UNIVERSIDAD DE GRANADA

EXPOSICIÓN PLACENTARIA A CONTAMINANTES AMBIENTALES EN UN ESTUDIO DE COHORTE

Memoria que presenta para aspirar al grado de Doctora en Ciencias Ambientales, dentro del programa de Medicina Clínica y Salud Pública, la Licenciada en Ciencias Ambientales ESPERANZA AMAYA GONZÁLEZ

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Granada, 24 de octubre de 2013

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La memoria de Tesis Doctoral que lleva por título **EXPOSICIÓN PLACENTARIA A CONTAMINANTES AMBIENTALES EN UN ESTUDIO DE COHORTE**, ha sido presentada por la Lda. Esperanza Amaya González para aspirar al grado de Doctora en Ciencias Ambientales dentro del programa de Medicina Clínica y Salud Pública, habiendo sido dirigida por D. Nicolás Olea Serrano, Catedrático del Departamento de Radiología y Medicina Física de la Facultad de Medicina de la Universidad de Granada, por Dña. Carmen Freire Warden, Investigadora Visitante en el Programa de Post-graduación en Salud Pública y Medio Ambiente de la Escuela Nacional de Salud Pública-FIOCRUZ en Rio de Janeiro (Brasil), y por D. José Manuel Molina Molina, Investigador del grupo 19 del Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBERESP).

Fdo. Esperanza Amaya González

La aproximación holística al estudio de la exposición a contaminantes ambientales que constituye esta tesis ha sido posible gracias a la colaboración multidisciplinar de un numeroso grupo de profesionales del Laboratorio de Investigaciones Médicas y la Unidad de Apoyo a la Investigación del Hospital Universitario San Cecilio de Granada, del grupo de investigación CTS 206 (Oncología Básica y Clínica) de la Universidad de Granada, del Centro de Investigación Biomédica de la Universidad de Granada, del Departamento de Medicina Legal, Toxicología y Antropología Física de la Universidad de Granada, del personal sanitario del Hospital Universitario San Cecilio de Granada y de la Unidad de Diagnóstico Neonatal Precoz de Metabolopatías del Hospital San Juan de Dios de Granada (Dra. Isabel Marín de la Delegación de Salud y colaboradores), así como la participación desinteresada de las familias incluidas en el estudio.

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El doctorando y los directores de la tesis *Exposición placentaria a contaminantes ambientales en un estudio de cohorte* garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

Granada, a 30 de septiembre de 2013.

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Carmen Freire Warden

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José Manuel Molina Molina

Fdo.:

Los siguientes coautores (no doctores) de las publicaciones incluidas en la presente tesis doctoral declaran renunciar a presentar dichas publicaciones en sus respectivas tesis doctorales en un futuro. Reconocen además la participación del doctorando en dicho trabajo:

In vitro study on the agonistic and antagonistic activities of bisphenol-S and other bisphenol-A congeners and derivatives via nuclear receptors.

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1. RESUMEN

Los estudios toxicológicos y epidemiológicos han demostrado que la exposición humana a contaminantes ambientales con actividad hormonal ha contribuido al incremento de ciertas enfermedades comunes de base endocrina. La gran diversidad estructural de los compuestos liberados al medioambiente y su fácil acceso al organismo, junto con las interacciones que pueden establecerse entre ellos y las hormonas endógenas, dificultan enormemente cualquier proceso de evaluación del riesgo.

En esta tesis se realiza una aproximación holística al estudio de la exposición materno-infantil a disruptores endocrinos, considerando cuatro aspectos distintos pero complementarios de la evaluación del riesgo: i) el estudio de la actividad hormonal de componentes del plástico policarbonato y las resinas epoxi, ii) la evaluación cuantitativa de la exposición materno-infantil a metales pesados a través de su medida en placentas, iii) la descripción de los determinantes de la exposición materno-infantil a pesticidas organoclorados, y iv) el estudio de la influencia de los niveles de TSH neonatal sobre el neurodesarrollo infantil.

De forma resumida, los resultados más relevantes se resumen de la siguiente manera:

i) Los bisfenoles testados BPS, BPF y BPA mostraron capacidad de activación de hERα y hERβ. TCBPA se comportó como un agonista débil del hERα. BPS mostró mayor actividad en hERβ que hERα, al contrario que BPF y BPA. BPF y BPA se comportaron como antagonistas de hAR, con mayor eficacia el BPA respecto a BPF. BPA y BPS mostraron una leve capacidad agonista de hAR. Y por último, sólo BPA, TCBPA y TBBP mostraron ser agonistas de hPXR (TCBPA > TBBPA > BPA).

ii) se confirmó la presencia de Cd y Mn en el 100% de las placentas analizadas de mujeres de la población general del área metropolitana de Granada, así como de Cr, Pb y Hg, detectados en un 98.5, 35.0 y 30.7% de las placentas, respectivamente. No se detectó As en las placentas estudiadas. El metal que presentó las mayores concentraciones placentarias fue Pb, seguido de Mn, Cr, Cd y Hg.

iii) En 257 mujeres de la cohorte INMA-Granada las concentraciones placentarias de *p,p'*-DDT fueron mayores entre las pertenecientes a clases sociales medias-bajas (III y IV) respecto a mujeres de clases más altas. La concentración de la

suma de metabolitos de DDT fue mayor en la clase social IV, mientras que la concentración del total de compuestos de endosulfán fue mayor en la clase social III. Las concentraciones de HCB fueron mayores en la clase IV y entre trabajadoras manuales (clases III y IV) respecto a ocupaciones no manuales. Las concentraciones placentarias de HCB mostraron una tendencia de aumento lineal estadísticamente significativa a lo largo de las categorías de clase social, de manera que las clases más desfavorecidas presentaron mayores niveles de HCB.

iv) En una submuestra de 178 niños de la INMA-Granada, evaluados mediante el test McCarthy a los 4 años de edad, se observó una asociación significativa entre mayores niveles de TSH neonatal y menores puntuaciones en el índice general cognitivo y en la función ejecutiva. Igualmente, niveles más elevados de TSH neonatal estuvieron asociados con mayor riesgo de tener una menor puntuación en el área numérica del test McCarthy. Los niños con niveles de TSH dentro del último cuartil (>4,19 mU/l) tuvieron mayor riesgo de tener una menor puntuación (< percentil 20) en el área de memoria de trabajo. Las asociaciones observadas entre el nivel de TSH neonatal y las puntuaciones obtenidas para la función ejecutiva y el índice general cognitivo se mantuvieron tras ajustar el modelo por las concentraciones de pesticidas organoclorados y la carga xenoestrogénica de las placentas.

El análisis de los resultados permite concluir que la exposición materno-infantil a disruptores endocrinos tales como bisfenoles, compuestos organoclorados y metales persistentes es un fenómeno frecuente, que ocurre de forma inadvertida para la población no profesionalmente expuesta y que tiene consecuencias para la salud.

2. INTRODUCCIÓN

2.1. Breve aproximación a la situación actual

Contaminación ambiental y efectos en la salud humana

En el 2011 la *Organización Mundial de la Salud* (OMS, WHO —*World Health Organization*—) estimaba que un 19% de los 12 millones de casos de cáncer que se diagnostican cada año en el mundo se pueden atribuir a exposiciones ambientales y ocupacionales, y que, por otra parte, son especialmente vulnerables a las exposiciones ambientales las mujeres embarazadas, el embrión/feto, los niños y la población ocupacionalmente expuesta.

Figura 1: Estimaciones sobre cáncer y medioambiente de la OMS (http://www.who.int/phe/news/events/international_conference/Call_for_action_en.pdf, abril 2013).

Durante las últimas décadas los estudios epidemiológicos han ido señalando una serie de efectos adversos ligados a la exposición a determinados contaminantes químicos, como el declive de la calidad y cantidad del semen, aumento de la incidencia de defectos en el tracto genitourinario masculino y problemas testiculares, cáncer de próstata y de mama, afección de la homeostasis tiroidea y problemas en el neurodesarrollo (Portefield, 2000; Daston *et al.*, 2003; Boas *et al.*, 2006; Maffini *et al.*, 2006; Fernández *et al.*, 2007; Chevrier *et al.*, 2008). Al mismo tiempo, diversos estudios en animales han confirmado el efecto que numerosas sustancias químicas antropogénicas pueden ocasionar en el desarrollo, y en los sistemas reproductivos y endocrinos (Maffini *et al.*, 2006; El-Shahawi *et al.*, 2010; WHO 2013).

Estos datos vienen a confirmar un hecho largamente sospechado, las enfermedades no contagiosas tienen, en muchos casos, como principal razón etiológica la exposición ambiental en su concepto más amplio: alimentación, hábitos y medio ambiente.

La investigación en este campo ha posibilitado caracterizar en los últimos años las propiedades físico-químicas de contaminantes liberados al medio ambiente que

interfieren con el sistema hormonal y las vías de exposición para los seres humanos, así como identificar algunos de sus efectos en el medio ambiente y su influencia en la salud humana.

Principio de precaución, biomonitorización y vigilancia de la salud

La postura de gran parte de la comunidad científica es defender la aplicación del *Principio de precaución*, basado en la idea de que las políticas y las decisiones en materia de medio ambiente y salud humana, como medida preventiva, deben estar orientadas a evitar o reducir tanto como sea razonablemente alcanzable la exposición a agentes potencialmente nocivos para la salud, mientras se llevan a cabo estudios que demuestren la seguridad en el uso del compuesto de que se trate. El objetivo es no tomar medidas únicamente para resolver los problemas de salud derivados de la acción de estas exposiciones en el organismo, sino prevenir la exposición antes de necesitar paliar el daño producido. La prevención primaria de la exposición constituye por ello el centro de eventos como la *Conferencia sobre Determinantes Medioambientales y Ocupacionales del Cáncer* de la OMS (2011). Con la afirmación *"Primary prevention keeps cancer from ever occurring"* ("La prevención primaria evita la aparición del cáncer"), la OMS pone de manifiesto su postura sobre la influencia que ejerce el *Principio de precaución* en materia de salud y medioambiente, y entorno a éste debe articularse la investigación y epidemiología de la salud ambiental.

Figura 2: Principio de precaución (Síntesis de la legislación de la Unión Europea, http://europa.eu/legislation_summaries/consumers/consumer_safetv/l32042_es.htm, octubre 2012).

Para ello, es necesario comprender los mecanismos de acción y efectos biológicos de los compuestos químicos, establecer curvas dosis-respuesta que ilustran cómo el sistema biológico estudiado responde a una sustancia o conjunto de sustancias químicas, y determinar la *dosis de referencia*, o concentración desprovista de riesgo, para compuestos particulares. Por otro lado, se necesitan los correspondientes estudios

epidemiológicos que establezcan relaciones entre la exposición y efectos en la salud de una población, sumando así pruebas a la evidencia de los efectos tóxicos de determinadas sustancias químicas.

El objetivo final de los planes de acción de los organismos de protección de la salud es que las concentraciones finales que llegan a los individuos estén por debajo de la dosis de referencia establecida como límite de seguridad a partir de los datos obtenidos de las curvas dosis-respuesta y el estudio de los efectos biológicos, donde mezclas reales son investigadas con objeto de conocer el efecto combinado. En este sentido, la *biomonitorización humana* permite tanto identificar grupos de riesgo como evaluar la eficacia de intervenciones de reducción de la contaminación ambiental, siendo por ello una herramienta útil en las estrategias de Vigilancia Ambiental y de Vigilancia de la Salud.

Exposición humana

La exposición humana a contaminantes químicos sucede por diferentes vías. En el caso de los trabajadores ocupacionalmente expuestos, se trata de poblaciones que están en contacto con *xenobióticos* —compuestos de síntesis antropogénica o que rara vez existen de manera natural— que han mostrado efectos nocivos en salud, siendo además muchos de ellos persistentes, acumulándose en el medio ambiente y/o en la cadena trófica, con lo que finalmente esta exposición alcanza así a toda la población (Fattore *et al.*, 2002; El-Shahawi *et al.*, 2010, Mrema *et al.*, 2012; WHO, 2013). Con este modelo de dispersión, las posibilidades de entrada al organismo cubren todas las vías. Los xenobióticos pueden ser transportados a través del aire, el suelo y el agua, llegando a entrar en contacto con la población general en forma de contaminación atmosférica o del agua de consumo, en vegetales cultivados en suelos con diferentes compuestos químicos o regados con agua de alta carga de contaminantes, en carnes procedentes de animales criados en zonas con problemas de contaminación, o alimentados con piensos contaminados. En el caso particular de los compuestos químicos no persistentes, resulta especialmente importante la exposición diaria a componentes de productos cosméticos, que pueden acceder al organismo por vía dérmica, así como los que forman parte del procesamiento o están contenidos en envases de uso alimentario, que pueden liberarse al producto de consumo y llegar al organismo a través de la dieta, o componentes de los productos textiles que pueden ser liberados al no estar estructuralmente incorporados.

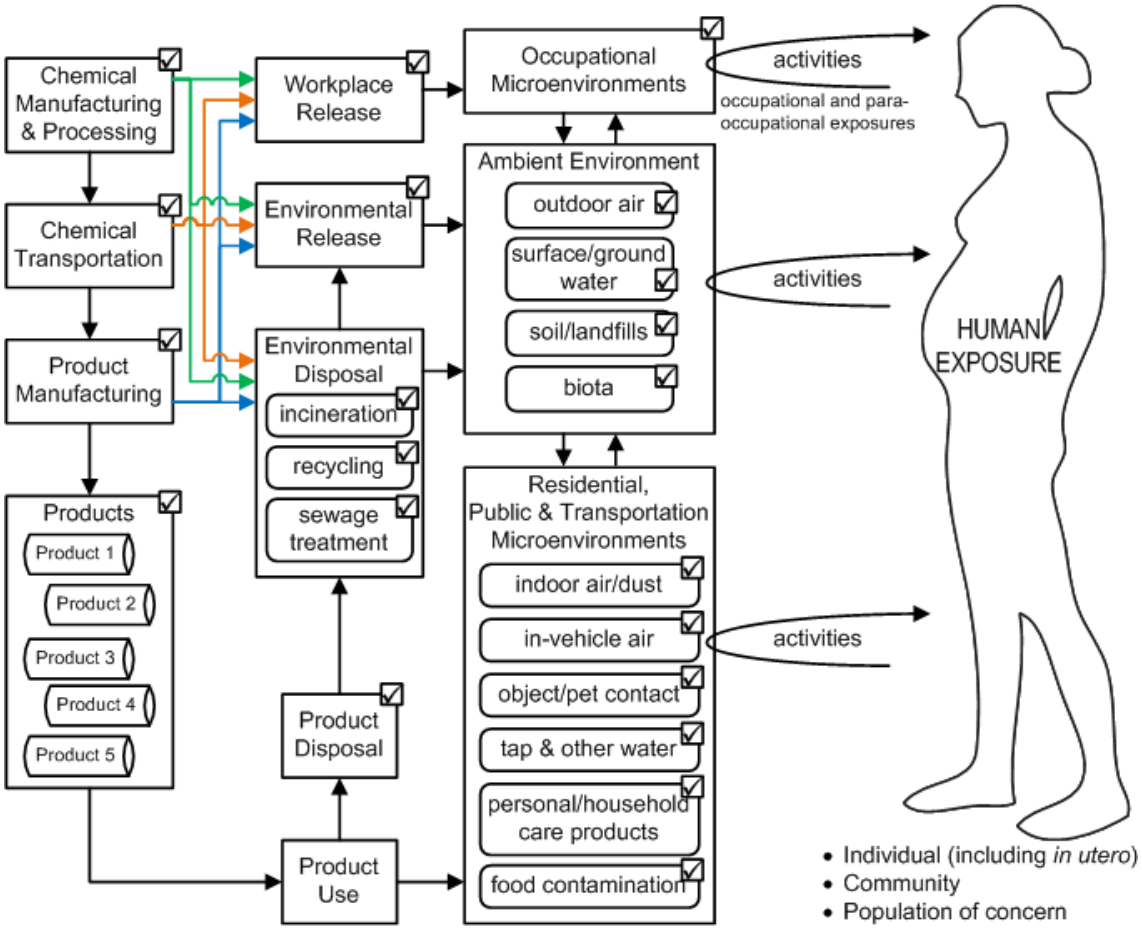


Figura 3: Vías de exposición humana (*The Center for Exposure and Risk Modeling*, http://ccl.rutgers.edu/cerm-alpha/, agosto 2013).

Según las características físico-químicas, los compuestos podrán o no ser acumulados en el organismo, ser metabolizados y excretados con mayor o menor facilidad. Los compuestos químicos fácilmente metabolizables, generalmente sustancias muy solubles en agua, podrán encontrarse, bien la molécula que es absorbida por el organismo o bien sus metabolitos, en sangre, orina, saliva, leche materna, heces y otros fluidos excretados por el organismo. Si la metabolización es más lenta y la velocidad de excreción es menor que la de incorporación, las sustancias contaminantes se acumularán en compartimentos del organismo como tejido graso, huesos, pelo, uñas o algunos órganos como el hígado o el cerebro.

De esta manera, el organismo humano puede constituir un reservorio de un gran número de contaminantes a los que las poblaciones están expuestas. Dependiendo de la

capacidad de bioacumulación del compuesto en particular, de la matriz biológica usada y del momento de la medición, las concentraciones determinadas en el organismo informarán sobre exposiciones recientes, a medio o a largo plazo, este último en el caso de contaminantes persistentes. La cuantificación de estos niveles constituye la *biomonitorización humana*, que proporciona información sobre qué contaminantes que rodean al hombre están siendo absorbidos realmente por el organismo y en qué medida, permitiendo identificar grupos de individuos bajo un mayor riesgo dentro de una población. A la información obtenida hasta ahora sobre niveles de contaminantes ambientales en población humana puede accederse a través de las bases de datos de publicaciones científicas, así como de organismos como la *Agencia Europea del Medioambiente* (*European Environmental Agency, EEA*) y el *Centro para el Control y la Prevención de Enfermedades (Center for Disease Control and Prevention, CDC)* de Estados Unidos y sus informes periódicos en materia de salud y medioambiente (EEA, 2013; CDC, 2013).

Caracterización del riesgo

Diferentes factores relacionados con el contexto social, cultural y económico, la alimentación, el ejercicio físico, y el estilo de vida del individuo en general determinan el grado o magnitud de exposición a los contaminantes ambientales y, en consecuencia, las concentraciones que podrán ser absorbidas por el organismo y estar presentes en las diferentes matrices biológicas. El estatus socioeconómico, determinado principalmente por el nivel de educación, ocupación y/o ingresos, se asocia con diferentes condiciones de salud, con la higiene, la nutrición, además de determinados patrones de hábitos de consumo, como la ingesta de alcohol, tabaquismo, consumo de medicamentos, uso de productos químicos en el hogar (plásticos, cosméticos, productos de limpieza, etc.). Por ello, es cada vez más obvio que las características socioeconómicas podrían ejercer un papel esencial en el binomio exposición-salud (Borrell *et al.*, 2004; Porta *et al.*, 2008a; González-Galarzo *et al.*, 2009).

Sinergias, exposición a bajas dosis y patrones de dosis-respuesta no lineales

La exposición humana a contaminación ambiental tiene un matiz que lo hace especialmente complejo. Excepto en situaciones agudas, accidentales, los seres humanos no se ven expuestos a un único contaminante a altas concentraciones, sino a

sino a mezclas complejas y heterogéneas de sustancias en muy bajas dosis que pueden interactuar entre sí produciendo diferentes combinaciones de efectos, desde los antagónicos a los aditivos y/o sinérgicos. Un ejemplo paradigmático es la exposición a *disruptores endocrinos* (WHO, 2013). Brevemente, son compuestos que presentan una acción agonista o antagonista de hormonas sexuales, interfiriendo así en el equilibrio del sistema endocrino. En este caso, el efecto combinado de la exposición a una mezcla de estas sustancias podría llegar a provocar un impacto mayor que el producido por la suma de las concentraciones individuales de cada uno de los contaminantes (Kortenkamp *et al.*, 2007). Se hipotetiza además que si los efectos adversos observados en relación a la exposición a contaminantes químicos persistentes se deben a mecanismos de disrupción endocrina, estos podrían ser más marcados ante concentraciones moderadas en comparación con niveles elevados (Daston *et al.*, 2003; Hamlin y Guillete, 2011).

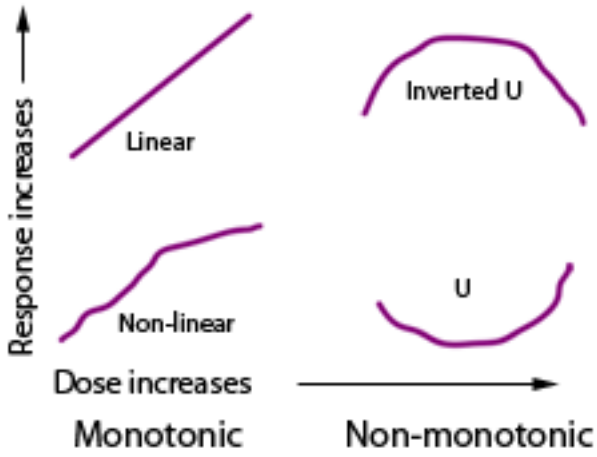


Figura 4: Diferentes curvas dosis-respuesta (*Environmental Health Sciences*, http://www.environmentalhealthnews.org/sciencebackground/2007/2007-0415nmdrc.html, agosto 2013).

De hecho, la respuesta biológica a disruptores endocrinos (o curva dosis-respuesta) podría tener forma de "U" invertida (Vandenberg *et al.*, 2012). Así lo observan diferentes estudios, entre ellos el de Lee *et al.* (2010), en el que se analiza la relación entre la exposición a contaminantes orgánicos persistentes y el riesgo de desarrollar diabetes: no se observa el patrón tradicional de dosis-respuesta, sino que el incremento del riesgo es mayor para niveles medios de los 31 contaminantes analizados (no obstante, los autores se plantean en este caso la posible acción de confusores como la obesidad, y si el efecto encontrado para compuestos individuales no es en realidad un indicador de exposición a mezclas, dado que las concentraciones de contaminantes persistentes suelen correlacionarse entre sí). El mismo comportamiento observan Arrebola *et al.* (2013a)

para el riesgo de diabetes a mayores concentraciones de contaminantes orgánicos persistentes en individuos obesos.

A pesar de estas evidencias y dada la complejidad de estudiar la exposición a múltiples compuestos, los estudios toxicológicos clásicos y los epidemiológicos evalúan generalmente la exposición a sustancias químicas individuales. Por esto, caracterizar la exposición humana a contaminantes ambientales con rigor supone un esfuerzo holístico de integración de diferentes campos que merece una especial atención.

2.2. Exposición infantil a contaminantes ambientales

Relevancia de la exposición temprana, evidencias epidemiológicas

La exposición a contaminantes ambientales durante el periodo preconcepcional y gestacional puede afectar el desarrollo embrionario y fetal y comprometer la estructura y funcionamiento de los sistemas del nuevo individuo. La etapa intrauterina y la infancia son un momento crítico en cuanto a la vulnerabilidad a las sustancias tóxicas debido, principalmente, a que las rutas metabólicas aún son inmaduras. Los daños potenciales de esta exposición no terminan con el nacimiento, ya que el efecto puede hacerse evidente no sólo en los sistemas no estructurados al nacer (como el neurológico, el inmunitario o el sexual) sino en órganos y aparatos ya estructurados pero cuya función y maduración posterior pueden verse alterados por estas exposiciones tempranas (Ramón *et al.,* 2005). De acuerdo con el aumento y diversificación de la exposición a tóxicos ambientales que ha supuesto la revolución química de las últimas décadas (EEA, 2010), es esperable observar que ésta tenga efectos adversos en la salud originados en los períodos críticos del desarrollo. La población de mujeres en edad fértil, gestantes, niños y adolescentes constituyen un grupo particularmente vulnerable a los efectos tóxicos de los contaminantes ambientales.

Los procesos biológicos de base hormonal juegan un papel clave en la etapa fetal para el desarrollo y función de diversos sistemas. En el caso del *Sistema Nervioso Central* (SNC) se especula que determinadas alteraciones cognitivas en la infancia o adolescencia pudiesen tener su origen en la exposición pre- o neonatal a contaminantes ambientales con actividad hormonal (*i.e.*, disruptores endocrinos) (Damstra, 2002; Rauh *et al.,* 2011; Gilbert *et al.,* 2012). De hecho, el desarrollo del sistema nervioso es altamente dependiente de las hormonas tiroideas desde el comienzo de la etapa fetal, siendo esencial un nivel adecuado de estas hormonas para el desarrollo y regulación neuropsicológica, por lo que su alteración puede comprometer la salud infantil (Williams, 2008). Por otra parte, la alteración de los niveles de hormonas tiroideas es un efecto potencial de determinados compuestos organoclorados, además de ser el SNC una diana importante de la acción de contaminantes con carácter estrogénico (Gabor *et al.,* 2003). De esta manera, no es descabellado especular que el incremento de la exposición a sustancias químicas observado en las últimas décadas esté en alguna medida relacionado con el aparente aumento de los trastornos neuroconductuales en la

población infantil, tales como dificultades de aprendizaje, trastornos de déficit de atención e hiperactividad, autismo o problemas de conducta, entre otros (Grandjean y Landrigan, 2006).

En España, trabajos realizados en tres de las cohortes pertenecientes al proyecto multicéntrico *Infancia y Medio Ambiente* (INMA) en Ribera d'Ebre, Menorca y Granada, ya han mostrado asociaciones inversas entre exposición prenatal e infantil a determinados contaminantes neurotóxicos y el desarrollo neuroconductual en edades preescolares (Ribas-Fitó *et al.*, 2003, 2007a, 2007b; Freire *et al.*, 2010a, 2010b).

Figura 5: Logo del proyecto INMA, http://www.proyectoinma.org/, mayo 2013

Evaluación de la exposición materno-infantil

En el diseño de la recogida de muestras biológicas de estudios de biomonitorización de contaminantes ambientales se opta tradicionalmente por la utilización de sangre, suero, orina y cabello. La sangre es la matriz de referencia para la cuantificación de la mayor parte de los contaminantes, por mantenerse en contacto con todos los órganos y tejidos, pero obtener sangre fetal o del recién nacido es un método invasivo que puede reducir la participación en estudios epidemiológicos (Esteban y Castaño, 2009).

Una estimación posible de los contaminantes transferidos de la madre al hijo es el análisis de la leche materna (Esteban y Castaño, 2009). Esta matriz se suele utilizar para evaluar la exposición materno-infantil durante el período neonatal a contaminantes liposolubles, dado el alto porcentaje de grasa que contiene. Debido a la lipofilicidad que caracteriza a este tipo de compuestos, son metabolizados lentamente acumulándose en tejidos grasos del organismo, siendo movilizados y liberados al torrente sanguíneo durante el embarazo por la redistribución de tejido graso que se produce durante éste. De este modo, pueden ser transferidos fácilmente a la leche materna para su excreción, razón por la que diversos estudios describen las concentraciones de varios contaminantes orgánicos persistentes en la leche materna (Lakind *et al.*, 2009; Todaka *et al.*, 2010; Needham *et al.*, 2011).

No obstante, el verdadero conocimiento de la exposición fetal pasa por el estudio del ambiente intrauterino y de la etapa perinatal, que permitiría conocer el entorno en el que se desarrolla el ser humano durante su etapa más vulnerable. Por esto, las determinaciones analíticas realizadas en muestras de placenta y sangre de cordón umbilical parecen ser más adecuadas para la evaluación de la exposición materno-fetal a contaminantes ambientales (Iyengar y Rapp, 2001; Myren *et al.,* 2007; Esteban y Castaño, 2009).

La barrera placentaria es parcialmente permeable a las sustancias exógenas (Osman *et al.*, 2000; Myren *et al.*, 2007; ATSDR 2007a). Muchos compuestos tóxicos, como por ejemplo algunos metales presentes en el humo del tabaco, pueden atravesarla e incluso interferir con los sistemas de transporte placentario (Wier *et al.*, 1990; Zhang *et al.*, 2004), por lo que la utilización de la placenta como matriz biológica para análisis de contaminantes es particularmente útil para caracterizar el grado de exposición fetal a través de la madre. Tanto es así, que el uso de dicho tejido ha sido validado por diferentes estudios que han descrito concentraciones de metales, pesticidas, ftalatos, *bifenilos policlorados* (PCBs) o compuestos bromados (PBDEs) en placenta (Falcón *et al.*, 2004; Chan *et al.*, 2007; Gómara *et al.*, 2007; López-Espinosa *et al.*, 2007; Mose *et al.*, 2007; Esteban y Castaño, 2009).

2.3. Red INMA – cohorte de Granada

La Red de investigación colaborativa *Infancia y Medio Ambiente* (proyecto INMA) es un estudio de base poblacional que se constituyó en el 2003 para estudiar los efectos del medio ambiente y la dieta en el desarrollo fetal e infantil en diversas zonas geográficas en España (Ribas-Fitó *et al.*, 2006; Guxens *et al.*, 2011). El estudio incluye siete cohortes (Menorca, Ribera d'Ebre, Granada, Valencia, Sabadell, Asturias y País Vasco), que están llevando a cabo el seguimiento de aproximadamente 4.000 mujeres y sus respectivos hijos.

Figura 6: Cohortes participantes en el proyecto INMA (http://www.proyectoinma.org/presentacion-inma/diseno.html, mayo 2013).

La cohorte INMA-Granada se constituyó en el período de octubre de 2000 a agosto de 2002, con el reclutamiento de 700 parejas madres-hijos varones. El objetivo inicial del estudio de Granada era evaluar la relación entre la exposición intrauterina a compuestos disruptores endocrinos y la presencia de malformaciones urogenitales en varones recién nacidos (Fernández *et al.*, 2007). Hasta 2013, el seguimiento de esta cohorte se ha completado en el momento del nacimiento, a los 4 años, y a los 9-10 años de los niños. De manera general, se han determinado los siguientes marcadores biológicos de exposición pre- y neonatal a contaminantes ambientales: mercurio, bisfenoles y metabolitos de los ftalatos en orina, pesticidas organoclorados en placenta y sangre de cordón, dioxinas, metales pesados y contaminantes no persistentes (parabenes, benzofenonas, bisfenoles y derivados salicílicos) en placenta, así como la *carga estrogénica* (biomarcador de exposición y efecto combinado de sustancias con actividad estrogénica) en tejido placentario. También, se dispone de información sobre los niveles en sangre de cordón de hormonas tiroideas (TSH y T4). En la visita de los 4-5 años (entre 2005 y 2006) se realizó la exploración antropométrica de los niños y la evaluación del neurodesarrollo infantil y se determinaron las concentraciones de mercurio total en pelo y la concentración en orina de marcadores de exposición a *hidrocarburos aromáticos policíclicos* (HAP) como 1-hidroxipireno y a humo de tabaco

como la cotinina. Entre 2010 y 2012 se realizó el seguimiento de los niños de la cohorte de Granada a la edad de 9-10 años mediante una nueva evaluación del crecimiento y de la dieta, del neurodesarrollo, del desarrollo sexual, y la determinación en orina de niveles de yoduria.

2.4. Evaluación de la exposición infantil: Contaminantes objeto de estudio

La exposición fetal e infantil a contaminantes ambientales es universal y a pesar de ello pasa desapercibida en casos no ligados a exposición laboral de la madre. Autores como Grandjean y Landrigan (2006) identificaron diversos contaminantes químicos de exposición inadvertida para los que existe evidencia suficiente de poseer potencial tóxico para causar trastornos en el neurodesarrollo humano. Entre los compuestos listados por estos autores se encuentran los pesticidas organoclorados, los PCBs, y determinados metales y disolventes orgánicos. Atendiendo a las características toxicocinéticas y toxicodinámicas de los contaminantes químicos, éstos pueden ser subclasificados para su estudio en varias categorías, no excluyentes, de manera que determinados compuestos podrán atenerse a las características de varios de los grupos. A continuación se presentan algunos grupos de contaminantes de especial relevancia para la salud humana abordados en este trabajo de tesis doctoral.

Disruptores endocrinos

Los disruptores endocrinos son sustancias exógenas, o mezclas, que alteran las funciones del sistema endocrino, causando diversos efectos adversos en la salud del individuo, o su progenie (WHO, 2013). Existen suficientes evidencias toxicológicas y epidemiológicas que indican que la exposición a este tipo de compuestos puede producir alteraciones en el sistema reproductivo, así como en la proliferación de neoplasias de base hormonal (como cáncer de mama, útero y próstata), alteraciones tiroideas y neuroendocrinas, trastornos metabólicos y obesidad (Maffini *et al.*, 2006; Diamanti-Kandarakis *et al.*, 2009).

El problema de salud ambiental asociado a este grupo de contaminantes químicos es complejo por motivos como la ubicuidad de estos compuestos, sus múltiples usos y aplicaciones, la heterogeneidad de sus estructuras, la complejidad de sus mecanismos de acción, y la exposición inadvertida y globalizada. Pueden encontrarse en la composición de formulaciones usadas como pesticidas, en componentes industriales o plásticos, envases alimentarios, productos textiles, cosméticos, medicamentos, así como formando parte de la composición de cualquier proceso o material de uso común.

Se ha observado que continúa aumentando la incidencia de desórdenes endocrinos, existiendo cerca de 800 compuestos de los que se conoce, o se sospecha, que son

capaces de provocar este tipo de afecciones. Además, el rápido incremento de la incidencia excluye factores genéticos como única causa posible. Sin embargo, determinar qué factores ambientales influyen en este tipo de efectos es complicado (WHO, 2013).

Mientras que los efectos biológicos de la exposición a dosis elevadas de sustancias con actividad hormonal parecen más que evidentes tanto en animales como en humanos (Collota *et al.*, 2013), la principal controversia hoy en día en cuanto a estas sustancias son los efectos de la exposición a bajas dosis, como ya hacía constar el panel de expertos del *National Toxicology Program's Report of the Endocrine Disruptors, Low dose peer review* (NTP) de la Agencia de Protección Ambiental (EPA) de los Estados Unidos en el 2001. A pesar de que el informe publicado al respecto establece la rigurosidad de los efectos observados como consecuencia de la exposición a bajas dosis de determinados disruptores endocrinos, aún existe controversia en el peso de las evidencias observadas.

Contaminantes orgánicos persistentes

Existe un grupo de compuestos conocidos como contaminantes orgánicos persistentes (COPs, también llamados POPs, del inglés *Persistent Organic Pollutants*), caracterizados por ser altamente tóxicos, persistentes en el medio durante mucho tiempo hasta su degradación, ser capaces de recorrer largas distancias a través de aire o agua, bioacumularse y biomagnificarse a lo largo de la cadena trófica, alcanzando las máximas concentraciones en animales en el último escalón de la cadena, como es el caso del ser humano. Debido a su persistencia y movilidad podemos encontrarlos en todos los lugares del planeta, incluso en las regiones menos habitadas. Su carácter bioacumulable se explica por la elevada afinidad de estas sustancias por los lípidos, acumulándose principalmente en

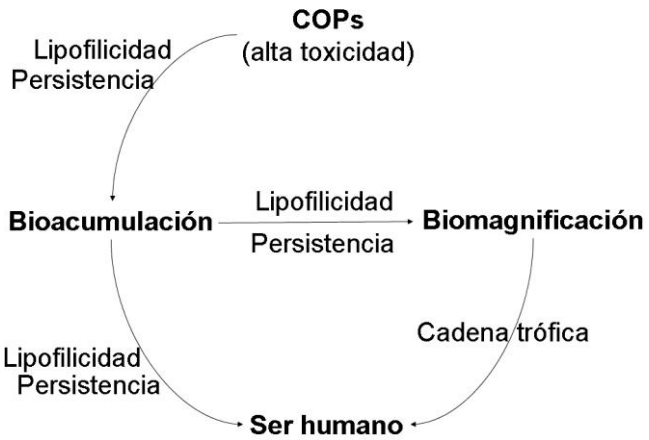


Figura 7: Simplificación de los mecanismos de llegada de los COPs al ser humano.

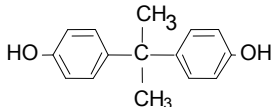
el tejido adiposo de los organismos vivos. Es frecuente que se transfieran a la siguiente generación durante el embarazo (a través de la placenta y el cordón umbilical) y la lactancia (EPA, 2013; UNEP, 2013).

