, Jeonghee, Lehmann, N., Lehtimäki, T., Lemogoum, D., Levitt, N.S., Li, Y., Lilly, C.L., Lim, W.Y., Lima-Costa, M.F., Lin, X., Lin, Y.T., Lind, L., Lingam, V., Linneberg, A., Lissner, L., Litwin, M., Lo, W.C., Loit, H.M., Lopez-Garcia, E., Lopez, T., Lotufo, P.A., Lozano, J.E., Lovrenčić, I.L., Lukrafka, J.L., Luksiene, D., Lundqvist, A., Lundqvist, R., Lunet, N., Lustigová, M., Luszczki, E., Ma, G., Ma, J., Machado-Coelho, G.L.L., Machado-Rodrigues, A.M., Macia, E., Macieira, L.M., Madar, A.A., Maggi, S., Magliano, D.J., Magriplis, E., Mahasampath, G., Maire, B., Majer, M., Makdisse, M., Malekzadeh, F., Malekzadeh, R., Malhotra, R., Rao, K.M., Malyutina, S.K., Maniego, L. V., Manios, Y., Mann, J.I., Mansour-Ghanaei, F., Manzato, E., Marcil, A., Margozzini, P., Mårild, S.B., Marinović Glavić, M., Marques-Vidal, P., Marques, L.P., Marrugat, J., Martorell, R., Mascarenhas, L.P., Matasin, M., Mathiesen, E.B., Mathur, P., Matijasevich, A., Matlosz, P., Matsha, T.E., Mavrogianni, C.,

Mbanya, J.C.N., Mc Donald Posso, A.J., McFarlane, S.R., McGarvey, S.T., McLachlan, S., McLean, R.M., McLean, S.B., McNulty, B.A., Mediene Benchekor, S., Medzioniene, J., Mehdipour, P., Mehlig, K., Mehrparvar, A.H., Meirhaeghe, A., Meisinger, C., Montano, C.M., Menezes, A.M.B., Menon, G.R., Mereke, A., Meshram, I.I., Metspalu, A., Meyer, H.E., Mi, J., Michels, N., Mikkel, K., Milkowska, K., Miller, J.C., Minderico, C.S., Mini, G.K., Mirjalili, M.R., Mirrakhimov, E., Mišigoj-Duraković, M., Modesti, P.A., Moghaddam, S.S., Mohajer, B., Mohamed, M.K., Mohamed, S.F., Mohammad, K., Mohammadi, M.R., Mohammadi, Z., Mohammadifard, N., Mohammadpourhodki, R., Mohan, V., Mohanna, S., Mohd Yusoff, M.F., Mohebbi, I., Mohebi, F., Moitry, M., Møllehave, L.T., Molnár, D., Momenan, A., Mondo, C.K., Monterrubio-Flores, E., Monyeki, K.D.K., Moon, J.S., Moosazadeh, M., Moreira, L.B., Morejon, A., Moreno, L.A., Morgan, K., Moschonis, G., Mossakowska, M., Mostafa, A., Mostafavi, S.A., Mota, J., Motlagh, M.E., Motta, J., Moura-Dos-Santos, M.A., Mridha, M.K., Msyamboza, K.P., Mu, T.T., Muhihi, A.J., Muiesan, M.L., Müller-Nurasyid, M., Murphy, N., Mursu, J., Musa, K.I., Milanović, S.M., Musil, V., Mustafa, N., Nabipour, I., Naderimagham, S., Nagel, G., Naidu, B.M., Najafi, F., Nakamura, H., Námešná, J., Nang, E.E.K., Nangia, V.B., Narake, S., Ndiaye, N.C., Neal, W.A., Nejatizadeh, A., Nenko, I., Neovius, M., Neuhauser, H.K., Nguyen, C.T., Nguyen, N.D., Nguyen, Q. V., Nguyen, Q.N., Nieto-Martínez, R.E., Niiranen, T.J., Nikitin, Y.P., Ninomiya, T., Nishtar, S., Njelekela, M.A., Noale, M., Noboa, O.A., Noorbala, A.A., Norat, T., Nordendahl, M., Nordestgaard, B.G., Noto, D., Nowak-Szczepanska, N., Nsour, M. Al, Nunes, B., O'Neill, T.W., O'Reilly, D., Ochimana, C., Oda, E., Odili, A.N., Oh, K., Ohara, K., Ohtsuka, R., Olinto, M.T.A., Oliveira, I.O., Omar, M.A., Onat, A., Ong, S.K., Ono, L.M., Ordunez, P., Ornelas, R.,

Ortiz, P.J., Osmond, C., Ostojic, S.M., Ostovar, A., Otero, J.A., Overvad, K., Owusu-Dabo, E., Paccaud, F.M., Padez, C., Pahomova, E., de Paiva, K.M., Pająk, A., Palli, D., Palmieri, L., Pan, W.H., Panda-Jonas, S., Panza, F., Paoli, M., Papandreou, D., Park, S.W., Park, S., Parnell, W.R., Parsaeian, M., Pasquet, P., Patel, N.D., Pavlyshyn, H., Pećin, I., Pednekar, M.S., Pedro, J.M., Peer, N., Peixoto, S.V., Peltonen, M., Pereira, A.C., Peres, K.G.D.A., Peres, M.A., Peters, A., Petkeviciene, J., Peykari, N., Pham, S.T., Pichardo, R.N., Pigeot, I., Pikhart, H., Pilav, A., Pilotto, L., Pitakaka, F., Piwonska, A., Pizarro, A., Pedro Plans-Rubió, Polašek, O., Porta, M., Poudyal, A., Pourfarzi, F., Pourshams, A., Poustchi, H., Pradeepa, R., Price, A.J., Price, J.F., Providencia, R., Puhakka, S.E., Puiu, M., Punab, M., Qasrawi, R.F., Qorbani, M., Queiroz, D., Quoc Bao, T., Radić, I., Radisauskas, R., Rahimikazerooni, S., Rahman, M., Raitakari, O., Raj, M., Rakhimova, E.M., Rao, S.R., Ramachandran, A., Ramos, E., Rampal, L., Rampal, S., Rangel Reina, D.A., Rarra, V., Ricardo Rech, C., Redon, J., Reganit, P.F.M., Regecová, V., Revilla, L., Rezaianzadeh, A., Ribeiro, R., Riboli, E., Richter, A., Rigo, F., Rinke de Wit, T.F., Ritti-Dias, R.M., Robitaille, C., Rodríguez-Artalejo, F., Rodriguez-Perez, M.D.C., Rodríguez-Villamizar, L.A., Roggenbuck, U., Rojas-Martinez, R., Romaguera, D., Romeo, E.L., Rosengren, A., Roy, J.G.R., Rubinstein, A., Ruidavets, J.B., Ruiz-Betancourt, B.S., Ruiz-Castell, M., Rusakova, I.A., Russo, P., Rutkowski, M., Sabanayagam, C., Sabbaghi, H., Sachdev, H.S., Sadjadi, A., Safarpour, A.R., Safi, S., Safiri, S., Saidi, O., Sakarya, S., Saki, N., Salanave, B., Salazar Martinez, E., Salmerón, D., Salomaa, V., Salonen, J.T., Salvetti, M., Sánchez-Abanto, J., Sans, S., Santos, D.A., Santos, I.S., Santos, L.C., Santos, M.P., Santos, R., Saramies, J.L., Sardinha, L.B., Sarganas, G., Sarrafzadegan, N., Sathish, T., Saum, K.U., Savva, S., Sawada, N.,

