TESIS DOCTORAL INTERNACIONAL / INTERNATIONAL PhD THESIS

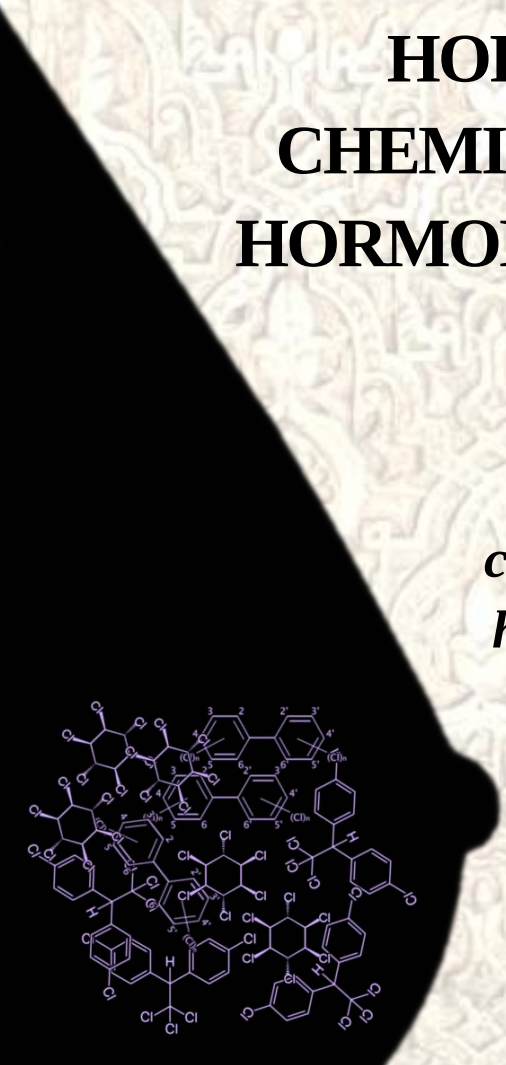
UNIVERSIDAD DE GRANADA

Programa Oficial de Doctorado en Medicina Clínica y Salud Pública

Universidad de Granada

ENVIRONMENTAL EXPOSURE TO HORMONE-MIMICKING CHEMICALS AND THE RISK OF HORMONE-DEPENDENT CANCER

Exposición medioambiental a compuestos químicos con actividad hormonal y cáncer dependiente de las hormonas esteroideas



Author:
Francisco Artacho Cordón

Granada, 2016

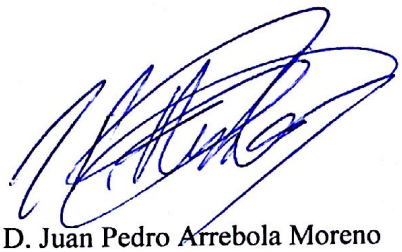
Editor: Universidad de Granada. Tesis Doctorales
Autor: Francisco Artacho Cordón
ISBN: 978-84-9163-143-9
URI: http://hdl.handle.net/10481/45402

El doctorando **Francisco Artacho Cordón** y los directores de la tesis **D. Nicolás Olea Serrano y D. Juan Pedro Arrebola Moreno**. 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, 20 de Abril de 2016

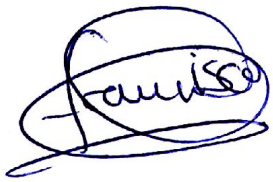
Director/es de la Tesis

Fdo.: D. Nicolás Olea Serrano



Fdo.: D. Juan Pedro Arrebola Moreno

Doctorando



Fdo.: Francisco Artacho Cordón

DEPARTAMENTO DE RADIOLOGÍA Y MEDICINA FÍSICA

FACULTAD DE MEDICINA

UNIVERSIDAD DE GRANADA

Dr. Nicolás Olea Serrano, Catedrático de Radiología y Medicina Física y Director del Departamento de Radiología y Medicina Física de la Universidad de Granada,

CERTIFICA:

Que el presente trabajo de Tesis Doctoral ha sido realizado por el Licenciado en Biología D. FRANCISCO ARTACHO CORDÓN en el Departamento de Radiología y Medicina Física de la Universidad de Granada. Y para que conste y surta efectos donde proceda, firmo el presente certificado en:

Granada a 20 de Abril de 2016

Fdo. Prof. Dr. D. Nicolás Olea Serrano

ACKNOWLEDGMENTS
AGRADECIMIENTOS

Acknowledgments / Agradecimientos

Although it is only my name that appears on the cover of this PhD thesis, many people have contributed to its production. I owe my gratitude to all the people who have made this thesis possible and have made my postgraduate experience an extraordinary life lesson.

First, I would like to express my sincere gratitude to my advisor Prof. Dr. Nicolás Olea for the continuous support during my Ph.D studies and during all the research carried out, for his patience, enthusiasm, and immense knowledge. Thanks for giving me the opportunity to improve myself, for teaching me that the size of the challenges you can face only depends on your motivation. Thanks for an entire life devoted to research.

I am also grateful to Dr. Juan Pedro Arrebola. I have been amazingly fortunate to have an advisor who gave me the freedom to explore on my own and at the same time the guidance and support to recover when my steps faltered. You have taught me how to question thoughts and express ideas. Your patience and support helped me overcome some crisis situations and finish this PhD thesis. I hope that one day I would become as good an advisor to my students as you have been to me. You are the mirror in which many people, including me, want to see themselves.

I would also like to express my gratitude to Prof. Dr. Marieta Fernandez. Thanks for the insightful comments and constructive criticisms. Your determination and efficiency helped me in difficult situations. Thanks for counting on me in every moment.

I am also thankful to the Radiology and Physical Medicine Department for including me in its institutional life, giving me the opportunity to teach my first lessons at the university. In particular, I am grateful to Prof. Dr. Maribel Núñez for enlightening me with the first glance of research, for the trust placed on me. Thanks for placing my dreams before yours. I will be forever in debt to you.

My sincere thanks go to Prof. Dr. Nils Skakkebaek, Anna-Maria Andersson, Hanne Frederiksen and Ole Nielsen, for offering me the opportunity to become part of your group during some months and letting me work on an exciting project. Thanks for the warm atmosphere you created that let me enjoy a really wonderful summer.

No menos importante, y por tanto, con la misma intensidad he de redactar mis agradecimientos a todos aquellas personas con las que he tenido el placer de compartir estos años de formación predoctoral. Como la gran Rachel Carson decía sobre la influencia de la química en el medio ambiente, mi pequeño microambiente laboral y social ha contribuido silenciosa, pero a la vez

sustancialmente (eso sí, de forma más que positiva) a algo mucho más sutil y gratificante como lo han sido cada uno de esos pequeños momentos especiales que hemos vivido juntos, tanto dentro como fuera de las paredes del laboratorio. No podría no comenzar estos agradecimientos sin destacar la continua y altruista ayuda de quien rápidamente pasó de ser mi compañera de trabajo a alguien imprescindible en mi vida. Gracias Sandra por tanta dulzura en cada uno de los sabios consejos que me has dado y me seguirás dando. Jamás imaginé tener la suerte de poder vivir esta experiencia al lado de alguien tan especial como tú.

Aunque en un primer momento se sintió sola en esa pequeña habitación de paso, numerosas fueron las oportunidades de compartir experiencias inolvidables con mi "Little monkey". No había mañana que no llegases con una gran sonrisa y de buen humor. Gracias por esos masajitos tan agradables y por aquella maravillosa "encerrona" en tu casa que cambió mi vida. Por supuesto agradecer a mi rubia peligrosa, por tantos momentos divertidos entre células y campos magnéticos. Espero poder algún día como doctor, ya que como predoctoral no lo hice, enseñarte de una vez por todas, el inquietante y misterioso mundo de la PCR. Gracias a Inma por resolverme tantas dudas técnicas, por las horas que has dedicado para conseguir que ese dichoso cromatógrafo funcionase a pesar de sus limitaciones. Gracias por los consejos y experiencias compartidas.