Los pesticidas organoclorados son un amplio grupo de COPs caracterizados por presentar en su estructura uno o varios átomos de cloro. Estudios experimentales han demostrado que este tipo de pesticidas, muy usados en el pasado en diversos países, tienen efectos estrogénicos y/o antiandrogénicos (Soto *et al.*, 1994; Fernández *et al.*, 2004; Kang *et al.*, 2004; Kojima *et al.*, 2004). Sus elevadas toxicidad y capacidad de bioacumulación condujeron a la prohibición de uso y comercialización de la mayoría de estos pesticidas aunque, por las características citadas, aún pueden detectarse algunos de ellos en la población general, como se ha ido observando en los sucesivos estudios del grupo de trabajo en el que se desarrolla esta tesis (Fernández *et al.*, 2004; Cerrillo *et al.*, 2005; Carreño *et al.*, 2007; López-Espinosa *et al.*, 2007; López-Espinosa *et al.*, 2008; López-Espinosa *et al.*, 2009a; Mariscal-Arcas *et al.*, 2010; Arrebola *et al.*, 2013a; Arrebola *et al.*, 2013b).

La importancia de la toxicidad de estos compuestos radica en que, no sólo a altas dosis, sino también a bajas concentraciones, pueden aumentar el riesgo de cáncer, trastornos del sistema inmunológico, en el sistema nervioso, daños hepáticos, alteraciones endocrinas, defectos congénitos y problemas reproductivos tanto a nivel celular como en animales y humanos (Benachour y Aris, 2009; Amano *et al.*, 2010; Goncharov *et al.*, 2010; Lee *et al.*, 2010, 2011; Hass *et al.*, 2012;).

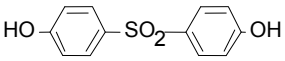
Contaminantes orgánicos no persistentes: Bisfenoles

Los *bisfenoles* son un grupo de compuestos utilizados ampliamente en la producción de policarbonato y resinas epoxi, utilizadas éstas en el sector de la alimentación como capa protectora de latas de conserva, por ejemplo. Uno de ellos, el *bisfenol A* (BPA), es uno de los químicos de mayor producción mundial en unidades de volumen y por su diseminación en el medio ambiente se considera ya ubicuo (Vandenberg *et al.*, 2010). Su amplia utilización se debe a que este compuesto confiere al plástico una gran resistencia al calor y a la luz solar (Fiege *et al.*, 2000). Los análisis toxicológicos del BPA han demostrado que éste actúa como disruptor endocrino a diferentes niveles (Moriyama *et al.*, 2002; Okada *et al.*, 2008; Richter *et al.*, 2007;



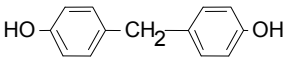
2,2-bis-(4-hidroxifenil)propano

BPA



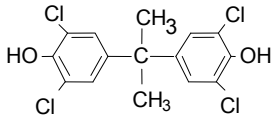
bis(4-hidroxifenil)sulfona

BPS



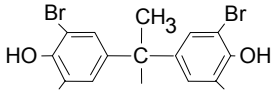
bis(4-hidroxifenil)metano

BPF



2,2-bis(4-hidroxi-3,5-diclorofenil)propano

TCBPA



2,2-bis(4-hidroxi-3,5-dibromofenil)propano

TBBPA

Figura 8: Bisfenoles.

Ropero *et al.*, 2008) y ha podido relacionarse con efectos promotores de la obesidad (Eng *et al.*, 2013; Li *et al.*, 2013).

Se ha observado que la exposición prenatal y/o perinatal a dosis relevantes de BPA produce en humanos alteraciones morfológicas y funcionales en el tracto genital masculino y femenino, y en glándulas mamarias, que podrían predisponer a los tejidos al desarrollo temprano de enfermedades como cáncer de mama, o producir efectos como la reducción de la fertilidad (Maffini *et al.*, 2006). Vom Saal y Hughes ya propusieron en 2005 una nueva evaluación del riesgo de exposición a BPA, basándose en que los efectos nocivos observados en ratones se producen a dosis menores de las que se encuentran en sangre humana, inclusive fetal. Por estos motivos, se están llevando a cabo en varios países campañas para su sustitución en plásticos de envases alimentarios. Son candidatos actuales a reemplazarlo el *bisfenol S* (BPS) y el *bisfenol F* (BPF), sin embargo, ambos están también en el punto de mira al haber mostrado menos biodegradabilidad (Ike *et al.*, 2006) y presentar también comportamiento hormonal en varios estudios preliminares (Chen *et al.*, 2002; Hashimoto *et al.*, 2000; Hashimoto *et al.*, 2001; Kuruto-Niwa *et al.*, 2005; Kitamura *et al.*, 2006; Grignard *et al.*, 2012).

En la actualidad, se encuentran también en uso algunos derivados halogenados del BPA, como son el *tetrabromobisfenol A* (TBBPA) y *tetraclorobisfenol A* (TCBPA), utilizados como retardantes de llama, además de como componentes en plásticos y

equipos electrónicos. Al igual que los anteriores, existen evidencias de su actividad como disruptores hormonales (Kitamura *et al.*, 2005).

Metales pesados

Los metales pesados son contaminantes muy extendidos, procedentes tanto de fuentes naturales como antropogénicas. En la población general, la exposición suele ocurrir a través de la dieta y el agua, aunque para algunos de ellos existen otras fuentes relevantes, como es el caso del cadmio (Cd) y el cromo (Cr), cuya principal fuente de exposición en población no expuesta ocupacionalmente es el tabaco (ATSDR, 2007a, b; 2008a, b, c). En España, varios estudios han descrito los niveles de algunos metales pesados en diferentes alimentos (Herreros *et al.*, 2008; Martí-Cid *et al.*, 2008; Fontcuberta *et al.*, 2011; Martorell *et al.*, 2011). Aunque, en general, se observa que las concentraciones de metales en alimentos se sitúan por debajo de los máximos tolerables establecidos, así como ocurre con los valores estimados de ingesta de metales (Fontcuberta *et al.*, 2011; Martorell *et al.*, 2011), el riesgo para los grupos poblacionales especialmente vulnerables no puede desestimarse. De hecho, estudios realizados en la Red INMA muestran una correlación entre el consumo de pescado y la exposición a mercurio (Hg) (Freire *et al.*, 2010a; Ramón *et al.*, 2011), observación que se ha visto reforzada por la descripción de valores altos de Hg en las conservas de pescado actualmente en el mercado (González-Estecha *et al.*, 2013).

Varios de estos metales podrían producir efectos adversos en el desarrollo fetal e infantil, como el plomo (Pb) y el Hg, que son tóxicos para el neurodesarrollo, el Cd y arsénico (As), de conocido carácter carcinogénico (IARC, 2012) y neurotóxico (Wasserman *et al.*, 2004; Hu *et al.*, 2007; Govil *et al.*, 2012), pudiendo también el Cd actuar como disruptor endocrino (Henson y Chedrese, 2004), o el cromo hexavalente (Cr^{+6}), mutagénico y carcinogénico (Zhitkovich, 2011). El interés en los efectos de estos metales en la salud humana está creciendo incluso para los que actúan como micronutrientes, como el manganeso (Mn), para el que aumenta la preocupación sobre sus efectos en el desarrollo del sistema nervioso durante la exposición intrauterina (Zoni *et al.*, 2007).

Se ha relacionado la exposición a metales pesados con diferentes efectos sobre la salud infantil, tanto a nivel neurológico (Després *et al.*, 2005, Lanphear *et al.*, 2005;

Oken *et al.*, 2005; Wright *et al.*, 2006; Freire *et al.*, 2010a), como antropométrico (Gundacker *et al.*, 2010) o endocrino (Gollenberg *et al.*, 2010; Stasenko *et al.*, 2010).

3. JUSTIFICACIÓN, HIPÓTESIS Y OBJETIVOS

3.1. Justificación

La exposición a contaminantes ambientales es ubicua y especialmente preocupante en etapas de gran vulnerabilidad, como son el desarrollo fetal y el crecimiento infantil. Por esta razón la caracterización y la cuantificación de la exposición materno-infantil son necesarias para implementar programas de protección de la salud de la población. La falta de conocimiento acerca del papel de los distintos factores ambientales que inciden sobre el neurodesarrollo infantil desde sus primeras fases, junto al reconocimiento de la vulnerabilidad de esas etapas, son razones más que suficientes para promover una investigación etiológica en este campo. La hipótesis patogénica que subyace en la disrupción endocrina se fundamenta en que la alteración de la homeostasis hormonal en momentos críticos del desarrollo puede tener consecuencias graves sobre el individuo expuesto aunque los efectos no sean apreciables en el momento de la evaluación de la exposición.

El estudio de base poblacional Infancia y Medio Ambiente (INMA), con un diseño epidemiológico de cohorte de seguimiento prospectivo, basado en la aplicación de una metodología común en las cohortes incluidas, constituye un marco ideal para llevar a cabo la biomonitorización de la exposición materno-infantil a contaminantes ambientales en diversas poblaciones de nuestro país, así como para investigar los diferentes factores de riesgo relacionados con la exposición temprana a contaminantes y con sus efectos tardíos en el desarrollo infantil. La placenta constituye una matriz perfecta para describir el ambiente intrauterino en el que se desarrolla el feto, permitiendo cuantificar de forma simultánea la exposición materna e infantil a compuestos químicos.

Los objetivos generales del proyecto INMA, asumidos en el presente trabajo, suponen un proceso de investigación de la exposición a contaminantes ambientales y sus consecuencias de marcado carácter holístico: i) describir las fuentes y vías de exposición humana; ii) determinar la dosis interna de contaminantes durante el embarazo, nacimiento e infancia; iii) evaluar la interacción entre contaminantes y las diferentes características sociodemográficas que puedan ser significativas en un incremento o disminución del riesgo de exposición o de efectos adversos; iv) evaluar los efectos de esta exposición en la salud infantil.

3.2. Hipótesis de estudio

La mujer gestante y la infancia representan grupos poblacionales de particular vulnerabilidad a los efectos tóxicos derivados de la exposición a contaminantes ambientales. Determinados contaminantes presentes tanto en el medio ambiente (agua, aire, suelo) como en diferentes productos de consumo (alimentos, envases, cosméticos, textiles, entre otros) son susceptibles de ejercer un efecto hormonal (disrupción endocrina) que puede ser perjudicial para el desarrollo del feto y del niño. Los contaminantes acumulados en el organismo materno pueden ser transferidos al feto a través de la placenta. Así, la concentración placentaria de un determinado contaminante será un indicador del grado de exposición materna y fetal con consecuencias determinadas por la influencia de factores diversos, tales como características socioeconómicas, alimentación y estilo de vida materno. La exposición prenatal y perinatal a disruptores endocrinos puede afectar negativamente al desarrollo cognitivo y conductual infantil mediante mecanismos de disrupción tiroidea, entre otros.

Una aproximación holística a este proceso permitiría explorar algunos de los pasos necesarios para una completa caracterización del riesgo: conocimiento del efecto hormonal y el mecanismo de acción, caracterización de la exposición, descripción de los determinantes y establecimiento de una asociación entre exposición y efecto adverso.

3.3. Objetivos

El objeto del presente trabajo de investigación es caracterizar la exposición materno-infantil a contaminantes ambientales disruptores endocrinos e investigar su efecto sobre la salud del niño.

Para esto, se plantean los siguientes objetivos específicos:

(i) Caracterizar la actividad hormonal *in vitro*, la toxicidad y el mecanismo de acción de los bisfenoles más comunes contenidos en plásticos y resinas epoxi.

(ii) Cuantificar la presencia de metales pesados en tejido placentario como forma de evaluar la exposición del binomio madre-hijo.

(iii) Describir los determinantes de la exposición a compuestos orgánicos persistentes encontrado en placenta.

(iv) Evaluar la asociación entre los disruptores endocrinos y el neurodesarrollo infantil a través de la homeostasis tiroidea.

4. MATERIAL Y MÉTODOS

4.1. Diseño y población de estudio

Los datos poblacionales incluidos en esta tesis forman parte de la Red de Investigación Colaborativa *INfancia y Medio Ambiente* (proyecto INMA), que se constituyó en 2003 para estudiar los efectos del medio ambiente y la dieta en el desarrollo fetal e infantil en diversas zonas geográficas de España (Ribas-Fitó *et al.*, 2006; Guxens *et al.*, 2011), participando tres cohortes preestablecidas (Menorca, Ribera d'Ebre y Granada) y cuatro cohortes *de novo* (Valencia, Sabadell, Asturias y País Vasco).

Los objetivos generales del proyecto INMA abarcan diferentes áreas de investigación: (i) describir las fuentes y vías de exposición a contaminantes ambientales, (ii) describir la dosis interna de éstos durante el embarazo, nacimiento e infancia; (iii) evaluar la interacción entre contaminantes, nutrientes y variables genéticas en el crecimiento, desarrollo y salud del niño; (iv) evaluar los efectos de la exposición en el crecimiento, desarrollo y salud del niño. Para ello se han realizado exámenes físicos y recogido muestras biológicas de la madre durante el embarazo, del recién nacido y del niño en diversas etapas de la infancia, y se ha recogido información mediante cuestionarios y entrevista personal con los participantes del estudio.

Los datos utilizados para esta tesis son los procedentes de la investigación del grupo de Granada. Los integrantes de ésta cohorte comenzaron a ser reclutados en el año 2000 en el Hospital Universitario San Cecilio de Granada, formando parte entonces del proyecto del 5º Programa Marco de la Unión Europea *"Environmental and Reproductive Health*", que tenía como objetivo estudiar los efectos (a corto y largo plazo) de la exposición intrauterina a contaminantes químicos con actividad hormonal en población masculina. Para ello, se estableció una cohorte formada por 700 pares de madres-hijos (varones), en la que se ha estudiado la exposición intrauterina a xenobióticos con efecto estrogénico y su relación con la prevalencia de malformaciones del tracto genito-urinario al nacimiento, entre otras alteraciones (Fernández *et al.*, 2007).

Los criterios de inclusión de las madres en esta cohorte fueron: (i) residir en el área de referencia del Hospital Universitario San Cecilio (HUSC), (ii) tener al menos 16 años, (iii) no haber seguido programas de reproducción asistida, y (iv) ausencia de

enfermedades crónicas como diabetes, hipertensión o enfermedad tiroidea, y no haber tenido complicaciones en el embarazo que pudiesen haber afectado al crecimiento y/o desarrollo fetal. Se obtuvo el consentimiento informado de todas las familias participantes en el proyecto. El estudio fue aprobado por el Comité Ético del Hospital Universitario San Cecilio, y toda la información fue codificada para mantener la confidencialidad.

4.2. Recogida de muestras biológicas

El contacto para el reclutamiento se llevó a cabo en el ingreso en el hospital de referencia (HUSC) para el parto, por lo que el historial de muestras biológicas recogidas comienza en ese momento. Se dispone así para la cohorte de Granada de muestras de placenta, sangre de cordón umbilical, leche materna, y orina y pelo del niño en diferentes etapas (ver tabla 1). En esta tesis doctoral se utilizarán indicadores de exposición determinados en muestras de placenta así como niveles de hormonas tiroideas en muestras de sangre de cordón umbilical de la cohorte.

Tras su recogida, las placentas se examinaron y pesaron, se tomó una porción triangular que se homogeneizó mecánicamente, incluyendo partes materna y fetal, central y periférica. Se codificaron y almacenaron inmediatamente a -86ºC hasta su procesado. Las muestras de sangre de cordón umbilical se tomaron durante el parto de forma rutinaria, según el programa de diagnóstico neonatal precoz de metabolopatías del Hospital San Juan de Dios de Granada.

4.3. Recogida de datos: información sociodemográfica y datos clínicos

El día posterior al parto, las madres de la cohorte de Granada completaron un cuestionario que incluía información sobre uso de medicamentos durante el embarazo, consumo de alcohol, tabaco y drogas durante el embarazo, historia reproductiva y obstétrica, historial médico, características sociodemográficas y del ambiente de trabajo, área residencial, dieta y uso de cosméticos. Los niveles de TSH de los recién nacidos fueron obtenidos a partir de los registros del Centro de Detección Neonatal Precoz de Metabolopatías del Hospital San Juan de Dios de Granada.

Además, durante los diferentes seguimientos, se han aplicado diferentes cuestionarios para completar y ampliar la información sociodemográfica y de

exposición ambiental, así como para conocer características del niño según los objetivos específicos (ver tabla 1).

Las diferentes variables de interés recogidas para la cohorte se describen en la tabla 2.

Tabla 1: Datos obtenidos de la cohorte INMA-Granada

	Nacimiento (2000/2002) N = 700	4 años (2005/2006) N = 220	9-11 años (2010/2012) N = 300
Muestras biológicas (determinaciones)	Placenta (DE[a], metales) Sangre cordón (DE[b], hormonas tiroideas[c]) Leche materna (DE[b])	Orina (1-OHP)[e] Pelo (Hg total)	Orina (yoduria, BPA) Sangre (hormonas tiroideas[c] y sexuales[d])
Cuestionarios	Historia/salud reproductiva, datos sociodemográficos y de exposiciones ambientales	Datos sociodemográficos Salud general familiar Alimentación (CFA) del niño[f] Exposición a contaminación atmosférica y del agua Psicológicos: Atención e impulsividad/hiperactividad del niño (padres y profesores)[g], competencia social del niño (padres y profesores)[h], salud mental de los padres[i] y vínculo afectivo (padres y profesores) [j]	Datos sociodemográficos Salud general familiar CFA del niño[f] Psicológicos: Atención e impulsividad/hiperactividad del niño (padres y profesores)[g], cuestionario de comportamiento del niño (padres y profesores, CBCL 6-18).
Exploraciones y tests	Antropometría Desarrollo sexual	Antropometría Neurodesarrollo (MSCA) [k]	Antropometría Desarrollo sexual Batería de evaluación neuropsicológica[n]
Mediciones ambientales		Contaminación atmosférica [l] Agua de consumo [m]	Radiación no ionizante

DE: Disruptores endocrinos; 1-OHP: 1-hidroxipireno; Hg: mercurio; BPA: bisfenol A; CFA: Cuestionario de Frecuencia Alimentaria; MSCA: Test McCarthy de aptitudes y psicomotricidad en niños (McCarthy, 1972); CBCL 6-18: *Child behaviour checklist* para niños de 6 a 18 años (Achenbach y Rescorla, 2001).

[a] Pesticidas organoclorados, BPA, parabenes, benzofenonas y oxicinamatos.
[b] Pesticidas organoclorados.
[c] T4 libre y TSH
[d] Testosterona, folículo estimulante (FSH) basal, hormona luteinizante (LH).
[e] Principal metabolito de los hidrocarburos aromáticos policíclicos (HAPs).
[f] CFA, versión adaptada a población infantil del cuestionario *Harvard* (Willet, 1985, validado en población española por Vioque, 2006).
[g] Cuestionario de Criterios diagnósticos para Trastorno de Déficit de Atención e Hiperactividad (DSM-IV) (APA, 2002).
[h] Escala California sobre competencia social del niño en edad preescolar.

[i] Salud mental de los padres, *General Mental Health Questionnaire* (GHQ) (Goldberg y Williams, 1998).

[j] Vínculo afectivo, *Parent-to-infant Attachment* (Condon y Corkindale, 1998).

[k] Mediante el MSCA se obtiene un Índice General Cognitivo (IGC) que se correlaciona con el coeficiente intelectual de la Escala Wechsler para niños en edad preescolar y primaria (WPPSI) y con el test de inteligencia Stanford-Binet para niños (Jacobson *et al.*, 1990).

[l] Niveles de contaminantes atmosféricos (dióxido de nitrógeno -NO_2- y compuestos orgánicos volátiles) medidos en un conjunto de puntos del área de estudio.

[m] Niveles de trihalometanos (subproductos de la cloración) en agua de consumo del área de estudio.

[n] Batería de test para la evaluación neuropsicológica:

Inteligencia o habilidad cognitiva general: Test Breve de Inteligencia de Kaufman (K-BIT, Kaufman y Kaufman, 1997, adaptación de Cordero y Calonge, 2000); Velocidad de Procesamiento: Símbolos y Claves de la Escala de Inteligencia de Weschler para niños IV (WISC-IV, Wechsler, 2007); Coordinación viso-motora: Trail Making Test-A; Lenguaje: subtests (vocabulario expresivo y definiciones) del K-BIT (Reitan, 1958); Atención, mantenida y selectiva: Continuous Performance Test (CPT, Conners, 1995); Memoria verbal: Test de Aprendizaje Verbal (TAVECI, Benedet *et al.*, 2001); Función Ejecutiva (Donders, 1969; Benton, 1989; Diamond, 2013), considerando las funciones de (i) función de monitorización/actualización: memoria de trabajo (test de letras y números, WISC-IV), razonamiento abstracto (subtest de matrices de K-BIT) y fluidez verbal (FAS); (ii) función de impulsividad/inhibición (test de colores y palabras (STROOP, Golden, 2005), test Go-No-Go; (iii) función de flexibilidad cognitiva (Trail Making Test, *TMT-B*); (iv) función de toma de decisiones (Hungry Donkey Test, *HDT*).

Tabla 2: Variables de interés para los objetivos de esta tesis recogidas en la cohorte INMA-Granada.

Variables de exposición	*Tipo*
Niveles de TSH en sangre de cordón umbilical	Continua: mU/l
Concentración de metales (Cd, Cr, Hg, Mn, Pb) y metaloide (As) en placenta	Continua: ng/g placenta (peso seco)
Concentración de pesticidas organoclorados en placenta (*o,p'*DDT, *o,p'*DDT, *o,p'*DDE, *o,p'*DDD, endosulfán I, endosulfán II, endosulfán éter, endosulfán sulfato, endosulfán lactona, aldrin, endrín, dieldrín, lindano, HCB, metoxicloro, mírex)	Continua: ng/g placenta Categórica: presencia (concentración superior al límite de detección) o ausencia
Carga estrogénica (TEXB-α y TEXB-β) en placenta	Continua: equivalentes de estradiol por gramo de placenta (Eeq/g) Categórica: presencia o ausencia de actividad estrogénica
Variables de efecto	*Tipo*
Puntuaciones derivadas del test McCarthy (4 años) para las áreas: cognición global, memoria, verbal, numérica, perceptivo-manipulativa, ejecutiva, motora gruesa/fina, memoria verbal, memoria a corto plazo.	Continuas (Estandarizadas: media = 100, desviación estándar = 15.)
Covariables	*Tipo*
Estado civil	Categórica: con o sin pareja estable
Área de residencia	Categórica: Rural (localidades < 10000 habitantes), suburbana (localidades de 10000 a 20000 habitantes), metropolitana (> 20000 habitantes, ciudad de Granada).

Nivel educativo del padre y la madre	Categórica: Primario, secundario, universitario
Paridad (número de hijos previos nacidos vivos)	Categórica: 1, 2, 3 o más hijos Categórica, dicotómica: Primípara o multípara
Índice de masa corporal (IMC) materno antes del embarazo	Continua: kg/m^2
Edad de la madre en el momento del parto	Continua: Años Categórica: < 32 años o ≥ 32 años
Ganancia materna de peso durante el embarazo	Continua: kg
Consumo de tabaco durante el embarazo	Categórica: Sí o no
Fumadora pasiva durante el embarazo	Categórica: Sí o no
Peso del niño al nacer	Continua: g Categórica: < 2500 g o ≥ 2500 g
Talla del niño al nacer	Continua: cm Categórica: < 49 cm o ≥ 49 cm
Edad gestacional	Continua: semanas Categórica: < 37 semanas o ≥ 37 semanas
Tipo de parto	Categórica: Espontáneo, cesárea o instrumental
Lactancia materna	Categórica: Sí o no. Continua: Semanas de lactancia materna
Edad del niño en el momento del test de neurodesarrollo	Continua: Meses
Trimestre escolar en el que se realizó el test de neurodesarrollo	Categórica: 3^{er} trimestre del tercer año, 1^{er} trimestre del cuarto año, 2^{o} o 3^{er} trimestre del cuarto año

Vínculo padre-hijo, vínculo madre-hijo	Continua (una puntuación mayor indica mayor vínculo afectivo)
Salud mental del padre y de la madre	Continua (una puntuación mayor indica desórdenes psicológicos)

4.4. Tratamiento de las muestras biológicas: protocolos de laboratorio.

Para la consecución de los objetivos fijados se llevaron a cabo diferentes determinaciones analíticas en las muestras biológicas, que se detallan brevemente a continuación (los protocolos ampliamente descritos se encuentran en los diferentes artículos resultantes del trabajo en cada uno de los objetivos):

4.4.1. Protocolos para la determinación de la actividad hormonal *in vitro* de bisfenoles (BPA, BPF, BPS, TCBPA y TBBPA).

Se utilizaron los siguientes ensayos *in vitro*: test de proliferación celular, test de modulación de la expresión génica y test de inhibición/competición por unión al receptor. La toxicidad de los compuestos en las diferentes líneas celulares utilizadas se estudió mediante un ensayo de viabilidad celular.

Ensayo de viabilidad/toxicidad celular (Denizot y Lang, 1986).

Se basa en la reducción metabólica del bromuro de 3-(4,5- dimetiltiazol-2-ilo)-2,5-difeniltetrazol (MTT) por la enzima mitocondrial succinato-deshidrogenasa en un compuesto coloreado azul (formazán), permitiendo determinar la funcionalidad mitocondrial de las células tratadas. Brevemente, las líneas celulares usadas (MCF-7, HELN-derivadas y PALM) se sembraron en placas de 96 pocillos ($5x10^4$ células/pocillo), se dejaron adherir y se trataron con los compuestos (en concentraciones que oscilan entre 0.01 y 10 µM) durante 24 h. Tras la incubación, las células se lavaron tres veces con buffer fosfato salino (PBS) y se añadieron 100 µl de solución de MTT (0.5 mg/ml) por pocillo. Tras 2 h adicionales de incubación, el medio con MTT se eliminó y se añadió dimetil sulfóxido (DMSO) para parar la reacción, midiendo la absorbancia a 540 nm. El resultado de número de células vivas es proporcional a la cantidad de formazán producido. El control consistió en un medio sin células.

Tests de proliferación celular

- Bioensayo de estrogenicidad/anti-estrogenicidad E-Screen (Soto *et al.,* 1995; Villalobos *et al.,* 1995): Emplea la línea celular MCF-7 (que expresa de forma endógena el receptor de los estrógenos alfa humano —hERα—), respondiendo al tratamiento con 17β-estradiol (E_2) incrementando su ritmo proliferativo,

sintetizando nuevas proteínas y procediendo a la transcripción de genes específicos (las células no proliferan cuando se exponen a un medio de cultivo desprovisto de estrógenos y solamente estrógenos naturales o sintéticos inducen proliferación celular). El test compara el número de células, o la proliferación celular obtenida tras 6 días de cultivo, de células que crecen en un medio suplementado con suero humano desprovisto de estrógenos, en presencia y/o ausencia de E_2, así como, de compuestos químicos de sospechada actividad estrogénica.

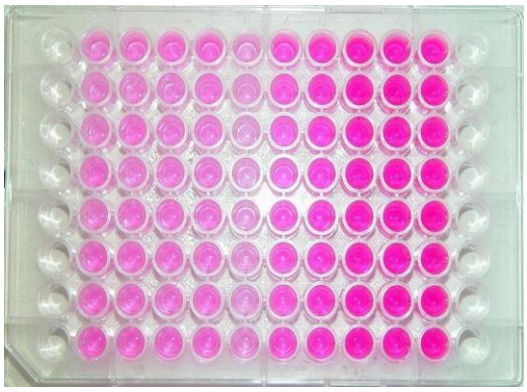


Figura 9: Placa de 96 pocillos para E-Screen (http://www.iswa.uni-stuttgart.de/ch/forschung/auftragsanalytik.en.html, abril 2013)

Las células MCF-7 tripsinizadas se sembraron en placas de 96 pocillos (4.5 x 10^3 células/pocillo, en medio de mantenimiento (DMEM, *—Dulbecco's Modified Eagle Medium—* con rojo fenol —+RF—, suplementado con 10% de suero fetal bovino —FBS—, L-glutamina, y bicarbonato sódico). Tras su adhesión (24 h) se retiró el medio de mantenimiento y se añadió medio experimental (DMEM sin RF, suplementado con 10% de FBS desprovisto de estrógenos —10% *dextran coated charcoal-treated,DCC - FBS*—), con los compuestos a ensayar. Se utilizó E_2 como control positivo. Tras 144 h de subcultivo (fase exponencial) se aspiró el medio y se fijaron y tiñieron las células con sulforrodamina-B, midiendo la absorbancia a 492 nm. Se estableció la tasa máxima de proliferación celular inducida por el compuesto, también llamada *efecto proliferativo* (EP), siendo ésta la relación existente entre la máxima tasa de proliferación obtenida para el compuesto problema y la tasa de proliferación alcanzada por el control negativo. Los datos de proliferación correspondientes a las sucesivas concentraciones del compuesto se expresaron como EP.

Para el estudio del efecto hormonal antagonista (anti-estrogénico) se añadió al medio de cultivo experimental el compuesto junto con E_2, a la concentración que la hormona produce proliferación máxima (100 pM). La reversibilidad del efecto anti-hormonal, ante la presencia del estrógeno natural, sirve para cuantificar la potencia del compuesto como anti-estrógeno.

- Bioensayo de androgenicidad A-Screen (Szelei *et al.,* 1997): Utiliza la línea celular MCF-7 AR1, obtenida mediante co-transfección de la línea celular MCF-7 con el gen del receptor humano de andrógenos (hAR). Éstas células expresan de forma exógena hAR, respondiendo al tratamiento con E_2 de igual manera que las células MCF-7, pero en presencia de andrógenos agonistas se produce una inhibición de la proliferación celular. El test compara la proliferación obtenida tras 5 días de cultivo en un medio suplementado con 10% DCC-FBS, en presencia de E_2, así como de los compuestos a testar. Su mantenimiento en cultivo es similar a la línea MCF-7 excepto por la adición al medio de 0.6 mg/ml de antibiótico geneticin sulfato® (G418). Las células se sembraron en 96 pocillos ($5x10^3$ células/pocillo en medio de mantenimiento), y se incubaron para permitir la adhesión (24 h). El medio de siembra fue sustituido por medio experimental junto con los compuestos a ensayar y con E_2 (100 pM). Para cada experimento se realizó una curva de dosis-respuesta (0.1- 1000 pM) del andrógeno sintético metiltrienolona (R1881), un control negativo (medio libre de hormonas) y uno positivo (100 pM de E_2). Tras 5 días de incubación, el proceso de fijación, tinción y colorimetría fue el mismo que el seguido para el ensayo E-Screen. Los datos de proliferación correspondientes a las diferentes concentraciones del compuesto se expresaron como EP.

Modulación de la expresión génica: Ensayos de transactivación vía hERα, hERβ, hAR, y hPXR (Molina-Molina *et al.* 2008).

Estos bioensayos se basan en el empleo de líneas celulares transfectadas con diversos receptores hormonales, y analizan la expresión de genes específicos. En este caso los compuestos fueron estudiados en células transfectadas con *hERα, hERβ, hAR, and hPXR* y un sistema de activación enzimático de luciferasa. Se emplearon las líneas celulares HELN-derivadas, MELN, PALM y HG_5LN-hPXR. Las células se sembraron (5×10^4 células/pocillo) en placas blancas opacas de 96 pocillos, en 150 µl de medio

experimental. Tras 8 h, los compuestos a testar en un rango de 0.01-10 µM, se añadieron a cada pocillo, disueltos en medio experimental (50 µl). Se incubó durante 30 h (40 h en el caso de la línea celular PALM) a 37°C. Se reemplazó el medio por medio fresco con 0,3 mM de luciferina (a esta concentración la luciferina difunde al interior celular produciendo una señal luminiscente que es estable tras 5 minutos y durante al menos 2 h), y se midió la luminiscencia durante 2 segundos. Para el estudio del efecto hormonal de carácter antagonista se procedió al ensayo añadiendo de forma simultánea al medio de cultivo el compuesto a testar junto con E_2 (0.1 nM) para hERs, R1881 (0.3 nM) para hAR y SR12813 (0.2 µM) para hPXR. Tras el ensayo de transactivación, se estableció la actividad luciferasa inducida por el compuesto, calculada como porcentaje de máxima actividad luciferasa (100%), obtenida en presencia del control positivo. Los resultados se expresaron como valores EC_{50} (concentración molar a la que el compuesto alcanza el 50% de su máxima actividad) e IC_{50} (concentración molar a la que el compuesto alcanza el 50% de su máxima inhibición) para cada compuesto testado.

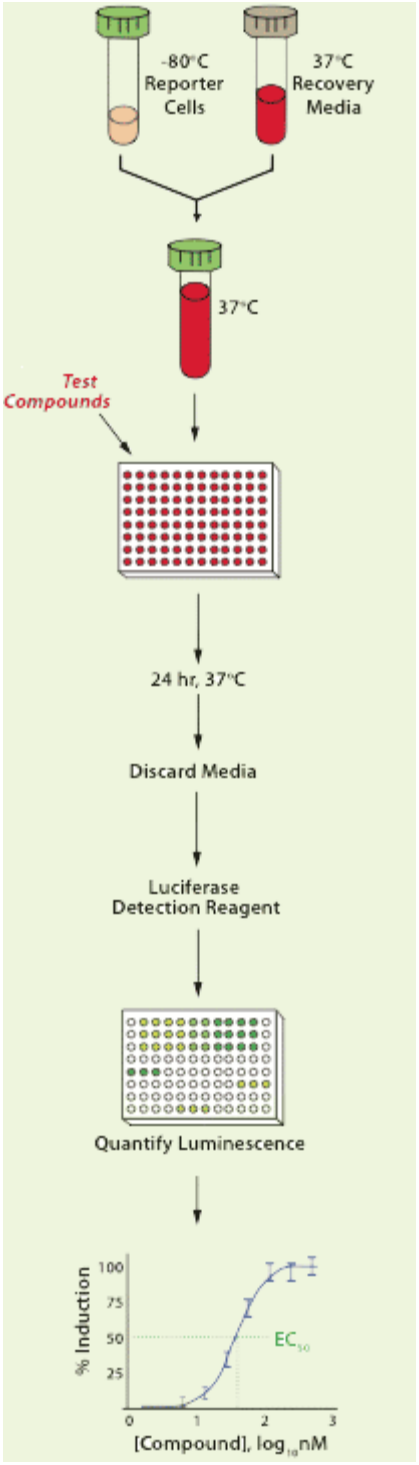


Figura 10: Ensayos de receptores nucleares (Indigo Biosciences, http://indigobiosciences.com/catalog, abril 2013)

Ensayos de inhibición/competición por unión al receptor (Molina-Molina *et al.*, 2008).

La afinidad de un compuesto por un determinado receptor se define como la habilidad para competir con un compuesto radiomarcado conocido por la unión con un receptor, y de esta forma inhibir la unión de éste. La concentración del radioligando es fija, mientras la concentración del compuesto no marcado es variable para evaluar la competencia por el enlace con el receptor. El parámetro de enlace

obtenido a partir de este experimento es la concentración del compuesto no marcado que desplaza el 50% de la unión específica del radioligando, lo que se denomina como valor de IC_{50}. Se sembraron células HELN-ERα y -ERβ (2×10^5 células/pocillo) en placas de 24 pocillos en 1 ml de medio experimental. Tras 24 h, los compuestos a testar (rango de 0.01-10 µM), se añadieron a cada pocillo disueltos en medio experimental junto con E_2 tritiado (0.1nM) en un volumen final de 500 µl. Tras 3 h a 37º C, se aspiró el medio y se lavaron las células tres veces con PBS frío. Posteriormente, las células se lisaron con 250 µl de buffer de lisis, y la radioactividad emitida se midió con contador de centelleo, expresada como *desintegraciones por minuto* (dpm). Los resultados se expresaron como valores IC_{50} para cada compuesto testado. Se calculó la afinidad relativa (RBA) de cada compuesto con respecto a E_2 por unión al receptor (ERα y ERβ).

4.4.2. Determinación de las concentraciones de los metales Cd, Cr, Hg, Mn, Pb y el metaloide As en las placentas de la cohorte INMA-Granada.