Sbaraini, M., Scazufca, M., Schaan, B.D., Schargrodsky, H., Schipf, S., Schmidt, C.O., Schnohr, P., Schöttker, B., Schramm, S., Schultsz, C., Schutte, A.E., Sebert, S., Sein, A.A., Sen, A., Senbanjo, I.O., Sepanlou, S.G., Servais, J., Shalnova, S.A., Shamah-Levy, T., Shamshirgaran, M., Shanthirani, C.S., Sharafkhah, M., Sharma, S.K., Shaw, J.E., Shayanrad, A., Shayesteh, A.A., Shi, Z., Shibuya, K., Shimizu-Furusawa, H., Shin, D.W., Shirani, M., Shiri, R., Shrestha, N., Si-Ramlee, K., Siani, A., Siantar, R., Sibai, A.M., Silva, C.R.D.M., Santos Silva, D.A., Simon, M., Simons, J., Simons, L.A., Sjöström, M., Slowikowska-Hilczer, J., Slusarczyk, P., Smeeth, L., So, H.K., Soares, F.C., Sobngwi, E., Söderberg, S., Soemantri, A., Sofat, R., Solfrizzi, V., Somi, M.H., Sonestedt, E., Song, Y., Sørensen, T.I.A., Sørgjerd, E.P., Sorić, M., Sossa Jérome, C., Soumaré, A., Sparboe-Nilsen, B., Sparrenberger, K., Staessen, J.A., Starc, G., Stavreski, B., Steene-Johannessen, J., Stehle, P., Stein, A.D., Stergiou, G.S., Stessman, J., Stieber, J., Stöckl, D., Stocks, T., Stokwiszewski, J., Stronks, K., Strufaldi, M.W., Suka, M., Sun, C.A., Sung, Y.T., Suriyawongpaisal, P., Sy, R.G., Syddall, H.E., Sylva, R.C., Szklo, M., Shyong Tai, E., Tammesoo, M.L., Tamosiunas, A., Tan, E.J., Tang, X., Tanser, F., Tao, Y., Tarawneh, M.R., Tarqui-Mamani, C.B., Taylor, A., Taylor, J., Tebar, W.R., Tell, G.S., Tello, T., Tham, Y.C., Thankappan, K.R., Theobald, H., Theodoridis, X., Thijs, L., Thinggaard, M., Thomas, N., Thorand, B., Thuesen, B.H., Timmermans, E.J., Tjandrarini, D.H., Tjonneland, A., Toft, U., Tolonen, H.K., Tolstrup, J.S., Topbas, M., Topór-Madry, R., Tormo, M.J., Tornaritis, M.J., Torrent, M., Torres-Collado, L., Touloumi, G., Traissac, P., Triantafyllou, A., Trichopoulos, D., Trichopoulou, A., Trinh, O.T.H., Trivedi, A., Tshepo, L., Tsugane, S., Tuliakova, A.M., Tulloch-Reid, M.K., Tullu, F., Tuomainen, T.P., Tuomilehto, J., Turley, M.L., Twig, G., Tynelius,

P., Tzourio, C., Ueda, P., Ugel, E., Ulmer, H., Uusitalo, H.M.T., Valdivia, G., Valvi, D., van Dam, R.M., van den Born, B.J., Van der Heyden, J., van der Schouw, Y.T., Van Herck, K., Van Minh, H., Van Schoor, N.M., van Valkengoed, I.G.M., van Zutphen, E.M., Vanderschueren, D., Vanuzzo, D., Varbo, A., Vasan, S.K., Vega, T., Veidebaum, T., Velasquez-Melendez, G., Veronesi, G., Verschuren, W.M.M., Verstraeten, R., Victora, C.G., Viet, L., Villalpando, S., Vineis, P., Vioque, J., Virtanen, J.K., Visvikis-Siest, S., Viswanathan, B., Vlasoff, T., Vollenweider, P., Voutilainen, A., Wade, A.N., Walton, J., Wambiya, E.O.A., Wan Bebakar, W.M., Wan Mohamud, W.N., Wanderley, R.D.S., Wang, M.D., Wang, N., Wang, Q., Wang, X., Wang, Y.X., Wang, Y.W., Wannamethee, S.G., Wareham, N., Wei, W., Weres, A., Werner, B., Whincup, P.H., Widhalm, K., Wiecek, A., Wilks, R.J., Willeit, J., Willeit, P., Williams, E.A., Wilsgaard, T., Wojtyniak, B., Wong-McClure, R.A., Wong, A., Wong, T.Y., Woo, J., Wu, F.C., Wu, S., Wyszynska, J., Xu, H., Xu, L., Yaacob, N.A., Yan, W., Yang, L., Yang, X., Yang, Y., Yasuharu, T., Ye, X., Yiallouros, P.K., Yoosefi, M., Yoshihara, A., You, S.L., Younger-Coleman, N.O., Yusoff, A.F., Zainuddin, A.A., Zakavi, S.R., Zamani, F., Zambon, S., Zampelas, A., Zapata, M.E., Zaw, K.K., Zejglicova, K., Zeljkovic Vrkic, T., Zeng, Y., Zhang, L., Zhang, Z.Y., Zhao, D., Zhao, M.H., Zhen, S., Zheng, Y., Zholdin, B., Zhu, D., Zins, M., Zitt, E., Zocalo, Y., Zoghlami, N., Zuñiga Cisneros, J., 2021. Worldwide trends in hypertension prevalence and progress in treatment and control from 1990 to 2019: a pooled analysis of 1201 population-representative studies with 104 million participants. Lancet (London, England) 398, 957–980. https://doi.org/10.1016/S0140-6736(21)01330-1

Zhou, B., Lu, Y., Hajifathalian, K., Bentham, J., Di Cesare, M., Danaei, G., Bixby, H., Cowan, M.J., Ali, M.K., Taddei, C., Lo, W.C., Reis-Santos,

B., Stevens, G.A., Riley, L.M., Miranda, J.J., Bjerregaard, P., Rivera, J.A., Fouad, H.M., Ma, G., Mbanya, J.C.N., McGarvey, S.T., Mohan, V., Onat, A., Pilav, A., Ramachandran, A., Ben Romdhane, H., Paciorek, C.J., Bennett, J.E., Ezzati, M., Abdeen, Z.A., Kadir, K.A., Abu-Rmeileh, N.M., Acosta-Cazares, B., Adams, R., Aekplakorn, W., Aguilar-Salinas, C.A., Agyemang, C., Ahmadvand, A., Al-Othman, A.R., Alkerwi, A., Amouyel, P., Amuzu, A., Bo Andersen, L., Anderssen, S.A., Anjana, R.M., Aounallah-Skhiri, H., Aris, T., Arlappa, N., Arveiler, D., Assah, F.K., Avdicová, M., Azizi, F., Balakrishna, N., Bandosz, P., Barbagallo, C.M., Barceló, A., Batieha, A.M., Baur, L.A., Benet, M., Bernabe-Ortiz, A., Bharadwaj, S., Bhargava, S.K., Bi, Y., Bjertness, E., Bjertness, M.B., Björkelund, C., Blokstra, A., Bo, S., Boehm, B.O., Boissonnet, C.P., Bovet, P., Brajkovich, I., Breckenkamp, J., Brenner, H., Brewster, L.M., Brian, G.R., Bruno, G., Bugge, A., De León, A.C., Can, G., Cândido, A.P.C., Capuano, V., Carlsson, A.C., Carvalho, M.J., Casanueva, F.F., Casas, J.P., Caserta, C.A., Castetbon, K., Chamukuttan, S., Chaturvedi, N., Chen, C.J., Chen, F., Chen, S., Cheng, C.Y., Chetrit, A., Chiou, S.T., Cho, Y., Chudek, J., Cifkova, R., Claessens, F., Concin, H., Cooper, C., Cooper, R., Costanzo, S., Cottel, D., Cowell, C., Crujeiras, A.B., D'Arrigo, G., Dallongeville, J., Dankner, R., Dauchet, L., De Gaetano, G., De Henauw, S., Deepa, M., Dehghan, A., Deschamps, V., Dhana, K., Di Castelnuovo, A.F., Djalalinia, S., Doua, K., Drygas, W., Du, Y., Dzerve, V., Egbagbe, E.E., Eggertsen, R., El Ati, J., Elosua, R., Erasmus, R.T., Erem, C., Ergor, G., Eriksen, L., Escobedo-De La Peña, J., Fall, C.H., Farzadfar, F., Felix-Redondo, F.J., Ferguson, T.S., Fernández-Bergés, D., Ferrari, M., Ferreccio, C., Feskens, E.J.M., Finn, J.D., Föger, B., Foo, L.H., Forslund, A.S., Francis, D.K., Do Carmo Franco, M., Franco, O.H., Frontera, G., Furusawa, T., Gaciong, Z., Garnett,