Como un rayo de luz (y nunca mejor dicho) llegaste, a pesar de mi tozuda insistencia al inicio para que valorases otras posibilidades laborales. Sin embargo, desde el primer día te convertiste en un tesoro preciado del que no podía prescindir. Gracias Luz por estar siempre dispuesta a echarme una mano. No pierdas jamás esa dulce timidez que te hace ser tan especial. Por supuesto agradecer a Irene, por darme pequeñas lecciones de vida que perdurarán más allá de estos momentos que hemos estado compartiendo. A mi ginecóloga preferida, amiga, compañera y hasta profe por momentos. A pesar de haber compartido pocas horas de laboratorio, grande ha sido la amistad que hemos forjado en estos años. Gracias por los inestimables consejos que me has dado. Y por los que vendrán.

Igualmente agradecer al resto de mis compañeros de trabajo. A Chepe y sus fantástica terraza que todos echamos de menos cuando el sol empieza a calentar; a nuestra médico de referencia Cris; a Fernando y Vicente, los bailarines que todos querrían tener en sus filas; a José Manuel por arrancar en la pecera una sonrisa cada mañana. A Elena, por mostrar tanta elegancia en el día a día, tanto en sus palabras como en sus formas. También a aquellos que dejaron de formar parte de mi día a día, pero que también contribuyeron a hacer de estos años una experiencia fabulosa. Gracias a Rosa, a Espe y a mi compañera de fatigas María. El HPLC tiembla cada vez que nos ve llegar al laboratorio.

Agradecer también a todos los que habéis conseguido que ir a trabajar se convierta en un placer y no en un sacrificio. Gracias a Pepi por su ternura y bondad, a mis "Conchis" (Conchi y Paqui) que desde el primer día me cuidaron como si de su hijo se tratase; a Blanca, a Bea y Sonia. A José Antonio por transmitirme sus conocimientos en aquellos primeros pasitos en este dulce a la par que amargo mundo de la investigación, También a esa parejita que tan buenos ratos nos han dado, Sergio y Auxi; a Jorge, Ángel, Esther y demás "Salmerones". Al equipo de Biobanco, especialmente a Beni, que con una sonrisa siempre han estado dispuestos a buscar las muestras más difíciles en tiempo record. De igual forma, mi más sincero agradecimiento a todos aquellos voluntarios anónimos que con su incalculable contribución en los momentos más difíciles de su vida han contribuido generosamente para la realización de esta tesis doctoral.

Quisiera hacer extensiva mi gratitud a todas aquellas personas que indirectamente han formado parte de todo este proceso. A mis biólogos (y asociados), quienes además de haberme hecho vivir 5 magníficos años de universidad, han seguido generando momentos especiales en estos años de estudiante predoctoral. Gracias a Ana, quien ha tenido que aguantarme y escuchar tantas frustraciones. Gracias por haber estado siempre presente, por haberme animado cuando más lo he necesitado. A Carlos, Juanjo, Jose (y no José) y Girela por seguir confirmándome que la verdadera amistad no entiende de distancias ni de tiempos. También a mis pequeños fisios, quienes en estos últimos años han invertido el rol y han ejercido de padres de un viejo gruñón, que como el conejo blanco, siempre iba pendiente del reloj. Gracias a JoseRa, Pepe, Cris, Álvaro, Esteban, Richi y demás promesas de la fisioterapia.

Sin embargo, los verdaderos artífices de este logro son los miembros de mi familia. No existen palabras en ningún idioma que sirvan para agradecer a los que no solo les debo la vida, les debo todo cuanto a día de hoy puedo decir que soy. Por ello, a mis padres no les debo mostrar únicamente mi gratitud, sino también dedicar todo el fruto de mi esfuerzo. Soy consciente de todo el esfuerzo que lleváis haciendo desde el mismo momento en que decidisteis formar esta gran familia. Sois un verdadero ejemplo de superación, de generosidad, de esfuerzo altruista. Ojalá algún día pueda ser, al menos, un vago reflejo de lo que vosotros sois. Gracias por inculcarme tan buenos valores y por depositar tanta confianza en mí. Espero que estéis orgullosos de lo que cada día voy consiguiendo con vuestra ayuda.