Se utilizaron los métodos validados por Gil *et al.* (2006, 2011) y Olmedo *et al.* (2010). Brevemente, consisten en el análisis mediante espectrofotometría de absorción atómica de las muestras previamente mineralizadas.

Para el análisis fue necesaria una digestión previa de las muestras. Para ello, éstas se desecaron a 80ºC. Se tomaron 0.3 g de la placenta ya desecada y se añadieron 2 ml de ácido nítrico, 0.5 ml de ácido clorhídrico y 4 ml de peróxido de hidrógeno; se mantuvieron durante 35 minutos a 1400 W de potencia en un horno microondas a 280 ºC y 80 bar para permitir así el proceso de digestión (rampa de 10 minutos, proceso de 20 minutos, enfriamiento de 15 minutos). La solución ya digerida se transfirió finalmente a un tubo descontaminado.

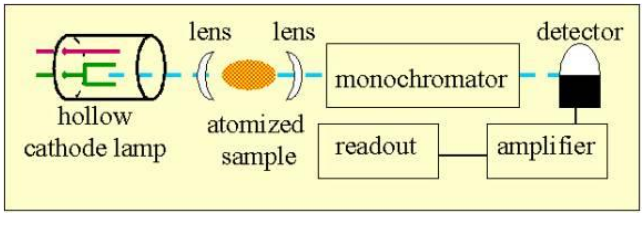


Figura 11: Esquema de proceso de AAS (New Mexico State University, http://web.nmsu.edu/~esevosti/scheme.htm, abril 2013).

La determinación de las concentraciones de As, Cd, Cr, Mn y Pb se realizó mediante un *espectrómetro de absorción atómica* (AAS), equipado con corrector de fondo Zeeman; El As se midió por inyección en flujo en un sistema de generación de hidruros con calentamiento previo de la célula de cuarzo; el Hg se determinó mediante

AAS con sistema de generación de hidruros de Hg en frío (técnica de vapor frío); para la determinación de Cd, Cr, Mn y Pb se utilizó un horno de grafito, así como tubos de grafito con plataforma L'vov integrada.

Para evaluar los resultados se construyeron curvas de calibrado con soluciones estándar de los metales analizados, preparadas a partir de una solución stock de 1000 mg/L de cada uno de los metales y sucesivas diluciones. Todas las disoluciones de agentes y estándares se prepararon con agua purificada mediante ósmosis inversa. Se utilizaron materiales de referencia certificados (CRM) para asegurar la exactitud de las medidas.

Los parámetros característicos del método se determinaron para cada metal utilizando análisis de blancos y soluciones estándar a diferentes concentraciones, incluyendo el límite de detección, de cuantificación, rango, precisión (mínima, intermedia y reproducibilidad), exactitud, recuperación, masa característica e incertidumbre. El límite de detección se calculó como $3s/m$, siendo s la desviación estándar de 10 inyecciones sucesivas del blanco y m la pendiente de la recta de calibrado. El límite de cuantificación se calculó como $10s/m$. La linealidad se evaluó comprobando el coeficiente de regresión (r^2) de la recta de calibrado (se consideró aceptable cuando se obtuvo un valor superior a 0.995). La precisión mínima se evaluó adicionando la matriz con estándares de las concentraciones mínimas y máximas de la recta de calibrado. La precisión intermedia se estudió en el intervalo de una a cinco semanas. La reproducibilidad del método se calculó utilizado la desviación estándar de diez inyecciones sucesivas. La exactitud del método fue establecida mediante materiales de referencia y el sistema de adición de muestras con una solución estándar del metal a analizar, realizando así mismo estudios de recuperación añadiendo una cantidad conocida de los elementos a las muestras. El parámetro de masa característica se utiliza para conocer la sensibilidad de las determinaciones por absorción atómica, proporcionado por la concentración de analito con una absorbancia de 0.0044; $m_0 = (C \times V \times 0.0044/Q_a - Q_{blanco})$, siendo C la concentración en el gráfico de calibrado, V el volumen de inyección, y finalmente Q_a y Q_{blanco} la absorbancia para la concentración dada y del blanco, respectivamente. La incertidumbre del método se estimó considerando cada fuente individual de incertidumbre y tratándola de manera independiente para obtener así su contribución al total.

Para la cuantificación se utilizaron modificadores de matriz adecuados (preparados en 0.2% [v/v] de ácido nítrico y 0.1% de Tritón X-100). Los límites de detección fueron 0.03, 0.19, 0.002, 0.12 y 0.83 µg/L para As, Cd, Cr, Hg, Mn y Pb, respectivamente. Las curvas de calibración fueron lineales hasta 4, 7, 30, 20, 20 y 200 µg/L, respectivamente.

4.4.3. Determinación de las concentraciones de pesticidas organoclorados en las placentas de la cohorte INMA-Granada.

Extracción de contaminantes placentarios: cromatografía líquida semipreparativa.

La preparación de las muestras de placenta de la cohorte INMA-Granada analizadas se realizó mediante el método validado por Rivas *et al.* (2001) y Fernández *et al.* (2004), con ligeras modificaciones:

Se comprimieron mecánicamente en cuatro tandas 400 mg de placenta homogeneizada diluida en 20 ml de *n*-hexano en homogeneizadores de vidrio con potter de teflón (peso total: 1.6 g de placenta). Se eluyó el extracto en una bureta de vidrio con Alúmina Merck 90 (desecada a 600º C durante 4 h y rehidratada con un 5% de agua destilada). La suma de los cuatro eluidos se concentró en rotavapor, se llevó a sequedad bajo corriente de N_2, y se resuspendió en 800 µl de *n*-hexano.

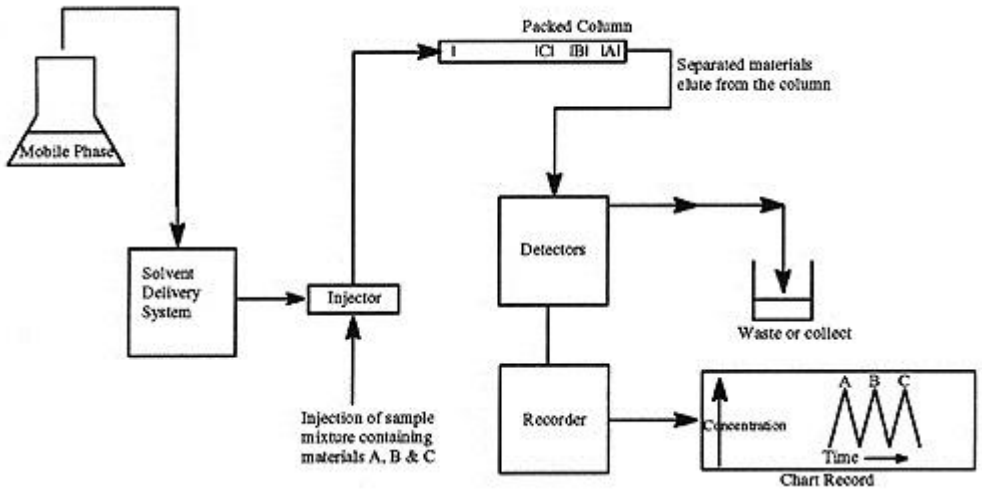


Figura 12: Diagrama general de componentes de HPLC (*Iowa State University*, http://www.protein.iastate.edu/hplc.html, abril 2013)

Mediante *cromatografía líquida* (HPLC) semipreparativa se procedió a separar xenoestrógenos (xenobióticos con actividad estrogénica) de los estrógenos naturales (hormonas endógenas) de la madre, siguiendo un método validado (Rivas *et al.*, 2001; Fernández *et al.*, 2004). Se utilizaron los 800 µl obtenidos en el proceso de extracción de la muestra, y se inyectaron (x 4 veces) en el HPLC.

De manera breve, el método utilizado consistió en un gradiente de dos fases móviles, fase A: *n*-hexano, y fase B: *n*-hexano:metanol:2-isopropanol (40:45:15) (v:v:v). El ciclo comienza con un flujo de 1 ml/min y 100% fase A, alcanza 60% fase A a los 17 minutos, el 100% de fase B a los 25 minutos, y vuelve al 100% de fase A a los 32 minutos. Se obtuvieron así tres fracciones: α (0-11 minutos), que recogió los compuestos organohalogenados, X (11-13 minutos) fue una fracción de seguridad para separar los compuestos obtenidos, y β (13-25 minutos) que recogió las hormonas naturales y compuestos no halogenados menos persistentes como alquilfenoles y bisfenoles.

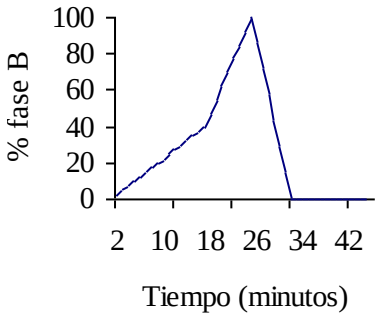


Figura 13: Gradiente usado en HPLC.

Determinación del contenido lipídico placentario.

Durante el desarrollo de la extracción se llevó a cabo de manera paralela una determinación lipídica mediante extracción líquido-líquido y gravimetría, para poder expresar las concentraciones de contaminantes en unidades por gramo de grasa, utilizando el método previamente validado (Rivas *et al.*, 2001; Fernández *et al.*, 2004; Cerrillo *et al.*, 2005). Se siguió el proceso de compresión mecánica manual seguido para extraer contaminantes, utilizando 200 mg de placenta homogeneizada, y 5 ml de cloroformo:metanol:ácido clorhídrico (20:10:0.1) (v:v:v) (en lugar de *n*-hexano). Tras repetir el proceso se añadieron 5 ml de 0.1 N de ácido clorhídrico a cada una de las réplicas y se centrifugaron a 3000 rpm durante 10 minutos. Se recogió la fase orgánica de ambas en un matriz previamente tarado y se llevó a sequedad en estufa (40ºC) durante 24 h. Se pesó el residuo seco y se expresó el total de lípidos en gramos de lípido por gramo de placenta.

Determinación de la concentración de pesticidas organoclorados en placenta: cromatografía de gases y masas.

Mediante *cromatografía de gases y captura de electrones* (GC/ECD) se analizó la fracción α del extracto de placenta (obtenida en el apartado 3.5.1., de cromatografía líquida), en la que se concentran los pesticidas organoclorados lipofílicos. Para ello, dicha fracción se llevó a sequedad y se resuspendió en 1 ml de *n*-hexano, se marcó con un patrón interno (*p,p'*-diclorodibenzofenona) y se inyectó 1 µl en GC/ECD.

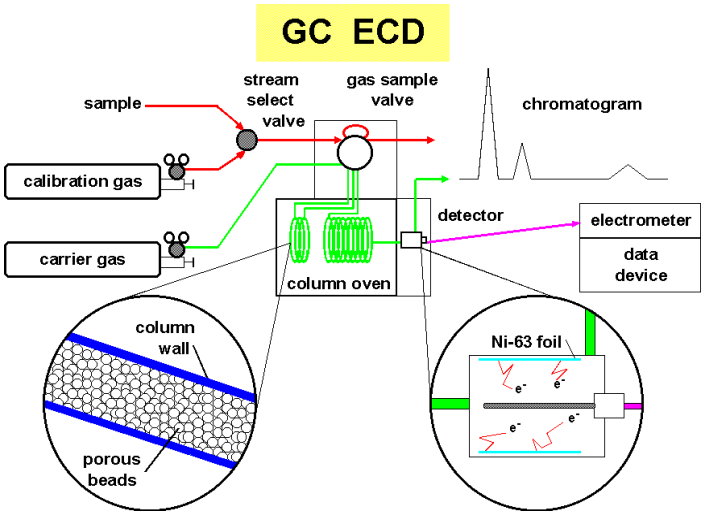


Figura 14: Esquema del proceso de GC/ECD (*National Oceanic & Atmospheric Administration, U.S. Department of Commerce*, http://www.esrl.noaa.gov/gmd/hats/insitu/insitu.html, abril 2013.

Para el análisis, las condiciones de trabajo fueron: ECD a 300ºC, inyector a 250ºC. El ciclo del programa partió de 130º, subiendo 20ºC/min hasta los 150ºC, 10ºC/min hasta los 200ºC y 20ºC/min hasta los 260ºC (20 minutos). El gas utilizado fue nitrógeno a 30ml/min, y como gas auxiliar, N_2 a 40 ml/min.

Se utilizó el patrón interno para la cuantificación de las concentraciones observadas en los cromatogramas.

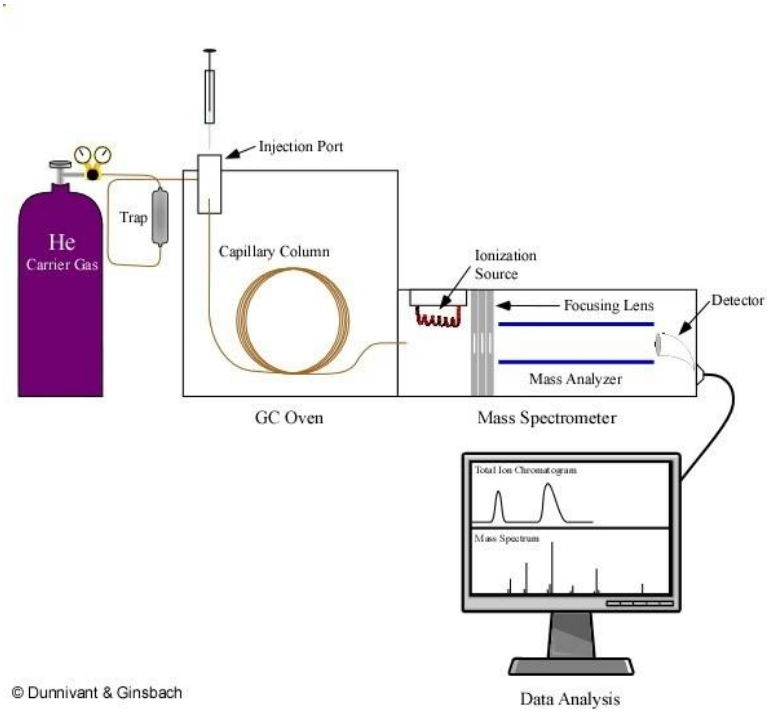


Figura 15: Esquema del proceso de GC/MS (*Whitman College*, http://people.whitman.edu/~dunnivfm/C_MS_Ebook/CH2/2_3.html, abril 2013).

Para confirmar la presencia de pesticidas organoclorados se utilizó la *cromatografía de gases y espectrometría de masas* (GC/MS). Se utilizaron 2 µl como volumen de inyección.

Para este análisis, las condiciones de trabajo partían de una temperatura del horno de 50 ºC (2 minutos), con un aumento de 30 ºC/min hasta 185 ºC (1 minuto), 2 ºC/min hasta 250 ºC, y 30 ºC/min hasta 300 ºC (5 minutos). El inyector se mantuvo a 250 ºC con un flujo de 1 ml/min. La trampa de iones del MS se mantuvo a 200 ºC, el colector a 50 ºC, la línea de transferencia a 280 ºC, el voltaje de modulación axial a 3.8 volts, y como gas portador se utilizó helio.

4.4.4. Determinación de los niveles de hormona estimulante del tiroides (TSH) en sangre de cordón umbilical de los niños de la cohorte INMA-Granada.

En el período de reclutamiento de la cohorte (2000-2002), los niveles neonatales de TSH fueron determinados rutinariamente en sangre de cordón umbilical, según el programa de diagnóstico neonatal precoz de metabolopatías llevado a cabo en el Hospital San Juan de Dios de Granada. Para ello, se utilizaba el protocolo validado por Cortés *et al.* (2002). Brevemente, la concentración de TSH en sangre de cordón umbilical se midió en papel de filtro mediante técnicas de inmunoanálisis, con un límite de detección de 0.01 mU/l. Según este protocolo, un valor igual o superior a 14 mU/l fue usado como criterio para confirmar la presencia de hipotiroidismo neonatal.

4.5. Fortalezas y limitaciones

Las principales limitaciones de esta tesis se deben a la imposibilidad de incluir toda la población general como población de estudio, así como describir al 100% la exposición ambiental a la que se ven sometidos:

- A pesar de que los cuestionarios utilizados recogen información del estilo de vida y diferentes variables sociodemográficas, caracterizar la exposición al detalle requiere un análisis mucho más específico del que es posible realizar a las familias que, voluntariamente, forman parte del proyecto. La caracterización completa de los compuestos y las diferentes dosis recibidas requeriría una toma de muestras ambientales (aire, agua, alimentos) del entorno familiar, así como seguir sus movimientos día a día. Además de las dificultades metodológicas de dicho seguimiento, las molestias ocasionadas y el grado de intimidad personal alcanzado podrían disminuir la participación y/o aumentar el abandono.

- Por otra parte, el origen de la cohorte para evaluar las malformaciones urogenitales en niños en relación a la exposición ambiental hace que no dispongamos de estos datos en niñas, con lo que también, el tamaño de la población de estudio es entorno a la mitad del que podía haber sido si se hubiesen incluido todos los partos producidos en la época del reclutamiento.

Las principales fortalezas radican en el diseño de los estudios:

- La utilización de placenta como matriz para la evaluación de la exposición intrauterina, permite cuantificar el paso de agentes nocivos al feto, ya que la concentración de contaminantes químicos en placentas no perfundidas es indicativa de la dosis interna de xenobióticos (Iyengar y Rapp, 2001). La ventaja en la utilización de éste tejido radica también en su fácil obtención, no invasiva, tras el parto, evitando la repetida toma de muestras como ocurre en los análisis de sangre u orina. Por otra parte, se ha sugerido que las enzimas presentes en el tejido placentario podrían detoxificar o activar diferentes compuestos, evitando, o promoviendo así su acumulación o paso a través del tejido (Myllynen y Vähäkangas, 2002), lo que hace que la placenta sea una matriz idónea para evaluar la carga de contaminantes que recibe el feto.

- La estructura de la red de investigación de la que forma parte la cohorte de INMA-Granada, y la amplia información existente tanto de ésta misma como de las otras cohortes que conforman la red, es una facilidad a la hora de contrastar hipótesis.

- La posibilidad de no sólo cuantificar los contaminantes, sino contar con un biomarcador de dosis interna que muestre la exposición a xenoestrógenos, constituye una aproximación holística a la situación real de las poblaciones, donde se pueda evaluar también el efecto sinérgico de los diferentes compuestos.

- La evaluación de la exposición a contaminantes presentes en productos de uso cotidiano, y su consecuente evaluación del riesgo.

- Tener información de los diversos seguimientos realizados a los niños de la cohorte INMA-Granada (a los 4 y a los 11 años), que posibilita relacionar la exposición con un "outcome".

5. RESULTADOS Y DISCUSION

Se presentan a continuación cuatro publicaciones en revistas internacionales que responden a los objetivos del presente trabajo. Para su lectura se han ordenado no por la fecha de publicación (2010-2013) sino por su posición en el proceso de evaluación del riesgo de la exposición a disruptores endocrinos.

Por esta razón, se presenta en primer lugar el trabajo de caracterización del riesgo centrado en el estudio de una serie de derivados bifenólicos, con actividad hormonal, ya que compiten por receptores nucleares diversos.

A continuación, se describe el trabajo de caracterización de la exposición, que presenta los resultados de concentraciones de metales pesados en placentas de la cohorte INMA.

Los aspectos de determinantes de la exposición se analizan en la publicación de organoclorados en placenta y clase social, y, por último, se presenta el trabajo de influencia de los valores de TSH perinatales sobre el desarrollo neuroconductual del niño.

Resultados del Objetivo 1: *Estudio de la actividad hormonal de contaminantes ambientales empleados en la fabricación de plásticos (resinas epoxi y policarbonato).*

La preocupación sobre los efectos adversos que pueden tener los disruptores endocrinos sobre la salud humana ha orientado la investigación hacia la caracterización de compuestos químicos con capacidad de enlace con los receptores nucleares. De forma particular la interacción con los receptores estrogénicos y androgénicos y otras hormonas esteroideas ha centrado la investigación en los últimos años, aunque más recientemente se han unido otras familias de receptores nucleares. Tal es el caso del *receptor X de pregnano* (hPXR), cuya activación ha mostrado incrementar los niveles de metabolitos ciertos disruptores endocrinos, además de alterar la biodisponibilidad de estrógenos y andrógenos endógenos.

El BPA ha sido caracterizado como disruptor endocrino por trabajos previos del grupo de investigación en el que se desarrolla este trabajo y por otros investigadores, lo que ha orientado la búsqueda de alternativas a los plásticos policarbonato y las resinas epoxi presentes en materiales plásticos de uso cotidiano. De hecho diferentes estrategias gubernamentales pretenden su sustitución por un material seguro. Entre los materiales candidatos se encuentran análogos estructurales como el BPS y BPF, ya utilizados como aditivos en algunos materiales como el papel térmico, o plásticos adhesivos, pero que también, al igual que ocurre con BPA, se detectan con frecuencia en orina de población general (Liao *et al.*, 2012) y estudios preliminares demuestran la actividad hormonal de ambos compuestos (Delfosse *et al.,* 2012; Cabaton *et al.,* 2009). Otros derivados halogenados como el TCBPA o TBBPA, de uso habitual como retardantes de llama, se han podido detectar también como contaminantes en la población general (Jimenez-Diaz *et al.,* 2010), así como han mostrado interferir en los receptores hormonales tiroideos (Kitamura *et al.,* 2002).

A pesar de esto, la evaluación toxicológica de estos análogos estructurales del BPA no ha sido tan exhaustiva como la realizada para éste. Por esta razón en el presente estudio se evalúa la interacción directa de un grupos de bisfenoles y derivados halogenados del BPA sobre los receptores de estrógenos (isoformas hERα, hERβ), de andrógenos (hAR) y el receptor hPXR, provenientes de tejidos humanos.

In vitro study on the agonistic and antagonistic activities of bisphenol-S and other bisphenol-A congeners and derivatives via nuclear receptors. Molina-Molina JM, Amaya E, Grimaldi M, Sáenz JM, Real M, Fernández MF, Balaguer P, Olea N. Toxicol Appl Pharmacol. 2013 1;272(1):127-36. doi: 10.1016/j.taap.2013.05.015.

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In vitro study on the agonistic and antagonistic activities of bisphenol-S and other bisphenol-A congeners and derivatives via nuclear receptors

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ABSTRACT

Bisphenols are a group of chemicals structurally similar to bisphenol-A (BPA) in current use as the primary raw material in the production of polycarbonate and epoxy resins. Some bisphenols are intended to replace BPA in several industrial applications. This is the case of bisphenol-S (BPS), which has an excellent stability at high temperature and resistance to sunlight. Studies on the endocrine properties of BPS have focused on its interaction with human estrogen receptor alpha (hERα), but information on its interaction with other nuclear receptors is scarce. The aim of this study was to investigate interactions of BPS, BPF, BPA and its halogenated derivatives, tetrachlorobisphenol A (TCBPA), and tetrabromobisphenol A (TBBPA), with human estrogen receptors (hERα and hERβ), androgen receptor (hAR), and pregnane X receptor (hPXR), using a panel of in vitro bioassays based on competitive binding to nuclear receptors (NRs), reporter gene expression, and cell proliferation assessment. BPS, BPF, and BPA efficiently activated both ERs, while TCBPA behaved as weak hERα agonist. Unlike BPF and BPA, BPS was more active in the hERβ versus hERα assay. BPF and BPA were full hAR antagonists (BPA > BPF), whereas BPA and BPS were weak hAR agonists. Only BPA, TCBPA, and TBBPA, were hPXR agonists (TCBPA > TBBPA > BPA). These findings provide evidence that BPA congeners and derivatives disrupt multiple NRs and may therefore interfere with the endocrine system. Hence, further research is needed to evaluate the potential endocrine-disrupting activity of putative BPA substitutes.

Introduction

Over the past few decades, concerns have grown about the possible health threat posed by endocrine-disrupting chemicals (EDCs), i.e., substances in our environment, food, and consumer products that interfere with hormone biosynthesis, metabolism, or activity and produce a deviation from normal homeostatic control or reproduction (Diamanti-Kandarakis et al., 2009). The direct interaction of chemicals, acting as receptor agonists or antagonists, with nuclear receptors (NRs), is a well-known mechanism of endocrine disruption. NRs are members of the steroid receptor superfamily, a large family of ligand-dependent transcriptional factors (Germain et al., 2006). Most research on EDCs has focused on their deleterious effects on sexual development and reproduction caused by interference with steroid signaling via human estrogen (hER) and androgen (hAR) receptors, because the outcome is readily identifiable and represents a sensitive health issue for a wide public (Henley and Korach, 2006). However, more recent reports have shown that several environmental chemicals can also affect hormone metabolism and synthesis by regulating their related enzymes, e.g., cytochrome P450, as activators of other NRs (Tabb and Blumberg, 2006), such as the human pregnane X receptor (hPXR). Indeed, activation of hPXR and up-regulation of their target genes by numerous compounds can increase the levels of endocrine-disrupting metabolites while at the same time altering the local bioavailability of endogenous androgens and estrogens. This provides a pathway for EDCs to alter steroid receptor activity without directly binding to steroid receptors. The problem posed by EDCs was addressed by European regulation (EU, 2006) on Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), which set out the steps for authorizing their use and called for the development of safer alternatives. Subsequently, the European Commission (EC) published a new Directive (EU, 2011) that amended Directive 2002/72/EC to restrict the use of bisphenol-A (BPA) in plastic infant feeding bottles. Currently, a law banning the use of BPA in food packaging has passed its final stage in the French Senate and is set to be implemented in 2013 for packaging for children under the age of three and for all food packaging

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in France in 2015. The National Assembly of France has asked the government to submit a report on the potential toxicity of possible alternatives to BPA before 1 July 2014, six months before the ban takes effect.

BPA [2,2-bis(4-hydroxyphenyl)propane], one of the highest production volume chemicals worldwide (Vandenberg et al., 2010), is an industrial chemical used to make a hard clear plastic known as polycarbonate (Fiege et al., 2000), a component of numerous consumer products. BPA is also found in epoxy resins, which act as a protective lining on the inside of metal-based food and beverage cans. BPA has been detected in the environment (Kang et al., 2007) and in human fluids and tissues (Calafat et al., 2008; Jiménez-Díaz et al., 2010), and its toxicity has been intensively studied since the 1970s. Despite possessing only modest estrogenic activity in comparison to the natural estrogen 17β-estradiol (E_2), BPA has produced a range of adverse effects in laboratory animals, and major concerns have been raised about its impact on reproductive systems (Richter et al., 2007). Further receptor-mediated biological activities have been reported in different model systems, e.g., binding to the orphan estrogen-related receptor gamma (ERRγ) (Okada et al., 2008), thyroid hormone disruption (Moriyama et al., 2002), altered pancreatic β-cell function (Ropero et al., 2008), and obesity promotion (Newbold et al., 2008). However, although BPA is a well-known EDC, the effects of low doses remain controversial (Vandenberg et al., 2012).

Several chemicals that are structurally similar to BPA are utilized in the manufacture of resins and plastics. They consist of two phenolic rings joined by a bridging carbon or other chemical structures (Fig. 1) and are designated BPA analogs, congeners or bisphenols. Some of these are considered candidates for the partial replacement of BPA in the industrial applications, including bisphenol-S [bis(4-hydroxyphenyl)sulfone (BPS)], whose two phenolic rings are linked by a sulfur dioxide (SO_2) group. BPS is of interest for the preparation of high temperature resistant thermosetting thermoplastic polymers (Spitsbergen et al., 1971). BPS-based epoxy resins resist deformation by heat and thermal stability and offer shorter gelling gel times, the more rapid development of mechanical properties in cured systems, improved resistance to organic solvent attack, increased dimensional stability, and better wetting of glass reinforcements (Rwei et al., 2003). As well as in epoxy resins, BPS is widely used as a monomer in the production of cyclic carbonates (Kim et al., 2001) and sulfonated poly(ether ketone ether sulfone) (Changkhamchom and Sirivat, 2010), and is a chemical additive in pesticides, dyestuffs, color-fast agents, leather tanning agents, dye dispersants, and fiber improvers. BPS replaced BPA as a developer in dyes for thermal paper in Japan (Watanabe et al., 2004) and China (Liu, 2005) and has been detected in canned food (Viñas et al., 2010) and in paper products and currency bills (Liao et al., 2012a). In fact, widespread exposure of the general population to BPS has been demonstrated in various countries, with the detection of BPS levels ranging from 0.02 to 21 ng/ml (0.8–84 nM) in urine samples from people living in the U.S. and seven Asian countries (Liao et al., 2012b). BPS is much less biodegradable than BPA (Danzl et al., 2009; Ike et al., 2006) and, given its annually increasing production, it is expected to become as widespread as BPA (Liao et al, 2012c). There has been less research on BPS than on BPA, but preliminary studies have shown that it also possesses hormone-mimicking properties (Chen et al., 2002; Delfosse et al., 2012; Grignard et al., 2012; Hashimoto et al., 2001; Kitamura et al., 2005; Kuruto-Niwa et al., 2005). However, studies on BPS as an endocrine disrupter have focused on its interaction with human estrogen receptor alpha (hERα), and much less is known about its interaction with other NRs.

Bisphenol-F, [bis(4-hydroxyphenyl)methane, (BPF)], which has no substituent at the bridging carbon (except with H atoms), has a broad range of industrial applications. The BPF monomer is polymerized to prepare epoxy resins and polycarbonates for use in the manufacture of lacquer, varnishes, coatings, adhesive plastics, and other products (Jana et al., 2005). Although no information is available on human exposure, BPF has been detected in the environment (Fromme et al., 2002; Stachel et al., 2003) and has demonstrated an estrogenic effect in various in vivo (Yamasaki et al., 2002) and in vitro studies (Cabaton et al., 2009; Hashimoto and Nakamura, 2000; Hashimoto et al., 2001). BPF has also shown anti-androgenic activity in several human recombinant cell lines carrying hAR (Cabaton et al., 2009; Satoh et al., 2004).

Halogenated derivatives of BPA, such as tetrabromobisphenol-A [2,2-bis(4-hydroxy-3,5-dibromophenyl)propane, (TBBPA)] and tetrachlorobisphenol-A [2,2-bis(4-hydroxy-3,5-dichlorophenyl)propane, (TCBPA)] are both widely used as flame-retardants for building material, paints, and epoxy resin-containing plastic products such as electronic circuit boards, and other electronic equipment. Like BPA, both compounds are considered environmental contaminants (de Wit et al., 2009; Fukazawa et al., 2001) and have also been reported in human fluids and/or tissues (Cariou et al., 2008; Fernandez et al., 2007; Jimenez-Diaz et al., 2010; Johnson-Restrepo et al., 2008). Moreover, these compounds have been found to interact with and disrupt thyroid hormone receptor signaling (Kitamura et al. 2002). TBBPA and TCBPA are also potent peroxisome proliferator-activated receptor gamma (PPARγ) agonists (Riu et al., 2011a).

As noted, research has focused mainly on the endocrine disrupting activity of BPA, and much less attention has been paid to the toxicity of the other bisphenols proposed as substitutes, such as BPS. The present study was designed to develop a comprehensive NR interaction profile of five bisphenols in current use (BPS, BPF, BPA, TCBPA and TBBPA) in order to contribute additional information on their endocrine disruptive activity. For this purpose, we investigated the direct interaction of these compounds with hERα, hERβ, hAR,

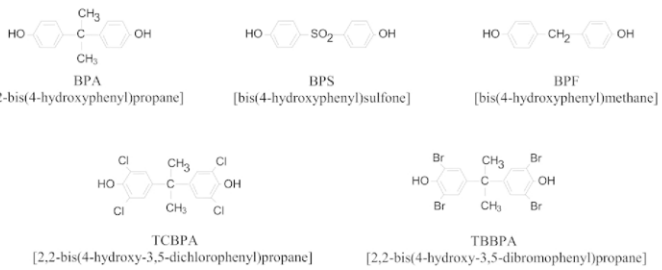

Fig. 1. Chemical structures of bisphenol-A (BPA) and derivatives.

and hPXR, using a panel of steroid hormone receptor cell based assays to measure different endpoints at distinct levels of biological complexity.

Materials and methods

Chemicals and materials. Culture medium and fetal bovine serum (FBS) were obtained from Gibco (Invitrogen, Barcelona, Spain). E_2, EE_2, BPA, BPS, BPF, TBBPA, TCBPA, puromycin, geneticin, luciferin, methyl thiazolyl diphenyl tetrazolium bromide (MTT) and sulforhodamine B (SRB) were obtained from Sigma-Aldrich Inc. (St Louis, MO, USA). [^{3}H]-E_2 (41.3 Ci/mmol specific activity), methyltrienolone (R1881) and tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate (SR12813) were purchased from NEN Life Science Products (Paris, France). Stock solutions (10 mM) of E_2 (≥98% purity), EE_2 (≥98% purity), R1881 (≥98% purity), SR12813 (≥98% purity), BPA (≥99% purity), BPS (98% purity), BPF (98% purity), TBBPA (97% purity), and TCBPA (98% purity) were prepared in ethanol (>99.7% purity), and successive dilutions were performed in culture medium. Stock solutions were kept at −20 °C and dilution series were freshly prepared before each experiment. All other chemicals were of the highest quality available from commercial sources. All cell culture plastics were obtained from Falcon (VWR International Eurolab, Barcelona, Spain). An infinite M200 luminometer (Tecan, Barcelona, Spain) was used to detect luciferase activity in intact cells.

Charcoal–dextran treatment of serum to remove sex steroids. Sex steroids were removed from FBS by dextran-coated charcoal (DCC) stripping. Briefly, a suspension of 5% charcoal with 0.5% dextran T-70 was prepared. Aliquots of the DCC suspension of a volume similar to the serum aliquot to be processed were centrifuged at 1000 ×*g* for 10 min. Supernatants were aspirated, and serum aliquots were mixed with the charcoal pellets. This DCC-serum mixture was maintained in suspension by rolling (6 cycles/min) at 37 °C for 1 h. The suspension was centrifuged at 2000 ×*g* for 20 min, and the supernatant was then filtered through a 0.22 mm filter (Millipore). DCC-treated FBS (DCC-FBS) was stored at −20 °C until needed.

Plasmids. The plasmids used have been described elsewhere: pSG_5-ERα-puro (aa 1–595), pSG_5-ERβ-puro (aa 1–530), pSG_5-AR-puro (aa 1–919), and $pGAL_4$RE-ERE-βGlobin-Luc-SV-Neo (Balaguer et al., 1999; Paris et al., 2002) and $p(GAL_4RE)_5$-βGlob-Luc-SVNeo and pSG_5-GAL_4(DBD)-hPXR(LBD)-puro (Lemaire et al., 2006; Seimandi et al., 2005).

Generation of stable reporter cell lines and culture conditions. The stably transfected luciferase reporter MELN cell line was obtained as previously reported (Balaguer et al., 2001). Briefly, MELN cells were obtained by transfecting ERα-positive breast cancer MCF-7 cells with the estrogen-responsive gene ERE-βGlob-Luc-SV-Neo (Balaguer et al., 1999). MELN cells were cultured in Dulbecco's modified Eagle medium (DMEM) F12 with phenol red supplemented with 10% FBS, 1% antibiotic (penicillin/streptomycin), and 1 mg/ml G418. Basal luciferase activity in MELN cells was around 15% of maximal activity (100% for 10 nM E_2).

Generation of HELN-hERα and -hERβ reporter cell lines was performed in two steps (Balaguer et al., 1999; Escande et al., 2006). The estrogen responsive reporter gene was first stably transfected into HeLa cells, generating the HELN cell line and, in a second step, these HELN cells were transfected with -hERα or -hERβ plasmid constructs to obtain the HELN-hERα or -hERβ cell lines, respectively. HELN cells were cultured in DMEM supplemented with 5% FBS, 1% antibiotic, and 1 mg/ml G418. HELN-ER cells were cultured in DMEM F12 without phenol red supplemented with 6% DCC-FBS, 1% antibiotic, 1 mg/ml G418, and 0.5 μg/ml puromycin. Basal luciferase activity in HELN-hERα and HELN-hERβ cells was around 10% of maximal activity (100% for 10 nM E_2).