S.P., Gaspoz, J.M., Gasull, M., Gates, L., Geleijnse, J.M., Ghasemian, A., Ghimire, A., Giampaoli, S., Gianfagna, F., Giovannelli, J., Giwercman, A., González-Gross, M.M., Rivas, J.P.G., Gorbea, M.B., Gottrand, F., Grafnetter, D., Grodzicki, T., Grøntved, A., Gruden, G., Gu, D., Guan, O.P., Guerrero, R., Guessous, I., Guimaraes, A.L., Gutierrez, L., Hambleton, I.R., Hardy, R., Kumar, R.H., Hata, J., He, J., Heidemann, C., Herrala, S., Hihtaniemi, I.T., Ho, S.Y., Ho, S.C., Hofman, A., Hormiga, C.M., Horta, B.L., Houti, L., Howitt, C., Htay, T.T., Htet, A.S., Htike, M.M.T., Hu, Y., Hussieni, A.S., Huybrechts, I., Hwalla, N., Iacoviello, L., Iannone, A.G., Ibrahim, M.M., Ikeda, N., Ikram, M.A., Irazola, V.E., Islam, M., Iwasaki, M., Jacobs, J.M., Jafar, T., Jamil, K.M., Jasienska, G., Jiang, C.Q., Jonas, J.B., Joshi, P., Kafatos, A., Kalter-Leibovici, O., Kasaeian, A., Katz, J., Kaur, P., Kavousi, M., Keinänen-Kiukaanniemi, S., Kelishadi, R., Kengne, A.P., Kersting, M., Khader, Y.S., Khalili, D., Khang, Y.H., Kiechl, S., Kim, J., Kolsteren, P., Korrovits, P., Kratzer, W., Kromhout, D., Kujala, U.M., Kula, K., Kyobutungi, C., Laatikainen, T., Lachat, C., Laid, Y., Lam, T.H., Landrove, O., Lanska, V., Lappas, G., Laxmaiah, A., Leclercq, C., Lee, Jeannette, Lee, Jeonghee, Lehtimäki, T., Rampal, L., León-Muñoz, L.M., Li, Y., Lim, W.Y., Lima-Costa, M.F., Lin, H.H., Lin, X., Lissner, L., Lorbeer, R., Lozano, J.E., Luksiene, D., Lundqvist, A., Lytsy, P., Machado-Coelho, G.L.L., Machi, S., Maggi, S., Magliano, D.J., Makdisse, M., Rao, K.M., Manios, Y., Manzato, E., Margozzini, P., Marques-Vidal, P., Martorell, R., Masoodi, S.R., Mathiesen, E.B., Matsha, T.E., McFarlane, S.R., McLachlan, S., McNulty, B.A., Mediene-Benchekor, S., Meirhaeghe, A., Menezes, A.M.B., Merat, S., Meshram, I.I., Mi, J., Miquel, J.F., Mohamed, M.K., Mohammad, K., Mohammadifard, N., Mohd Yusoff, M.F., Møller, N.C., Molnár, D., Mondo, C.K., Morejon, A., Moreno, L.A., Morgan, K., Moschonis, G.,

Mossakowska, M., Mostafa, A., Mota, J., Motta, J., Mu, T.T., Muiesan, M.L., Müller-Nurasyid, M., Mursu, J., Nagel, G., Námešná, J., Nang, E.E.K., Nangia, V.B., Navarrete-Muñoz, E.M., Ndiaye, N.C., Nenko, I., Nervi, F., Nguyen, N.D., Nguyen, Q.N., Nieto-Martínez, R.E., Ning, G., Ninomiya, T., Noale, M., Noto, D., Al Nsour, M., Ochoa-Avilés, A.M., Oh, K., Ordunez, P., Osmond, C., Otero, J.A., Owusu-Dabo, E., Pahomova, E., Palmieri, L., Panda-Jonas, S., Panza, F., Parsaeian, M., Peixoto, S.V., Peltonen, M., Peters, A., Peykari, N., Pham, S.T., Pitakaka, F., Piwonska, A., Piwonski, J., Plans-Rubió, P., Porta, M., Portegies, M.L.P., Poustchi, H., Pradeepa, R., Price, J.F., Punab, M., Qasrawi, R.F., Qorbani, M., Radisauskas, R., Rahman, M., Raitakari, O., Rao, S.R., Ramke, J., Ramos, R., Rampal, S., Rathmann, W., Redon, J., Reganit, P.F.M., Rigo, F., Robinson, S.M., Robitaille, C., Rodríguez-Artalejo, F., Del CristoRodriguez-Perez, M., Rodríguez-Villamizar, L.A., Rojas-Martinez, R., Ronkainen, K., Rosengren, A., Rubinstein, A., Rui, O., Ruiz-Betancourt, B.S., Horimoto, A.R.V.R., Rutkowski, M., Sabanayagam, C., Sachdev, H.S., Saidi, O., Sakarya, S., Salanave, B., Salonen, J.T., Salvetti, M., Sánchez-Abanto, J., Santos, D., Dos Santos, R.N., Santos, R., Saramies, J.L., Sardinha, L.B., Sarrafzadegan, N., Saum, K.U., Scazufca, M., Schargrodsky, H., Scheidt-Nave, C., Sein, A.A., Sharma, S.K., Shaw, J.E., Shibuya, K., Shin, Y., Shiri, R., Siantar, R., Sibai, A.M., Simon, M., Simons, J., Simons, L.A., Sjostrom, M., Slowikowska-Hilczer, J., Slusarczyk, P., Smeeth, L., Snijder, M.B., So, H.K., Sobngwi, E., Söderberg, S., Solfrizzi, V., Sonestedt, E., Soumare, A., Staessen, J.A., Stathopoulou, M.G., Steene-Johannessen, J., Stehle, P., Stein, A.D., Stessman, J., Stöckl, D., Stokwiszewski, J., Stronks, K., Strufaldi, M.W., Sun, C.A., Sundström, J., Sung, Y.T., Suriyawongpaisal, P., Sy, R.G., Tai, E.S., Tamosiunas, A., Tang, L., Tarawneh, M., Tarqui-

Mamani, C.B., Taylor, A., Theobald, H., Thijs, L., Thuesen, B.H., Tolonen, H.K., Tolstrup, J.S., Topbas, M., Torrent, M., Traissac, P., Trinh, O.T.H., Tulloch-Reid, M.K., Tuomainen, T.P., Turley, M.L., Tzourio, C., Ueda, P., Ukoli, F.A.M., Ulmer, H., Uusitalo, H.M.T., Valdivia, G., Valvi, D., Van Rossem, L., Van Valkengoed, I.G.M., Vanderschueren, D., Vanuzzo, D., Vega, T., Velasquez-Melendez, G., Veronesi, G., Verschuren, W.M.M., Verstraeten, R., Viet, L., Vioque, J., Virtanen, J.K., Visvikis-Siest, S., Viswanathan, B., Vollenweider, P., Voutilainen, S., Vrijheid, M., Wade, A.N., Wagner, A., Walton, J., Wan Mohamud, W.N., Wang, F., Wang, M.D., Wang, Q., Wang, Y.X., Wannamethee, S.G., Weerasekera, D., Whincup, P.H., Widhalm, K., Wiecek, A., Wijga, A.H., Wilks, R.J., Willeit, J., Wilsgaard, T., Wojtyniak, B., Wong, T.Y., Woo, J., Woodward, M., Wu, F.C., Wu, S.L., Xu, H., Yan, W., Yang, X., Ye, X., Yoshihara, A., Younger-Coleman, N.O., Zambon, S., Zargar, A.H., Zdrojewski, T., Zhao, W., Zheng, Y., Cisneros, J.Z., 2016. Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4.4 million participants. Lancet (London, England) 387, 1513–1530. https://doi.org/10.1016/S0140-6736(16)00618-8

Zhou, R., Cheng, W., Feng, Y., Wei, H., Liang, F., Wang, Y., 2017. Interactions between three typical endocrine-disrupting chemicals (EDCs) in binary mixtures exposure on myocardial differentiation of mouse embryonic stem cell. Chemosphere 178, 378–383. https://doi.org/10.1016/J.CHEMOSPHERE.2017.03.040

Zoeller, R.T., Brown, T.R., Doan, L.L., Gore, A.C., Skakkebaek, N.E., Soto, A.M., Woodruff, T.J., Vom Saal, F.S., 2012. Endocrine-disrupting chemicals and public health protection: a statement of principles

from The Endocrine Society. Endocrinology 153, 4097–4110. https://doi.org/10.1210/EN.2012-1422