Debo agradecer también a mi hermana Toñi, con quien tantos días he convivido, quien me ha visto crecer como persona. A quien he transmitido mis preocupaciones y en quien he buscado consejo y ayuda. A pesar de que cada día la distancia física entre nosotros aumentará, estoy seguro que la emocional siempre nos mantendrá estrechamente unidos. También a Isa y Rubén, mis pequeños, que ya no lo son tanto. Gracias por tanta paciencia cuando he intentado daros algún que otro consejo, al lado de la chimenea, por teléfono, e incluso a miles de kilómetros de

distancia vía Skype. A mi tío Salvador, quien siempre ha estado cuidando de mí como si de un padre se tratase.Sin cuestionar, sin opinar. Gracias por tanto apoyo. También agradecer al resto de mi gran familia, abuelos, tías, tíos, primos y primas. A mi campanilla, quien guía cada uno de nuestros pasos.

Mis últimas líneas deben ir dirigidas a quien ha sabido sacarme una sonrisa aún en los momentos más difíciles. Quien me ha dado fuerzas cuando no las había. Quien ha puesto luz a mis días más oscuros. Quien ha conseguido que no me rinda. Quien ha esperado pacientemente. Quien ha puesto algo de cordura en esta mente tan caótica. Gracias por esa comprensión infinita, por tantos momentos inolvidables. Gracias por anteponer mis proyectos a tus necesidades. Por esas paellas maravillosas. Por tantos sueños cumplidos. Y por tantos otros que nos quedan por vivir juntos. Gracias por tanto amor.

A mis padres,

A mis hermanos,

A mi campanilla de ojos negros.

A ti.

"All our dreams can come true if we have the courage to pursue them."

"Todos nuestros sueños pueden hacerse realidad si tenemos el coraje de perseguirlos"

Walt Disney

The following research projects have supported this PhD thesis:

1. "**Puesta a punto del biomarcador Carga Estrogénica Total Efectiva (TEXB) en muestras de suero humano y estudio de su relación con tejido adiposo mamario en pacientes de cáncer de mama**." Founded by the Andalusian Regional Government (PI-0513-2012)

2. "**Consecuencias bioquímicas, metabólicas y genéticas de la exposición humana a disruptores endocrinos "obesógenos" en sujetos con obesidad y normopeso"** founded by the Fondo de Investigaciones Sanitarias del Instituto de Salud Carlos III (PI13-02406)

3. "**Exposición múltiple a disruptores endocrinos en la cohorte de Infancia y Medioambiente (INMA): nuevos marcadores de exposición hormonal**" founded by the Fondo de Investigaciones Sanitarias del Instituto de Salud Carlos III (PI11-00610)

ÍNDICE / INDEX

1. RESUMEN

Dentro de los cánceres hormondependientes, el cáncer de mama es, tanto en España como en la mayoría de los países desarrollados, el más frecuente en la mujer y la primera causa de muerte por cáncer en la población femenina. A pesar del intenso esfuerzo diagnóstico y terapéutico, la incidencia de cáncer de mama continúa incrementándose, principalmente, aquellos subtipos hormonodependientes, no solo en los países industrializados sino también en los países en vías de desarrollo. Además de factores de riesgo ampliamente conocidos como la edad, menarquia precoz, menopausia tardía, número de embarazos y lactancia acumulada, se ha postulado que la exposición a sustancias químicas disruptoras endocrinas (DEs) con actividad xenoestrogénica podrían estar contribuyendo al incremento de nuevos casos que se están registrando con el tiempo. Entre las sustancias descritas como DEs se encuentran los denominados compuestos orgánicos persistentes (COPs) [entre los que se incluye el *p,p'*-diclorodifenildicloroetileno (*p,p'*-DDE), el hexaclorobenceno (HCB), el hexaclorociclohexano (HCH) y congéneres de los bifenilos policlorados (PCBs)] y los denominados como disruptores endocrinos no persistentes (npDEs) [entre los que se encuentran el bisfenol-A (BPA), parabenes y benzofenonas]. Sin embargo, los estudios epidemiológicos realizados hasta la fecha han mostrado resultados contradictorios, mostrando tanto asociaciones positivas como ausencia de asociación. Dichas discrepancias podrían estar causadas, al menos en parte, a diferencias metodológicas, la naturaleza del estudio, el biomarcador de exposición utilizado, el momento de la exposición o la población de estudio analizada.