PALM cells were obtained as already described (Terouanne et al., 2000). Briefly, PC3 cells were co-transfected with an androgen responsive gene, MMTV-Luc-SV-Neo, and an androgen receptor expressing plasmid, pSG_5AR-puro. PALM cells were cultured in Ham's F12 supplemented with 10% FBS, 1 mg/ml G418, and 1 μg/ml puromycin. Basal luciferase in PALM cells was around 10% of maximal activity (100% for 10 nM R1881).

The HG_5LN-hPXR cell line was generated in two steps (Lemaire et al., 2006). In a first step, HeLa cells were stably transfected with a GAL_4RE_5-βGlob-Luc-SVNeo plasmid to produce the HG_5LN cell line, which expresses constitutively luciferase activity. Then, HG_5LN cells were stably transfected with the pSG_5-GAL_4(DBD)-hPXR(LBD)-puro plasmid to obtain the HG_5LN-hPXR cell line. HG_5LN and HG_5LN-hPXR cells were cultured in DMEM supplemented with 5% FBS, 1% antibiotic, and 1 mg/ml G418. Additionally, 0.5 μg/ml puromycin was added in HG_5LN-hPXR cell medium.

Because of the estrogenic activity of phenol red and FBS, experiments were performed in a test culture medium, i.e., DMEM F12 without phenol red supplemented with 6% DCC-FBS (for MELN, HELN-hERα, -hERβ, and HG_5LN-hPXR cells) or Ham's F12 supplemented with 6% DCC-FBS (for PALM cells) and 1% antibiotic in a 5% CO_2 humidified atmosphere at 37 °C. The test culture medium was used in transactivation and competitive binding assays.

Living cell luciferase assay. Reporter cells were seeded at a density of 5×10^4 cells per well in 96-well white opaque tissue culture plates in 150 μl test culture medium. Test compounds were prepared at 4× concentration in the same medium, and 50 μl was added per well 8 h after seeding. Cell lines were incubated for 16 h (except for PALM cells, which were incubated for 40 h) with the compounds at 37 °C. At the end of incubation, the medium containing test compounds was removed and replaced with test culture medium containing 0.3 mM luciferin. At this concentration, luciferin diffused into the cell and produced a stable luminescent signal 5 min later. This signal is approximately 10-fold less intense than the signal obtained after cell lysis but is perfectly stable for several hours. The 96-well plate was then introduced into a luminometer, and luminescence was measured in intact living cells for 2 s.

Agonist and antagonist assays. Agonistic activities of hERα, hERβ, hAR, and hPXR in HELN-derived, MELN, PALM, or HG_5LN-hPXR cells were tested in the presence of increasing concentrations (0.01–10 μM) of BPS, BPF, BPA, TCBPA, and TBBPA. Tests were performed in quadruplicate for each concentration. Results were expressed as a percentage of maximal luciferase activity. Maximal luciferase activity (100%) was obtained in the presence of 10 nM E_2, 10 nM R1881, and 3 μM SR12813 (for hERs, hAR, and hPXR, respectively). For each compound, the potency corresponding to the concentration yielding half-maximal luciferase activity (EC_{50} value) was calculated. The antagonistic activities of these compounds (tested at 0.01–10 μM) were determined by coincubation with E_2 (0.1 nM), R1881 (0.3 nM), and SR12813 (0.2 μM) agonists for hERs, hAR, and hPXR, respectively. At these concentrations, activities reach approximately 90, 60, 80 and 60% of maximal luciferase activity (for hERα, hERβ, hAR, and hPXR, respectively). Data were expressed as half-maximal inhibitory concentration (IC_{50} value) for each compound tested.

Whole-cell hERα and hERβ competitive binding assays. Briefly, HELN-hERα and -hERβ cells were seeded at a density of 2×10^5 cells per well in 24-well tissue culture plates and grown in test culture medium. After 24 h, HELN-hERα and -hERβ cells were labeled with 0.1 nM [^{3}H]-E_2 (41.3 Ci/mmol specific activity) at 37 °C for 3 h in the absence or presence of BPS, BPF, BPA (0.01–10 μM), or unlabelled E_2 (100 nM). The final incubation volume was 500 μl, and each well was

tested in duplicate. After incubation, unbound material was aspirated and cells washed three times with 500 µl of cold PBS. Then, 250 µl lysis buffer (400 mM NaCl, 25 mM Tris phosphate pH 7.8, 2 mM DTT, 2 mM EDTA, 10% glycerol, 1% triton X-100) was added, and plates were shaken for 5 min. Total cell lysate (200 µl) was mixed with 4 ml of LSC-cocktail (Emulsifier-Safe, Packard BioScience), and [^{3}H] bound radioactivity was liquid scintillation-counted (LS-6000-SC, Beckman-Coulter, Roissy, France). Non-specific binding was determined in the presence of 100 nM unlabeled E_2. Specific binding was calculated by subtracting non-specific binding from total binding. Bound radioactivity values were expressed in disintegrations per minute (dpm). In the absence of a competitor, specific bound radioactivity was 750–1000 dpm.

Results were plotted as dpm versus concentration of tested compounds. IC_{50} values were defined as the compound concentration required to decrease maximal [^{3}H]-E_2 binding by 50%. Compound selectivity towards hERα or hERβ was evaluated using the relative binding affinity (RBA) to E_2. The RBA for each competitor was calculated as the ratio of E_2 to competitor concentration required to reduce specific radiolabeled binding by 50% (ratio of IC_{50} values). The RBA value for E_2 was arbitrarily set at 100.

MCF-7 cell lines. Human breast cancer MCF-7 cells were cultured for routine maintenance in DMEM with phenol red supplemented with 10% FBS. MCF-7 AR1 cells, which are stable transfectants of MCF-7 cells expressing the wild-type hAR, were obtained as already described (Szelei et al., 1997). MCF7-AR1 cells were cultured in DMEM with phenol red supplemented with 10% FBS and 0.6 mg/ml G418. Cell proliferation experiments were performed in test culture medium (DMEM without phenol red supplemented with 10% DCC-FBS) in a 5% CO_2 humidified atmosphere at 37 °C.

E-Screen bioassay. MCF-7 cells were used in the test of estrogenicity according to a technique slightly modified from that originally described by Soto (Soto et al., 1995). Briefly, MCF-7 cells were trypsinized and plated in 96-well culture plates at initial concentrations of 4×10^3 cells per well. Cells were allowed to attach for 24 h and the seeding medium was then removed and replaced with the test culture medium. A range of concentrations of the test compound was added to this medium in the sample wells. In each experiment, a dose–response curve (0.1 pM–1000 pM) for E_2 and a negative control (cell treated only with hormone-free medium) were included. The bioassay was ended on day 6 (late exponential phase) by removing the media from the wells, fixing the cells, and staining them with SRB. The cells were treated with cold 10% trichloracetic acid and incubated at 4 °C for 30 min, washed five times with tap water, and left to dry. Trichloroacetic-fixed cells were stained for 10 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Wells were rinsed with 1% acetic acid and air dried. Bound dye was solubilized with 10 mM Tris base (pH 10.5) in a shaker for 20 min. Finally, the absorbance was read in a Titertek Multiscan apparatus (Flow, Irvine, CA) at 492 nm. Linearity of the SRB assay with cell number was verified prior to cell growth experiments.

Agonistic assays were performed in the presence of increasing concentrations (0.01–10 µM) of the test compounds. For each compound, the ratio between the cell yield obtained and the proliferation of hormone-free control cells (negative control) was calculated. Tests were done in triplicate for each concentration. Results were expressed as proliferative effect (PE) [MCF-7 cell proliferation (fold-over control)]. The antagonistic activities of these compounds were determined by coincubation with the agonist E_2 at 100 pM.

A-Screen bioassay. A slightly modified version of the protocol described previously by Szelei (Szelei et al., 1997) was employed to evaluate the androgenic activity of the test compounds in the A-screen assay. Briefly, MCF-7 AR1 cells were trypsinized and plated in 96-well culture plates at initial concentrations of 5×10^3 cells per well and allowed to attach for 24 h. On the second day, the seeding medium was removed and replaced with the test culture medium. Assays were performed in the presence of increasing concentrations (0.01–10 µM) of the test compounds together with 100 pM E_2. In each experiment, a dose–response curve (0.1 pM–1000 pM) of R1881, a negative control (cell treated only with hormone-free medium), and a positive control (E_2 at 100 pM) were included. Cells were incubated for 5 days. The bioassay was terminated by removing the media from the wells, fixing the cells, and staining them with SRB. The fixation protocol and SRB colorimetric assay were done as described above for the E-screen assay. Results were also expressed as PE [MCF-7 cell proliferation (fold-over control)].

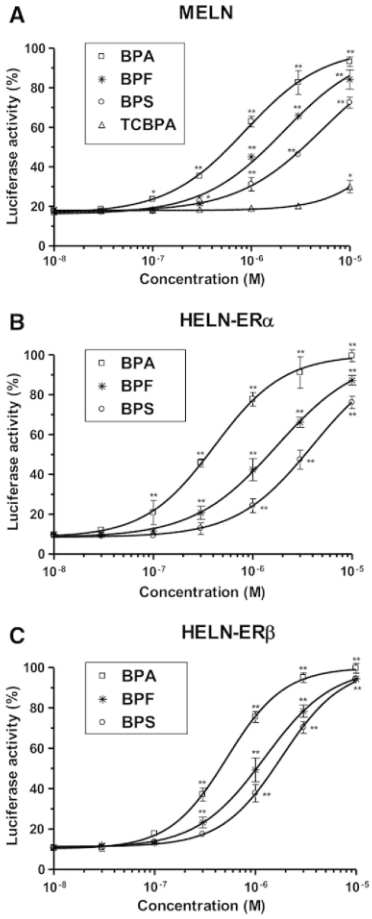

Fig. 2. Induction of luciferase activity in MELN, HELN-hERα, and -hERβ cells by BPA and derivatives. Cells were treated with BPA, BPF, BPS and TCBPA for 16 h at the indicated concentrations. Maximal luciferase activity (100%) was obtained with 10 nM E_2. Results are expressed as a percentage of maximal E_2 induction. Values were the mean ± SD from three separate experiments. *$p < 0.05$ and **$p < 0.01$ (versus 0.1% ethanol used as a control).

Table 1
Effective and inhibitory concentrations for half-maximal luciferase activity (EC_{50} and IC_{50}) of BPA and derivatives on transcriptional activation through hERs, hAR and hPXR.

Compounds	MELN	HELN-ERα	HELN-ERβ	PALM	PALM	HG_5LN-PXR
	EC_{50} (μM)	EC_{50} (μM)	EC_{50} (μM)	EC_{50} (μM)	IC_{50} (μM)	EC_{50} (μM)
BPS	12.10 ± 0.92	3.96 ± 0.33	1.72 ± 0.27	70.54 ± 2.21	ne	ne
BPF	0.98 ± 0.05	1.73 ± 0.46	1.43 ± 0.19	ne	6.98 ± 0.15	ne
BPA	0.47 ± 0.03	0.41 ± 0.11	0.52 ± 0.09	55.38 ± 6.46	0.92 ± 0.02	17.72 ± 2.43
TCBPA	47.60 ± 5.26	nt	nt	ne	ne	8.49 ± 0.63
TBBPA	ne	nt	nt	ne	ne	11.97 ± 5.38

(ne) no effect.
(nt) no tested.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) toxicity assay. The effects of BPA, BPS, BPF, TBBPA, and TCBPA on cell viability were assessed with the MTT test, using Denizot and Lang's modified technique (1986). In short, cell lines (MCF-7, MCF-7 AR1, MELN, HELN-derived, PALM, and HG_5LN-hPXR) were seeded at a density of 5×10^4 cells per well in 96-well tissue culture-grade plate for 8 h, followed by treatment with different concentrations (0.01–10 μM) of each compound for a further 24 h. Cells were washed with PBS three times, and 100 μl of MTT solution (0.5 mg/ml) was then added to each well. After incubation (2 h), viable cells cleaved the MTT tetrazolium ring into a dark blue formazan reaction product, whereas dead cells remained colorless. The MTT-containing medium was gently removed, and DMSO was added to each well. After shaking, the plates were read in absorbance at 540 nm. Medium alone with no cells served as an additional control. Data were expressed as the mean of three wells.

Data analysis. For all assays, each compound was tested at various concentrations in at least three independent experiments, and data were expressed as mean ± SD. Individual dose–response curves, in the absence and presence of agonist, were fitted using the sigmoid dose–response function of a graphics and statistics software package (Graph-Pad Prism, version 4.0, 2003, Graph-Pad Software Inc., San Diego, CA, USA). Results are presented as EC_{50} and IC_{50} values. Data were analyzed for significant differences using one-way ANOVA followed by Dunnett's post-comparison test (vs. control). Differences were considered statistically significant at $p < 0.05$.

Results

Transcriptional activation of hERs by BPA congeners and derivatives

BPS, BPF, BPA, TCBPA, and TBBPA were tested on the MELN cell line, which stably expresses an estrogen-responsive luciferase reporter under the control of endogenous hERα. In this cell line, all compounds except the halogenated bisphenols, TCBPA and TBBPA, induced luciferase expression in a concentration-response manner (Fig. 2A) but with different potencies, in the order BPA > BPF > BPS, as indicated by their EC_{50} values (Table 1). TCBPA showed only 30% transactivation at 10 μM concentrations, whereas TBBPA, was found inactive in MELN cells.

We next explored whether the most hERα-active compounds, BPA, BPF, and BPS, could act as specific ER modulators. We used the stably transfected HELN-hERα and -hERβ cell lines, which allow characterization of ER selectivity (between subtypes) and activity (antagonistic, partial or full agonistic) within the same cellular context (Escande et al., 2006). As in previous studies (Molina-Molina et al., 2008), dose–response curves in these cells showed a slight difference in assay sensitivity for the natural estrogen E_2 and for the synthetic estrogen EE_2, with EC_{50} values of 0.019 and 0.007 nM for hERα and 0.067 and 0.24 nM for hERβ, respectively.

When BPS, BPF, and BPA were applied on HELN-hERα cells, the estrogenic responses were very similar to those obtained in MELN cells. BPA was the most effective agonist, exhibiting a full dose–response curve, followed by BPF and BPS, which induced 87 and 76% of maximal luciferase activity, respectively, at the highest concentration tested (Fig. 2B). BPS, BPF, and BPA behaved as full hERβ agonists, and BPA was again the most effective agonist (Fig. 2C), compared to other compounds. Interestingly, unlike BPS, which was more active in HELN-hERβ than in HELN-hERα cells, BPA and BPF were equally active in the hERβ than in hERα assay (Table 1). Finally, all three compounds were tested for non-specific modulation of luciferase expression on the HELN parental cell line, finding that luciferase expression was not induced at concentrations up to 10 μM (data not shown).

Effect of BPS, BPF and BPA on E_2 binding to hERα and hERβ

Whole-cell competitive binding assays were performed with HELN-hERα and -hERβ cells to determine whether the estrogenic effects observed in transactivation assays reflected the abilities of BPS, BPF and BPA (the most effective compounds) to bind to hERα and hERβ. Table 2 summarizes IC_{50} and RBA values for the two hERs. All three compounds showed subtype-selective differences in ligand binding to the two hER subtypes, with an approximately 2-fold greater affinity to hERβ than to hERα, the opposite effect to that of E_2. In fact, BPA was able to completely displace [^{3}H]-E_2 from hERα at 10 μM concentration, whereas BPF and BPS were less active, inhibiting [^{3}H]-E_2 binding by approximately 85 and 70%, respectively, at this concentration (Fig. 3A). In HELN-hERβ cells, BPF and BPS inhibited the binding of [^{3}H]-E_2 to this receptor in a concentration-dependent and competitive manner, although less effectively than BPA, which was also able to completely displace [^{3}H]-E_2 from hERβ at the highest concentration tested (Fig. 3B). These findings indicate that the ability of these compounds to act as hER agonists derives from receptor binding and the greater affinity of BPS for hERβ than for hERα correlated with the preferential agonism of hERβ activity in transactivation assays. However, BPA and BPF compete more effectively for binding to hERβ, but induce hERα and hERβ mediated gene expression with comparable efficacy.

Table 2
IC_{50} and relative binding affinity (RBA) values of BPA, BPS and BPF for hERα and hERβ.

Competitors	hERα		hERβ	
	IC_{50} (nM)	RBA (%)	IC_{50} (nM)	RBA (%)
E_2	0.12 ± 0.03	100	0.21 ± 0.01	100
BPS	6560 ± 530	0.001	3452 ± 878	0.006
BPF	2182 ± 87	0.005	1452 ± 261	0.014
BPA	839 ± 270	0.014	401 ± 126	0.052

(IC_{50}) Competitor concentration required to decrease maximal [^{3}H]-E_2 binding by 50%.
(RBA) Relative binding affinity of each competitor for hER subtypes. The RBA was calculated as the ratio of E_2 to competitor concentration required to reduce specific radiolabeled binding by 50% (ratio of IC_{50} values). The RBA value for E_2 was arbitrarily set at 100.

Estrogenic effects of BPA congeners and derivatives in the E-Screen bioassay

The estrogenic potential of BPS, BPF, BPA, TCBPA, and TBBPA was further characterized by using the E-Screen bioassay to investigate their ability to stimulate cell proliferation in MCF-7 cells. In this cell line, the full ER agonist E_2 strongly induced significant proliferation in a dose-dependent manner, with an EC_{50} value of 0.018 nM. All tested compounds except TBBPA also increased cell proliferation (BPA > BPF > BPS > TCBPA), but their potency was very low in comparison to E_2 (Fig. 4). BPA and BPF showed full dose–response curves (EC_{50} = 0.47 and 1.01 μM, respectively), and a 6.8- and 6.3-fold increase in cell number, respectively, versus control-treated cells (hormone-free medium). BPS and TCBPA (both at 10 μM) also increased cell number by approximately 3.7- and 2.0-fold, respectively (EC_{50} = 12.1 and 45.8 μM, respectively). By contrast, TBBPA did not stimulate MCF-7 cell proliferation in the concentration range of 0.01–10 μM.

hAR in vitro activation by BPA congeners and derivatives

The potential androgenic and anti-androgenic activities of BPS, BPF, BPA, TCBPA, and TBBPA were examined by using PALM cells. The synthetic androgen R1881 was previously found to exert marked androgenic activity, with an EC_{50} value of 0.1 nM (Molina-Molina et al., 2006, 2008). BPA and BPS showed weak agonistic activity at 10 μM concentrations (20 and 15% of maximal activity, respectively) while BPF, TCBPA, and TBBPA did not (Fig. 5A). When the antagonistic activity of these compounds was tested, BPA and BPF at 10 μM concentrations proved to be potent hAR antagonists that strongly inhibited the luciferase activity induced by 0.2 nM of R1881 (Fig. 5B). Despite its weak agonistic activity, BPA was a better antagonist (IC_{50} = 0.92 μM) than BPF (IC_{50} = 6.98 μM), whereas BPS, TCBPA, and TBBPA showed no antagonistic activity towards hAR.

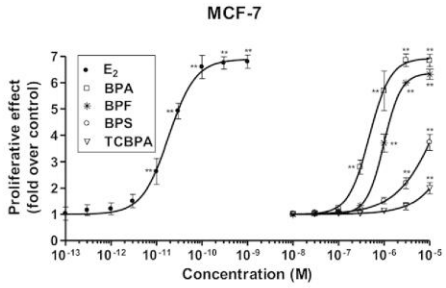

Fig. 4. Dose–proliferative response curves in MCF-7 cells. Cells were incubated for 144 h at 37 °C in the presence of E_2, BPA, BPF, BPS and TCBPA at the indicated concentrations. Results are expressed as proliferative effect (ratio between the highest cell yield obtained with the chemical and the proliferation of hormone-free control cells). Values were the mean ± SD from three separate experiments. *p < 0.05 and **p < 0.01 (versus hormone-free control).

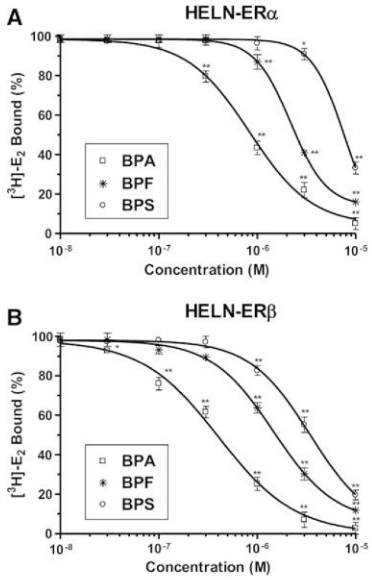

Fig. 3. Competitive inhibition of [^{3}H]-E_2 binding to hERα and hERβ by BPA, BPF and BPS. HELN-hERα and -hERβ cells were incubated with different concentrations (0.01–10 μM) of BPA, BPF and BPS in the presence of 0.1 nM [^{3}H]-E_2. Values were the mean ± SD from three separate experiments. *p < 0.05 and **p < 0.01 (versus 0.1 nM [^{3}H]-E_2).

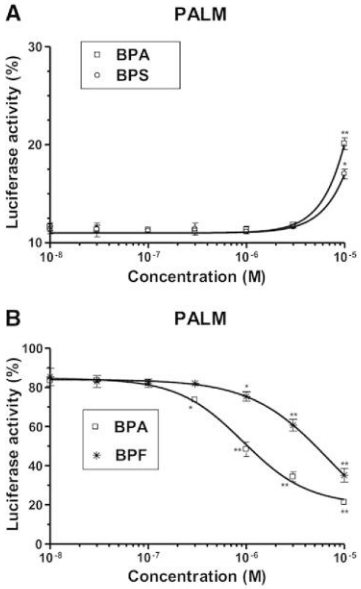

Fig. 5. Induction of luciferase activity in PALM cells by BPA and derivatives. Panel (A). PALM cells were treated with BPA and BPS for 40 h at the indicated concentrations. Maximal luciferase activity (100%) was obtained with 10 nM R1881. Values were the mean ± SD from three separate experiments. *p < 0.05 and **p < 0.01 (versus 0.1% ethanol). Panel (B). PALM cells were treated with 0.2 nM R1881 in the presence of increasing concentrations of BPA and BPF for 40 h. Maximal luciferase activity (100%) was obtained with 10 nM R1881. Values were the mean ± SD from three separate experiments. *p < 0.05 and **p < 0.01 (versus R1881 0.2 nM).

Anti-proliferative potential of BPA congeners and derivatives on MCF-7 AR1 cells

The androgenic response of these compounds to hAR was further characterized by studying the effects of BPS, BPF, BPA, TCBPA, and TBBPA on MCF-7 AR1 cells, which are stable transfectants of MCF-7 cells that express wild-type hAR. Androgen agonists inhibit cell proliferation in MCF-7 AR1 cells, which serves as an end-point to assess the endogenous cell response to androgens (von Bueren et al., 2008). As expected, the full agonist R1881 strongly inhibited the proliferation induced by 100 pM E_2 in a dose-dependent manner in this cell line, with an IC_{50} value (concentration required for 50% of maximal inhibition of E_2-induced proliferation) of 44.5 pM. When the test compounds were applied to MCF-7 AR1 cells, BPA and BPS showed weak but significant inhibitory effects in these cells at 10 μM concentrations (Fig. 6). Consistent with their transactivation assay results, BPF, TCBPA, and TBBPA had no effect on this cell line, indicating that they are not androgenic.

Gene expression modulation via the human pregnane X receptor (hPXR)

We used two HeLa-derived reporter cell lines: the HG_5LN-hPXR line, to detect hPXR agonists or antagonists; and the parental HG_5LN cell line, which constitutively expresses luciferase activity, as a control for non-PXR-specific activities. As previously reported (Creusot et al., 2010), the cholesterol-lowering drug SR12813 exhibited marked hPXR agonistic activity in HG_5LN-hPXR cells, with an EC_{50} value of 69 nM. When BPS, BPF, BPA, TCBPA, and TBBPA were tested for their ability to activate hPXR, only BPA and its halogenated derivatives, TCBPA and TBBPA, were found to be weak-to-moderate hPXR activators (Fig. 7). TCBPA was the most potent of these compounds, activating hPXR with an EC_{50} of 8.49 μM. BPA and TBBPA also induced significant activation of luciferase activity (40 and 50%, respectively). By contrast, BPS and BPF were unable to activate hPXR after 16 h of exposure at concentrations up to 10 μM (Table 1). None of the compounds tested were able to activate luciferase expression in HG_5LN cells, demonstrating that the activity observed in HG_5LN-hPXR cells was hPXR-specific. Finally, prompted by a report by Dring et al. (2010), the test compounds were tested for their ability to antagonize hPXR in our cell model. However, no antagonistic activity was detected (data not shown).

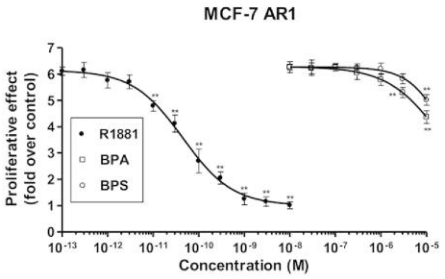

Fig. 6. Anti-proliferative response curves in MCF-7 AR1 cells. Cells were treated with 100 pM E_2 in the presence of increasing concentrations of R1881, BPA and BPS for 5 days at 37 °C. Results are expressed as proliferative effect (ratio between the highest cell yield obtained with the chemical and the proliferation of hormone-free control cells). Values were the mean ± SD from three separate experiments. *p < 0.05 and **p < 0.01 (versus hormone-free control).

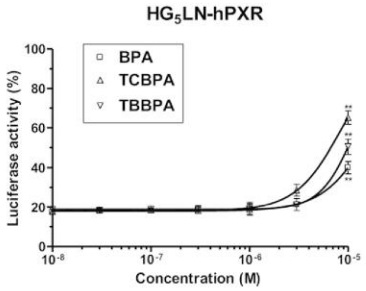

Fig. 7. Induction of luciferase activity in HG_5LN-hPXR cells by BPA and derivatives. HG_5LN-hPXR cells were treated with BPA, TCBPA and TBBPA for 16 h at the indicated concentrations. Maximal luciferase activity (100%) was obtained with 3 μM SR12813. Results are expressed as percentage of maximal SR12813 induction. Values were the mean ± SD from three separate experiments. *p < 0.05 and **p < 0.01 (versus 0.1% ethanol).

Cell viability

The MTT test was used to assess the cytotoxicity of BPA, BPS, BPF, TBBPA, and TCBPA was assessed for the six cell lines used in this study. In all assays, the tested compounds were devoid of any cytotoxicity (cell survival ranging from 95 to 100%) in the 0.01–10 μM range (data not shown).

Discussion

This study contributes evidence that BPS and BPF disrupt the function of several NRs and may therefore interfere with the endocrine system in humans. It was found that BPS, BPF, and BPA all compete with natural ligands for binding to NRs, trigger the expression of cell type-specific genes, and promote cell proliferation in in vitro bioassays. This interference in NR signaling has been considered crucial for assessing the toxicology of BPA and is of no less importance for BPS and BPF, which have been proposed to replace BPA in many of its multiple applications. Given the disrupting effects of BPS observed in different in vitro test systems and its higher resistance to environmental degradation in comparison to BPA or BPF, the proposal to utilize BPS instead of BPA in various products (e.g., baby bottles, food and beverage cans, thermal paper, currency bills) should be viewed with caution (Danzl et al., 2009; Ike et al., 2006). Most of the reported effects of EDCs are attributed to their interference with hormonal signaling mediated by NRs; hence, before any decision is taken on appropriate BPA alternatives, the complete characterization of their NR-mediated effects is essential.

In the present study, the agonistic and antagonistic activities of BPA congeners and derivatives were assessed by using a panel of in vitro bioassays for the detection of different steroid receptor-mediated activities (hERα, hERβ, hAR and HPXR). The estrogenic responses of BPA congeners and derivatives in HELN-ERα cells were highly similar to those obtained in MELN cells, as previously reported for other ERα ligands (Molina-Molina et al., 2008). All of the bisphenols tested except for TBBPA exhibited a marked estrogenic activity (BPA > BPF > BPS > TCBPA), with EC_{50} values in the micromolar range, showing a 25,000- to 250,000-fold less potent transactivation activity, compared to E_2 or the potent pharmaceutical estrogen EE_2. Although fewer data are available on BPS than on BPA and other bisphenols, various in vitro assays have confirmed its estrogenic activity via hERα (Chen et al., 2002; Hashimoto et al., 2001; Kitamura et al., 2005; Kuruto-Niwa et al., 2005). Our results indicate that the ability of BPS to act as hERα agonist derives from its receptor binding affinity. We also studied the effects of the tested

bisphenols on cell proliferation using the E-screen bioassay to further characterize the estrogenic response of these compounds towards hERα. The ranking order of estrogenic potency in MCF-7 cells was BPA > BPF > BPS > TCBPA, whereas TBBPA showed no estrogenic activity in this cell line, confirming the results observed with ours stably transfected cells. In this context, when a wide range of bisphenols were ranked by proliferative potency in MCF-7 cells (Perez et al., 1998), experimental data suggested that not only the distance between *para* hydroxyl groups but also the nature of the bridging carbon substituent determined their estrogenicity. In this regard, bis(4-hydroxyphenyl)ketone, which is more polar than the other bisphenols because of its bridging carbonyl group, has shown the poorest proliferative effect (Perez et al., 1998) and weakest cell-type protein induction in MCF-7 cells (Rivas et al., 2002). This implies that a higher polarity, as in BPS, reduces the estrogenicity. This is consistent with the earlier report by Dodds and Lawson (1936), who used an uterotrophic assay and found bis(4-hydroxyphenyl)ketone to be the least active estrogenic compound out of a series of bisphenol derivatives. The bridging carbon in BPA is replaced with a SO_2 group in BPS, conferring a higher polarity and consequently a lower estrogenicity in this hERα-driven effects model.

Previous reports on halogenated derivatives of BPA (Meerts et al., 2001) indicated that the inclusion of chlorine or bromine atoms in the *meta* position of the aromatic ring of bisphenols had no significant effect on the estrogenic potency. However, the introduction of two atoms in two *meta* positions of one aromatic ring drastically decreased the estrogenic potency (Riu et al., 2011a; Rivas et al., 2001). In agreement with these findings, the results of our reporter gene and MCF-7 cell proliferation bioassays showed a tendency for TCBPA to exert a weak hERα agonistic activity.

Although some attention has been paid to relationship between the structure/function of bisphenols and ERα activation, very little information is available on the binding or activation of ERβ. We have found that BPS can efficiently stimulate hERβ-mediated gene expression and show a higher affinity for binding to hERβ than to hERα. The binding affinity of BPS for hERβ was consistent with the estrogenic activity in the reporter gene assay, demonstrating a good correlation between binding affinity and agonistic activity. The ligand polarity may again explain these results. In a study of selective ligands for α and β ER isoforms, Hillisch et al. (2004) suggested that bulky substitutions below the so-called D-ring in the E_2 molecule lead to ERα agonists, whereas substitutions above the so-called B and C rings preferentially yield ERβ agonists, suggesting that ligand polarity modifies affinity to ER isoforms. Moreover, Nilsson and Gustafsson (2011) reported that each class of ER ligands induces a unique ER conformation that promotes specific co-regulator protein interactions and associations of ER N- and C-terminal activation functions (AF-1 and AF-2, respectively). In this regard, when BPS was tested in HELN cells stably transfected with AF-1 deleted hERs, deletion of the A/B domain in hERβ markedly altered its transactivation potency, indicating that BPS is dependent on AF-1 (Delfosse et al., 2012). In summary, the response of a given cell or tissue to E_2 and to synthetic agonists and antagonists is influenced not only by the relative levels of ERα and ERβ but also by their ligand polarity, which modifies their affinity for ER isoforms.

Beside BPS, other bisphenols such BPF, bisphenol B (BPB), and bisphenol E (BPE) are being considered as putative BPA substitutes and have been investigated in food stimulants and materials in contact with food (Gallart-Ayala et al., 2011). BPF diglycidyl ether (BFDGE) and BPA diglycidyl ether (BADGE) are the building blocks of epoxy resins that coat food and beverage cans and are additives in organosol resins. When in contact with aqueous solution and acidic food, both BADGE and BFDGE are partly converted to BADGE.2HCl and BFDGE.2HCl and to BADGE.$2H_2O$ and BFDGE.$2H_20$, among other compounds. The migration of BADGE and BFDGE from food contact materials was recently investigated (Coulier et al., 2010), and the European commission has set a limit of 1 mg/kg for BADGE and its hydrolytic and chlorinated derivatives (EU, 2005). BADGE and its hydrolysis products are common contaminants in indoor dust (Wang et al., 2012b). Their inhalation, alongside the consumption of canned food, contributes to the exposure of children and adults to bisphenol derivatives. Concerns raised by the presence of BPF residues in canned food are related not only to its estrogenic effects but also its anti-androgenic effects, attributable to its ability to bind to the AR. In 2004, the anti-androgenic activity of BPF was observed in AR-luciferase reporter gene assays using Chinese hamster ovary cells (Satoh et al., 2004) and human breast cancer cells (Stroheker et al., 2004). Furthermore, anti-androgenic effects were also shown by some BPF derivatives (BFDGE and hydrolytic byproducts) (Satoh et al., 2004). In the present study, the potential activity of BPS, BPF, BPA, TCBPA, and TBBPA via hAR was investigated by using PALM cells. As expected, BPA and BPF showed potent anti-androgenic activity (BPA > BPF), whereas only BPA and BPS out of the above compounds evidenced weak agonistic activity at the highest concentration tested. The findings for BPA were consistent with reports of the mixed agonistic/antagonistic activity of some compounds, e.g., cyproterone acetate, chlormadinone acetate, and hydroxyflutamide (Kemppainen and Wilson, 1996; Wilson et al., 2002; Wong et al., 1995). At high concentrations, these ligands appear to induce a receptor conformation that is compatible with AR DNA binding and transcriptional activation.

Given that androgen agonists inhibit cell proliferation in MCF-7 AR1 cells (von Bueren et al., 2007), we assessed the ability of BPA congeners and derivatives to inhibit cell proliferation in this cell line. The cells were stably transfected with a full hAR (Szelei et al., 1997) and expressed approximately 5-fold more hAR in comparison to wild-type MCF-7 cells. Although MCF-7 AR1 cells retain the capacity to proliferate in response to estrogen treatment, androgens inhibit estrogen-induced proliferation and the cells arrest in G0/G1 phase. Only BPA and BPS inhibited cell proliferation at 10 μM concentrations in this cell line, indicating that both compounds are weakly androgenic and confirming the results of our transactivation assays.

It is now well-known, that the metabolic inactivation and excretion of xenobiotics are promoted by the activation of multiple signaling pathways to trigger hepatic biotransformation, biliary excretion, and renal elimination (Wang et al., 2012a). Some of these clearance mechanisms are coordinated by NRs such as PXR. PXR is an important transcription factor controlling xenobiotic detoxification and is strongly expressed in the liver and the intestine, the primarily exposed organs (Lamba et al., 2004). The ligand-binding pocket of PXR accommodates a wide range of structurally unrelated endogenous and exogenous ligands (Di Masi et al., 2009). For instance, hPXR is activated by: endogenous ligands, e.g., bile acids and steroid hormones (Timsit and Negishi, 2007); xenobiotics, e.g., pharmaceuticals (Berthier et al., 2012); endocrine disrupters, e.g., BPA and phthalates (DeKeyser et al., 2001; Mnif et al., 2007); and natural plant compounds, e.g., zearalenone (Ayed-Boussema et al., 2001). Hence, we investigated whether they could be PXR activators. The ability of BPA and derivatives to activate transcription via hPXR was examined using HG_5LN-hPXR cells. We found that only BPA and its halogenated derivatives TCBPA and TBBPA, but not BPS or BPF, were able to activate the hPXR (TCBPA > TBBPA > BPA). In a cell-based reporter assay using HepG2 cells, Sui et al. (2012) found that BPA exhibited agonistic activity for hPXR and that BPF and BPS were inactive, as observed in the present study. They reported that a key structural requirement for the hPXR-mediated activity of BPA and its derivatives is the presence of at least one *para* phenolic group. Moreover, the number and position of methyl groups in the bridge between the two phenolic rings appeared to play a significant role in hPXR activity. In fact, its agonistic activity was abolished by the loss of both methyl groups, as in BPF, or by their replacement with a SO_2 group, as in BPS. However, unlike in the present study, BPA halogenated derivatives TCBPA and TBBPA were unable to activate the hPXR in HepG2 cells, which may be explained by

the ability of human hepatoblastoma cells to metabolize TCBPA and TBBPA, as demonstrated by Riu et al. (2011b).