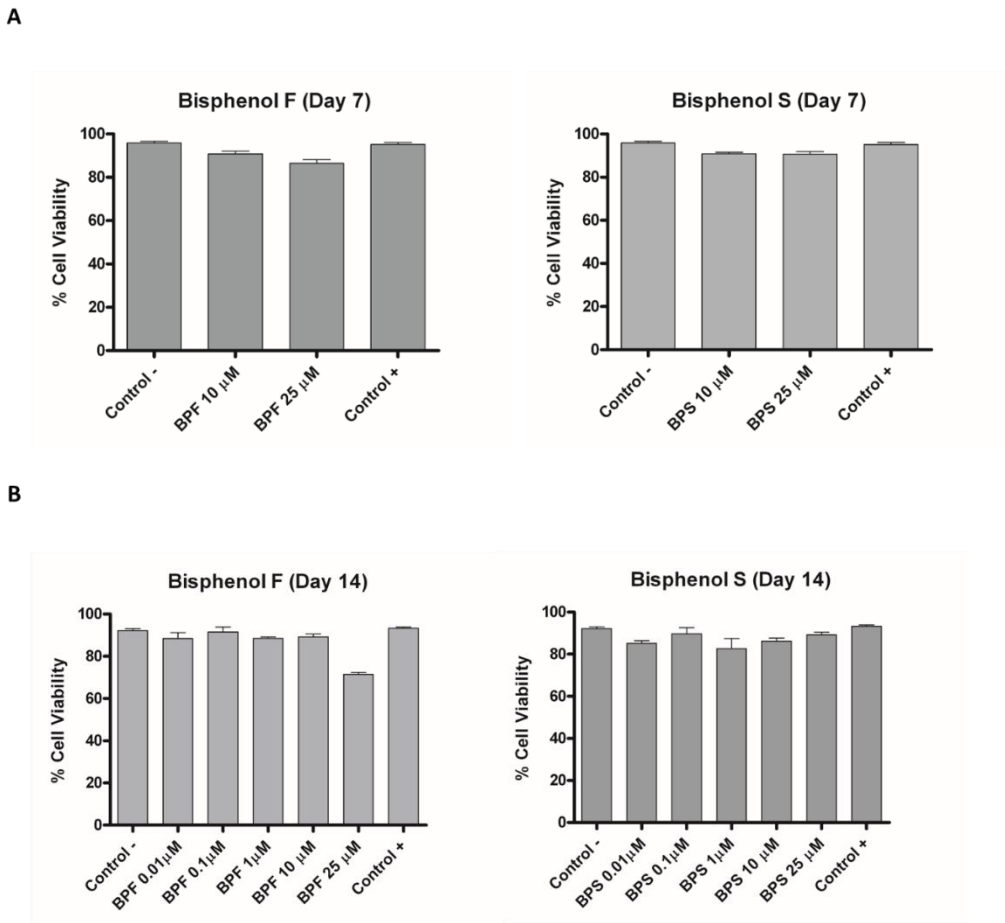
Zolezzi, J.M., Santos, M.J., Bastías-Candia, S., Pinto, C., Godoy, J.A., Inestrosa, N.C., 2017. PPARs in the central nervous system: roles in neurodegeneration and neuroinflammation. Biol. Rev. Camb. Philos. Soc. 92, 2046–2069. https://doi.org/10.1111/BRV.12320

Annexes

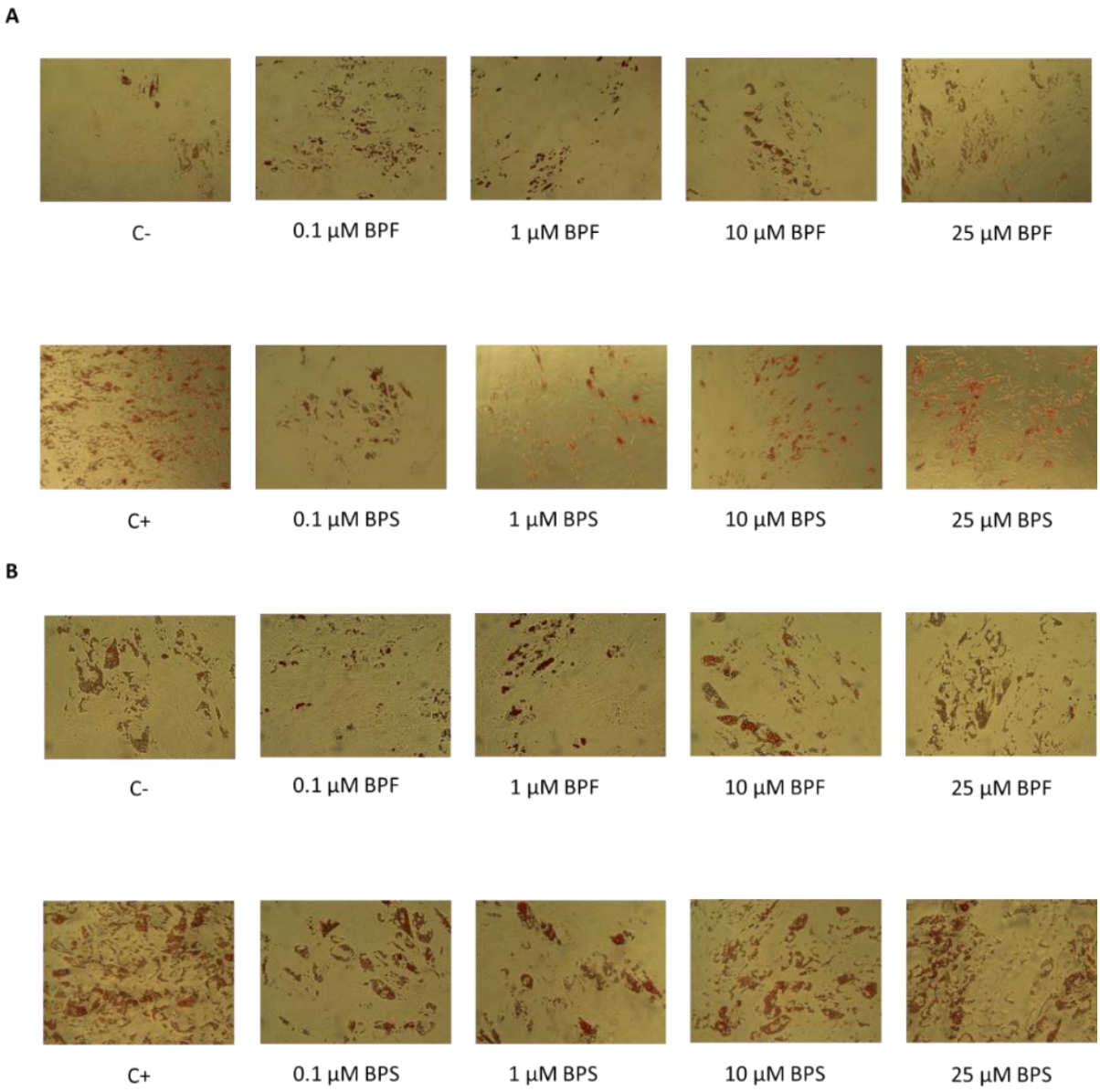
Supplementary Material. Article #1

Supplementary Table 1. Brief description of primary and secondary antibodies used in western blot

Peptide/Protein Target	Manufacturer, Catalog number	Species	Dilution
PPARγ	Cell Signaling Technology, 2435	Rabbit	1:1000 (5 % BSA in 1X TBS with 0.05 % Tween 20)
C/EBPα	Cell Signaling Technology, 2841		
FABP4	Cell Signaling Technology, 3544		
LPL	Abcam, ab172953		1:1000 (5 % non-fat milk in 1X TBS with 0.05 % Tween 20)



Supplementary Fig. 1. Effect of BPF and BPS on the viability of hASCs at days 7 and 14 during adipogenic differentiation. hASCs were differentiated for 7 (A) and 14 days (B), and their viability was assessed by trypan blue assay. Data were expressed as means ± SEM from three experiments with multiple replicates for each experimental condition.



Supplementary Fig. 2. hASCs visualized by Oil Red O staining assay at day 14 of adipogenic differentiation. (Fig.2A at 20X and Fig.2B at 40X). C-, negative control; C+, positive control.

Supplementary Table 2. Physicochemical properties of BPA, BPF and BPS

Common name	Abbreviation	Systematic name	Molecular formula	Structure	Molecular weight	Solubility	log P	Dissociation Constants
Bisphenol A	BPA	2,2-bis(4-hydroxyphenyl)propane or 4,4'-dihydroxy-2,2-diphenylpropane or 4-[2-(4-hydroxyphenyl)propan-2-yl]phenol	C15H16O2		228.29 g/mol	less than 1 mg/mL at 70.7° F (NTP, 1992); 5.26e-04 M; In water, 300 mg/L at 25 °C; In water solubility, 120 mg/L at 25 °C; Insoluble in water; Very soluble in ethanol, ether, benzene, alkali; soluble in acetic acid; Soluble in aqueous alkaline solution, alcohol, acetone; slightly soluble in carbon tetrachloride; 0.12 mg/mL at 25 °C; Solubility in water, g/100ml: 0.03 (very poor)	LogP= 3.32; log Kow = 3.32	pKa = 9.6
Bisphenol F	BPF	4,4'-methylenediphenol or 4,4'-Dihydroxydiphenylmethane or 4-[(4-hydroxyphenyl)methyl]phenol	C13H12O2		200.23 g/mol	soluble in ethanol, ether, chloroform, alkali; slightly soluble in DMSO; insoluble in carbon disulphide	LogP= 2.91; log Kow = 2.91	pKa1 = 7.55, pKa2 = 10.80 at 25 °C

Bisphenol S	BPS	4,4'-sulfonyldiphenol or bis(4-hydroxyphenyl)sulfone or 4-(4-hydroxyphenyl)sulfonylphenol	C12H10O4S		250.27 g/mol	insoluble in water; Soluble in ethanol, ether; slightly soluble in benzene, DMSO	log Kow = 1.65 (est)	pKa = 8.2 at 25 °C (est)

References:

National Center for Biotechnology Information. PubChem Compound Summary for CID 6623, Bisphenol A. https://pubchem.ncbi.nlm.nih.gov/compound/Bisphenol-A. Accessed Mar. 18, 2021.
National Center for Biotechnology Information. PubChem Compound Summary for CID 12111, 4,4'-Methylenediphenol. https://pubchem.ncbi.nlm.nih.gov/compound/4_4_-Methylenediphenol. Accessed Mar. 18, 2021.
National Center for Biotechnology Information. PubChem Compound Summary for CID 6626, 4,4'-Sulfonyldiphenol. https://pubchem.ncbi.nlm.nih.gov/compound/4_4_-Sulfonyldiphenol. Accessed Mar. 18, 2021.