Así, no existe actualmente consenso internacional sobre la matriz biológica más adecuada para valorar la exposición a DEs lipofílicos en pacientes de cáncer de mama, ya que los niveles en tejido adiposo son considerados como indicativos de exposición acumulada, pero es una muestra de difícil obtención. Por otro lado, las concentraciones séricas (el biomarcador más accesible y ampliamente utilizado) podrían verse más afectadas por ciertas condiciones fisiológicas. Para cumplir con el primer objetivo específico de esta tesis doctoral, que era evaluar las diferencias del tejido adiposo y el suero como biomarcadores de exposición a DEs en pacientes con cáncer de mama, se cuantificaron las concentraciones en suero y tejido adiposo de un grupo de COPs en una muestra de 103 pacientes diagnosticados de cáncer de mama en el Complejo Hospitalario de Granada. Tanto en tejido adiposo como en suero, el COP que mostró la concentración más elevada fue el *p,p'*-DDE (mediana 194.34 y 173.84 ng/g lípido, respectivamente). En general, se detectaron correlaciones positivas entre los COP estudiados en tejido adiposo, así como en suero. Sin embargo, sólo HCB y *p,p'*-DDE mostraron una correlación significativa entre tejido adiposo y suero, mientras que los PCBs no mostraron correlación entre ambas matrices. Además, observamos que mientras la edad se asociaba con la mayoría de COPs en ambas matrices, la asociación de determinados COPs con el índice de masa corporal (IMC) dependía del marcador utilizado. Así, se encontró una asociación positiva

en tejido adiposo, mientras que en suero la asociación encontrada fue negativa. En la misma línea, la pérdida reciente de peso se asoció con mayores niveles de COPs en suero y menores en tejido adiposo. Finalmente, las concentraciones séricas de HCB y PCB-180 eran menores entre aquellas pacientes que habían recibido quimioterapia preoperatoria, mientras que los niveles en tejido adiposo no se asociaban con el tratamiento neoadjuvante. Por tanto, nuestros datos sugieren que las concentraciones de COPs en suero y tejido adiposo podrían verse afectadas diferencialmente por factores externos, y por tanto, evidencian la utilidad que podría tener la obtención de información de los niveles de COPs en las dos matrices en lugar de una sola en el caso de los estudios que abordan el riesgo de cáncer de mama.