The present study demonstrates the ability of BPA congeners and derivatives to act at different levels of the NR signal transduction pathway, modulating reporter gene expression, competitive binding, and cell proliferation. The combination of multiple assays offers a rational and informative approach for assessing the disruption capacity of these compounds. Although these bisphenols are active at micromolar concentrations (concentrations unlikely to be leached from bisphenol-containing products or reported in human fluids and tissues), some studies have confirmed that lower concentrations of these compounds appear to affect non-genomic signaling in estrogen-responsive cells, with potential consequences for cell function. For example, BPA binds to both ERs, triggering non-classical estrogenic effects at nanomolar concentrations and altering the function of key cell types involved in human metabolism, such as pancreatic β cells and adipocytes (Soriano et al., 2012; vom Saal et al., 2010). Moreover, BPS is active via non-genomic signaling pathways in pituitary cells (Viñas and Watson, 2013a) at low-dose ranges likely to be present in food items and human fluids. Furthermore, in the real world, environmental and even occupational exposures are rarely due to a single chemical but rather involve complex chemical mixtures. Therefore, it cannot be ruled out that synergistic effects exerted via different NRs bound to bisphenols alone or to other EDCs may mediate the putative in vivo endocrine disrupting effect of these compounds (Viñas and Watson, 2013b). These findings call into question the advisability of replacing BPA with other bisphenols and underscore the need for further investigation of putative BPA substitutes.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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Discusión del Objetivo 1: *Estudio de la actividad hormonal de contaminantes ambientales empleados en la fabricación de plásticos (resinas epoxi y policarbonato).*

La caracterización del riesgo en lo referente a los bisfenoles empleados para el plástico policarbonato y polisulfonato y las resinas epoxi se ha llevado a cabo investigando la capacidad de los bisfenoles para competir con los receptores nucleares. Así, hemos podido poner de manifiesto que todos los compuestos ensayados, con la excepción de TBBPA, mostraron actividad estrogénica en el rango micromolar, con una potencia en la transactivación entre $25 \cdot 10^3$ y $25 \cdot 10^5$ veces menor que el estradiol o el estrógeno sintético etinil estradiol (EE2). Los bisfenoles quedan ordenados según su actividad estrogénica de esta manera BPA > BPF > BPS > TCBPA.

La información existen sobre la actividad estrogénica de los bisfenoles es escasa, a excepción de la que corresponde al BPA. No obstante, varios ensayos habían señalado con anterioridad su afinidad al receptor estrogénico α (Hashimoto *et al.*, 2001; Chen *et al.*, 2002; Kitamura *et al.*, 2005; Kuruto-Niwa *et al.*, 2005). En el presente estudio se observa que la habilidad del BPS para actuar como agonista del receptor estrogénico α corresponde fielmente con su afinidad de unión al receptor.

Cuando se investigó la respuesta proliferativa de los bisfenoles en el ensayo E-Screen, la clasificación en cuanto a potencia resultante es la siguiente: BPA > BPF > BPS > TCBPA. De nuevo TBBPA no mostró actividad sobre las células de cáncer mamario MCF-7. Los datos experimentales en la línea MCF-7 sugieren que no sólo la distancia entre los grupos para-hidroxilo sino también la naturaleza del sustituto del carbono central son importantes determinantes de la actividad biológica. Así, la di(4-hidroxifenil)-cetona, que es más polar que otros bisfenoles debido a la sustitución del grupo carbonilo, ha mostrado siempre una menor actividad en la inducción de proteínas estrógeno específicas (Rivas *et al.*, 2002); esto implica que una mayor polaridad, como es el caso del BPS, reduce la estrogenicidad. Otros estudios también han demostrado que la inclusión de átomos de cloro o bromo en la posición *meta* del anillo aromático de los bisfenoles afectan a la actividad estrogénica (Meerts *et al.*, 2001), fenómeno que se ve confirmado en los experimentos de expresión génica y proliferación celular con el TCBPA, que presenta una débil actividad agonista del receptor estrógenico α.

La información existente respecto a la competencia de estos bisfenoles con el receptor estrogénico β es muy limitada. Por esto es interesante hacer notar que el BPS

puede estimular la expresión génica a través de dicho receptor, mostrando además una mayor afinidad por la unión a esta isoforma que al receptor α. Ésta afinidad concuerda con la actividad estrogénica observada en el ensayo de expresión génica, encontrándose una buena correlación entre afinidad por el receptor y actividad agonista. Esta observación podría estar relacionada con la polaridad del ligando ya que en un estudio con ligandos selectivos para las isoformas α y β, Hillisch *et al.* (2004) sugieren que las sustituciones "abultadas" en el anillo D de la molécula de estradiol dan lugar a agonistas del receptor α, mientras que esas sustituciones en los anillos B y C dan lugar, preferentemente, a agonistas del receptor β; en ambos casos la polaridad del ligando modifica la afinidad a las dos isoformas del receptor estrogénico. Es interesante resaltar que Nilsson y Gustaffson (2011) asocian cada tipo de ligando del receptor estrogénico a una única conformación molecular, la cual promueve la interacción de una proteína co-reguladora específica del receptor. En este mismo sentido Delfosse *et al.*, (2012) asocia la supresión del dominio A/B del hERβ con la potencia de transactivación, sugiriendo que bisfenoles como el BPS son dependientes de AF-1. En resumen, la respuesta celular o del tejido al estradiol y a los agonistas y antagonistas sintéticos depende no sólo de los niveles relativos de las isoformas α y β de los receptores sino también de la polaridad de los ligandos, que modifica su afinidad por el receptor.

Además del BPS, se están planteando como sustitutos del BPA otros bisfenoles como el BPF, el bisfenol B (BPB) o el bisfenol E (BPE) (Gallart-Ayala *et al.*, 2011). De hecho, el bisfenol F diglicidil éter (BFDGE) y el bisfenol A diglicidil éter (BADGE) son una parte significativa de las resinas empleadas en envases alimentarios. Estas resinas tapizan el interior de envases alimentarios metálicos y al entrar en contacto con soluciones acuosas y alimentos ácidos, se hidrolizan parcialmente en forma de BADGE.2HCl y BFDGE.2HCl, y en BADGE.2H_2O y BFDGE.2H_2O, entre otras moléculas. Por esta razón se ha sugerido que no solo la actividad hormonal de los monómeros merece nuestra atención sino que es interesante estudiar la migración a los alimentos de éstos compuestos intermediarios de la degradación (Coulier *et al.*, 2010). Por esta razón, la Comisión Europea ha establecido un límite de migración de 1 mg/kg para el BADGE y sus derivados (EU, 2005). Tanto BADGE como los productos de hidrólisis son contaminantes habituales del polvo de interiores (Wang *et al.*, 2012b) y su inhalación, además del consumo de alimentos enlatados, contribuye a la exposición a derivados bifenólicos.

La preocupación sobre la presencia de BPF como contaminante proveniente de las resinas empleadas en latas de conservas se debe no sólo a sus efectos estrogénicos, bien conocidos, sino también a su actividad antiandrogénica relacionada con su capacidad para competir con el receptor de andrógenos. Ya en 2004 se publicó la actividad antiandrogénica en un ensayo de luciferasa vinculado a un gen reporter en células de ovario de hámster chino (Satoh *et al.*, 2004) y en células tumorales humanas (Stroheker *et al.*, 2004). Esta actividad antiandrogénica atañe también a algunos derivados del BPF (Satoh *et al.*, 2004). En el presente trabajo se ha evaluado la actividad de BPS, BPF, BPA, TCBPA y TBBPA en modelos que contienen el receptor androgénico empleando las células de próstata de origen humano PALM. BPA y BPF se comportaron como potentes antiandrógenos (BPA > BPF) mientras que sólo BPA y BPS, de entre todos los compuestos estudiados, mostraron una débil actividad agonista a la mayor concentración testada. Los resultados para el BPA coinciden con los informes de actividad agonística y antagonística de este compuesto (Wong *et al.*, 1995; Kemppainen y Wilson, 1996; Wilson *et al.*, 2002).

Dado que los agonistas androgénicos inhiben la proliferación celular en la línea MCF-7 AR1 utilizada (von Bueren *et al.*, 2007), se testó la habilidad del BPA y sus congéneres y derivados para inhibir la proliferación celular en ésta línea celular. Las células MCF-7 AR1 están transfectadas de manera estable con un receptor androgénico completo (Szelei *et al.*, 1997) y expresan aproximadamente 5 veces más hAR que las células MCF-7 originales. Aunque las células transfectadas mantienen la capacidad de proliferar ante tratamiento estrogénico, los andrógenos inhiben la proliferación inducida por estrógenos y el ciclo celular se detiene en la fase G0/G1. En este modelo sólo BPA y BPS inhibieron la proliferación celular a concentraciones de 10 µM, demostrando que ambos compuestos son débilmente androgénicos y confirmando los resultados de los ensayos de transactivación.

La inactivación metabólica y la excreción de xenobióticos está relacionada por la activación de vías de biotransformación hepática, excreción biliar y eliminación renal (Wang *et al.*, 2012a). Algunos de esos mecanismos están coordinados por receptores nucleares, como PXR, que es un factor de transcripción que controla la detoxificación de xenobióticos y se expresa en el hígado y el intestino (Lamba *et al.*, 2004). El grupo de ligandos para PXR está representado por un amplio grupo de compuestos endógenos y exógenos (Di Masi *et al.*, 2009), entre los que se incluyen hormonas esteroideas

(Timsit y Negishi, 2007), xenobióticos (Berthier *et al.*, 2012), disruptores endocrinos como BPA y ftalatos (DeKeyser *et al.*, 2001; Mnif *et al.*, 2007) y fitocomponentes como la zearalenona (Ayed-Boussema *et al.*, 2001). Por esta razón, en el presente trabajo se investigó si los bisfenoles seleccionados podían activar el PXR, utilizando para ello la línea celular HG_5LN-hPXR. Los resultados mostraron que sólo el BPA y los derivados halogenados (TCBPA y TBBPA) activaban el receptor PXR (TCBPA > TBBPA > BPA). Utilizando células HepG2 transfectadas, Sui *et al.* (2012) observaron actividad agonista del BPA en hPXR pero no del BPF ni BPS. Estos autores sugieren que la clave para la actividad a través de hPXR del BPA y sus derivados es la presencia de, al menos, un grupo fenólico en posición *para.* Además, el número y posición de los grupos metilo en el carbono central de enlace entre los dos anillos fenólicos parecería jugar un papel significativo en la actividad de hPXE. De hecho, su actividad agonista desaparece cuando se pierden ambos grupos metilo, como es el caso de la estructura de BPF, o es reemplazado por un grupo SO_2, como en el caso del BPS. Sin embargo, al contrario de lo observado en el presente estudio, los derivados halogenados del BPA (TCBPA y TBBPA) no mostraron actividad en hPXR en las células HepG2, lo que podría explicarse por la habilidad de las células de hepatoblastoma para metabolizar TCBPA y TBBPA (Riu *et al.*, 2011b).

En resumen, se ha podido demostrar que los congéneres del BPA y sus derivados pueden actuar a diferentes niveles en las vías de señalización hormonal, compitiendo por el enlace al receptor, modulando la expresión génica y afectando a la proliferación celular. La combinación de diferentes propiedades en la batería de ensayos empleada proporciona una aproximación global a la evaluación de la capacidad disruptora de los bisfenoles. Aunque muchos de ellos son activos a concentraciones micromolares —que serían fáciles de alcanzar en algunas matrices biológicas humanas a través de la migración de diferentes productos—, algunos estudios han confirmado que bajas concentraciones de estos compuestos podrían afectar a la señal no-genómica de las células sensibles a los estrógenos con consecuencias importantes para la función celular. Así, BPA se une a ambas isoformas del receptor estrogénico, promoviendo los efectos estrogénicos no clásicos a concentraciones nanomolares y alterando la función de tipos celulares concretos implicados en el metabolismo, como es el caso de las células pancreáticas β y los adipocitos (vom Saal *et al.*, 2010; Soriano *et al.*, 2012). Además, el BPS es activo por vías no genómicas en células hipofisarias (Viñas y Watson, 2013a)

actuando a bajas dosis, como las que se pueden encontrar en productos alimenticios o en tejidos humanos. Por último, es necesario resaltar que tanto las exposiciones ambientales como las ocupacionales rara vez se deben a un único compuesto, sino a una mezcla compleja de compuestos químicos, por lo que no puede descartarse un efecto sinérgico de los diferentes bisfenoles y demás disruptores endocrinos en los receptores nucleares (Viñas y Watson, 2013b).

Resultados del Objetivo 2: *Estudio de la exposición materno-infantil a metales pesados.*

Los metales pesados son contaminantes ambientales ampliamente extendidos con conocidos efectos adversos sobre la salud humana. La exposición en poblaciones no ocupacionalmente expuestas suele ocurrir a través de la dieta y el agua (ATSDR 1999, 2007a, b, 2008a, b, c), aunque existen fuentes adicionales de exposición habitual como es el hábito tabáquico pare el caso del Cd y Cr (ATSDR 2008a, b). Diversos estudios en alimentos han mostrado que los niveles de metales se mantienen dentro de los límites de seguridad establecidos, si bien determinados productos pueden suponer un riesgo para poblaciones especialmente susceptibles (Herreros *et al.*, 2008; Martí-Cid *et al.*, 2008; Fontcuberta *et al.*, 2011; Martorell *et al.*, 2011) Mención especial merece el Hg, cuyos niveles alcanzan máximos preocupantes en el pescado envasado (González-Estecha *et al.*, 2013); de hecho, en la cohorte estudiada en esta tesis se ha observado previamente una relación directa entre las concentraciones de este metal en niños (pelo) y la ingesta de pescado (Freire *et al.*, 2010a).

Los metales Cd y As están catalogados por la IARC como carcinógenos (IARC, 2012) y descritos como neurotóxicos (Govil *et al.*, 2012), e incluso el Cd ha mostrado capacidad para actuar como disruptor endocrino (Henson y Chedrese, 2004). Numerosos estudios epidemiológicos muestran una asociación entre exposiciones tempranas a diferentes metales pesados y efectos adversos en el neurodesarrollo infantil, o desórdenes endocrinos (Wright *et al.*, 2006; Gundacker *et al.*, 2010; Stasenko *et al.*, 2010).

Debido a que se ha demostrado la capacidad de algunos metales de atravesar la barrera placentaria (ATSDR 2007a) e interferir con los sistemas de transporte de ésta (Zhang *et al.*, 2004), la exposición intrauterina a estos contaminantes podría ser de especial relevancia para el correcto desarrollo fetal. El objeto del presente trabajo es poner a punto una metodología y caracterizar la exposición intrauterina en lo que respecta a cinco metales pesados y un metaloide empleando como material de estudio las placentas de la cohorte INMA de Granada.

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Placental concentrations of heavy metals in a mother–child cohort

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ABSTRACT

Heavy metals are environmental contaminants with properties known to be toxic for wildlife and humans. Despite strong concerns about their harmful effects, little information is available on intrauterine exposure in humans. The aim of this study was to evaluate prenatal exposure to As, Cd, Cr, Hg, Mn, and Pb and its association with maternal factors in a population-based mother–child cohort in Southern Spain. Between 2000 and 2002, 700 pregnant women were recruited and 137 placentas from the cohort were randomly selected and analyzed for the selected metals by atomic absorption. Maternal sociodemographic and lifestyle factors were obtained by questionnaire after delivery. Bivariate analysis and multivariate linear regression were performed. Cd and Mn concentrations were detected in all placentas, while Cr, Pb, and Hg were found in 98.5%, 35.0%, and 30.7% of samples, respectively. The highest concentrations were observed for Pb (mean: 94.80 ng/g wet weight of placenta), followed by Mn (63.80 ng/g), Cr (63.70 ng/g), Cd (3.45 ng/g), and Hg (0.024 ng/g). Arsenic was not detected in any sample. Gestational age and smoking during pregnancy were associated with placental Cd concentrations, while no factor appeared to influence concentrations of Cr, Hg, Mn, or Pb. In comparison to results of European studies, these concentrations are in a low-intermediate position. Studies are required to investigate the factors contributing to early exposure to heavy metals and to determine how placental transfer of these toxic compounds may affect children's health.

1. Introduction

Heavy metals are ubiquitous environmental pollutants with known toxic properties. Human exposure to these chemicals may occur occupationally, environmentally, or through dietary intake (ATSDR, 1999, 2007a,b, 2008a,b,c). In the general population, food and water are the most common sources of exposure (ATSDR, 2007a,b, 2008a,b,c), while cigarette smoking is an additional relevant source of exposure to heavy metals such as Cd and Cr (ATSDR, 2008a,b).

In Spain, levels of As, Cd, Hg, and Pb were analyzed in various marketed food items (Fontcuberta et al., 2011; Martí-Cid et al., 2008; Martorell et al., 2011). Martorell et al. (2011) reported that the estimated intake of these metals was lower than maximum tolerable intakes. Herreros et al., (2008) investigated Cd, Pb, and Hg concentrations in fish consumed in Spain and reported that the consumption of certain species (*i.e.*, sword-fish and louvar) may pose a risk to fertile women from high Hg exposure. Accordingly, a study of pregnant women from a Spanish Mediterranean region found that the intake of fish, especially large oily fish, was related to higher cord blood Hg levels, which were above the current US-EPA (United States Environmental Protection Agency) reference dose in 75% of the study population (Ramon et al., 2011). Moreover, a positive relationship was observed between hair Hg concentrations in infants and their fish intake in a cross-sectional analysis of the present cohort birth study (Freire et al., 2010).

Although the estimated dietary intake of heavy metals in Spain may not represent a potential health risk for the general population (Fontcuberta et al., 2011; Martorell et al., 2011), this hazard cannot be ruled out in particular populations with a higher consumption of the food items with greater metal concentrations. Furthermore, fetuses and neonates are especially vulnerable to toxic chemicals because of the immaturity of their detoxification systems. In fact, intake by children of heavy metals per unit of body weight is expected to be higher than in adults (Martí-Cid et al., 2007).

A large number of epidemiological studies have associated early exposure to Pb, Hg, As, and Cd with infant health effects, including neurological (Després et al., 2005; Lanphear et al., 2005; Oken et al., 2005; Wright et al., 2006), developmental (Gundacker et al., 2010; Zhang et al., 2004), and endocrine disorders (Gollenberg et al., 2010; Stasenko et al., 2010). Pb and Hg are established neurodevelopmental

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toxicants, while Cd and As are well-known human carcinogens (IARC, 2012). Both Cd and As are also neurotoxicants (Govil et al., 2012; Hu et al., 2007; Wasserman et al., 2004), and Cd may act as an endocrine disrupter (Henson and Chedrese, 2004). There is less evidence on the human health effects of Cr and Mn. Hexavalent Cr is known to be mutagenic and carcinogenic (Zhitkovich, 2011), but little information is available on adverse developmental effects in humans, while there is growing epidemiological concern about the potential effects of Mn on the developing nervous system (Zoni et al., 2007).

Because some heavy metals may reach and cross the placental barrier (ATSDR 2007a; Osman et al., 2000) and interfere with placental transport systems (Wier et al., 1990; Zhang et al., 2004), prenatal exposure to these toxic compounds should be a matter of special concern. The placenta appears to be the optimal biological matrix to assess environmental risk and maternal transfer to the fetus (Esteban and Castaño, 2009), however, few data are available on the levels of heavy metals in human placenta, and most studies have analyzed Cd, Cu, Fe, Pb, and Zn (Singh et al., 2010; Tekin et al., 2012; Zadrozna et al., 2009; Zagrodzki et al., 2003).

The objective of this study was to investigate placental concentrations of As, Cd, Cr, Hg, Mn, and Pb, and their association with maternal factors in a mother–child birth cohort from Granada (Southern Spain) within the framework of the INMA project (*Infancia y Medio Ambiente*—Environment and Childhood).

2. Materials and methods

2.1. Study population

The study population was drawn from the INMA-Granada birth cohort established in the geographical area covered by the San Cecilio University Hospital of Granada, in Southern Spain (Fernandez et al., 2007). General criteria for inclusion of the mothers and their children in the study were: (i) to be resident in the study area, (ii) to be at least 16 years old at the time of delivery, (iii) to not have followed any program of assisted reproduction, and (iv) absence of serious chronic diseases, such as diabetes, hypertension, or thyroid disease, those developing a pregnancy complication that could affect fetal growth or development. From October 2000 to July 2002, 700 mother–child pairs were recruited after delivery with the initial aim of investigating chronic exposure to endocrine disrupting chemicals and urogenital malformations in newborn boys. All pregnant women attending the San Cecilio University Hospital for delivery in this period (a total of 1741 giving birth to boys) were invited to participate in the study. Among these, 40.3% agreed to participate. The study was approved by the Institutional Ethical Committee of the San Cecilio University Hospital. Informed consent was obtained from all subjects who participated in the study.

2.2. Data collection

Sociodemographic and lifestyle information was gathered on participants from face-to-face interview questionnaires administered within the first 48 h after delivery, including: maternal age (years), place of residence (urban: city of Granada and towns of >20000 inhabitants in city residential belt; rural: towns with <20000 inhabitants), maternal education (university; secondary; primary), smoking during pregnancy (no; yes), and parity (primiparous; multiparous). Gestational age (weeks calculated from the date of last menstrual period), and newborn birth weight (g) were collected from medical records.

2.3. Placentas collection

A total of 680 placentas from the cohort were collected at the time of delivery, examined, and weighed. The samples were immediately coded, frozen, and anonymously stored at -86 °C until analysis. For processing, placentas were defrosted and mechanically homogenized, including maternal and fetal sides and central and peripheral parts. Approximately one in five placentas ($n=137$) were randomly selected to analyze concentrations of As, Cd, Cr, Hg, Mn, and Pb. Differences in characteristics between participants with metal exposure data and those with no metal information were not statistically significant, except for educational level (Table 1).

Table 1
Characteristics of cohort population.

	Participants included ($N=137$)	Participants not included ($N=543$)[†]
Maternal age (years), mean (SD)	30.7 (4.7)	33 (3.8)
Gestational age (weeks), mean (SD)	39 (2)	39 (1)
Birth weight (g), mean (SD)	3249 (505)	3356 (327)
Birth length (cm), mean (SD)	50.2 (2.6)	49.0 (2.1)
Parity, *N* (%)		
Primiparous	62 (45.3)	268 (49.4)
Multiparous	75 (54.7)	275 (50.6)
Smoking during pregnancy, *N* (%)		
No	102 (74.5)	422 (77.7)
Yes	35 (25.5)	121 (22.3)
Area of residence, *N* (%)		
Rural	50 (36.5)	162 (29.8)
Urban	87 (63.5)	381 (70.2)
Educational level, *N* (%)		
University	23 (16.8)	102 (18.7)
Secondary	97 (70.8)	331 (61.0)
Primary	17 (12.4)	110 (20.3)[*]

SD: standard deviation.

[*] $p<0.05$ (difference between mothers included and not included)

[†] Cohort population not included in the present study because of not available data on metal concentrations in placenta.

2.4. Determination of heavy metal concentrations

2.4.1. Digestion procedure

Placenta homogenates were dried at 80 °C. From each dried placenta, an aliquot of 0.3 g was transferred to a quartz vessel, which, after addition of 2 mL HNO_3, 0.5 mL HCl, and 4 mL H_2O_2, was closed to allow digestion for 35 min in a 1400 W microwave oven at 280 °C and 80 bar. The digested solution was transferred to a decontaminated tube.

2.4.2. Equipment

Total As, Cd, Cr, Mn, and Pb concentrations were determined using a Perkin–Elmer AAnalyst 800 Atomic Absorption Spectrometer (Perkin–Elmer, Norwalk, CT) equipped with Zeeman background correction and an AS-800 auto-sampler. Total As was measured with direct flow injection through a hydride generation system (Perkin–Elmer FIAS-100). A graphite furnace and graphite tubes with integrated L'vov platform were used for Cd, Cr, Mn, and Pb. Hg was determined in a Perkin–Elmer 560 Atomic Absorption Spectrometer (Perkin Elmer) equipped with Power Supply Lamp System and MHS-10 Mercury Hydride System.

Calibration curves were constructed using atomic absorption spectrometry standard solutions for As, Cd, Cr, Hg, Mn, and Pb, prepared from a stock solution of 1000 mg/L for each metal by successive dilutions. All aqueous solutions of reagents and standards were prepared using water of reverse-osmosis quality produced by a Milli-RO 12 plus Milli-Q purification system (Millipore, Bedford, Massachusetts).

All chemicals were of analytical reagent grade.

2.4.3. Validation of analytical methods

The analytical methods were previously validated (Gil et al., 2006, 2011; Olmedo et al., 2010). According to the recommendations of the IUPAC (International Union of Pure and Applied Chemistry), parameters included the limits of detection and quantification, linear range, precision (minimal, intermediate, and reproducibility), accuracy, recovery, and characteristic mass. The uncertainty of methods was calculated for each metal (Table 2).

2.4.4. Quantification of metals

Appropriate matrix modifiers prepared in 0.2% (v/v) nitric acid and 0.1% Triton X-100 were used for the heavy metals studied. An appropriate dilution of each sample was necessary to obtain the best results. The limit of detection was 0.03, 0.03, 0.19, 0.002, 0.12, and 0.83 μg/L for As, Cd, Cr, Hg, Mn, and Pb, respectively. Calibration graphs were linear up to 4, 7, 30, 20, 20, and 200 μg/L, respectively (more details in Gil et al., 2006, 2011, and Olmedo et al., 2010).

Table 2
Summary of parameters of the instrumental method for the determination of As, Cd, Cr, Hg, Mn, and Pb in placenta.

	As	Cd	Cr	Hg	Mn	Pb
Limit of detection (μg/L)	0.03	0.03	0.19	0.002	0.12	0.83
Limit of quantification (μg/L)	0.10	0.09	0.64	0.007	0.39	2.80
Linear range (μg/L)	4	7	30	20	20	200
Linear correlation coefficient	0.99	0.99	0.99	0.99	0.99	0.99
Precision (%)						
Minimal	3.51	2.62	4.36	1.98	1.76	3.21
Intermediate	6.70	4.94	2.32	3.56	2.57	2.63
Reproducibility	5.09	3.45	3.29	2.20	4.46	2.98
Accuracy (%)	1.33	1.70	1.60	5.80	3.71	1.20
Characteristic mass (pg)	25.37	2.96	12.70	12.00	5.16	11.00

Precision, accuracy and recovery data are based on the average of replicate determinations ($n=10$); accuracy data are expressed as % difference with certified value. Intermediate precision analyses were carried out over 1-week period.

Table 3
Placental concentrations of heavy metals (ng/g wet weight)[†].

	Cd	Cr	Hg	Mn	Pb
Limit of detection	0.16	1.49	0.016	0.94	0.65
% > limit of detection	100	98.54	30.66	100	35.04
Geometric mean	3.45	63.70	0.024	63.80	94.80
Geometric SD	1.97	3.16	0.01	1.61	4.45
Median	3.80	62.70	0.022	67.10	70.10
Minimum–maximum	0.70–13.60	8.40–5708	0.016–0.080	11.40–204.6	2.90–2073
25th percentile	2.15	37.70	0.018	49.90	25.50
75th percentile	5.80	118.1	0.028	85.35	309.0

SD: standard deviation.

[†] Concentrations above the limit of detection.

2.5. Statistical analysis

Concentrations of heavy metals in placenta were expressed in ng/g wet weight of placenta and did not fit a normal distribution. Geometric mean, geometric standard deviation, median, range, and 25th and 75th percentiles of concentrations above the limit of detection were calculated. Spearman's correlation analysis was conducted between metal concentrations. Median values were obtained for metal concentrations as a function of characteristics of the study population. A value equal to half the limit of detection was used for non-detected concentrations. The nonparametric Mann–Whitney and Kruskal–Wallis tests were performed in bivariate analyses between exposure variables and covariates. Multivariate linear regression models were conducted to examine potential factors contributing to exposure levels, with natural logarithm-transformed concentrations of heavy metals as dependent variables. All covariates were tested in multivariate analysis and they were retained in the final model on the basis of bivariate associations ($p<0.10$) with exposure variables.

3. Results

Mean age (standard deviation) of the 137 women was 30.7 (4.71) years, ranging from 18 to 42 years. Mean gestational age of newborns was 39 weeks (range: 32–42) and birth weight was 3249 g (range: 1900–4420 g). Primiparous and multiparous mothers were almost equally represented in the study population, *i.e.* 45.3% and 54.7%, respectively. A quarter of the women smoked during pregnancy, 36.5% were residents in rural areas, and 16.8% had a university education (Table 1).

Concentrations of Cd and Mn were detected in all placentas, whereas Cr, Pb, and Hg were detected in 98.5%, 35.0%, and 30.7% of placenta extracts, respectively. Arsenic was not investigated in the whole study population because no detectable concentrations were found in a preliminary study of 50 of these placentas. Table 3 summarizes frequencies and wet weight adjusted concentrations of metals in placentas. The highest concentrations were observed for Pb (geometric mean: 94.80 ng/g; maximum value: 2073 ng/g), Mn (geometric mean: 63.80 ng/g; maximum: 204.6 ng/g), and Cr (geometric mean: 63.70 ng/g; maximum: 5708 ng/g). Concentrations were particularly low for Hg, *i.e.* close to the limit of detection (geometric mean: 0.024 ng/g). Spearman's correlation analysis between concentrations of metals (detected and non-detected) showed a significant positive correlation between Mn and Cd, and Mn and Cr, whereas negative correlation was found between Pb and Cr (Table 4). The remaining metal concentrations were not correlated.

Bivariate associations between maternal characteristics and placental metal levels were only significant for Cd concentrations, which were higher in mothers smoking during pregnancy and those with primary education alone than in nonsmoking mothers and mothers with university studies (Table 5). Placental Cd concentrations were significantly lower in mothers with premature labor (6.6%) than in those with full-term delivery. When Spearman correlation was conducted, maternal age resulted positively correlated with Cd concentrations (Spearman correlation coefficient: 0.17; 95% confidence interval: 0.02; 0.25) and marginally correlated with placental Cr levels (correlation coefficient: 0.14; 95% confidence interval: −0.001; 0.18) (data not shown).

After testing variables in multivariate analysis, no relevant relationships were observed between maternal characteristics and Cr, Hg, Mn, or Pb concentrations (data not shown). Regarding Cd, linear regression analysis showed that, independently of age and maternal education, non-premature labor (> 37 weeks) and smoking during pregnancy were significantly associated with 1.65- and 1.26-fold higher placental concentrations of Cd, respectively (Table 6). The interaction between smoking and gestational age was tested in the model and no significant effects were observed (data not shown).

4. Discussion

In this study of 137 mothers in the INMA-Granada mother–child cohort (Southern Spain), with no reported occupational

Table 4
Spearman correlation coefficients and corresponding 95% confidence intervals for heavy metal concentrations[†].

	Cd	Cr	Hg	Mn	Pb
Cd	1				
Cr	0.001 (−0.17; 0.17)	1			
Hg	0.107 (−0.06; 0.27)	0.080 (−0.09; 0.24)	1		
Mn	0.172 (0.004; 0.33)	0.413 (0.26; 0.54)	0.145 (−0.02; 0.31)	1	
Pb	0.162 (−0.006; 0.32)	−0.670 (−0.75; −0.56)	0.075 (−0.09; 0.24)	0.037 (−0.13; 0.20)	1

[†] Concentrations above and below the limit of detection.

Table 5
Median concentrations (ng/g) of heavy metals in placentas by characteristics of study population[†].

	N (%)	Cd	Cr	Mn
Maternal age (years)				
<32 years	76 (55.5)	3.50	56.35	64.35
≥32 years	61 (44.5)	4.00	66.70	69.10
Gestational age (weeks)				
≥37	128 (93.4)	3.90	58.55	65.50
<37	9 (6.6)	2.20	83.90	78.20
Birth weight (g)				
≥2500	124 (90.5)	3.85	58.55	65.50
<2500	13 (9.5)	3.70	69.70	77.90
Parity				
Primiparous	62 (45.3)	3.50	71.30	68.20
Multiparous	75 (54.7)	3.90	48.80	64.60
Smoking during pregnancy				
No	102 (74.5)	3.30	69.40	66.30
Yes	35 (25.5)	4.90	48.80	70.50
Area of residence				
Rural	50 (36.5)	3.85	49.95	66.95
Urban	87 (63.5)	3.70	71.30	67.10
Educational level				
University	23 (16.8)	4.70	71.30	76.40
Secondary	97 (70.8)	3.30	57.90	64.50
Primary	17 (12.4)	6.70	56.70	67.10

[†] Median of concentrations above and below the limit of detection.

Table 6
Multivariate linear regression analysis for Cd concentrations in placenta.

Variables in the model	Rate of change in Cd concentrations	95% confidence interval
Maternal age ≥32 yrs (reference: <32 yrs)	1.17	0.93; 1.48
Gestational age ≥37 weeks (reference: <37 weeks)	1.65	1.05; 2.59
Smoking pregnancy (reference: not smoking)	1.26	0.97; 0.61
Maternal education (reference: university)		
Secondary	0.85	0.60; 1.21
Primary	1.28	0.82; 2.03

exposure to metals, the presence of Cd, Cr, and Mn was detected in more than 98% of placentas, while Pb and Hg were detected in one-third. Arsenic was not detected. Pb showed the highest placental concentrations, followed by Mn and Cr. Smoking during pregnancy and gestational age were associated with placental Cd concentrations, while no characteristic of the population appeared to influence concentrations of Cr, Hg, Mn or Pb.

Various studies in different countries have reported placental concentrations of heavy metals, mostly in populations living near industrial activities or in areas with heavy air pollution. However, little information is available on levels in the general population. Table 7 summarizes the results of the main studies on placental concentrations of heavy metals. The great majority of studies have reported on placental Cd, Hg, and Pb concentrations (Al-Saleh et al., 2011; Gundacker et al., 2010; Guo et al., 2010; Llanos and Ronco, 2009; Needham et al., 2011; Stasenko et al., 2010), and very few have examined placental exposure to Cr, Mn, or As (Guo et al., 2010; Kippler et al., 2010; Leino et al., in press; Llanos and Ronco, 2009; Odland et al., 2003). In general, reported concentration levels have been lower in European than Asiatic populations, *e.g.* in India or China. Except for Pb, the median values of heavy metal concentrations in the present study were within the low-intermediate range of those reported in the main European studies.

4.1. Cadmium

Cd concentrations were in concordance with findings in studies of Swedish, Finnish, and Croatian mothers (Akesson et al., 2002; Klapec et al., 2008; Leino et al., in press; Stasenko et al., 2010) but at the low end of the scale in comparison to reports from Saudi Arabia, China, and India (Al-Saleh et al., 2011; Guo et al., 2010; Kippler et al., 2010). In agreement with the results of other cohort studies (Kutlu et al., 2006; Osman et al., 2000; Piasek et al., 2001; Ronco et al., 2005; Sorkun et al., 2007; Stasenko et al., 2010; Zhang et al., 2004), smoking during pregnancy significantly contributed to higher Cd concentrations in placenta.

The observed association between gestational age and Cd levels may be a biased result due to the small number of mothers with premature labor in our study population. Another possible explanation may be that smoking mothers might have been more likely to have a short time pregnancy. However, interaction between these two variables was not significant.

4.2. Mercury

There have been few studies on Hg concentrations in placenta. Levels in our cohort were much lower than those reported elsewhere (Ask et al., 2002; Gundacker et al., 2010; Hsu et al., 2007; Llanos and Ronco, 2009; Marques et al., 2007). Reference levels in cord blood and maternal hair were set by the US-EPA (2012a) to protect the developing fetus from the neurotoxic effects of Hg. In a recent study, Needham et al., (2011) found close correlations among Hg concentrations in placenta, cord blood, cord tissue, and hair in mothers from the Faroe Islands. Nevertheless, low Hg concentrations in placenta should not be considered safe to the fetus, since a reference level in this tissue has not been established.

Because exposure to inorganic Hg does not appear to be relevant in our study area, Hg accumulation in the placenta might have occurred through maternal fish consumption. In Spain, fish is known to be the main source of exposure to methyl-Hg (Ramon

Table 7
Summarized results of main studies published in the past decade (2001-2011) on placental concentrations of heavy metals (ng/g).