Supplementary Material. Article #2

Table S1. Standard stock solutions of a mixture of three bisphenols (BPA, BPF, and BPS)

Mixture of three bisphenols (MIX)	
1.5 mL of MIX 0.01 mM	500 µL of BPA 0.01 mM + 500 µL of BPF 0.01 mM + 500 µL of BPS 0.01 mM
1.5 mL of MIX 0.1 mM	500 µL of BPA 0.1 mM + 500 µL of BPF 0.1 mM + 500 µL of BPS 0.1 mM
1.5 mL of MIX 1 mM	500 µL of BPA 1 mM + 500 µL of BPF 1 mM + 500 µL of BPS 1 mM
1.5 mL of MIX 10 mM	500 µL of BPA 10 mM + 500 µL of BPF 10 mM + 500 µL of BPS 10 mM

Table S2. Brief description of primary and secondary antibodies used in Western Blot

Peptide/Protein Target		**Manufacturer, Catalog number**	**Species**	**Dilution**
Primary Antibodies	PPARγ	Cell Signaling Technology, 2435	Rabbit	1:1000 (5% BSA in 1X TBS with 0.5% Tween 20)
	C/EBPα	Cell Signaling Technology, 2841		
	FABP4	Cell Signaling Technology, 3544		
	LPL	Abcam, ab172953		1:1000 (5% non-fat milk in 1X TBS with 0.5% Tween 20)
	HSC-70 (B6)	Santa Cruz Biotechnology, SC-7298	Mouse	
Secondary Antibodies	Goat Anti-Rabbit IgG (H+L)-HRP Conjugate	BIO-RAD, 1706515	Anti-Rabbit	
	Goat Anti-Mouse IgG (whole molecule)–Peroxidase Conjugate	Sigma, A4416	Anti-Mouse	

Table S3. Intracellular lipids assessed by Oil Red O bioassay in hASCs after 14 days

	BPA				BPF				BPS				Mixture			
	0.01 µM	**0.1 µM**	**1 µM**	**10 µM**	**0.01 µM**	**0.1 µM**	**1 µM**	**10 µM**	**0.01 µM**	**0.1 µM**	**1 µM**	**10 µM**	**0.01 µM**	**0.1 µM**	**1 µM**	**10 µM**
No ICI	1.050 ± 0.003	*1.101 ± 0.004	1.049 ± 0.002	*1.126 ± 0.005	1.010 ± 0.003	0.970 ± 0.008	1.023 ± 0.009	*1.322 ± 0.007	1.030 ± 0.003	1.046 ± 0.004	*1.268 ± 0.005	*1.422 ± 0.004	1.050 ± 0.003	1.025 ± 0.004	*1.066 ± 0.001	*1.134 ± 0.005
ICI	0.880 ± 0.003	*0.886 ± 0.003	0.923 ± 0.004	0.955 ± 0.003	0.560 ± 0.001	*0.569 ± 0.001	*0.599 ± 0.001	*0.667 ± 0.002	1.159 ± 0.009	1.164 ± 0.009	*1.319 ± 0.011	*1.539 ± 0.013	0.842 ± 0.004	*0.848 ± 0.004	*0.840 ± 0.004	*0.986 ± 0.004
%	88.00	80.49	87.97	84.74	55.44	58.63	58.50	50.45	114.75	111.3	104.07	108.20	84.20	82.71	78.82	86.97

Cells were exposed to BPA, BPF, BPS or the mixture of the three bisphenols. Lipid content was normalized using the negative control and expressed as fold-changes. Differences were analyzed using the Mann-Whitney U test and defined (*p<0.05). BPA, bisphenol A; BPF, bishpenol F; BPS, bisphenol S; ICI 182,780. % inhibition percentage.

Table S4. Gene expression of the adipogenic markers PPARγ, C/EBPα, LPL, and FABP4, in hASCs after 14 days of adipogenic differentiation

	BPA				BPF				BPS				Mixture			
	0.01 µM	**0.1 µM**	**1 µM**	**10 µM**	**0.01 µM**	**0.1 µM**	**1 µM**	**10 µM**	**0.01 µM**	**0.1 µM**	**1 µM**	**10 µM**	**0.01 µM**	**0.1 µM**	**1 µM**	**10 µM**
PPAR-γ	*1.40 ±0.09	*1.19 ±0.070	*1.35 ±0.250	*1.09 ±0.240	1.73 ±0.062	1.34 ±0.295	1.42 ±0.199	*1.72 ±0.113	1.04 ±0.185	1.23 ±0.004	*2.35 ±0.008	*2.43 ±0.094	*2.85 ±0.342	*1.84 ±0.151	*1.26 ± 0.09	*2.75 ± 1.09
C/EBPα	*1.70 ±0.120	*1.48 ±0.100	0.78 ±0.350	*0.65 ±0.290	1.62 ±0.081	1.34 ±0.427	*1.49 ±0.297	*1.82 ±0.148	0.63 ±0.173	1.44 ±0.031	*1.46 ±0.037	*2.13 ±0.181	*2.2 ±0.295	*1.93 ±0.169	*0.56 ±0.100	*3.17 ±0.156
LPL	*0.44 ±0.220	*4.540 ±0.360	*2.16 ±0.300	*2.10 ±0.300	1.71 ±0.099	1.52 ±0.050	1.85 ±0.301	1.97 ±0.193	*1.90 ±0.251	1.77 ±0.041	*1.93 ±0.324	*2.19 ±0.148	*2.98 ±0.265	*2.90 ±0.140	0.83 ±0.208	*3.67 ±0.180
FABP4	*6.47 ±0.100	*6.30 ±0.590	*3.19 ±0.160	*5.11 ±0.440	0.95 ±0.431	1.11 ±0.180	1.31 ±0.274	3.83 ±0.409	1.83 ±0.118	1.01 ±0.281	1.92 ±0.296	3.06 ±0.374	*5.22 ±0.238	*3.36 ±0.193	*2.81 ±0.127	*19.34 ±0.210

Cells were exposed to BPA, BPF, BPS or the mixture of the three bisphenols. Data were expressed as fold-changes ± SEM of three independent experiments with multiple replicates for each condition. Significant differences were analyzed using the Mann-Whitney U test and defined as *p<0.05. BPA, bisphenol A; BPF, bishpenol F; BPS, bisphenol S.

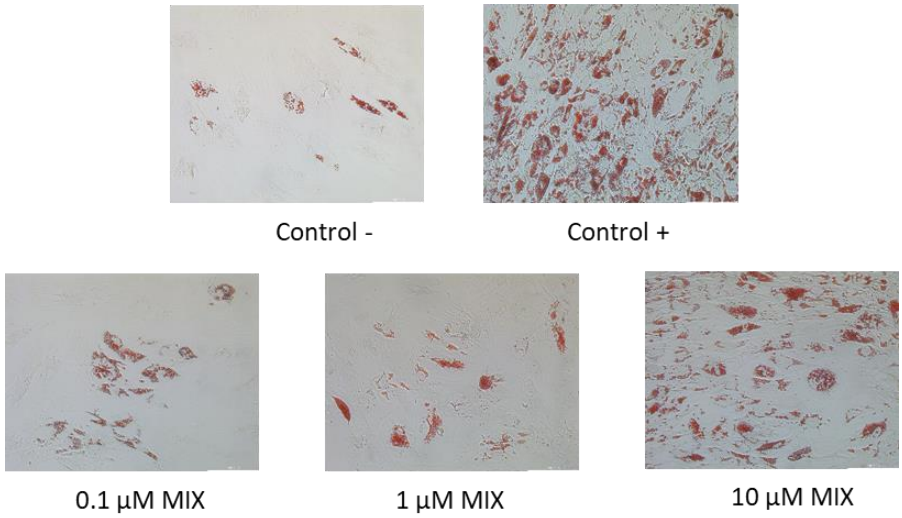


Figure S1. hASCs visualized by Oil Red O staining assay after 14 days of adipogenic differentiation. Human adipose derived stem cells were cultured in the presence of a mixture of three bisphenols (MIX: BPA, BPS and BPF) at different concentration (0.1, 1, or 10 µM MIX) and visualized at 20X under Leica DMi8 microscope (Leica Microsystems). hASCs, human adipose-derived stem cells.