Por otro lado, la problemática de la exposición humana a DEs y sus efectos carcinogénicos se agrava en los países en vías de desarrollo, donde los niveles de exposición de la población pueden ser distintos, debido a la prohibición tardía de estos compuestos, el comercio ilegal o las legislaciones inadecuadas, lo que podría suponer una exposición sustancial e inadvertida de las poblaciones. Para dar respuesta al segundo y tercer objetivo de esta tesis doctoral, se caracterizó la exposición inadvertida a DEs (COPs y npDEs), así como la asociación de COPs con el riesgo de desarrollar cáncer de mama, en un estudio caso-control compuesto por 125 mujeres residentes en Túnez, un país donde la legislación para el manejo de los almacenes de residuos no ha sido lo suficientemente estricta. La caracterización de la exposición a DEs (objetivo 2) se llevó a cabo entre el grupo control. Del total de COPs analizados, HCB, *p,p'*-DDE y PCBs (-138, -153 y -180) mostraron frecuencias de detección cercanas al 100%. Las concentraciones séricas de PCB138, PCB-153 y PCB-180 fueron 26.08, 119.10 y 29.84 ng/g lípido, mientras que las de HCB y *p,p'*-DDE fueron 19.98 y 127.59 ng/g lípido, respectivamente. La edad se asoció positivamente con los niveles séricos de todos los COPs estudiados. Se detectaron niveles más elevados de PCBs en aquellas mujeres residentes en la mitad norte del país, mientras que los niveles de *p,p'*-DDE se asociaron a las mujeres que trabajan fuera de casa y a un mayor consumo de cereales. Finalmente, el tiempo de lactancia acumulado se relacionó con menores niveles de *p,p'*-DDE y HCB. Simultáneamente, la media geométrica de los valores detectados en orina de metil- etil- propil- y butilparaben fue 30.10, 1.43, 2.03 y 0.47 ng/mL, respectivamente, mientras que la media geométrica de BPA encontrada fue 0.44 ng/mL y de benzofenona-1 y benzofenona-3 fueron 1.33 y 1.10 ng/mL. Así mismo, se observó que los niveles en orina de benzofenona-1, benzofenona-3, metilparaben y propilparaben eran más elevados en aquellas mujeres premenopáusicas. Por tanto, los niveles de exposición a DEs, especialmente los altos niveles de PCB-153, así como los determinantes de dicha exposición encontrados en mujeres tunecinas demuestran la necesidad de desarrollar programas más extensos de biomonitorización con objeto de identificar fuentes de exposición y grupos de población especialmente expuestas.

La asociación entre la exposición a COPs y el riesgo de desarrollar cáncer de mama en la población tunecina (objetivo 3) se evaluó mediante modelos de regresión logística incondicional. Tras ajustar los modelos por edad, IMC, clase ocupacional, residencia, educación, tiempo de lactancia acumulado, paridad, estado menopáusico, historia familiar de cáncer de mama y contenido lipídico del suero, se detectó que las concentraciones séricas de β-HCH y *p,p'*-DDE se asociaron positivamente con el riesgo de desarrollar cáncer de mama (OR =1.10 y OR=1.37), mientras que la asociación encontrada con el heptacloro fue cercana a la significación (p=0.078). Además, los análisis realizados usando las concentraciones de COPs en terciles corroboraron la relación dosis-respuesta encontrada para el *p,p'*-DDE (OR=6.26 y OR=9.65 para los terciles 2 y 3, respectivamente). Los individuos con niveles detectables de β-HCH mostraron una OR de 3.44 de desarrollar cáncer de mama. Finalmente, la influencia relativa de cada compuesto en presencia de los otros se analizó mediante la introducción de los tres compuestos en el modelo ajustado, observando que solo β-HCH se mantenía asociado positivamente con el riesgo de cáncer de mama (OR=1.18, 95%IC 1.05-1.34). En conjunto, nuestros resultados sugieren que la exposición a COPs (al menos la exposición a β-HCH) se asocia con un mayor riesgo de desarrollar cáncer de mama en la población tunecina, aunque estos resultados deben interpretarse con cautela, necesitándose más estudios que confirmen estos hallazgos.

Finalmente, considerando (1) que la evidencia científica disponible es inconsistente, (2) que la potencia xenoestrogénica es particular de cada DE, (3) que los humanos estamos expuestos a una mezcla compleja de DEs con gran variabilidad inter-individual y (4) que podrían ejercer efectos aditivos, sinérgicos y/o antagónicos; se intuye que pueda ser el efecto combinado de varios DEs el que resulte ser un factor causal en la génesis de la enfermedad tumoral. Para cumplir con el cuarto y último objetivo de esta tesis doctoral, evaluar la relación entre la carga total xenoestrogénica (TEXB) en suero y su relación con el riesgo de cáncer de mama, se llevó a cabo un estudio casos-control compuesto por 382 mujeres procedentes del estudio multicaso-control en España (MCC-Spain) en el que se determinó la carga estrogénica total atribuible a compuestos organohalogenados xenoestrogénicos (TEXB-α) y a las hormonas endógenas y compuestos xenoestrogénicos más polares (TEXB-β).