Country	N	Weight basis	Statistic	As	Cd	Cr	Hg	Mn	Pb	Reference
Spain	137	Wet	Detection (%)	ND	100	98.5	30.7	100	35.0	**Present study**
			Mean*		3.45	63.70	0.024	63.80	94.80	
			Median*		3.80	62.70	0.022	67.10	70.10	
Finland	130	Wet	Detection (%)	100	100	NA	98.5	NA	25.4	Leino et al. (in press)
			Median	5.68	3.70		2.31		13.1	
Turkey	83†	Dry	Detection (%)	NA	100	NA	NA	NA	NA	Tekin et al. (2012)
			Mean		20.83					
			Median		13.34					
Saudi Arabia	1578	Dry	Detection (%)	NA	70.2	NA	47.9	NA	96	Al-Saleh et al. (2011)
			Mean		45		64		579	
			Median		35		31		450	
Faroe Islands	15	Wet	Detection (%)	NA	100	NA	100	NA	100	Needham et al. (2011)
			Median		35		87		53	
Austria	31	Wet	Detection (%)	NA	NA	NA	45	NA	100	Gundacker et al. (2010)
			Median				1.9		25.8	
China	119†	Wet	Detection (%)	NA	100	100	NA	NA	100	Guo et al. (2010)
			Median		104.15	228.4			165.82	
India	44	Dry	Detection (%)	100	100	NA	NA	100	NA	Kippler et al. (2010)
			Mean	153	130			590		
			Median	61	110			530		
India	60	Wet	Detection (%)	NA	NA	NA	NA	NA	100	Singh et al. (2010)
			Mean						350	
			Median						300	
Croatia	109†	Wet	Detection (%)	NA	100	NA	NA	NA	100	Stasenko et al. (2010)
			Mean		10.3				20.0	
			Median		9.70				13.8	
Chile	20†	Dry	Detection (%)	100	100	NA	100	NA	100	Llanos & Ronco (2009)
			Mean	170	23		200		40	
Croatia	36†	Wet	Detection (%)	NA	100	NA	NA	NA	100	Klapec et al. (2008)
			Mean		7.99				46.59	
			Median		8.03				45.23	
Taiwan	65	Wet	Detection (%)	NA	NA	NA	100	NA	NA	Hsu et al. (2007)
			Mean				19			
			Median				18			
Brazil	100	Wet	Detection (%)	NA	NA	NA	100	NA	NA	Marques et al. (2007)
			Median				8.10			
Turkey	30†	Wet	Detection (%)	NA	100	NA	NA	NA	NA	Sorkun et al. (2007)
			Mean		38					
Turkey	30†	Wet	Detection (%)	NA	100	NA	NA	NA	100	Kutlu et al. (2006)
			Mean		0.29				2.79	
Chile	10†	Wet	Detection (%)	NA	100	NA	NA	NA	NA	Ronco et al. (2006)
			Median		4					
Chile	20	Dry	Detection (%)	NA	100	NA	NA	NA	NA	Ronco et al. (2005)
			Mean		20					
China	47	Dry	Detection (%)	NA	100	NA	NA	NA	NA	Zhang et al. (2004)
			Median		140					
Spain	71†	Dry	Detection (%)	NA	NA	NA	NA	NA	100	Falcón et al. (2003)
			Mean						103.2	
			Median						84.7	
Russia	249	Dry	Detection (%)	NA	100	NA	NA	100	100	Odland et al. (2003)
			Mean		40			260	-	
			Median		-			-	110	
Norway	322	Dry	Detection (%)	NA	100	NA	NA	100	100	
			Mean		30			190	-	
			Median		-			-	70	
Sweden	106	W et	Detection (%)	NA	100	NA	NA	NA	NA	Akesson et al. (2002)
			Median		4.8					
Sweden	119	Wet	Detection (%)	NA	NA	NA	100	NA	NA	Ask et al. (2002)
			Median				1.3 (I-Hg)			
							1.8 (Me-Hg)			
Croatia	27†	Wet	Detection (%)	NA	100	NA	NA	NA	100	Piasek et al. (2001)
			Mean		16.4				48.1	

* Concentrations above the limit of detection.
† Concentrations correspond to control subjects (*e.g.* without low birth weight); ND: Not detected; NA: Not analyzed; I-Hg: inorganic mercury; Me-Hg: methyl mercury.

et al., 2011), the form that contributes most to the accumulated body burden of Hg. In the 4-year follow-up of the present birth cohort, hair levels of total Hg were found to be higher than in children from other countries and were positively related to their fish intake (Freire et al., 2010). These hair Hg levels may also reflect exposure to the same dietary mercury sources as the mother, as it has been suggested (Marques et al., 2007).

4.3. Lead

Pb levels were in general higher than levels found in other European countries but were still lower than those reported in non-European regions. Thus, higher placental Pb concentrations have been reported in heavily-polluted areas in China, India, and Saudi Arabia (Al-Saleh et al., 2011; Guo et al., 2010; Singh et al., 2010),

whereas lower concentrations have been found in Finland, Austria, and Turkey (Gundacker et al., 2010; Kutlu et al., 2006; Leino et al., in press). Levels of placental Pb in the only study identified in Spain (Falcón et al., 2003) were in the same order of magnitude (median value of 84.7 ng/g dry weight basis) as the present results (median of 70.1 ng/g wet weight).

The low prevalence of Pb (35%) in the placentas, collected between 2000 and 2002, may be at least in part explained by the limitation on Pb in gasoline in Spain since 1999 (to 0.005 g/L) and its total prohibition since 2001 (Real Decreto 1728/1999, 1999). In this regard, a study on prenatal exposure to Pb in four other INMA mother–child cohorts detected Pb in only 5.9% of 1462 cord blood samples collected between 2004 and 2008 (Llop et al., 2011).

4.4. Chromium and Manganese

Few data are available on placental Cr and Mn concentrations. In the present study, these metals were both detected in almost all placentas. This is reasonable since Mn and Cr (trivalent form) are essential elements in humans. Cr and Mn were found at similar concentrations, which were lower than previous reports (Guo et al., 2010; Kippler et al., 2010; Odland et al., 2003).

Exposure to Cr and Mn may occur from natural or industrial sources and from hazardous waste sites (US-EPA, 2012b, 2012c) Regarding Cr, the general population is exposed mostly to trivalent Cr by eating food, drinking water, and inhaling air. Hence, considering that the population in the INMA-Granada cohort lives in an non-industrialized area, total Cr placenta levels may be mostly trivalent Cr, much less toxic than hexavalent Cr. The lack of information on human developmental toxicity of this metal prevents us from drawing stronger conclusions.

4.5. Arsenic

There are virtually no data on presence of As in human placenta, with only three published studies between 2000 and 2011. As was detected in 10 out of 200 placentas from the Ukraine (data not shown, mean value not available) (Zadorozhnaja et al., 2000) and in all 130 placenta samples (median of 5.68 ng/g wet weight) from a study in Finland (Leino et al., in press), while mean concentrations of 170 ng/g and 153 ng/g dry weight were found in 20 and 44 placentas in Chile (no prevalence data) and India, respectively (Kippler et al., 2010; Llanos and Ronco, 2009). Because no As was detected in the first third of the placenta samples, no further analysis of this metalloid was carried out.

According to the ATSDR (Agency for Toxic Substances and Disease Registry) (ATSDR, 2007a), food and water are by far the most important sources of human exposure to As, which can be naturally found in air, soil, and water. Our study area has no naturally occurring As contamination of groundwater, which is a serious public health problem in some countries (Wasserman et al., 2004). Other potential sources of As (industrial manufacture of batteries, military activities) have not been identified in the Granada metropolitan area or surroundings.

The main study limitation was the lack of information on potential sources of exposure to these heavy metals, such as maternal dietary intake and other lifestyle factors.

The use of placenta samples to investigate intrauterine exposure to heavy metals can be considered a strength of this study, largely because the placental passage represents the main access of chemical agents to the fetus. This chemical delivery relies primarily on blood flow to the placenta (Ginsberg et al., 2004), so that concentrations of chemicals in non-perfused placentas may be indicative of the internal dose of xenobiotics (Iyengar and Rapp, 2001). Moreover, because this highly accessible organ accumulates metals during pregnancy, it can be used to assess chronic metal exposure, avoiding repeated maternal blood sampling and other invasive biomonitoring (Esteban and Castaño, 2009). On the other hand, it has been suggested that placental xenobiotic metabolizing enzymes can detoxify or activate foreign chemicals by either enhancing or preventing cellular accumulation or transfer across the placenta (Myllynen and Vähäkangas, 2002). This makes the placenta a very sensitive matrix to examine the fate and effects of chemicals on the fetus.

5. Conclusion

Results of the present study confirm the feasibility of using the placenta to assess intrauterine exposure to heavy metals. Cd, Cr, and Mn were detected in almost all placenta samples from Spanish women with no history of occupational exposure. Pb and Hg, which are highly neurotoxic for the developing fetus, were detected in 35 and 31% of placentas, respectively. Placental concentrations of the five heavy metals studied were within the low-intermediate range of European reports. Further research is warranted to investigate the factors contributing to intrauterine exposure to heavy metals and the impact on infant development of the placental transfer of these toxic compounds.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2012.09.009.

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Discusión del Objetivo 2: *Estudio de la exposición materno-infantil a metales pesados.*

En el presente trabajo se ha caracterizado la exposición materno-infantil a metales pesados mediante la determinación de las concentraciones en un grupo de placentas seleccionadas aleatoriamente de las que constituyen la cohorte INMA de Granada. Los resultados obtenidos confirman la validez de la utilización de la placenta como matriz biológica para la evaluación de la exposición intrauterina a metales pesados. Además, ponen de manifiesto la presencia de Cd, Cr y Mn en prácticamente el 100 % de las placentas de mujeres sin antecedentes de exposición ocupacional, así como la presencia de metales neurotóxicos (Pb y Hg) en un tercio de ellas. La concentración más alta del grupo de metales investigados la alcanzó el Pb, seguida de Mn y Cr. Entre los factores de riesgo de exposición, fumar durante el embarazo y la edad gestacional presentaron una asociación con los niveles de Cd, mientras que no se encontró asociación entre los demás metales y otras características poblacionales.

La comparación de los resultados obtenidos con otras series de población general no es tarea fácil, fundamentalmente porque la información existente en la bibliografía científica corresponde principalmente a poblaciones con exposiciones ocupacionales o residentes en zonas contaminadas. No obstante es posible afirmar que en general, con la excepción del Pb, las concentraciones halladas se situaron en una posición intermedia-baja respecto a otros países europeos, y en una posición inferior con respecto a los datos obtenidos en países asiáticos. De forma detallada se presentan a continuación los resultados para cada uno de los residuos:

Cadmio: Los niveles obtenidos para este metal fueron similares a los observados en estudios de países europeos (Akesson *et al.*, 2002; Klapec *et al.*, 2008; Leino *et al.*, 2013; Stasenko *et al.*, 2010), y muy por debajo de los observados en países asiáticos (Guo *et al.*, 2010; Kippler *et al.*, 2010; Al-Saleh *et al.*, 2011). Se observó una asociación entre los niveles de Cd y el consumo materno de tabaco durante el embarazo, así como con la edad gestacional. Al igual que muestran otros estudios (Sorkun *et al.*, 2007; Stasenko *et al.*, 2010), el consumo materno de tabaco durante el embarazo contribuyó significativamente al aumento de los niveles de Cd en placenta. La asociación —que no alcanza la significación estadística— entre Cd y edad gestacional no permite establecer una hipótesis firme dado el bajo número de partos prematuros en la cohorte.

Mercurio: En la zona de estudio no existen fuentes relevantes de Hg inorgánico, por lo que los niveles hallados proceden, probablemente, de la dieta de la madre en forma de Hg orgánico. Ramón *et al.* (2011) señalan el pescado como una fuente de exposición a Hg orgánico. González-Estecha *et al.* (2013) confirman los altos niveles de este residuo en pescado enlatado. Es interesante destacar que durante el seguimiento realizado a los 4 años de los niños de la cohorte INMA de Granada se procedió a analizar los niveles de Hg en pelo infantil. Los resultados obtenidos mostraron concentraciones mayores a las de otros países y positivamente asociadas con el consumo de pescado (Freire *et al.*, 2010a). Sin embargo, en el presente estudio realizado en placentas, los niveles de Hg fueron menores a los observados en otros estudios (Ask *et al.*, 2002; Hsu *et al.*, 2007; Marques *et al.*, 2007; Llanos y Ronco, 2009; Gundacker *et al.*, 2010). A pesar de esto, una baja concentración de un metal con propiedades neurotóxicas no debería ser considerada como segura para el feto, dado que no existe un nivel de referencia establecido para la placenta.

Plomo: Los niveles observados fueron superiores a los encontrados en otros países europeos (Kutlu *et al.*, 2006; Gundacker *et al.*, 2010; Leino *et al.*, 2013), y del mismo orden que los valores encontrados previamente en España (Falcón *et al.*, 2003). No obstante la prevalencia fue solo del 35%, hecho que podría deberse, al menos en parte, a que el reclutamiento de la población se produjo entre 2000 y 2002, y la disminución de uso de la gasolina plomada en España comenzó en 1999 hasta prohibirse finalmente en 2001 (Real Decreto 1728/1999, 1999), determinando así diferencias sustanciales en la probabilidad de exposición. De hecho, estudios sobre los niveles de Pb en sangre de cordón umbilical en otras cohortes INMA reclutadas entre 2004 y 2008 mostraron una prevalencia de tan sólo el 5.9% (N = 1462) (Llop *et al.*, 2011).

Cromo y Manganeso: Por su comportamiento como elementos esenciales es razonable su alta prevalencia, cercana al 100%. Las concentraciones obtenidas son menores que las observadas en estudios similares, que pueden deberse a la falta de fuentes específicas de exposición a estos metales. En el caso del Cr, la población está generalmente expuesta a Cr en su forma trivalente a través de la dieta, agua y aire, por lo que al ser la cohorte INMA-Granada no ocupacionalmente expuesta y residente en zona no industrializada, los niveles encontrados deben corresponder en su mayor parte a Cr^{3+}, forma menos tóxica que la hexavalente.

Arsénico: Un análisis preliminar de estas placentas no reveló la presencia de As, lo que parece compatible con la falta de fuentes de exposición en la zona de estudio. La información sobre la prevalencia o frecuencia de éste metaloide en placenta es escasa, pudiendo observarse una prevalencia de sólo el 5% en placentas de Ucrania (Zadorozhnaja *et al.*, 2000), pero en el 100% de las placentas analizadas en Finlandia (Leino *et al.*, 2013), con unas concentraciones de 5.68 ng/g de placenta mientras que en Chile e India se encontraban unos niveles de 170 ng/g y 153 ng/g en peso seco, respectivamente (Llanos y Ronco, 2009; Kippler *et al.*, 2010).

Los resultados obtenidos, y la comparación con lo publicado al respecto en la literatura científica, permiten enunciar que es necesario continuar investigando los factores que condicionan la exposición intrauterina a metales pesados, así como el impacto en el neurodesarrollo infantil que puede suponer la transferencia placentaria de éstos contaminantes.

Resultados del Objetivo 3: *Estudio de los determinantes de la exposición materno-infantil a pesticidas organoclorados.*

Diferentes características sociodemográficas y de estilo de vida pueden conducir a disparidades en el riesgo de exposición a contaminantes ambientales. Se ha observado que variables como el estatus socioeconómico, determinado según la educación, ocupación y/o nivel de ingresos, condicionan la situación nutricional y de higiene. Por ello, las características socioeconómicas podrían suponer un papel importante en la caracterización del riesgo de exposición a contaminantes ambientales en población general (Borrell *et al.*, 2004; Porta *et al.*, 2008a; González-Galarzo *et al.*, 2009).

Desde 1970 se ha ido prohibiendo o limitando paulatinamente la utilización de muchos pesticidas organoclorados en Europa, aunque aún es legal el uso de algunos de ellos en países limítrofes, o se han prohibido recientemente, como es el caso de los endosulfanes (Endosulfan Preliminary Dossier, 2003; UNEP, 2003; Beard, 2006, PNA Convenio Estocolmo y Reglamento 850/2004, 2007). El sureste español es un área con una intensa actividad agrícola, por lo que la utilización de pesticidas ha sido muy amplia. Por esto, la población residente en estas zonas constituye un caso muy concreto de exposición a éstos contaminantes (Cerrillo *et al.*, 2005). La exposición de la población general ocurre principalmente por la dieta, pero se deben considerar fuentes diversas como la ambiental y farmacológica, así como otras específicas (Crinnion, 2009).

La persistencia de este tipo de compuestos en el tejido graso de las mujeres constituye un factor de riesgo para la descendencia, tanto durante la gestación como en la lactancia, lo que puede ocasionar efectos adversos en el desarrollo infantil o en el estado adulto (García, 2003).

En el presente trabajo se investiga la posible influencia de la clase social (y las variables sociodemográficas que la definen) sobre el riesgo de exposición a compuestos orgánicos persistentes durante el embarazo, por ser este un periodo de alta vulnerabilidad para el embrión/feto.

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Relationship between occupational social class and exposure to organochlorine pesticides during pregnancy

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ABSTRACT

Background: Little evidence is available on the influence of socioeconomic factors on exposure to persistent organic pollutants, especially during vulnerable periods such as pregnancy and early life.
Objective: To investigate the relationship of maternal social class with placental concentrations of organochlorine pesticides (OCPs) and their combined estrogenic activity measured with a biomarker of exposure.
Methods: Exposure to 16 OCPs (DDTs, endosulfans, and seven other compounds) and the total effective xenoestrogenic burden (TEXB) were analyzed in placentas from a mother–child cohort. OCP concentrations were quantified by gas chromatography and mass spectrometry, and TEXB was assessed with the E-Screen bioassay. Social class was classified according to maternal occupation. Multivariate regression analysis was conducted to examine variations in pesticide exposure and TEXB as a function of maternal social class in 257 subjects.
Results: Placental *p,p'*-DDT concentrations were higher in social classes III and IV than in classes I–II (the most affluent); concentrations of the sum of DDTs were higher in class IV; and exposure to the sum of endosulfans was greater in class III. HCB concentrations were higher among women in class IV than in classes I–II and among manual (classes III–V) than non-manual workers. However, the trend across social classes was only statistically significant for HCB. Social class significantly explained 10% of the variability in concentrations of the sum of endosulfans.
Conclusion: There is a need to explore whether more disadvantaged populations suffer higher levels of exposure to pesticides or other environmental chemicals and how different social processes contribute to this exposure.

1. Introduction

Persistent organic pollutants (POPs) are highly lipophilic and degradation-resistant synthetic chemicals that bioaccumulate in living organisms and the environment. Human exposure to POPs has been widely investigated and has been associated with adverse clinical effects at concentrations that were traditionally considered safe (UNEP, 2003; Porta et al., 2008a). Organochlorine pesticides (OCPs) are a group of POPs some of which have been demonstrated to interfere with the hormonal system, showing estrogenic (Soto et al., 1994; Fernández et al., 2004) and anti-androgenic (Kang et al., 2004; Kojima et al., 2004) activities. Worldwide and intensive use of OCPs has led to widespread contamination of the environment, and their presence is still detected in tissues, e.g., in Southern Spanish adult populations (Carreño et al., 2007), pregnant and breastfeeding women (Cerrillo et al., 2005; Lopez-Espinosa et al., 2007), and infants (Cerrillo et al., 2005; Lopez-Espinosa et al., 2008; Mariscal-Arcas et al., 2010).

Most OCPs have been restricted or banned in Europe since the 1970s, although some remain in legal use in various countries or have only recently been banned, including endosulfans (Endosulfan Preliminary Dossier, 2003; Beard, 2006; Stockholm Convention on POPs, Regulation 850/2004, 2007; UNEP, 2003). Southern Spain has the largest area of intensive greenhouse agriculture in Europe, where very large amounts of pesticides are used. Therefore, the

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population living in this area constitutes a special case of exposure to these chemical compounds (Cerrillo et al., 2005).

The relationship between environmental pollution and social disparities has become a priority research objective (O'Neill et al., 2003; Morello-Frosch and Shenassa, 2006; Weiss and Bellinger, 2006). Socioeconomic status, as determined by education, occupation and/or income, is associated with inequalities in hygiene and nutritional conditions and in residential and home environments, among others. It has been suggested that social and economic circumstances play an important role in exposure to POPs (Borrell et al., 2004; Porta et al., 2008b; González-Galarzo et al., 2009). However, there are few data on the social determinants and mechanisms of this exposure (Borrell et al., 2004; Porta et al., 2008b) or on the potential confounding effect of socioeconomic conditions on the association between POP exposure and health.

Furthermore, the factors that influence the bioaccumulation of POPs in humans are poorly understood. In the case of OCPs, exposure largely derives from the ingestion of contaminated food, but environmental, occupational and other domiciliary sources have also been implicated (Crinnion, 2009). Exposed occupational groups include agricultural employees and pesticide production workers, among others (García, 1998, 2003). The life-long accumulation of these compounds in the fat tissue of women is a major source of exposure for offspring, both during gestation and breast-feeding, which may lead to harmful effects on development and health during childhood and adult life (García, 2003).

The present study aimed to investigate the relationship of maternal occupational social class with placental concentrations of 16 OCPs and their combined estrogenic activity (TEXB) in a mother–child cohort from Granada province (Southern Spain). We hypothesized that mothers with a lower socioeconomic level would have higher exposure to OCPs. This investigation is part of the INMA – *Infancia y Medio Ambiente* (Child Health and Environment) network of population-based birth cohorts in Spain (Ribas-Fitó et al., 2006).

2. Material and methods

2.1. Subject recruitment

Data for this study derived from the INMA cohort established in the geographical area served by the San Cecilio University Hospital of Granada (Lopez-Espinosa et al., 2007). Briefly, from October 2000 to July 2002, 700 mother–son pairs were recruited at the time of delivery with the initial aim of investigating chronic exposure to endocrine disrupting chemicals and urogenital malformations in newborn boys. Exclusion criteria were: the maternal presence of serious chronic disease, such as diabetes, hypertension, or thyroid disease; a pregnancy complication that could affect growth or development; and non-residence in the hospital reference area (Fernández et al., 2007). The study was approved by the Institutional Ethical Committee of the Hospital, and signed informed consent was obtained from the eligible women who agreed to participate.

2.2. Laboratory analysis

2.2.1. Extraction and quantification of OCPs

Placentas were collected at delivery and were immediately coded, frozen, and stored at −86 °C until processing. Bioaccumulated compounds were extracted from samples by a previously described method (Fernández et al., 2007; Lopez-Espinosa et al., 2007). Briefly, 1.6 g of placenta homogenate was dissolved in hexane and eluted in a glass column filled with Alumine (Merck, Darmstadt, Germany). The tissue extract obtained was concentrated at reduced pressure under nitrogen stream and then injected into the preparative high-pressure liquid chromatography (HPLC) and eluted by a specific gradient with two mobile phases, collecting the alpha (min − 1 to min − 11) and beta (min − 13 to min − 32) fractions. This methodology was designed to extract all accumulated lipophilic pollutants and separate them from endogenous hormones (Fernández et al., 2004, 2007, 2008). It has been demonstrated that the alpha fraction contains DDT isomers and metabolites, endosulfan isomers and metabolites, aldrin, endrin, dieldrin, lindane, hexachlorobenzene (HCB), methoxychlor, and mirex, among other persistent environmental chemicals with estrogenic effect (Fernández et al., 2007, 2008). The beta fraction contains natural endogenous hormones together with phytoestrogens and non-halogenated xenoestrogens (Fernández et al., 2004; Lopez-Espinosa et al., 2009).

One microliter of the α fraction was dried, dissolved in *n*-hexane, and then injected into a gas chromatography apparatus with electron-capture detection (GC/ECD) and mass spectrometry (GC/MS) to analyze the presence and concentration of 16 OCPs in 308 randomly selected placenta tissue samples (Fernández et al., 2007; Lopez-Espinosa et al., 2007). The limits of detection (LD) for the studied chemicals ranged from 0.1 to 3.0 ng mL^{-1}. All exposures are expressed in ng g^{-1} placenta. Placental lipid content was gravimetrically quantified.

2.2.2. Evaluation of estrogenicity in placenta tissue samples

A biomarker of estrogenicity, the total effective xenoestrogenic burden (TEXB), was developed to measure the cumulative estrogenic effect of chemicals extracted from human specimens by determining the combined effect of the xenoestrogens (Fernández et al., 2004). The proliferative effect of the fractions (TEXB-α and TEXB-β) is estimated using the E-Screen bioassay, comparing the cell yield between cultures of MCF-7 human breast cancer treated with estradiol (positive control) and those treated with different dilutions of tissue extracts (Fernández et al., 2004). Finally, the xenoestrogenic effect was calculated as the ratio between the highest cell yield obtained with 100 pM of estradiol and the proliferation of hormone-free control cells, and expressed as estradiol equivalent units (Eeq) per gram of placenta. The limit of quantification (LQ) of the TEXB was defined as the concentration needed to produce a significantly different proliferative effect from that observed in control cells.

2.3. Information on characteristics of study population and occupational social class

Sociodemographic, health, and reproductive data were collected using a questionnaire administered after delivery to the mothers and by reviewing medical records. Covariates considered in the statistical analysis were: maternal age and education, place of residence (urban: city of Granada; metropolitan: towns of >20 000 inhabitants in city residential belt; sub-urban: towns of 10 000–20 000 inhabitants; rural: <10 000 inhabitants), marital status, tobacco habit and passive smoking during pregnancy, gestational age (calculated from the date of last menstrual period), parity (number of previous liveborn infants), body mass index (BMI) (calculated from pre-pregnancy weight and height), and weight gain during pregnancy.

Occupations were coded according to the Spanish National Classification of Occupations 1994 (CNO-94), which is based on the international ISCO88 coding system (www.ine.es). Occupational social class was assigned from the Spanish classification (Domingo-Salvany et al., 2000), which is based on the Goldthorpe scheme (Regidor, 2001). Five social class categories were then created: I, managers of companies with ⩾10 employees, senior technical staff, free professionals; II, managers of companies with <10

employees, intermediate occupations; III, administrative personnel and financial management supporting professionals, self-employed professionals, supervisors of manual workers, other skilled non-manual workers; IV, skilled and partly skilled manual workers; and V, unskilled manual workers. When multiple classifications of the occupation were possible, the highest class was assigned. Due to the low proportion of women in social class I, categories I and II were merged. In statistical analyses, subjects were grouped in two ways: in four categories (social classes I–II, III, IV and V); or as non-manual (classes I, II and III) or manual (classes IV and V) workers (Álvarez-Dardet et al., 1995).

For women employed during pregnancy ($n = 247$), the longest held job was considered when coding their social class; for women not employed during pregnancy ($n = 80$), the last occupation before pregnancy was used; and for women not employed in the 3 years previous to conception ($n = 148$), the most recent occupation of the father was taken. Information on social class was obtained from 475 women out of the 506 women in the cohort ($n = 700$) who completed the questionnaire.

The present analyses were limited to women for whom data were available on social class and covariates and on placental OCP concentrations and TEXB levels ($n = 257$). No differences in any study characteristics were found between this subset and the women for whom exposure information was not available ($n = 249$) (data not shown).

2.4. Statistical analysis

Descriptive statistics were obtained for each covariate, calculating means and standard deviations (SD) for continuous variables and proportions for categorical variables. Bivariate analysis with occupational social class was conducted by one-way analysis of variance and Kruskal–Wallis or Mann–Whitney test for normally and non-normally distributed quantitative variables, respectively. Chi-square test was applied to assess the relationship between two categorical variables. Concentrations of *p,p′*-DDE, the sum of DDT isomers/metabolites (∑DDTs), and sum of endosulfan isomers/metabolites (∑endosulfans), which all had a detection frequency ⩾80%, showed non-normal distributions and were transformed into natural logarithms and treated as continuous variables (a value of half the LD was considered for levels below the LD). Concentrations of the remaining pesticides and TEXB were treated as dichotomous variables, using the LD as cut-off.

Linear and unconditional logistic regression analyses were performed to explore variations in placental levels of OCPs and TEXB. Models were built with (i) covariates, (ii) covariates and social class in four categories (I–II, III, IV and V) and (iii) covariates and social class in two categories (non-manual and manual workers). The main effects of all predictors were independently explored in the models. Covariates evaluated were those associated with social class in bivariate analysis and those reported to influence levels of organochlorine compounds in the literature: maternal age (years), duration of gestation (weeks), smoking during pregnancy (yes/no), place of residence (urban/metropolitan/sub-urban/rural), parity (primiparous/multiparous), and BMI ($kg\,m^{-2}$). These variables were retained in the final adjusted models as confounders if they yielded a change of >10% in the effect estimate of social class. Maternal education was analyzed separately as a surrogate for social class. OCP concentrations (as continuous variable) were adjusted by g of placenta ($ng\,g^{-1}$), and the lipid content of placenta extracts was included as a separate term in the regression models, since it was recently shown that lipid standardization models are highly prone to bias (Schisterman et al., 2005). The variability in exposure explained by social class and covariates was compared by ANOVA *F*-test (R^2 change) in linear regression, while the likelihood ratio chi-square test (LR test) was used to evaluate the improvement in logistic model fit before and after adjustment for social class. The level of statistical significance was set at 0.05. STATA version 9.0 (Corporation, College Station, Texas) was used for the analyses.

3. Results

Table 1 shows the characteristics of the women by social class; out of the 257 mothers, 37 (14.4%) were classified in social classes I–II (15 in class I and 22 in class II), 35 (13.6%) in social class III, 151 women (58.8%) in class IV, and 34 (13.2%) in social class V. Hence, there was a higher prevalence of manual workers (72%) than non-manual workers (28%). Most of the mothers in this study were 25–35 years of age and had a stable partner. Around half of them had only primary education, were primiparous and declared that they were not passive smokers during pregnancy; 26% of women smoked during pregnancy; and 20% lived in a rural setting. Mean (SD) pre-pregnancy BMI was 23.5 (4.1) $kg\,m^{-2}$, weight gain during pregnancy was 13.0 (5.1) kg, and gestational length was 39.5 (1.3) weeks. Some characteristics of study population varied among social class categories. Among women in social classes IV and V, a lower proportion had university education and lived in an urban area, and their mean age was lower compared with those in classes I–II and III.

Percentage of placenta samples with detectable levels of OCPs ranged from 20.2% (dieldrin) to 92.2% (*p,p′*-DDE). There were statistically significant differences in concentrations of most OCPs and TEXB between primiparous and multiparous women (data not shown). BMI showed marginally-significant associations ($p < 0.1$) with *p,p′*-DDT, endosulfan-sulfate, and endosulfan-ether levels and a significant association ($p = 0.004$) with ∑endosulfans (data not shown). Higher levels of exposure to *p,p′*-DDT, HCB, ∑endosulfans and ∑DDTs were found in intermediate and upper social classes (*p*-trend for HCB = 0.03), and the placenta extracts were less frequently positive for TEXB-β in women from social class V than in those from lower classes (Table 2). There were no substantial differences across classes in concentrations of the other OCPs or in TEXB-α levels.

Multivariate linear regression analysis showed that adjusted concentrations of ∑DDTs were higher in class IV than in classes I–II (regression coefficient, 1.60), whereas concentrations of ∑endosulfans were higher in class III (regression coefficient, 0.48) (Table 3). The odds of having a higher *p,p′*-DDT level were greater in women in social classes III and IV than in classes I–II (odds ratios, 3.34 and 2.14, respectively); HCB was more frequent in class IV (odds ratio, 3.05), and TEXB-β was higher in class III (odds ratio, 1.25) than in classes I–II. In contrast, endosulfan-lactone and dieldrin showed lower odds for concentrations > LD in class IV (odds ratios, 0.46 and 0.40, respectively). The linear trend across social classes was significant for HCB ($p = 0.04$) and marginally significant for TEXB-β ($p < 0.1$). Furthermore, manual workers had significantly higher concentrations of HCB in placenta (odds ratio of 2.19 for concentration > LD) in comparison to non-manual workers (Table 4).

Covariates alone explained 5% or less of the variability in placental concentrations of *p,p′*-DDE, ∑DDTs, and ∑Endosulfans. When maternal social class (four categories) was entered in the models, coefficients of determination (R^2) were higher, but the R^2 change was only significant for concentrations of ∑Endosulfan, for which 10% of the variability was explained by covariates jointly with social class. The inclusion of social class also improved the fit of logistic models for HCB (LR test *p*-value = 0.05), and TEXB-β ($p = 0.04$) in comparison to models with covariates alone. Consideration of social class as non-manual/manual worker only

Table 1
Characteristics of study population by social class, INMA-Granada cohort, 2000–2002.

Characteristics of women	Total	Occupational social class				*p*-value
	(*N* = 257)	I–II (*N* = 37)	III (*N* = 35)	IV (*N* = 151)	V (*N* = 34)	
Age (years) (mean, SD)	30.2 (4.9)	32.4 (3.8)	31.2 (4.6)	29.6 (4.9)	29.4 (5.6)	0.009[a]
Education (%)						<0.001[c]
Primary	130 (50.6)	16.2	20.0	61.6	70.6	
Secondary	89 (34.6)	35.1	48.6	33.8	23.5	
University	38 (14.8)	48.6	31.4	4.6	5.9	
Marital status (%)						0.42[c]
With stable partner	241 (93.8)	94.6	91.4	95.4	88.2	
Without stable partner	16 (6.2)	5.4	8.6	4.6	1.8	
Place of residence (%)						0.002[c]
Rural	51 (19.8)	5.4	17.1	25.8	11.8	
Sub-urban	50 (19.4)	16.2	11.4	23.8	11.8	
Metropolitan	106 (41.2)	37.8	48.6	36.4	58.8	
Urban	50 (19.4)	40.5	22.9	13.9	17.6	
Smoking during pregnancy (%)						0.22[c]
No	191 (74.3)	73.0	88.6	71.5	73.5	
Yes	66 (25.7)	27.0	11.4	28.5	26.5	
Passive smoking during pregnancy (%)						0.70[c]
No	118 (45.9)	48.6	54.3	43.7	44.1	
Yes	139 (54.1)	51.3	45.7	56.3	55.9	
Parity (%)						0.73[c]
Primiparous	114 (44.4)	51.3	42.9	43.7	50.0	
Multiparous	143 (55.6)	48.6	54.3	56.3	50.0	
Body mass index (kg m^{-2}) (mean, SD)	23.5 (4.1)	23.1 (4.2)	22.4 (2.7)	23.9 (4.2)	23.4 (4.4)	0.23[b]
Weight gain during pregnancy (kg) (mean, SD)	13.0 (5.1)	12.5 (4.5)	12.5 (4.6)	13.1 (5.2)	13.9 (5.6)	0.61[b]
Gestational age (weeks) (mean, SD)	39.5 (1.3)	39.5 (1.4)	39.5 (1.3)	39.5 (1.3)	39.1 (1.2)	0.36[b]

SD: standard deviation.
Social class groups: I, managers of companies with ⩾10 employees, senior technical staff, free professionals; II, managers of companies with <10 employees, intermediate occupations; III, administrative personnel and financial managers, self-employed professionals, supervisors of manual workers, other skilled non-manual, workers; IV, skilled and partly skilled manual workers; and V, unskilled manual workers.
[a] ANOVA *p*-value.
[b] Kruskal–Wallis test *p*-value.
[c] Chi-square *p*-value for the comparison of social class categories I–II, III, IV, V.

Table 2
Placental concentrations of organochlorine pesticides (OCPs) and their combined estrogenic effect (TEXB) (% > limit of detection) by maternal social class (*N* = 257).