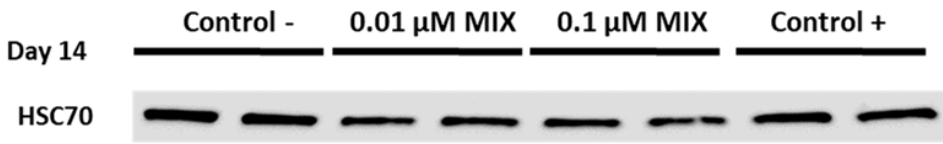


Figure S2. Western Blot of the control protein (HSC70) at the lowest concentrations of the mixture tested (0.01 and 0.1 µM). After 14 days of hASCs differentiation, protein levels of HSC70 were assessed to normalize the selected adipogenic markers. MIX, mixture of three bisphenols (BPA, BPF and BPS).

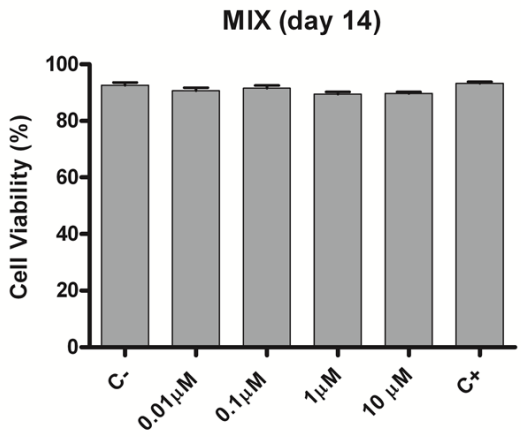


Figure S3. Effect of the mixture of three bisphenols (BPA, BPS, and BPF) on the viability of hASCs after 14 days of adipogenic differentiation. hASCs were differentiated in the presence of different concentrations of the mixture, and trypan blue assay was used to assess their viability. Cell viability (%) was expressed as means ± SEM from three independent experiments with multiple replicates for each experimental condition. BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S; C−, negative control; C+, positive control; hASCs, human adipose-derived stem cells; MIX, mixture of three bisphenols (BPA, BPF and BPS); SEM, standard error of the mean.

Supplementary Material. Article #3

Supplementary Table 1. Adipose tissue concentrations of selected POPs (ng/g tissue)

	N (%) > LOD	Mean	SD	Percentiles		
				25	50	75
HCB	61 (49.6%)	36.52	65.31	<LOD	5.26	49.31
***p,p'*-DDE**	77 (62.6%)	228.93	672.67	<LOD	51.00	236.20
***o,p'*-DDT**	69 (56.1%)	15.88	36.46	<LOD	6.68	12.74
PCB-138	96 (78.0%)	50.77	137.73	6.16	22.71	53.35
PCB-153	98 (79.7%)	61.17	161.72	6.39	27.82	64.31
PCB-180	77 (62.6%)	35.98	107.15	<LOD	9.96	42.54
α-HCH	0 (0.0%)	<LOD	-	<LOD	<LOD	<LOD
β-HCH	22 (17.9%)	4.50	13.91	<LOD	<LOD	<LOD
γ-HCH	67 (54.5%)	41.62	80.50	<LOD	13.16	42.21
ALDRIN	0 (0.0%)	<LOD	-	<LOD	<LOD	<LOD
ISODRIN	0 (0.0%)	<LOD	-	<LOD	<LOD	<LOD
ENDRIN	0 (0.0%)	<LOD	-	<LOD	<LOD	<LOD
DIELDRIN	0 (0.0%)	<LOD	-	<LOD	<LOD	<LOD
HEPTACHLOR	2 (1.6%)	<LOD	3.39	<LOD	<LOD	<LOD
VINCLOZOLINA	0 (0.0%)	<LOD	-	<LOD	<LOD	<LOD
ENDOSULFAN-I	1 (0.8%)	<LOD	1.19	<LOD	<LOD	<LOD
ENDOSULFAN-II	12 (9.8%)	<LOD	2.66	<LOD	<LOD	<LOD

o,p'-DDT: *o,p*'-dichlorodiphenyltrichloroethane; *p,p'*-DDE: *p,p'*-dichlorodiphenyldichloroethylene; HCB: hexachlorobenzene; HCH: hexachlorohexane; LOD: limit of detection; Mean: arithmetic mean; PCB: polychlorinated biphenyl; POPs: persistent organic pollutants; SD: standard deviation

Supplementary Table 2. Bivariate linear regression analyses between MetS components and log-transformed adipose tissue POP concentration (ng/g tissue)

	Unadjusted Model				
	β	exp(β)	95% CI		p-value
Waist circumference (cm)					
HCB	**0.06**	**1.06**	**1.04**	**1.08**	**<0.001**
p,p'-DDE	**0.02**	**1.02**	**1.00**	**1.04**	**0.021**
o,p'-DDT	**0.06**	**1.06**	**1.03**	**1.10**	**0.001**
γ-HCH	**0.05**	**1.05**	**1.03**	**1.07**	**<0.001**
PCB-138	0.01	1.01	0.99	1.04	0.303
PCB-153	0.00	1.00	0.97	1.03	0.915
PCB-180	-0.02	0.98	0.96	1.00	0.110
Fasting blood glucose (mg/dL)					
HCB	**0.03**	**1.03**	**1.01**	**1.05**	**0.002**
p,p'-DDE	0.01	1.01	1.00	1.03	0.168
o,p'-DDT	0.01	1.01	0.98	1.04	0.587
γ-HCH	**0.03**	**1.03**	**1.01**	**1.05**	**0.001**
PCB-138	0.02	1.02	1.00	1.04	0.081
PCB-153	0.02	1.02	1.00	1.04	0.134
PCB-180	0.01	1.01	0.99	1.04	0.161
Triglycerides (mg/dL)					
HCB	**0.06**	**1.06**	**1.02**	**1.11**	**0.006**
p,p'-DDE	0.00	1.00	0.97	1.04	0.847
o,p'-DDT	-0.04	0.96	0.89	1.03	0.254
γ-HCH	**0.07**	**1.07**	**1.02**	**1.12**	**0.003**
PCB-138	0.01	1.01	0.95	1.06	0.800
PCB-153	0.00	1.00	0.95	1.05	0.929
PCB-180	0.00	1.00	0.95	1.05	0.915
HDL cholesterol (mg/dL)					
HCB	-0.02	0.98	0.96	1.00	0.098
p,p'-DDE	0.01	1.01	0.99	1.03	0.519
o,p'-DDT	-0.01	0.99	0.95	1.03	0.671
γ-HCH	0.00	1.00	0.97	1.02	0.766
PCB-138	0.03	1.03	1.00	1.06	0.067
PCB-153	0.03	1.03	1.00	1.06	0.060
PCB-180	0.03	1.03	1.00	1.05	0.053
Diastolic Blood Pressure (mmHg)					
HCB	**0.02**	**1.02**	**1.00**	**1.03**	**0.021**
p,p'-DDE	0.00	1.00	0.99	1.01	0.911
o,p'-DDT	0.01	1.01	0.99	1.04	0.285
γ-HCH	**0.02**	**1.02**	**1.00**	**1.03**	**0.028**
PCB-138	0.02	1.02	1.00	1.03	0.088
PCB-153	0.01	1.01	1.00	1.03	0.131
PCB-180	0.01	1.01	0.99	1.02	0.558
Systolic Blood Pressure (mmHg)					
HCB	**0.02**	**1.02**	**1.01**	**1.04**	**0.004**
p,p'-DDE	0.01	1.01	1.00	1.03	0.098
o,p'-DDT	0.02	1.02	0.99	1.05	0.218
γ-HCH	**0.03**	**1.03**	**1.01**	**1.04**	**<0.001**
PCB-138	**0.03**	**1.03**	**1.01**	**1.05**	**0.001**
PCB-153	**0.02**	**1.02**	**1.01**	**1.04**	**0.013**
PCB-180	0.02	1.02	1.00	1.03	0.101