Los casos mostraron una media geométrica de TEXB-α mayor que los controles (8.32 y 2.99 pM/mL, respectivamente), al igual que de TEXB-β (9.94 y 5.96 pM/mL, respectivamente). La OR para el desarrollo de cáncer de mama (ajustada por lugar de residencia, edad, IMC, nivel educativo, hábito tabáquico, paridad, edad del primer embarazo, menopausia, uso de terapia hormonal sustitutiva, historia familiar de cáncer de mama y cantidad de lípidos) fue de 1.77 y 3.45 para los terciles 2 y 3 de TEXB-α, respectivamente. Por tanto, nuestros resultados demuestran que la exposición a DEs xenoestrogénicos podría estar relacionada con el riesgo de

desarrollar cáncer de mama, confirmando la importancia de evaluar mezclas de DEs en lugar de compuestos individuales durante el estudio de enfermedades hormono-dependientes.

2. ABSTRACT

Among all the hormone-dependent cancers, breast cancer (BC) is the most frequently diagnosed in women and is the leading cause of death in women in developed countries, including Spain. Despite all the diagnostic and therapeutic effort made in the last decades, the incidence of BC is still increasing, in particular in the hormone-dependent subtypes (in developed and developing countries). In addition to well-known risk factors for BC such as age, precocious menarche, late menopause, number of pregnancies and cumulative duration of lactation, it has been postulated that exposure to endocrine disrupting chemicals (EDCs) with xenoestrogenic activity may contribute to this increased incidence. This group includes those known as persistent organic pollutants (POPs) [e.g. hexachlorobenzene (HCB), *p,p'*-dichlorodiphenyl-dichloroethylene (*p,p'*-DDE), β-hexachlorocyclohexane (β-HCH) and polychlorinated biphenyls (PCBs)] and non-persistent EDCs (npEDCs) [e.g. bisphenol-A (BPA), parabens and benzophenones]. However, available epidemiological evidence has shown conflicting results, with some studies showing positive associations and others no association between exposure and risk of BC. These discrepancies between studies may be caused, at least in part, by differences in the methodologies applied, the study design, the biomarker of exposure, the time of the exposure, or the study population used.

Firstly, there is not international agreement on which biological matrix is the most adequate to evaluate exposure to lipophilic EDCs in BC patients. Adipose tissue (AT) levels are considered indicators of cumulative exposure, but AT is difficult to sample. In the other hand, serum concentrations (the most accessible and widely used biomarker) may be affected by some physiological conditions such as changes in fat metabolism. Hence, the first specific objective of this PhD thesis is the evaluation of the differences between AT and serum as biomarkers of exposure to POPs in BC patients. To this end, we quantified serum and AT concentrations of a group of POPs in a cross-sectional study including 103 newly diagnosed BC patients at the San Cecilio University Hospital in Granada. In both matrices, *p,p'*-DDE was the most abundant POP (194.34 and 173.84 ng/g lipid in AT and serum, respectively). In general terms, we found significant positive correlation coefficients between pairs of POPs in AT, as well as in serum, which were higher in AT in all the cases. However, we only found positive and statistically significant correlations between serum and AT concentrations for *p,p'*-DDE and HCB but not for PCBs. Age was positively associated with most POPs in AT and serum, while body mass index (BMI) was positively associated with AT HCB concentrations and negatively associated with serum PCB-153 and PCB-138 concentrations. Weight loss was inversely associated with POP residues in AT and positively associated with POP residues in serum. Moreover, serum HCB and PCB-180 concentrations were lower in patients who had received preoperative chemotherapy, while AT concentrations were not influenced by this treatment. Therefore, our results suggest that POP concentrations in AT and serum may be differentially affected by

external predictors in BC patients. Hence, our findings demonstrate that the determination of POP concentrations in both matrices, rather than in one, may be potentially useful for those studies aimed at evaluating the risk of BC.