OCPs/TEXB	% > LD	Occupational social class				*p*-value[a]	*p* for trend
		I–II (*N* = 37)	III (*N* = 35)	IV (*N* = 151)	V (*N* = 34)		
o,p'-DDT	51.4	45.9	57.1	50.3	47.1	0.74	0.61
p,p'-DDT	45.1	29.7	60.0*	47.0*	38.2	0.06	0.58
p,p'-DDE (median, SD)[b]	1.6 (6.5)	1.2 (2.3)	1.3 (3.6)	1.9 (7.5)	1.9 (6.4)	0.33	0.12
o,p'-DDD	47.1	51.3	48.6	48.3	35.3	0.51	0.27
∑DDTs (median, SD)[b]	4.2 (10.1)	2.6 (6.7)	4.6 (9.9)	4.6 (10.6)*	4.9 (8.3)	0.27	0.09
E-I	52.9	48.6	51.4	55.0	50.0	0.91	0.80
E-II	32.7	35.1	40.0	31.8	26.5	0.66	0.32
E-ether	48.6	54.0	37.1	52.3	38.2	0.20	0.61
E-sulfate	48.6	48.6	34.3	51.7	50.0	0.33	0.43
E-lactone	35.0	45.9	25.7*	33.1	41.2	0.25	0.68
∑Endosulfans (median, SD)[b]	4.8 (10.0)	4.9 (11.5)	1.9 (6.1)	6.1 (10.1)*	4.5 (22.5)	0.16	0.55
Aldrin	28.0	18.9	28.6	29.8	29.4	0.62	0.26
Endrin	34.6	32.4	25.7	37.7	32.4	0.56	0.55
Dieldrin	20.2	29.7	17.1	18.5	20.6	0.47	0.30
Lindane	74.3	75.7	71.4	72.2	85.3	0.44	0.54
HCB	42.4	24.3	34.3	49.7*	38.2	0.02	0.03
Methoxychlor	30.7	29.7	22.9	31.8	35.3	0.69	0.43
Mirex	25.3	29.7	31.4	23.8	20.6	0.65	0.25
TEXB-α	72.0	64.9	77.1	72.2	73.5	0.69	0.50
TEXB-β	83.3	86.5	91.4	84.1	70.6*	0.16	0.10

SD: standard deviation; LD: limit of detection.
DDT: 1,1,1-trichloro-2,2-bis(4-chlorophenyl)-ethane; ∑DDTs: sum of *o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDE, and *o,p'*-DDD; E: endosulfan; ∑Endosulfans: sum of E-I, E-II, E-ether, E-sulfate, and E-lactone; HCB: hexachlorobenzene; TEXB-α: total effective xenoestrogenic burden of the α fraction; TEXB-β: TEXB of the β fraction.
[a] Kruskal–Wallis or chi-square test *p*-value for the comparison of social class categories I–II, III, IV, V.
[b] Continuous concentrations (ng g^{-1} placenta); values <LD were assigned a value half the LD.
* Mann Whitney or chi-square *p*-value <0.05 for the comparison with social class categories I–II.

Table 3
Association of maternal social class with placental concentrations of organochlorine pesticides (OCPs) and their combined estrogenic effect (TEXB)*.

OCPs/TEXB	β	95% CI	OCPs/TEXB	OR	95% CI
p,p′-DDE		*o,p′*-DDT			
Class III	1.00	−0.59; 1.70	Class III	1.48	0.57; 3.85
Class IV	1.30	−0.85; 1.97	Class IV	1.23	0.57; 2.65
Class V	1.25	−0.74; 2.10	Class V	1.36	0.51; 3.58
	p-trend	0.20		*p*-trend	0.68
∑DDTs		*p,p′*-DDT			
Class III	1.24	−0.72; 2.14	Class III	3.34	1.23; 9.10
Class IV	1.60	0.94; 2.21	Class IV	2.14	1.01; 4.87
Class V	1.30	−0.76; 2.23	Class V	1.58	0.57; 4.39
	p-trend	0.20		*p*-trend	0.50
∑Endosulfans		*o,p′*-DDD			
Class III	0.48	0.22; 1.02	Class III	0.96	0.37; 2.50
Class IV	0.84	−0.46; 1.54	Class IV	0.89	0.41; 1.93
Class V	0.76	−0.36; 1.62	Class V	0.51	0.19; 1.39
	p-trend	0.94		*p*-trend	0.24
	OR	95% CI		OR	95% CI
E-I			E-sulfate		
Class III	1.05	0.40; 2.76	Class III	0.48	0.18; 1.29
Class IV	1.25	0.57; 2.72	Class IV	1.01	0.47; 2.19
Class V	0.96	0.36; 2.54	Class V	0.93	0.35; 2.46
	p-trend	0.92		*p*-trend	0.63
E-II			E-lactone		
Class III	1.05	0.39; 2.84	Class III	0.36	0.13; 0.99
Class IV	0.69	0.30; 1.57	Class IV	0.46	0.21; 0.91
Class V	0.51	0.17; 1.48	Class V	0.62	0.23; 1.68
	p-trend	0.13		*p*-trend	0.34
E-ether			Aldrin		
Class III	0.56	0.21; 1.48	Class III	1.64	0.52; 5.15
Class IV	0.98	0.45; 2.12	Class IV	1.85	0.72; 4.76
Class V	0.55	0.20; 1.46	Class V	1.89	0.61; 5.92
	p-trend	0.62		*p*-trend	0.24
Endrin			Dieldrin		
Class III	0.65	0.22; 1.90	Class III	0.37	0.11; 1.27
Class IV	1.41	0.62; 3.17	Class IV	0.40	0.16; 0.99
Class V	1.00	0.36; 2.80	Class V	0.47	0.15; 1.53
	p-trend	0.48		*p*-trend	0.15
Lindane			HCB		
Class III	0.66	0.22; 1.96	Class III	1.57	0.54; 4.58
Class IV	0.66	0.27; 1.61	Class IV	3.05	1.27; 7.31
Class V	1.72	0.49; 6.01	Class V	1.94	0.67; 5.63
	p-trend	0.67		*p*-trend	0.04
Methoxychlor			Mirex		
Class III	0.68	0.22; 2.05	Class III	1.24	0.43; 3.52
Class IV	1.23	0.53; 2.85	Class IV	0.71	0.29; 1.71
Class V	1.46	0.52; 4.09	Class V	0.71	0.23; 2.20
	p-trend	0.30		*p*-trend	0.32
TEXB-α			TEXB-β		
Class III	1.59	0.55; 4.64	Class III	1.25	1.12; 5.37
Class IV	1.20	0.52; 2.76	Class IV	0.83	0.27; 2.59
Class V	1.37	0.47; 3.96	Class V	0.39	0.11; 1.40
	p-trend	0.73		*p*-trend	0.09

β: regression coefficient; OR: odds ratio; CI: confidence interval.
Logistic regression models used concentrations > LD as dependent variable. Linear models used log-transformed concentrations of *p,p′*-DDE, ∑DDTs and ∑Endosulfans (ng g^{-1} placenta; g of lipids as a separate term in the model; concentrations < LD were assigned a value half the LD).
* Social classes I–II as reference. Adjusted for maternal age, place of residence, parity and BMI.

significantly improved the fit of logistic models for HCB (LR test *p*-value = 0.02).

Although related to social class (Table 1), formal achieved education (primary, secondary or university) was not associated with concentrations of the compounds in multivariate models, and neither variable altered estimates of models (data not shown).

Table 4
Association of maternal social class (non-manual vs. non-manual) with placental concentrations of organochlorine pesticides (OCPs) and their combined estrogenic effect (TEXB)*.

OCPs/TEXB	Manual workers (social classes IV–V)	
	β	95% CI
p,p′-DDE	1.28	0.94; 1.76
∑DDTs	1.26	0.92; 1.75
∑Endosulfans	1.19	0.75; 1.88
	OR	95% CI
o,p′-DDT	1.03	0.57; 1.85
p,p′-DDT	1.07	0.60; 1.93
o,p′-DDD	0.81	0.45; 1.46
E-I	1.15	0.64; 2.07
E-II	0.64	0.34; 1.18
E-ether	1.16	0.64; 2.08
E-sulfate	1.42	0.79; 2.55
E-lactone	0.79	0.43; 1.46
Aldrin	1.43	0.73; 2.82
Endrin	1.60	0.85; 3.02
Dieldrin	0.65	0.31; 1.33
Lindane	0.97	0.50; 1.88
HCB	2.19	1.17; 4.11
Methoxychlor	1.53	0.79; 2.95
Mirex	0.64	0.33; 1.23
TEXB-α	0.98	0.52; 1.89
TEXB-β	0.61	0.26; 1.43

β: regression coefficient; OR: odds ratio; CI: confidence interval.
Logistic regression models used concentrations > LD as dependent variable. Linear models used log-transformed concentrations of *p,p′*-DDE, ∑DDTs and ∑Endosulfans (ng g^{-1} placenta; g of lipids as a separate term in the model; concentrations < LD were assigned a value half the LD).
LD: limit of detection; DDT: 1,1,1-trichloro-2,2-bis(4-chlorophenyl)-ethane; ∑DDTs: sum of *o,p′*-DDT, *p,p′*-DDT, *p,p′*-DDE, and *o,p′*-DDD; E: endosulfan; ∑Endosulfans: sum of E-I, E-II, E-ether, E-sulfate, and E-lactone; HCB: hexachlorobenzene; TEXB-α: total effective xenoestrogenic burden of the α fraction; TEXB-β: TEXB of the β fraction.
* Non-manual workers (social classes I–II–III) as reference. Adjusted for maternal age, place of residence, parity and BMI.

4. Discussion

This study found some association between occupational social class and exposure to certain OCPs during pregnancy in a subsample of women from the INMA-Granada cohort in Southern Spain. Placental levels of *p,p′*-DDT, HCB, ∑DDTs and ∑endosulfans were higher in more disadvantaged social classes (III and IV), independently of the maternal age, the current place of residence, BMI, and parity. In addition, the women in social class III showed a higher xenoestrogenic levels in the β fraction (TEXB-β) in comparison to classes I–II. However, only HCB appeared to show an increasing pattern in chemical concentrations across social class categories.

To our knowledge, few studies have explicitly evaluated the association between socioeconomic position and organochlorine compounds in pregnant women (James et al., 2002; Borrell et al., 2004; Glynn et al., 2007). Epidemiologic studies have long classified social class on the basis of occupation, but there has been little research on the relationship between body concentrations of organochlorine compounds and socioeconomic status in the general population (Porta et al., 2008a), and it has mainly focused on exposure to polychlorinated biphenyls (PCBs) (Daniels et al., 2003; Choi et al., 2006; Porta et al., 2008b). Moreover, epidemiological studies on pesticides have largely addressed exposure and health-related problems in males (García, 2003). The few available studies in populations non-occupationally exposed to organochlorine compounds have published controversial results, finding positive, inverse or no relationships between concentrations of the pollutants and education, income or other indicators of socioeconomic position.

Cerrillo et al. (2006) investigated the presence of multiple OCPs in adipose tissue in a sample of 458 women from the same region and found higher concentrations of *p,p′*-DDE in women with lower education. Higher *p,p′*-DDE levels were found in non-whites in the USA (Rogan et al., 1986), and greater concentrations of *p,p′*-DDT, *p,p′*-DDE, *o,p′*-DDT and ∑DDTs were reported in non-white pregnant women and in those with higher social position (James et al., 2002). Racial differences in body levels of contaminants in the two former studies were found to be clearly mediated by social class, although family income was not associated with DDE levels in African–American pregnant women (Borrell et al., 2004). In agreement with the present findings, Porta et al. (2008b) reported that exposure levels of *p,p′*-DDE, *p,p′*-DDT – among other chemicals – were higher among Spanish pancreatic cancer patients ($N = 135$) in social classes IV and V. Likewise, higher exposure to *p,p′*-DDT and *p,p′*-DDE was reported in social classes IV–V in women ($N = 520$) from the general population of Catalonia, Northern Spain (Porta et al., 2010). The production and use of DDT as an insecticide was banned in Spain in 1977, but it can still be utilized for vector control in some neighboring countries and is employed in the manufacture of some pesticides currently produced and used in Spain, such as dicophol (Porta et al., 2002). DDT was extensively used in Spain between the 1950s and the 1970s and it has been repeatedly shown that its residues remain present in human populations (Porta et al., 2002, 2008a). Nowadays, diet accounts for almost 90% of human exposure to DDTs (UNEP, 2003), and the differences in exposure found in our study could be explained by differences in dietary habits (both current and past) among social classes rather than by occupation-related factors themselves. For instance, the intake of fatty foods such as red meat or milk products may have contributed to cumulative exposure levels in the general population of this southern Spanish region (Mariscal-Arcas et al., 2007, 2010). The influences of age, parity and BMI, reported to be associated with *p,p′*-DDE levels (Wolff et al., 2007), were controlled for in our analysis.

Manual workers presented greater risk of higher exposure to HCB during pregnancy (OR = 2.19) than non-manual workers, in line with findings by Porta et al. (2008b, 2010) of higher HCB exposure levels in social classes IV–V. By contrast, higher levels of HCB were found in Swedish pregnant women with higher educational level (Glynn et al., 2007). A study in the same area as the present study found that adipose tissue HCB concentrations in women were predicted ($R^2 = 0.50$) by age, BMI, consumption of milk and cheese, and occupation in industry (Arrebola et al., 2009). Although the use of HCB was long ago phased out in most countries, it is still formed as a by-product or impurity in the manufacture of chlorinated solvents, chlorinated aromatics, and pesticides as well as in thermal processes, e.g., incineration (Barber et al., 2005; EFSA, 2006).

We found the sum of placental concentrations of endosulfans (commercial isomers I and II, and metabolites) to be higher in women in the intermediate class (class III). Exposure to endosulfan is of particular interest in our study area due to its recent use as an insecticide in agriculture in Southern Spain. Although endosulfan was banned in 2008 in Spain, the women in our study gave birth between 2000 and 2002; therefore, their placental levels could be explained by agriculture-related activities or residence in the vicinity of farming areas as well as by the intake of contaminated fruits and vegetables (Martinez Vidal et al., 1998; Arrebola et al., 1999; Cerrillo et al., 2005). Thus, a positive association between cord blood endosulfan-I levels and fruit intake was reported in 318 newborns from the same area as the one in the present study (Mariscal-Arcas et al., 2010). The relationship between exposure to endosulfan and social status has only been investigated in the aforementioned study by Cerrillo et al., 2006. They found that exposure to endosulfan-I was not associated with education level but with a longer period of residence in an urban setting. Interestingly, a study of populations from several Spanish regions found that the highest score of adherence to the Mediterranean diet (e.g., high intake of vegetables, fiber and grains, and low intake of saturated fat) was obtained by subjects of the intermediate social class (González et al., 2002). Taken together, these data suggest that the higher endosulfan level we observed in social class III is likely due to differences in dietary habits, i.e., a higher intake of fruit and vegetables.

Exposure to some of the studied OCPs in the present women may depend on specific sources of exposure (e.g., industrial HCB emissions) and on their socioeconomic background. Food and water contamination is expected to be the main source of pesticide exposure in the general population, but occupational exposure is more likely in women from low social classes – manual workers – than in those from higher classes (García, 2003). However, associations between socioeconomic status and past exposure to POPs may be complex and inconsistent, and differences in OCP levels by occupational social class in our study could be due to chance.

We found no association between social class and the xenoestrogenic burden of the α fraction, which contains no endogenous sex-hormones and is considered a marker of exposure to environmental organohalogenated estrogens (Fernández et al., 2007). One explanation of our finding is that differences in exposure among social classes were not observed for all of the OCPs analyzed. A case-control study in breast cancer performed in the same geographical area found no association between the TEXB-α and any of the 16 individual pesticide residues quantified in adipose tissue (Ibarluzea et al., 2004). Interestingly, it has been reported that TEXB-β levels were higher among women with secondary and university studies (Fernández et al., 2007). In our study, the women in social class III, who were less likely to have primary education, presented a higher TEXB-β level than classes I–II after adjustment for age and other covariates. The beta fraction contains endogenous sex steroids and more polar xenoestrogens, distinct from those eluted in the alpha-fraction, such as alkylphenols, phytoestrogens and bisphenol-A (Fernández et al., 2004). Therefore, TEXB-β is the result of interaction among endogenous estrogens and also contributes to the total environmental estrogenic burden. Hence, a plausible explanation is that women in intermediate and higher socioeconomic positions are more likely to use oral contraceptives, known to contribute to the TEXB-β. However, the biological meaning of TEXB-β has yet to be fully elucidated (Fernández et al., 2008). The HPLC extraction methodology favors the removal of lipophilic xenoestrogens; hence it may not be as effective for extracting endogenous sex steroids and more polar xenoestrogens.

Study strengths include the large sample size, the population-based design, the assessment of a large number of pesticides, and the use of a biomarker of exposure to estrogenic xenobiotics. However, we cannot rule out the possible confounding effect of characteristics of the women that were not taken into account in our study.

5. Conclusion

Placental concentrations of certain pesticides were higher in less affluent social classes among this group of women, although no clear pattern of distribution was observed. Further research is warranted to determine whether more disadvantaged populations suffer higher levels of exposure environmental chemicals and how different social processes contribute to this exposure.

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Discusión del Objetivo 3: *Estudio de los determinantes de la exposición materno-infantil a pesticidas organoclorados.*

En este trabajo, realizado para caracterizar el riesgo de exposición, se demostró la influencia de diferentes factores sociodemográficos y de estilo de vida sobre los niveles de *p,p'*-DDT, HCB, el total de DDTs y el total de endosulfanes medidos en placentas. Las concentraciones de todos ellos fueron mayores en mujeres pertenecientes a las clases sociales más bajas (III y IV) independientemente de la edad materna, el lugar de residencia, el IMC y la paridad. Además las mujeres pertenecientes a la clase social III mostraron mayores niveles de actividad estrogénica atribuible al efecto combinado de los xenoestrógenos colectados en la fracción β (en el ensayo TEXB), cuando se compararon con las mujeres de las clases sociales I y II. La actividad estrogénica de esta fracción está relacionada con los compuestos más polares y las hormonas endógenas.

La asociación entre estatus socioeconómico y compuestos organoclorados en embarazadas ha sido escasamente estudiada (James *et al.,* 2002; Borrell et al, 2004; Glynn *et al.*, 2007). Los estudios epidemiológicos han clasificado tradicionalmente la clase social según la ocupación laboral, pero no se ha incidido en la relación entre la carga interna de organoclorados y el estatus socioeconómico en la población general (Porta *et al.*, 2008a), y cuando se ha realizado se ha centrado principalmente en la exposición a PCBs (Daniels *et al.*, 2003; Choi *et al.*, 2006; Porta *et al.*, 2008b). Por otra parte, los estudios epidemiológicos sobre pesticidas se han centrado históricamente en los problemas de salud derivados de la exposición en hombres (García, 2003). Por todas estas razones, la información existente en población no ocupacionalmente expuesta a organoclorados muestra resultados dispares, pudiendo observarse relaciones positivas, negativas, o falta de asociación entre las concentraciones de contaminantes y la educación, ingresos u otros indicadores del estatus socioeconómico.

En 2006, el grupo de investigación en el que se desarrolla la tesis (Cerrillo *et al.* 2006) describió cómo en el tejido adiposo de mujeres de la misma área geográfica del presente estudio se encontraban mayores concentraciones de *p,p'*-DDE cuando el nivel educativo era inferior. Por el contrario, en 2002 James *et al.* encontraron concentraciones mayores de *p,p'*-DDT, *p,p'*-DDE, *o,p'*-DDT y total de DDTs en embarazadas de clases sociales más altas, así como diferencias raciales (mayores niveles en embarazadas no caucásicas). Este mismo patrón racial se había observado ya en 1986

(Rogan *et al.*), pudiendo comprobarse en ambos estudios que la carga interna de contaminantes estaba mediada por la clase social, a pesar de que el nivel de ingresos no se asocia con los niveles de DDE en embarazadas afroamericanas (Borrell *et al.,* 2004). En 2008 (b), Porta *et al.* encontraron en pacientes de cáncer pancreático que los niveles de *p,p'*-DDE y *p,p'*-DDT, entre otros contaminantes, fueron mayores entre las clases sociales IV y V. Así mismo se encontraron mayores niveles de éstos pesticidas en las clases IV y V en mujeres de población general de Cataluña (Porta *et al.,* 2010).

A pesar de que la utilización de DDT como insecticida se prohibió en España en 1977, aún puede utilizarse en varios países, y se emplea en la manufactura de otros pesticidas de uso común (Porta *et al.,* 2002). El DDT tuvo una amplia utilización en España entre los años 50 y 70, y se ha mostrado que sus residuos permanecen aún en la población (Porta *et al.,* 2002, 2008a). Hoy en día la principal fuente de exposición a DDTs es la dieta (UNEP, 2003), por lo que las diferencias encontradas entre las clases sociales podrían deberse a diferentes hábitos nutricionales entre éstas, más que a causas relacionadas con la ocupación. A este respecto, la alta ingesta de grasas podría contribuir a una exposición acumulada en la población general mayor en esta región (Mariscal-Arcas *et al.,* 2010). La edad, paridad e IMC se asocian con los niveles de *p,p'*-DDE (Wolff *et al.,* 2007), por lo que se controlaron en el presente análisis.

Las trabajadoras de ocupaciones manuales mostraron un riesgo mayor de altos niveles de HCB (OR = 2.19) cuando se compararon con las dedicadas a ocupaciones no manuales, en consonancia con las mayores concentraciones de HCB observadas por Porta *et al.* (2008b, 2010) en las clases sociales IV y V. En cambio, en embarazadas suecas los mayores niveles de éste contaminante se observaron en mujeres con mayor nivel educativo (Glynn *et al.,* 2007).

Más recientemente, un estudio del grupo de investigación en el que se desarrolla esta tesis (Arrebola *et al.,* 2009), realizado en la misma área geográfica (sureste español), muestra que en mujeres las concentraciones de HCB en tejido adiposo son dependientes (R^2 = 0.50) de la edad, el IMC, el consumo de leche y queso, y una ocupación industrial. A pesar de que se ha sustituido el uso de HCB en muchos países, aún forma parte de la manufactura de diversos compuestos, o como subproducto de procesos como la incineración (EFSA, 2006).

La concentración total de los diferentes isómeros del endosulfán y sus metabolitos fue mayor en las mujeres pertenecientes a la clase social III. La exposición a éstos en el área de estudio es de particular interés debido a su reciente utilización como insecticidas, prohibida en el 2008. Dado que el reclutamiento de la cohorte, y por tanto el embarazo, tuvo lugar entre 2000 y 2002, los niveles en placenta podrían estar relacionados con la proximidad a zonas agrícolas, o actividades relacionadas con la agricultura, así como por la ingesta de frutas y vegetales con gran carga de éstos contaminantes (Martínez Vidal *et al.,* 1998; Arrebola *et al.,* 1999; Cerrillo *et al.,* 2005). De hecho, un estudio en sangre de cordón umbilical de recién nacidos de la misma área geográfica mostraba una asociación entre los niveles de endosulfán-I y la ingesta de fruta (Mariscal-Arcas *et al.,* 2010).

Cerrillo *et al.* (2006) encontraron que la exposición a endosulfán-I no se asociaba con el nivel educativo, sino con largos periodos de residencia en zonas urbanas. Un estudio en varias regiones españolas que investigaba la adherencia a la dieta Mediterránea (alta ingesta de vegetales y fibra, y bajo consumo de grasas saturadas) mostraba que el mayor seguimiento de ésta se producía en las clases sociales intermedias. Estos datos sugieren que los altos niveles de endosulfan observados en la clase social III podrían deberse a hábitos alimenticios, como son un alto consumo de frutas y verduras. La dieta y el agua son las principales fuentes de exposición en población general, pero cabe destacar que la exposición ocupacional es más probable en mujeres de clases sociales bajas, por incluir trabajadoras manuales que puedan entrar en contacto con emisiones industriales (García, 2003). Sin embargo, la asociación entre el estatus socioeconómico y exposiciones pasadas a organoclorados puede ser compleja e inadvertida, y las diferencias observadas en el presente estudio podrían deberse al azar.

No se observa asociación entre la clase social y la carga de xenoestrógenos en la fracción α (en el ensayo TEXB), que dado que no contiene hormonas endógenas de la madre se puede considerar un marcador de la exposición ambiental a estrógenos órgano-halogenados (Fernández *et al.,* 2007). Un estudio que utilizó tejido adiposo de pacientes de cáncer de mama, residentes en la misma área geográfica, no encontró asociación entre TEXB-α y ninguno de los 16 pesticidas cuantificados (Ibarluzea *et al.,* 2004). Sin embargo, se ha podido constatar que los niveles de TEXB-β son mayores en mujeres con estudios secundarios y universitarios (Fernández *et al.,* 2007). En el presente estudio, las mujeres de la clase social III (más propensas a niveles educativos bajos)

mostraron mayores niveles de TEXB-β que las de clases sociales I y II, tras ajustar por edad y otras covariables. Ésta fracción β contiene las hormonas sexuales y xenoestrógenos más polares, como son los alquilfenoles, fitoestrógenos y BPA (Fernández *et al.*, 2004). Por esto, TEXB-β es el resultado de los estrógenos endógenos y una contribución a la carga estrogénica total. Una posible explicación a esta relación es que las mujeres de clases intermedias y altas utilicen más frecuentemente anticonceptivos orales, que contribuyen a la carga medida en TEXB-β. A pesar de esto el significado real de TEXB-β aún debe ser clarificado (Fernández *et al.*, 2008). La metodología de extracción mediante HPLC favorece la obtención de xenoestrógenos lipofílicos, no siendo quizá tan efectiva con la extracción de hormonas endógenas y xenoestrógenos más polares.

Resultados para Objetivo 4: *Estudio de la influencia de los niveles de TSH neonatal sobre el neurodesarrollo infantil.*

Las hormonas tiroideas juegan un papel fundamental en el neurodesarrollo fetal e infantil, así como en la regulación de la función neuropsicológica en niños y adultos (Williams, 2008). Diferentes estudios epidemiológicos señalan que una mala función tiroidea durante el embarazo, especialmente en etapas tempranas, puede afectar negativamente al neurodesarrollo fetal (Santiago-Fernández *et al.,* 2004). La deficiencia de yodo implica una inadecuada secreción de tiroxina (T4) por el tiroides, y es la causa más frecuente de déficit de hormonas tiroideas en la madre y en el feto, siendo por ello la suplementación del yodo una forma de prevenir retrasos mentales (De Escobar *et al.*, 2000).

Durante las últimas décadas se ha prestado atención al funcionamiento sub-óptimo a nivel cognitivo o de comportamiento en hijos de madres con moderadas deficiencias de yodo (Haddow *et al.,* 1999; Pop *et al.,* 1999; Santiago-Fernández *et al.,* 2004; Vermiglio *et al.,* 2004; De Escobar *et al.,* 2007; Berbel *et al.,* 2009; Velasco *et al.,* 2009), así como las implicaciones en el desarrollo del niño de una ligera elevación neonatal de los niveles de TSH (Oakley *et al.,* 1998; Simpson *et al.,* 2005). Por otra parte, las deficiencias tiroideas durante los últimos dos trimestres del embarazo y los primeros meses tras el parto pueden ocasionar también retrasos mentales y físicos, así como déficits neurológicos (Anderson, 2001). Las implicaciones neurológicas son menos severas en casos de hipotiroidismo neonatal que en prenatal, aunque los déficits en memoria e inteligencia parecen persistir (Zoeller y Rovet, 2004). A pesar de esto pocos estudios evalúan la influencia de desviaciones moderadas en la función tiroidea de neonatos, máxime en variaciones dentro del rango normal de hormonas tiroideas.

Se ha observado que la exposición a ciertos contaminantes ambientales puede interferir en el estatus tiroideo de la madre durante el embarazo, y con ello en la función tiroidea del recién nacido (Takser *et al.,* 2005; Wang *et al.,* 2005; Maervoet *et al.,* 2007; Chevrier *et al.,* 2008; Álvarez-Pedrerol *et al.,* 2009; López-Espinosa *et al.,* 2009b, 2009c). Por ello se ha especulado que algunos de los efectos neurotóxicos de la exposición temprana puedan resultar de la disrupción tiroidea (Portefield, 2000; Boas *et al.,* 2006). A pesar de ello, la influencia de la exposición temprana a disruptores

endocrinos en la función tiroidea y en el neurodesarrollo infantil no está suficientemente estudiada.

En un estudio previo del grupo de investigación en el que se desarrolla la presente tesis se analizó la influencia de la exposición a contaminantes organoclorados en el estatus de hormonas tiroideas neonatales (resultados no publicados). Se procede ahora a examinar la asociación entre los niveles neonatales de TSH y el desarrollo cognitivo infantil a los 4 años en los niños de la cohorte INMA-Granada, hipotetizando que los niños con niveles de TSH mayores al nacimiento (indicativos de peor funcionamiento tiroideo) podrían tener peores resultados en tests cognitivos.

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CLINICAL STUDY

Newborn TSH concentration and its association with cognitive development in healthy boys

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Abstract

Objective: An association between thyroid function during pregnancy or infancy and neurodevelopment in children has been demonstrated. We aimed to investigate whether newborn TSH concentrations are related to subsequent neurocognitive development.
Design: We conducted a longitudinal study on 178 children from a general population birth cohort in Granada (Spain) born in 2000–2002.
Methods: TSH concentrations were measured in umbilical cord blood, and cognitive functions were assessed at 4 years of age using the McCarthy's scales of children's abilities (MSCA). Organochlorine (OC) compound concentrations and the combined oestrogenicity (total effective xeno-oestrogenic burden (TEXB)) were also determined in the placentae.
Results: Mean newborn TSH was 3.55 mU/l (range=0.24–17 mU/l). In multivariate regression analyses, adjusting for maternal and child characteristics, higher newborn TSH concentrations showed a decrease of 3.51 and 3.15 points on the MSCA general cognitive and executive function scores respectively and were associated with a higher risk of scoring below the 20th percentile (P20) on the quantitative score (odds ratio (OR)=2.64). Children with TSH in the upper quartile (4.19–17.0 mU/l) were at higher risk of scoring <P20 on span memory (OR=5.73), whereas children with TSH in the second quartile (2.05–2.95 mU/l) were at lower risk of scoring <P20 on the verbal scale (OR=0.24). Neonatal TSH status was also associated with general cognitive and executive function outcomes when controlling for prenatal exposure to OCs or placental TEXB.
Conclusions: Newborn thyroid hormone status expressed by TSH in cord blood may adversely affect later cognitive function. A more thorough screening for neonatal thyroid deficiency is warranted.

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Introduction

Thyroid hormones (THs) are essential for the fetal and postnatal human development and for the regulation of neuropsychological function in children and adults (1). THs regulate the processes of neurogenesis, myelination, dendrite proliferation and synapse formation (2, 3). Although THs are required throughout gestation, the fetal thyroid gland does not produce its own TH in appreciable amounts until the third trimester (4, 5). Accordingly, an increasing number of epidemiological studies and case reports have strongly supported the notion that impaired maternal thyroid function during early gestation may result in poor fetal neurodevelopment (6–10).

Iodine deficiency (ID), which compromises adequate production and secretion of thyroxine (T_4) by the thyroid, remains the most frequent cause of maternal and fetal TH deficit and therefore of preventable mental retardation (5). Over the past two decades, attention has been drawn to the sub-optimal cognitive or behavioural functioning (which may be sub-clinical) observed in children born to mothers with even mild or moderate ID (10–16) or the developmental implications for children with slight neonatal elevations of TSH (17, 18). Thyroid deficiency during the last two trimesters of pregnancy and the first few months post delivery can also result in mental and physical retardation and sometimes neurological deficits, a condition known as cretinism (19). Neurological features are less severe in neonatal hypothyroidism than in prenatal hypothyroidism, although deficits in memory and intelligence quotient may persist (2). Overall, few studies have addressed whether subsequent development can be influenced by moderate thyroid dysfunction in neonates or even by variations within the normal range of TH levels.

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Recent studies indicate that exposure to certain environmental contaminants may also interfere with maternal thyroid status during pregnancy and with thyroid function in newborns (20–26). Hence, it has been speculated that some of the neurotoxic effects of early exposure to the environmental chemicals may result from thyroid disruption (27, 28). Nonetheless, the influence of early exposure to endocrine disruptors (ED) on thyroid function and therefore children's neurodevelopment remains to be elucidated.

In a previous study of a mother–child cohort, we analysed the influence on neonatal TH status of placental exposure to certain organochlorine (OC) pesticides with known ED activity (C Freire, M J Lopez-Espinosa, M F Fernández, J M Molina-Molina, R Prada and N Olea, unpublished observations). In this study, we examined the association of newborn TSH concentrations with cognition at 4 years of age in the same cohort (in Granada, Southern Spain). We hypothesised that infants with poorer thyroid status, manifested by higher TSH levels, would have lower scores in subsequent cognitive testing. This investigation is part of the 'Infancia y Medio Ambiente (Environment and Childhood) (INMA) Project', a prospective multicentre study in Spain (29).

Materials and methods

Subject recruitment

From 2000 to 2002, 700 eligible mother–son pairs registered at the San Cecilio University Hospital were enrolled at delivery, establishing the INMA-Granada cohort, with the initial aim of investigating chronic exposure to ED and urogenital malformations in newborn boys. Exclusion criteria were the maternal presence of serious chronic disease, such as diabetes, hypertension or thyroid disease; a pregnancy complication that could affect growth or development and a non-residence in the hospital referral area (30). In 2005–2006, 220 of the 700 boys aged 4 years and their mothers were randomly invited to participate in the physical examination and cognitive testing (31, 32).

Approval by committee for human subjects A written informed consent was obtained from parents before the study, which was approved by the Ethics Committee of the San Cecilio University Hospital, in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

The study was approved by the Institutional Ethical Committee of the Hospital, and signed informed consent was also obtained from the women who agreed to participate.

TSH determination

TSH was measured in a cord blood sample spotted on a filter paper (Schleicher & Schuell no. 2992), which is routinely obtained at delivery for the screening programme of neonatal congenital hypothyroidism (33). TSH concentrations were determined by using time-resolved sandwich fluoroimmunoassay (Auto-DELFIA, Perkin Elmer/Wallac, Turku, Finland) at the Centre for the Early Detection of Metabolopathies in Neonates in San Juan de Dios Hospital (Granada, Spain). The limit of detection (LD) was 0.01 mU/l. A cord blood level ≥ 14 mU/l is established in the Centre's laboratory to trigger the protocol for the study and for the confirmation of neonatal hypothyroidism.

Quantification of OC pesticides and oestrogenicity in the placenta

Placentas were collected at delivery from the cohort, and 17 OC pesticides (dichlorodiphenyltrichloroethane (DDT) isomers and metabolites, endosulphan isomers and metabolites, aldrin, endrin, dieldrin, lindane, hexachlorobenzene, methoxychlor and mirex) were extracted from tissue samples by a previously described method, which was developed to separate natural oestrogens (α fraction) from more lipophilic xeno-oestrogens (β fraction) without destroying either (34, 35).

OC pesticide concentration was determined in 308 randomly selected placenta samples by gas chromatography (GC) with electron-capture detection. The compounds were confirmed by GC and mass spectrometry (30). The LD for the studied chemicals ranged from 0.1 to 3.0 ng/ml. For levels below the LD, we considered a value of half the LD.

The total effective xeno-oestrogenic burden (TEXB) of the α fraction (α-TEXB) and the β fraction (β-TEXB) was estimated in the placenta samples by using the E-Screen bioassay (34, 35). The α-TEXB can be considered a marker of the TEXB of environmental organohalogenated oestrogens (35). The LD of TEXB was defined as the concentration needed to produce a significantly different proliferative effect from that observed in the control cells.

Cognitive testing

The neurocognitive evaluation of the children was performed by two specifically trained psychologists in the Paediatrics Department of our Hospital. Cognitive and motor abilities were assessed using a Spanish adaptation of the McCarthy scales of children's abilities (MSCA) (36), which gives standardised test scores for five domains (quantitative, verbal, memory, perceptual performance and motor). A general cognitive score, which estimates global intellectual function, was calculated by combining the verbal, perceptual

performance and quantitative scores. A strict protocol was applied to avoid inter-observer variability (37), which was <5%. Psychologists involved in the cognitive testing of the children were unaware of the design sequence of the study.

At the same time as the children were evaluated, the parents completed a self-reported questionnaire on parent-to-infant attachment and another questionnaire on mental health, considered as effect modifiers on infant mental development (38). The parent-to-infant attachment questionnaire consisted of 19 items that assessed the emotional bond of affection experienced by the parent towards the infant (39). The 12-item version of the general mental health questionnaire was used to identify psychological distress and short-term changes in parental mental health.