CI: confidence interval

Supplementary Table 3. Linear regression analyses between MetS components and dichotomized log-transformed adipose tissue POP concentration (ng/g tissue) (detected *vs.* non-detected)

	Unadjusted Model				Adjusted Modela			
	β	CI95%		p-value	β	CI95%		p-value
Waist circumference (cm)								
HCB	**0.23**	**0.15**	**0.30**	**0.000**	**0.27**	**0.19**	**0.35**	**0.000**
o,p'-DDT	**0.14**	**0.05**	**0.22**	**0.002**	**0.13**	**0.04**	**0.22**	**0.005**
γ-HCH	**0.16**	**0.07**	**0.24**	**0.000**	**0.24**	**0.15**	**0.33**	**0.000**
Fasting blood glucose (mg/dL)								
HCB	**0.08**	**0.01**	**0.15**	**0.029**	0.06	-0.02	0.13	0.152
o,p'-DDT	-0.01	-0.09	0.06	0.751	0.00	-0.07	0.08	0.934
γ-HCH	**0.12**	**0.05**	**0.19**	**0.001**	**0.09**	**0.01**	**0.18**	**0.023**
Triglycerides (mg/dL)								
HCB	**0.19**	**0.02**	**0.37**	**0.032**	0.11	-0.07	0.30	0.223
o,p'-DDT	-0.12	-0.30	0.06	0.181	-0.08	-0.26	0.11	0.407
γ-HCH	**0.23**	**0.05**	**0.40**	**0.013**	0.12	-0.08	0.32	0.252
HDL cholesterol (mg/dL)								
HCB	-0.08	-0.17	0.01	0.079	**-0.12**	**-0.22**	**-0.03**	**0.010**
o,p'-DDT	0.02	-0.07	0.12	0.651	0.03	-0.06	0.13	0.510
γ-HCH	-0.01	-0.10	0.09	0.878	-0.06	-0.17	0.04	0.224
Diastolic Blood Pressure (mmHg)								
HCB	**0.07**	**0.02**	**0.13**	**0.010**	**0.08**	**0.01**	**0.14**	**0.015**
o,p'-DDT	0.04	-0.02	0.10	0.221	0.04	-0.02	0.10	0.212
γ-HCH	**0.07**	**0.02**	**0.13**	**0.011**	**0.09**	**0.02**	**0.16**	**0.012**
Systolic Blood Pressure (mmHg)								
HCB	**0.09**	**0.03**	**0.15**	**0.005**	**0.07**	**0.00**	**0.13**	**0.042**
o,p'-DDT	0.03	-0.04	0.09	0.387	0.05	-0.02	0.11	0.147
γ-HCH	**0.12**	**0.06**	**0.18**	**0.000**	**0.10**	**0.03**	**0.17**	**0.006**

aAdjusted for age (yr) and sex (male/female); CI: confidence inte

[a]Adjusted for age (yr) and sex (male/female)

CI: confidence interval. MetS: metabolic syndrome; POPs: persistent organic pollutants; HCB, *o,p'*-DDT, and γ-HCH (POPs around 50% detection)

Supplementary Table 4. ORs (95%CI) of the risk of MetS components according to adipose tissue POP concentration (ng/g tissue)

	Unadjusted Model			
	OR	**95% CI**		**p-value**
Elevated waist circumference (≥ 94 cm men or ≥ 80 cm women)				
HCB	**1.93**	**1.38**	**2.70**	**<0.001**
p,p'-DDE	1.13	0.93	1.37	0.210
o,p'-DDT	1.35	0.90	2.03	0.148
γ-HCH	**1.76**	**1.32**	**2.34**	**<0.001**
PCB-138	1.22	0.93	1.59	0.153
PCB-153	1.07	0.83	1.39	0.603
PCB-180	0.92	0.72	1.18	0.516
Elevated fasting blood glucose (≥100 mg/dL)				
HCB	**1.29**	**1.04**	**1.59**	**0.020**
p,p'-DDE	1.07	0.89	1.29	0.463
o,p'-DDT	0.93	0.65	1.33	0.678
γ-HCH	**1.29**	**1.04**	**1.60**	**0.022**
PCB-138	1.08	0.83	1.41	0.555
PCB-153	1.07	0.83	1.38	0.605
PCB-180	1.10	0.87	1.38	0.446
Elevated triglycerides (≥ 150 mg/dL)				
HCB	**1.24**	**1.01**	**1.51**	**0.036**
p,p'-DDE	0.89	0.75	1.05	0.168
o,p'-DDT	0.71	0.49	1.02	0.064
γ-HCH	**1.28**	**1.04**	**1.57**	**0.018**
PCB-138	0.95	0.75	1.21	0.694
PCB-153	0.96	0.76	1.20	0.698
PCB-180	1.04	0.84	1.29	0.747
Reduced HDL cholesterol (<40 mg/dL in men or <50 mg/dL in women)				
HCB	**1.24**	**1.03**	**1.50**	**0.026**
p,p'-DDE	1.06	0.90	1.25	0.492
o,p'-DDT	1.25	0.91	1.73	0.165
γ-HCH	1.12	0.92	1.35	0.257
PCB-138	0.86	0.68	1.09	0.225
PCB-153	0.87	0.69	1.09	0.229
PCB-180	0.83	0.67	1.02	0.079
Elevated blood pressure (SBP ≥130mmHg and/or DBP ≥85mmHg)				
HCB	**1.25**	**1.02**	**1.53**	**0.033**
p,p'-DDE	1.03	0.87	1.21	0.772
o,p'-DDT	1.16	0.83	1.63	0.381
γ-HCH	**1.30**	**1.06**	**1.59**	**0.011**
PCB-138	1.24	0.97	1.59	0.088
PCB-153	1.13	0.90	1.43	0.301
PCB-180	1.07	0.86	1.32	0.552

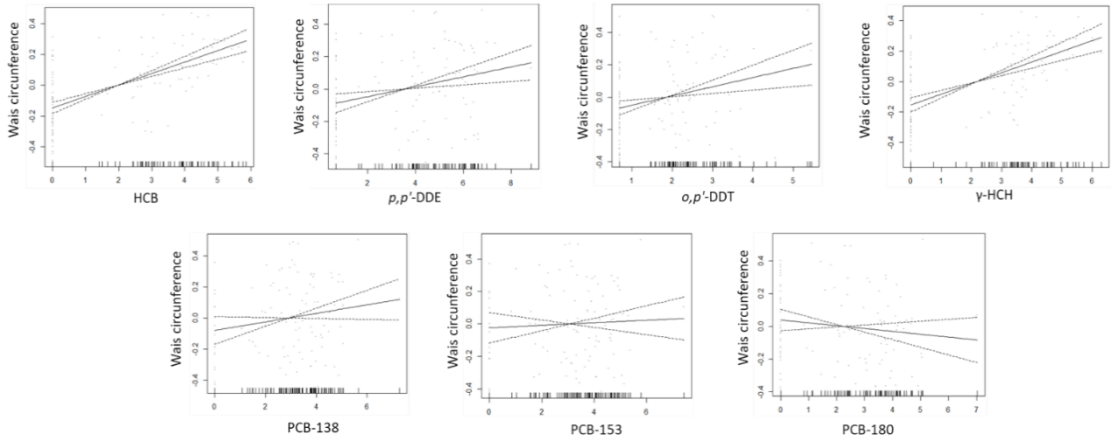
CI: confidence interval; MetS: metabolic syndrome; POPs: persistent organic pollutants; SBP, DBP: systolic and diastolic blood pressure, respectively.