To further improve our understanding of the specific functions associated with neonatal TSH, the MSCA items were reorganised into the following new outcomes for tasks highly associated with specific neurocognitive functions: verbal memory (items 3 and 7II), working memory (items 5 and 14II), memory span or short-term memory (items 6, 7I and 14I), gross motor (items 9, 10 and 11), fine motor (items 12 and 13) and executive function (items 2, 5, 6, 14II, 15, 17 and 18) (32, 37).

Covariates

The attending paediatrician and trained interviewers gathered information at delivery and at the 4-year visit respectively on maternal age, alcohol consumption and cigarette smoking during pregnancy, reproductive history, parity, pre-pregnancy body mass index (BMI), duration of breastfeeding, maternal and paternal education, marital status and area of residence (urban: city of Granada; metropolitan: towns of >20 000 inhabitants in city residential belt; sub-urban: towns of 10 000–20 000 inhabitants; rural: <10 000 inhabitants). Information on gestational age and anthropometric measurements at birth were obtained from medical records. Covariates considered for inclusion in the statistical analysis were expressed as shown in Table 1.

Complete cognitive outcome data and information on cord blood TSH concentration and covariates were available for 178 subjects from the cohort ($n=220$). Information on the former and prenatal exposure to OCs and TEXB in the placenta was available for a subset of 101 of the 178 children (57%) in this study. No differences in any study characteristics were found between this subset and the children without TSH measurements ($n=42$) (data not shown).

Statistical analysis

MSCA general cognitive, perceptual performance and executive function scores were normally distributed and were standardised to a mean of 100 points with an S.D. of 15 to homogenise the scales. A cut-off point corresponding to the 20th percentile (P20) was used to categorise the outcomes with a non-normal distribution. We used simple linear regression or ANOVA to examine the relationship of covariates with general cognitive scores.

TSH values were transformed into natural logarithms (log transformed) to improve the normality. We used adjusted general additive models (GAM) to evaluate the linearity of the relationship between general cognitive scores and TSH levels, comparing models with TSH in a linear and a non-linear manner (a cubic smoothing spline with 2–4 degrees of freedom) by means of likelihood ratio tests (Fig. 1). Because no significant improvement in the model was obtained with non-linear models, we first treated TSH as a continuous variable. In a second analysis, TSH was categorised into quartiles.

The strength of the unadjusted and adjusted associations between the outcome scores and TSH levels was measured by calculating coefficients (β) and odds ratios (ORs) for linear and logistic regression models. Variables associated with the general cognitive score at a significance level of $P<0.20$ in the bivariate analysis or whose inclusion in the models changed TSH effect estimates by >10% were considered confounders. All multivariate models controlled for maternal age (≥32 years) and gestational age (continuous), regardless of their statistical significance. The potential confounding of exposure to OC and TEXB levels in the placenta was examined in a further regression analysis of the association of TSH with general cognitive and executive function outcomes. Concentrations of OCs and TEXB values were categorised using the LD cut-off points, except for *p,p′*-dichlorodiphenyldichloroethylene (*p,p′*-DDE) (detected in ≥90% of the placenta extracts), which was introduced as continuous (ng/g of placenta). In all the cases, the level of significance was $P\leq0.05$. STATA version 9.2 (STATA Corporation, College Station, TX, USA) was used for the analyses.

Results

Mean (S.D., range) newborn TSH was 3.55 (2.54, 0.24–17) mU/l, and only three newborns had a TSH level above 14 mU/l (laboratory reference value). Repeated measurement analyses confirmed the TSH levels of these three babies, none of whom were diagnosed with thyroid disorder. Mean (S.D.) infant birth weight was 3289 (487) g, and gestational length was 39 (1.8) weeks; maternal age at delivery was 31 (5) years, and pre-pregnancy BMI was 23.5 (4.0) kg/m^2. Around 22% of women smoked during pregnancy, 47% were primiparous, 85% breastfed the child, 15% of mothers and 16% of fathers had university education and 64% of the families lived in urban or metropolitan settings. TSH concentrations were slightly higher in infants with lower birth weight (by −0.66 mU/l per kg; 95% confidence interval (CI)=−1.4, 0.11;

Table 1 MSCA general cognitive scores by characteristics of the study population from the INMA-Granada cohort, 2000–2006 ($n=178$).

		General cognitive score[a]	
		Mean (s.d.)	*P* value*
Child variables			
Gestational age (weeks) (median)	39	–	0.05
Birth weight (g) (mean)	3289	–	0.17
Birth length (cm) (median)	51	–	0.04
Cord blood TSH (mU/l) (median)	2.95	–	0.52
Age at evaluation (months) (mean)	51	–	0.008
School term at evaluation (%)			0.002
3rd year, 3rd term	38.2	96.6 (15.2)	
4th year, 1st term	29.8	99.1 (13.0)	
4th year, 2nd or 3rd term	32.0	105.4 (13.5)	–§
Area of residence at evaluation (%)			0.05
Rural	15.2	97.8 (15.2)	
Sub-urban	20.8	96.8 (16.4)	
Metropolitan	48.3	100.3 (13.3)	
Urban	15.7	106.4 (13.0)	–†
Maternal variables			
Age at delivery (years) (median)	31	–	0.84
Parity (%)			<0.001
0	46.6	105.3 (12.0)	
1	38.8	95.5 (15.0)	–§
≥2	14.6	95.9 (15.1)	–‡
Educational level (%)			0.03
Up to primary school	15.2	95.8 (15.6)	
Secondary school	70.2	99.8 (13.7)	–†
University	14.6	106.3 (15.4)	–‡
Smoking during pregnancy (%)			0.74
No	77.5	100.3 (14.6)	
Yes	22.5	99.5 (14.1)	
Breastfeeding (%)			0.31
No	14.6	96.9 (16.3)	
Yes	85.4	100.1 (14.1)	
Mother-to-infant attachment score (mean)[b]	74.6	–	0.02
Maternal mental health score (median)[c]	10.0	–	0.67
Paternal variables			
Educational level (%)			<0.001
Up to primary school	20.8	96.5 (15.8)	
Secondary school	63.5	100.1 (13.3)	
University	15.7	105.4 (16.3)	–†
Father-to-infant attachment score (mean)[b]	74.6	–	0.13
Paternal mental health score (median)[c]	9.1	–	0.44

*P value for simple linear regression or ANOVA. †$P<0.05$; ‡$P<0.01$; §$P<0.001$ for test of difference in means with the first category as reference.
[a]Mean of the general cognitive score is 100, with a s.d. of 15.
[b]A higher score indicates a closer bond of affection.
[c]A higher score indicates greater psychological distress.

$P=0.09$), and infants of fathers with university education were substantially more likely to have lower TSH (by -1.27 mU/l; 95% CI$=-2.20, -0.34$; $P=0.007$) (data not shown). Newborn TSH levels did not differ by maternal age, gestational age, parity or other covariates. Mean (s.d., range) age of the boys at psychological testing was 51 (2, 47–58) months, and their general cognitive score was 100.1 (14.5, 56.9–131) points. This score was significantly higher among older children, urban children, those from primiparous mothers and those from parents with university education, and it was positively associated with gestational age, birth length and mother-to-infant attachment score at 4 years of age (Table 1). Perceptual performance scores ranged between 53.1 and 135; the range of the executive function was 58.3–133; verbal, 57.2–133 (22.5%, <P20); quantitative, 61.5–155 (23.6%, <P20); memory, 60.4–146 (19.7%, <P20); motor, 45.7–131 (20.2%, <P20); span memory, 59.2–133 (15.7%, <P20); working memory, 76.8–159 (42.7%, <P20); verbal memory, 54.4–161 (29.2%, <P20); gross motor, 69.2–137 (15.2%, <P20) and fine motor, 52.8–134 (15.7%, <P20) (data not shown).

Table 2 shows the unadjusted associations between the cord blood TSH levels and children's cognitive test scores. In accordance with our hypothesis, a higher TSH was related to lower MSCA scores. The risk of scoring

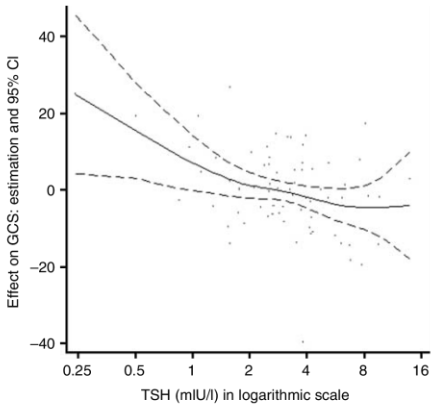

Figure 1 Cubic smoothing association between log-transformed TSH levels and general cognitive scores (GCS) in multivariate analysis.

<P20 on the quantitative scale was greater in children with higher TSH levels (OR=2.25; 95% CI=1.20, 4.25; $P=0.01$), but no significant association was found with any MSCA outcome score in crude analyses. By contrast, multivariate models adjusted for maternal and child characteristics showed an association between elevated newborn TSH levels as a continuous measure and poorer child functioning in general cognitive, executive function and quantitative areas (Table 3). Thus, higher TSH predicted lower child general cognitive score (by −3.52; 95% CI=−6.81, −0.23; $P=0.04$) and executive function score (by −3.15; 95% CI=−6.66, −0.19; $P=0.05$), and infants with higher cord blood TSH were more likely to score <P20 on the quantitative scale (OR=2.64; 95% CI=1.16, 5.54; $P=0.02$). These associations with the general cognitive and the quantitative outcomes were also observed when TSH status quartiles were considered. Lower test scores (general cognitive, −5.42; 95% CI=−11.30, −0.61; $P=0.05$; quantitative, OR=4.92; 95% CI=1.30, 16.47; $P=0.02$) were found for children with newborn TSH concentrations in the upper quartile (>4.19 mU/l) versus the first quartile (<2.05 mU/l), and the risk of delayed span memory function (<P20) was higher for children with newborn TSH in the upper quartile (OR=5.73; 95% CI=0.72, 24.67; $P=0.03$). Interestingly, newborn TSH in the second quartile had a positive effect on verbal scores (risk for scores <P20=0.24) in comparison with children with TSH in the lower quartile. Figure 1 depicts the GAM for the relationship between TSH concentrations and general cognitive scores, showing a positive linear trend.

With the exception of *p,p′*-DDE, detected in 92.1% of the placenta samples and with a mean (S.D.) concentration of 3.09 (6.50) ng/g placenta, the percentage detection of OC pesticides was <90% ($n=101$), ranging from 24.8% (dieldrin) to 84.2% (lindane) (data not shown). The TEXB of the α and β fractions were above the LD in 67.3 and 83.2% of the placenta extracts respectively. Table 4 shows that the negative associations of cord blood TSH status with the general cognitive score (by −6.34; 95% CI=−12.32, −0.36; $P=0.04$) and executive function score (by −7.85; 95% CI=−14.04, −1.67; $P=0.009$) were also present after simultaneous adjustment for prenatal exposure to the 17 OCs measured. After controlling for TEXB, the association remained significant for the general cognitive score (by −5.35, 95% CI=−10.24, −0.45; $P=0.03$) and was marginally significant for executive function score (by −5.05; 95% CI=−10.27, 0.18; $P=0.06$). However, contrary to expectations, the magnitude of the effect of TSH on cognitive functions was strengthened after adjustment for prenatal OC exposure (change in regression coefficient >10%).

Table 2 Crude coefficients and odds ratios (95% confidence intervals) for the association between cord blood TSH levels and MSCA outcomes, INMA-Granada cohort, 2000–2006 ($n=178$)[a].

MSCA outcomes at 4 years of age	TSH (mU/l)[b]	*P* value
General cognitive (β)	−1.71 (−4.90, 1.49)	0.29
Verbal (OR)	1.11 (0.62, 1.99)	0.72
Perceptual performance (β)	−0.04 (−3.30, 3.22)	0.98
Quantitative (OR)	2.26 (1.20, 4.25)	0.01
Memory (OR)	1.17 (0.63, 2.16)	0.61
Span (OR)	1.19 (0.61, 2.31)	0.62
Verbal memory (OR)	1.40 (0.82, 2.39)	0.22
Working memory (OR)	1.55 (0.95, 2.55)	0.08
Motor skills (OR)	0.71 (0.39, 1.29)	0.26
Fine motor skills (OR)	1.12 (0.57, 2.18)	0.75
Gross motor skills (OR)	1.19 (0.58, 2.44)	0.63
Executive function (β)	−1.62 (−4.79, 1.56)	0.32

OR, logistic regression odds ratio; β, linear regression coefficient. For ORs, the cut-off points correspond to the 20th percentile (reference group >P20). Normal distributed data are standardised (mean: 100; S.D.: 15).
[a]Each row represents a model adjusted for child's age and school term and psychologist administering the test.
[b]Log-transformed TSH levels.

Table 3 Adjusted regression coefficients and odds ratios (95% confidence intervals) for the effect of cord blood TSH levels (mU/l) on MSCA outcomes, INMA-Granada cohort, 2000–2006 ($n=178$)[a].

MSCA outcomes at 4 years of age	Continuous TSH[b]	TSH quartiles		
		2.05–2.95	2.96–4.18	4.19–17.0
General cognitive (β)	−3.52 (−6.81, −0.23)*	−0.36 (−6.29, 5.67)	−3.03 (−8.78, 2.74)	−5.42 (−11.30, −0.61)*
Verbal (OR)	1.48 (0.66, 3.30)	0.24 (0.05, 1.05)*	0.55 (0.15, 2.05)	1.48 (0.38, 5.39)
Perceptual performance (β)	−1.41 (−4.87, 1.91)	−1.12 (−7.28, 4.99)	−4.04 (−9.99, 1.84)	−2.17 (−8.31, 3.92)
Quantitative (OR)	2.64 (1.16, 5.54)†	1.21 (0.31, 4.25)	1.28 (0.32, 4.73)	4.92 (1.30, 16.47)†
Memory (OR)	1.61 (0.60, 3.80)	0.83 (0.13, 3.60)	0.92 (0.18, 3.74)	3.51 (0.65, 15.26)
Span (OR)	1.51 (0.52, 3.94)	0.53 (0.07, 3.23)	0.32 (0.05, 2.50)	5.73 (0.72, 24.67)*
Verbal memory (OR)	1.72 (0.86, 3.41)	0.69 (0.21, 2.29)	1.22 (0.39, 3.71)	1.98 (0.62, 6.30)
Working memory (OR)	1.35 (0.76, 2.49)	1.03 (0.35, 2.93)	1.34 (0.50, 3.77)	1.47 (0.54, 4.27)
Motor skills (OR)	0.65 (0.32, 1.47)	0.39 (0.11, 1.87)	0.42 (0.13, 1.73)	0.80 (0.24, 3.10)
Fine motor skills (OR)	1.48 (0.63, 3.66)	1.68 (0.35, 9.12)	2.14 (0.54, 9.28)	2.31 (0.53, 11.39)
Gross motor skills (OR)	0.69 (0.26, 1.99)	4.27 (0.69, 28.84)	0.24 (0.02, 2.55)	2.49 (0.44, 16.67)
Executive function (β)	−3.15 (−6.66, −0.19)*	−0.30 (−6.51, 5.96)	−3.37 (−9.46, 2.56)	−4.29 (−10.56, 1.86)

OR, logistic regression odds ratio; β, linear regression coefficient. For ORs, the cut-off points correspond to the 20th percentile (reference group >P20).
*$P<0.05$; †$P<0.01$. Normal distributed data are standardised (mean: 100; S.D.: 15).
[a]Each row represents two models: one using continuous TSH and the other using TSH quartiles. All models are adjusted for birth length, gestational age, maternal age, parity, breastfeeding, maternal and paternal education, mother-to infant attachment, child's age and school term and psychologist administering the test.
[b]Log-transformed TSH levels.

Discussion

This study of 178 children in Southern Spain born with normal thyroid function yielded evidence of an association between neonatal TSH and the cognitive development of children at 4 years of age, supporting our study hypothesis. Thus, MSCA general cognitive, quantitative and executive function scores appeared to be impaired by higher TSH cord blood levels. Limited data are available on the effect of newborn thyroid status on neurodevelopment, and most reports of associations have described the influence of thyroid status during pregnancy, specifically in relation to reduced T_4 levels (5). In addition, infants of fathers with university education had lower TSH, suggesting that neonatal thyroid status may be affected by social conditions. Paternal education and cord blood TSH levels contributed to predict cognitive performance at 4 years of age, consistent with the findings in research on ID that maternal education has a protective role in infant development (40).

The relationship between TH or TSH and cognitive function has mainly been studied in children with congenital hypothyroidism, children of mothers with low TH concentrations during pregnancy or children living in ID areas (41). A number of case–control studies have reported associations between decreased maternal or neonatal T_4 and/or triiodothyronine (T_3) levels and poorer neurodevelopment in children born to mothers with hypothyroxinaemia during pregnancy or in ex-preterm infants, among others (11, 12, 42, 43). Detected neurocognitive deficits include attention deficits (11, 43), reduced mental and motor development scores (12, 42, 43), impaired intelligence and language skills and difficulties in school performance at later ages (11). Interestingly, a cross-sectional analysis of 334 healthy children from two general population cohorts at 4 years of age (from Menorca and Ribera d'Ebre; INMA study) found an association between higher serum TSH levels (2.43–5.01 mU/l) and delayed general cognitive, quantitative, memory, verbal and

Table 4 Regression coefficients (95% confidence interval) for the effect of cord blood TSH levels (mU/l) on the MSCA general cognitive score and executive function score, adjusted for the placental organochlorine (OC) pesticide and combined oestrogenicity (TEXB) in a sub-sample of 101 children from the INMA-Granada cohort, 2000–2006[a].

MSCA outcomes at 4 years of age	TSH (mU/l)	OC pesticides[b]	α-TEXB + β-TEXB[c]
General cognitive	−5.51 (−10.54, −0.48)*	−6.34 (−12.32, −0.36)*	−5.35 (−10.24, −0.45)*
Executive function	−5.20 (−10.50, −0.11)*	−7.85 (−14.04, −1.67)†	−5.05 (−10.27, 0.18)

*$P<0.05$; †$P<0.01$. MSCA outcome data are standardised (Mean: 100; S.D.: 15).
[a]Each row represents three models of the association between log-transformed TSH concentrations and MSCA normal-distributed outcomes, adjusted for birth length, gestational age, maternal age, parity, breastfeeding, maternal and paternal education, mother-to-infant attachment, child's age and school term and psychologist administering the test.
[b]Additionally adjusted for placental concentration of 17 OC (≥LD, excepting for *p,p'*-DDE, which was introduced as continuous).
[c]Additionally adjusted for the combined xeno-oestrogenic effect of the α fraction (α-TEXB) and the β fraction (β-TEXB) of placenta samples (≥LD).

perceptual performance MSCA outcomes (44), consistent with the present findings.

By contrast, other authors reported that higher newborn T_4 was unexpectedly associated with lower scores on the visual recognition memory test at the age of 6 months but not with scores for verbal abilities, intelligence or visual motor abilities at the age of 3 years (45). Another study observed no neurological impairment in infants aged <1 year born to mothers with elevated TSH during pregnancy (46). Furthermore, neonatal T_4 levels were not associated with the risk of a heterogeneous group of developmental diagnoses in 5–12-year-old children, including attention deficit disorder, autism spectrum disorder, behavioural disorder, cognitive disorder, developmental delay, emotional disorder, learning disability and speech/language disorder (47). A recent case–control study in Southern Spain observed a superior psychometric and behavioural development among children whose mothers had received iodine supplementation compared with the children of non-supplemented mothers; cord blood TSH was significantly higher in infants of supplemented mothers (16). A study of Sicilian children reported that a mild to moderate ID was associated with a reduced IQ and attention deficit hyperactivity disorder (13). Finally, increases in the risk of delay in gross and fine motor coordination and socialisation in 18-month infants were found to result from a period of isolated hypothyroxinaemia in pregnant women from a coastal region in Spain with mild ID (15).

The timing of TH action is crucial for neurodevelopment, and the effects of TH status may therefore differ among pregnant women, neonates and children. In the neonatal and postnatal periods, neurological development still depends on THs, whose supplies to the brain are entirely derived from the child and are critical for continuing maturation (6). It has been demonstrated that THs during early pregnancy influence later child development, although the neurological effects of THs may be less severe in neonates. This study demonstrated that higher newborn TSH levels within a normal reference range were related to lower intelligence, as measured by the general cognitive score, and to impairment of higher psychological processes (executive function) at the age of 4 years. These findings support the view that moderate alterations in neonatal thyroid status may play a role in subsequent neurodevelopment. Even subtle cognitive delays at this age may lead to sub-clinical but permanent decreases in IQ and to long-lasting effects on educational and social development (48). Hence, they should be considered clinically relevant, since early identification of sub-optimal cognitive functioning is necessary to adopt preventive measures.

The study limitations include the fact that only boys were studied; the non-assessment of behavioural or psychopathological outcomes such as attention deficit, social or emotional disorders, which have also been associated with early thyroid status (15, 43, 44) and the absence of measurements of newborn T_4, T_3 or thyroid axis hormones other than TSH, which would have yielded complete information on the newborn's thyroid regulatory system. In fact, recent studies have reported the unexpected finding of a positive correlation between TSH and free T_4 levels in cord blood correlate (14, 49), suggesting that TSH elevations should not necessarily be interpreted as indicating a potentially harmful effect on the child. There is a progressive modulation of the set point for T_4 negative feedback regulation of TSH secretion in infants, which implies that TSH production is overstimulated during gestation, decreasing from postnatal age of around 2 weeks (49). Owing to this decline in TSH levels over the first days of life, newborns with elevated TSH should be evaluated for congenital hypothyroidism with repeat TSH and free T_4 measurements. However, it is recognised that TSH measurement offers higher sensitivity to detect thyroid dysfunction in comparison with T_4 or T_3 testing, since subtle alterations in T_4 or T_3 within the normal reference range may result in an amplified alteration of TSH (50). The study strengths include the longitudinal design, the considerable number of covariates considered (e.g. breastfeeding and parental attachment) and, most importantly, the fact that we examined the association between TSH and neurodevelopment in typically developing children from the general population. In addition, this is the first report to evaluate the potential confounding effect of prenatal exposure to a wide range of ED, environmental chemicals and their combined oestrogenic effect on the association under study.

To date, epidemiological studies have described associations of early exposure to OC compounds with TH levels (20–26) and with neurodevelopment impairment (51–53). However, a consistent influence on thyroid status and neurodevelopment of many OC compounds at environmental background levels has not been established yet. Humans may be exposed to mixtures of these and numerous other compounds, hampering the prediction of effects on TH levels. We have previously demonstrated the ubiquity of exposure to OC xeno-oestrogens in the INMA-Granada cohort (54). In this study, prenatal exposure to 17 OC pesticides or the xeno-oestrogenic burden in the placentas were observed to modify the impact of neonatal TSH on neurodevelopment, in agreement with the suspected capacity of OCs to interfere with the thyroid system (20–26).

In conclusion, this study of a birth cohort in Southern Spain revealed an impaired mental development at 4 years of age in children with higher neonatal TSH levels compared with children with lower neonatal TSH levels within the normal reference range. These findings indicate that a more thorough screening for neonatal thyroid deficiency is required to prevent long-term developmental effects. Further research is warranted into the influence on neurodevelopment of marginally altered TSH concentrations in newborns and into

causal relationships between ED, environmental chemicals and TH status.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Discusión para Objetivo 4: *Estudio de la influencia de los niveles de TSH neonatal sobre el neurodesarrollo infantil.*

El resultado más interesante del presente trabajo es la demostración de una asociación entre la medida de la función tiroidea en el neonato y las funciones neuroconductuales del niño. La hipótesis que subyace en el proceso es que una elevación ligera de TSH, indicativa de una situación de hipotiroidismo subclínico en el recién nacido, determina que las funciones cuantitativas y ejecutivas den puntuaciones más bajas cuando el niño es explorado a los cuatro años de edad, y esa disfunción hormonal tan sutil pidiera estar asociada a la exposición ambiental de la madre durante el embarazo.

Los estudios de relación entre el estatus tiroideo al nacimiento y el neurodesarrollo del niño no son frecuentes, centrándose la mayor parte de ellos en la determinación del estatus hormonal de la madre durante el embarazo, especialmente a través de la medida de los niveles de la hormona T4 (De Escobar *et al.,* 2004). Por esta razón este trabajo da un paso más en la demostración de una hipótesis hasta el momento no bien explorada. Resulta interesante resaltar que se ha observado que los niños cuyos padres tienen educación universitaria tuvieron menores niveles de TSH, sugiriendo que el estatus tiroideo del recién nacido podría verse afectado por variables sociales. De hecho, la educación paterna y los niveles de TSH en sangre de cordón umbilical se comportaron como predictores del desarrollo cognitivo, reafirmando la idea de que el nivel educativo de la madre puede tener un papel protector en el desarrollo infantil (Choudhury y Gorman, 2003).

Los estudios de asociación entre TH y/o TSH y función cognitiva se han realizado, fundamentalmente, en niños con hipotiroidismo congénito, hijos de madres con bajos niveles de TH durante el embarazo o niños residentes en áreas de deficiencia de yodo. Así, se ha encontrado una asociación entre niveles más bajos, maternos o neonatales, de T4 y/o T3 y un neurodesarrollo deficiente (Haddow *et al.,* 1999; Pop *et al.,* 1999, 2003; Simic *et al.,* 2009), que incluye problemas de atención (Haddow *et al.,* 1999; Simic *et al.,* 2009), peor desarrollo mental o motor (Pop *et al.,* 1999, 2003; Simic *et al.,* 2009), disminución de inteligencia y habilidades del lenguaje o dificultades en la escuela (Haddow *et al.,* 1999). Un estudio en dos cohortes pertenecientes al proyecto INMA observó una asociación entre altos niveles de TSH en suero de niños a los 4 años y

retraso general cognitivo, y peores resultados para las áreas cuantitativa, de memoria, verbal y perceptual en el test MSCA (Álvarez-Pedrerol *et al.,* 2007). En cambio, otros autores han observado que mayores niveles de T4 en el recién nacido se asociaban con menores puntuaciones en test de memoria visual a los 6 meses pero no en habilidades verbales, inteligencia o habilidades visomotoras a los 3 años (Oken *et al.,* 2009). Por otra parte, no se observó empeoramiento neurológico en niños menores de un año cuyas madres tuvieron altos niveles de TSH durante el embarazo (Orito *et al.,* 2009) ni se pudieron asociar altos niveles de T4 neonatal con el riesgo de desarrollar diversos desórdenes entre niños de 5 a 12 años (Soldin *et al.,* 2003).

En la misma región donde se ha llevado a cabo este estudio, Velasco *et al.* (2009) observaban un mayor desarrollo cognitivo y conductual en niños cuyas madres recibieron suplementos de yodo respecto a los madres que no los tomaron; a pesar de que los niveles de TSH en sangre de cordón umbilical fueron significativamente mayores en madres suplementadas y el grupo control tuvo mayores niveles de T4 libre en el tercer trimestre del embarazo. En Sicilia pudo observarse que un déficit de yodo se asociaba con menor IQ y TDAH (Vermiglio *et al.,* 2004), así como en España un riesgo mayor de déficit de la coordinación motora y socialización en niños de 18 meses cuyas madres tuvieron periodos de hipotiroxinemia (Berbel *et al.,* 2009).

La acción de las hormonas tiroideas es vital para el neurodesarrollo, pero las consecuencias de un estatus tiroideo alterado pueden ser distintas en embarazadas, neonatos y niños. En los periodos neonatales y postnatales, el neurodesarrollo depende de las hormonas tiroideas, cuyos niveles dependen del niño y son críticos para la maduración cerebral (De Escobar *et al.,* 2000). Se ha demostrado que los niveles de estas hormonas durante las primeras fases del embarazo marcan el desarrollo del niño, aunque los efectos neurológicos pueden ser menos severos en neonatos. Así, en el presente trabajo, los recién nacidos con mayores niveles de TSH en un rango de referencia normal tuvieron peores puntuaciones en test de inteligencia (medidos como puntuación general cognitiva) y peor función ejecutiva a los 4 años. Estos hallazgos confirman la idea de que alteraciones moderadas en el estatus tiroideo neonatal pueden tener una influencia en el neurodesarrollo posterior. Incluso retrasos cognitivos leves en esta edad pueden conducir a disminuciones permanentes del IQ y efectos en el desarrollo social y educación (Grandjean y Landrigan, 2006). Por ello, estos hallazgos deberían ser considerados clínicamente relevantes, ya que la identificación temprana de

un funcionamiento cognitivo sub-óptimo es necesaria para adoptar medidas preventivas. De hecho, diversos desórdenes psicopatológicos han podido relacionarse con alteraciones tiroideas (Álvarez-Pedrerol *et al.,* 2007; Berbel *et al.,* 2009; Simic *et al.,* 2009).

Algunos estudios muestran además una correlación positiva entre TSH y T4 libre en sangre de cordón umbilical (Anderson, 2001; De Escobar *et al.,* 2007), sugiriendo que incrementos en los niveles de TSH no necesariamente deben interpretarse como potencialmente dañinos. Hay una modulación progresiva que implica que la producción de TSH se sobreestimula durante el embarazo, decreciendo de nuevo desde el nacimiento durante dos semanas (Hume *et al.,* 2004). Debido a esta caída en los primeros días de vida, los recién nacidos con niveles elevados de TSH deberían ser evaluados para detectar hipotiroidismo congénito con medidas repetidas de TSH y T4 libre, sin embargo, la medida de TSH proporciona una alta sensibilidad para detectar desórdenes tiroideos en comparación con la medida de T3 o T4, ya que alteraciones sutiles de T3 o T4 en un rango de referencia normal pueden resultar en una alteración amplificada de la TSH (*The National Academy of Clinical Biochemistry,* 2007).

Por último, diversos estudios epidemiológicos han descrito asociaciones entre la exposición temprana a organoclorados y los niveles de hormonas tiroideas (Takser *et al.,* 2005; Wang *et al.,* 2005; Maervoet *et al.,* 2007; Chevrier *et al.,* 2008; Álvarez-Pedrerol *et al.,* 2009; López-Espinosa *et al.,* 2009b; López-Espinosa *et al.,* 2009c) así como con efectos adversos en el neurodesarrollo (Eskenazi *et al.,* 2006; Ribas-Fitó *et al.,* 2006; Puertas *et al.,* 2010), a pesar de lo cual no se ha enunciado aún cómo influye la exposición a contaminantes ambientales en el estatus tiroideo y el neurodesarrollo. Establecer una asociación clara es difícil dado que la exposición ocurre para mezclas complejas en el mundo real y no a un único compuesto. En la cohorte INMA-Granada ya se ha descrito previamente la ubicuidad de la exposición a organoclorados (López-Espinosa *et al.,* 2007), y puede observarse ahora cómo los 17 pesticidas cuantificados modifican el impacto de los niveles neonatales de TSH en el neurodesarrollo, de acuerdo con la sospecha de la capacidad de los organoclorados para interferir en la homeostasis tiroidea (Takser *et al.,* 2005; Wang *et al.,* 2005; Maervoet *et al.,* 2007; Chevrier *et al.,* 2008; Álvarez-Pedrerol *et al.,* 2009; López-Espinosa *et al.,* 2009b; López-Espinosa *et al.,* 2009c).

6. CONCLUSIONES

El análisis de los resultados obtenidos y su confrontación con la literatura científica permite concluir que la exposición materno-infantil a disruptores endocrinos tales como bisfenoles, compuestos organoclorados y metales persistentes es un fenómeno frecuente, que ocurre de forma inadvertida para la población no profesionalmente expuesta y que tiene consecuencias para la salud.

1. Los bisfenoles objeto de estudio tienen actividad disruptora debido a su capacidad para activar receptores nucleares, pudiendo por ello interferir en el sistema endocrino, compitiendo con los ligandos naturales por la unión al receptor, modificando la expresión de genes específicos o promoviendo la proliferación celular. Los bisfenoles testados BPS, BPF y BPA mostraron capacidad de activación de hERα y hERβ. TCBPA se comportó como un agonista débil del hERα. BPS mostró mayor actividad en hERβ que hERα, al contrario que BPF y BPA. BPF y BPA se comportaron como antagonistas de hAR, con mayor eficacia el BPA respecto a BPF. BPA y BPS mostraron una leve capacidad agonista de hAR. Y por último, BPA, TCBPA y TBBP mostraron ser agonistas de hPXR (TCBPA > TBBPA > BPA). Los valores de EC_{50} se sitúan en el rango micromolar, pero a pesar de que estas concentraciones son difíciles de alcanzar en tejidos o fluidos humanos, algunos estudios confirman que bajas concentraciones (nanomolares o picomolares) de éstos contaminantes actúan mediante señalización "no genómica", y no es descartable un efecto combinado, ya sea sumatorio, sinérgico o antagónico.

2. La demostración de la exposición durante la etapa intrauterina a metales pesados con propiedades neurotóxicas y de disrupción endocrina remarca la necesidad de investigar la presencia de estos elementos en el organismo materno y del niño, así como las posibles consecuencias negativas para la salud de la exposición temprana a dosis moderadas o incluso bajas de estos metales y otros contaminantes ambientales. Los resultados confirman la presencia de Cd y Mn en el 100% de las placentas analizadas de mujeres de la población general del área metropolitana de Granada, así como de Cr, Pb y Hg, detectados en un 98.5, 35.0 y 30.7% de las placentas, respectivamente. Las mayores concentraciones placentarias son las de Pb, seguido de Mn, Cr, Cd y Hg. De manera general, estas concentraciones se situaron en un nivel intermedio-bajo comparado con estudios realizados en muestras poblacionales similares, con la excepción del Pb, que presentó mayores niveles en las mujeres granadinas. Mujeres que tuvieron una gestación más prolongada y que fumaron durante

el embarazo tuvieron mayores concentraciones placentarias de Cd. Los niveles de este metal, además, fueron mayores en mujeres de mayor edad y con gestaciones más largas, probablemente debido a un mayor tiempo de bioacumulación. Los niveles de Pb encontrados en un grupo significativo de madres –un 25% tenían concentraciones superiores a 309 ng/g– junto con la frecuencia de detección relativamente baja, podrían estar relacionados con la eliminación en España de la gasolina con plomo durante el reclutamiento de la cohorte (2000-2002). Por último, los niveles de Hg observados en placenta resultaron ser menores que en estudios similares en otras poblaciones.

3. En la población general no ocupacionalmente expuesta a pesticidas organoclorados persistentes, la dieta parece ser la principal vía de exposición. Dado que la mayoría estaban ya prohibidos en el periodo de reclutamiento, los niveles encontrados en las placentas de las mujeres de la cohorte deben ser reflejo de la liberación al torrente sanguíneo durante el embarazo de los residuos acumulados en el tejido adiposo. Las mayores concentraciones de pesticidas observadas en clases sociales medias y bajas se deberían a una mayor exposición acumulada a lo largo de la vida. En este sentido, es muy probable que la mayor parte de estas mujeres procedan de un entorno familiar en el que el patrón de alimentación y estilo de vida sea similar al de sus padres, para los que la exposición ambiental a organoclorados fue más que probable. La concentración de la suma de metabolitos de DDT fue mayor en la clase social IV, mientras que la concentración del total de compuestos de endosulfán fue mayor en la clase social III. Los niveles placentarios de HCB mostraron una tendencia de aumento lineal estadísticamente significativa a lo largo de las categorías de clase social, de manera que las clases más desfavorecidas presentaron mayores niveles de HCB.

4. Se ha observado una asociación significativa entre mayores niveles de TSH neonatal y menores puntuaciones en el índice general cognitivo y en la función ejecutiva evaluadas a los 4 años de edad. Esta asociación fue observada tras controlar por los factores de confusión. Igualmente, niveles más elevados de TSH neonatal estuvieron asociados con mayor riesgo de tener una menor puntuación en el área numérica del test McCarthy. Los niños con niveles de TSH en el último cuartil (>4,19 mU/l) tuvieron mayor riesgo de tener una menor puntuación en el área de memoria de trabajo. Se observaron menores niveles de TSH en niños cuyos padres poseían nivel educativo universitario, en comparación con niños de padres sin estudios superiores. Las asociaciones observadas entre el nivel de TSH neonatal y las puntuaciones

obtenidas para la función ejecutiva y el índice general cognitivo se mantuvieron tras ajustar el modelo por las concentraciones de pesticidas organoclorados y la carga xenoestrogénica de las placentas.

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8. ANEXOS

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