Supplementary Table 5. ORs (95%CI) of the risk of MetS according to adipose tissue POP concentration (ng/g tissue)

	Unadjusted Model			
	OR	**95% CI**		**p-value**
HCB	**1.55**	**1.26**	**1.92**	**<0.001**
p,p'-DDE	1.05	0.89	1.24	0.539
o,p'-DDT	1.15	0.84	1.58	0.392
γ-HCH	**1.42**	**1.16**	**1.75**	**0.001**
PCB-138	1.24	0.97	1.59	0.083
PCB-153	1.23	0.97	1.56	0.093
PCB-180	1.14	0.92	1.41	0.235

CI: confidence interval

A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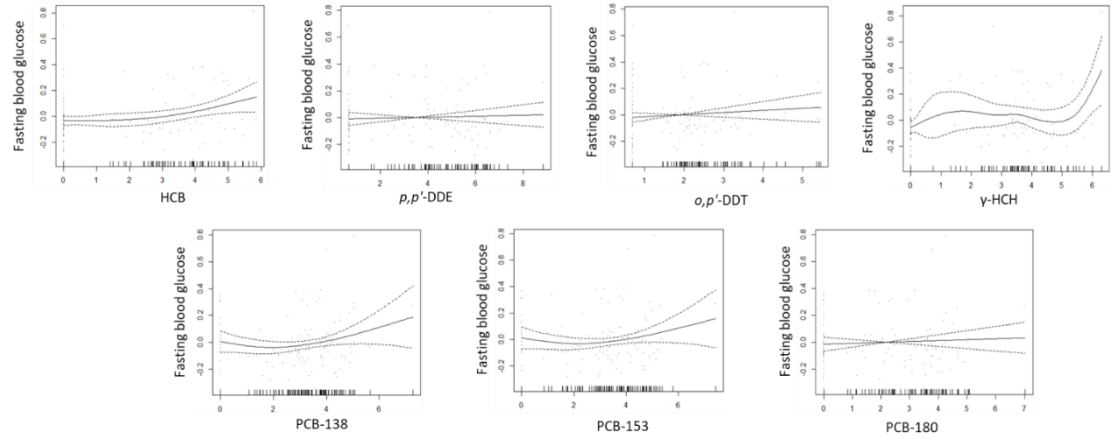
HCB
p,p'-DDE
o,p'-DDT
γ-HCH
PCB-138
PCB-153
PCB-180

B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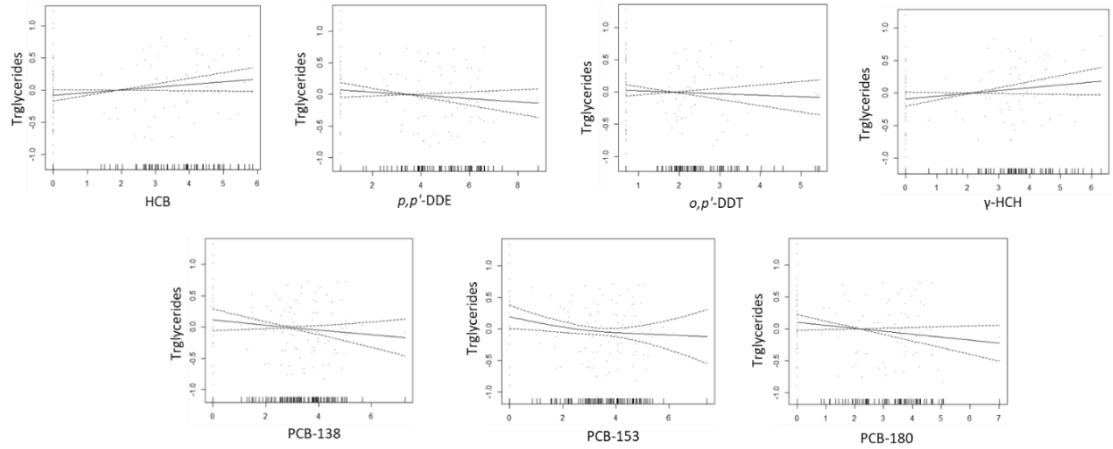
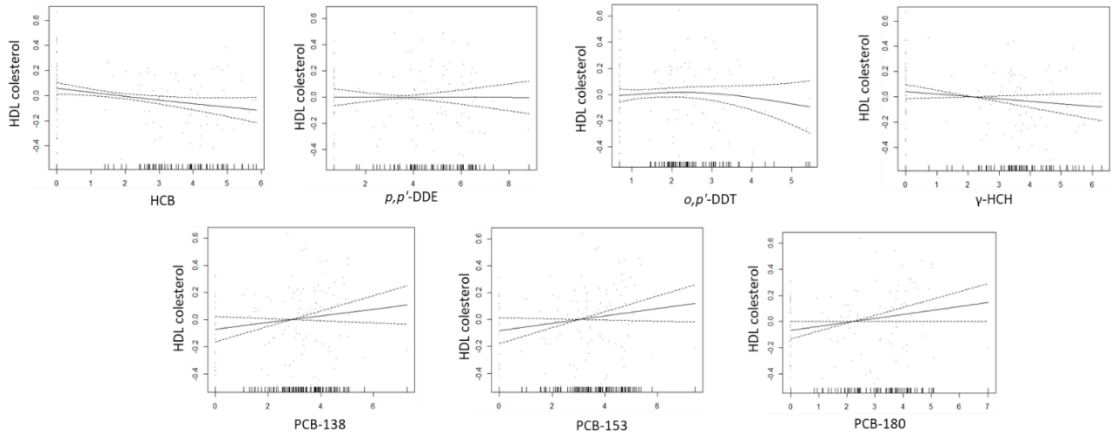
o,p'-DDT
γ-HCH
PCB-138
PCB-153
PCB-180

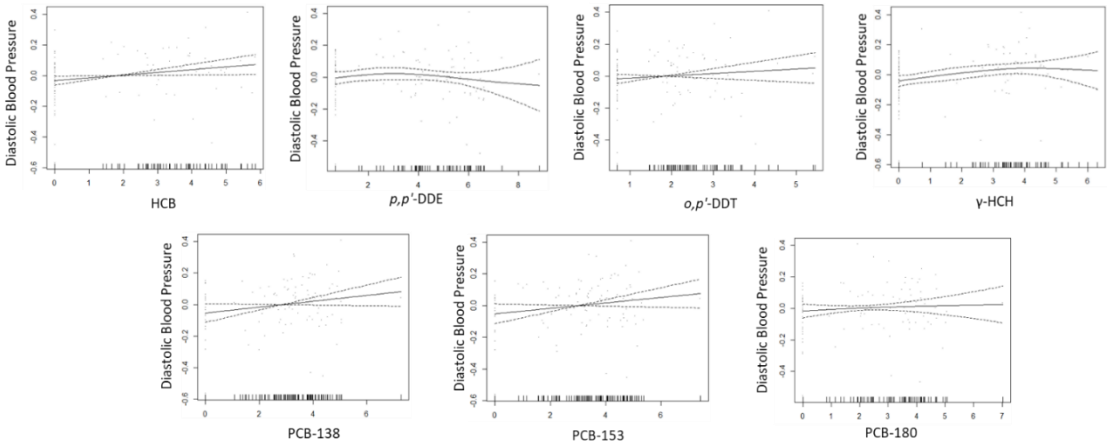
C


Trglycerides
HCB
p,p'-DDE
o,p'-DDT
γ-HCH
PCB-138
PCB-153
PCB-180

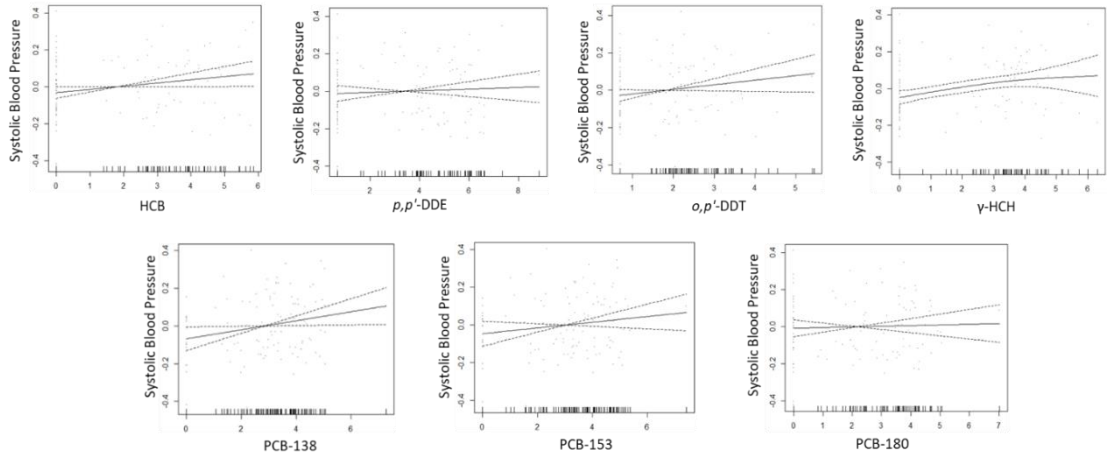
D


HDL colesterol
HCB
p,p'-DDE
o,p'-DDT
γ-HCH
PCB-138
PCB-153
PCB-180

E



F



Supplementary Figure 1. Generalized additive models (GAMs) for the relationship between adipose tissue POPs concentrations and MetS components.
A: waist circumference (cm); B: fasting blood glucose (mg/dL); C: triglycerides (mg/dL); D: HDL cholesterol (mg/dL); E: diastolic blood pressure, and F: systolic blood pressure (mmHg)

Appendix