Second, human exposure to EDCs and their carcinogenic effects is a major public health concern particularly in developing countries, where levels of exposure may be different due to the late prohibition of EDCs, their illegal use or inadequate legislation, which may result in inadvertent and substantial exposure in these populations. To address the second and third objectives of this PhD thesis, EDC exposure (POPs and npEDCs) was characterized and its association between POPs and risk of BC was investigated in a case-control study involving 125 women living in Tunisia, where old stockpiles of these compounds have been poorly managed. Characterization of EDC exposure in Tunisia (objective 2) was carried out in the control group of this study. Among the ten POPs analyzed, HCB, *p,p'*-DDE and PCBs (-138, -153 and -180 congeners) showed detection frequencies close to 100%. Median serum concentrations of PCB congeners (-138, -153 PCB-180) were 26.08, 119.10 and 29.84 ng/g lipid, respectively. Median concentrations of HCB and *p,p'*-DDE were 19.98 and 127.59 ng/g lipid, respectively. Age was positively correlated with serum levels of the selected POPs. Women living in Northern Tunisia showed higher serum levels of all the PCBs analyzed. Working outside home and cereal consumption were positively associated to serum levels of *p,p'*-DDE. Cumulative lactation time was also related to lower serum levels of *p,p'*-DDE and HCB. Simultaneously, urinary geometric mean concentrations of methyl-, ethyl-, propyl- and butylparaben were 30.10, 1.43, 2.03 y 0.47 ng/mL, respectively. Geometric mean level of urinary BPA was 0.44 ng/mL, while geometric mean levels of urinary benzophenone-1 and benzophenone-3 were 1.33 and 1.10 ng/mL, respectively. Furthermore, higher urinary levels of benzophenone-1, benzophenone-3, methyl-paraben and propyl-paraben were found in premenopausal women. Therefore, the levels of exposure to EDCs and the determinants of the exposure found in Tunisian women warrant the introduction of biomonitoring programs in order to identify sources of exposure and population groups at higher risk.

The association between POP exposure and risk of BC in Tunisian women (objective 3) was evaluated by unconditional logistic regression analyses. After adjustment for age, BMI, occupational class, residence, educational level, cumulative duration of lactation, parity, menopausal status, family history of BC and total serum lipids, β-HCH and *p,p'*-DDE were positively associated with BC (OR=1.10 and OR=1.37), while heptachlor was borderline associated (p=0.078). Moreover, models using POP concentration tertiles corroborated a positive dose-response relationship for *p,p'*-DDE (OR=6.26 and OR=9.65 for second and third tertiles, respectively). Women with detectable levels of β-HCH showed an OR=3.44 for BC. Finally, the relative influence of each chemical in the presence of the others was assessed by

entering the three chemicals in a single model with all the covariates, and only β-HCH remained positively associated with the risk of BC (OR=1.18). Taken together, our results suggest that exposure to POPs (at least the exposure to β-HCH) may be associated to risk of BC in the Tunisian population, although these results should be confirmed in future studies.

Finally, considering that (1) the available scientific evidence in inconsistent; (2) the endocrine potency is specific for each EDC; (3) human beings are globally exposed to complex mixtures of EDCs, with high inter-individual variability; and (4) EDCs can act cumulatively but also exerting synergistic or antagonistic effects; it seems plausible that the combined effect may be a risk factor in BC development. To explore the last objective of this PhD thesis, the evaluation of the relationship between the combined estrogenic effect of mixtures of xenoestrogens in serum and risk of BC, a case-control study was carried out. The study involved 382 women from a large population-based multicase-control study in Spain (MCC-Spain) where the total effective xenoestrogen burden attributable to organohalogenated xenoestrogens (TEXB-α) and endogenous hormones and more polar xenoestrogens (TEXB-β) were quantified. Cases had higher geometric mean TEXB-α and TEXB-β levels (8.32 and 9.94 pM/mL, respectively) than controls (2.99 and 5.96 pM/mL, respectively). The OR for BC (adjusted for place of residence, age, BMI, educational level, smoking habits, parity, age at first birth, menopause, hormone replacement therapy, family history of BC and total lipid content) were 1.77 and 3.45 for second and third tertiles of TEXB-α, respectively. Therefore, our results demonstrate that exposure to xenoestrogenic EDCs may be related to higher risk of BC, confirming the importance of evaluating mixtures of EDCs, rather than evaluating single compounds, when studying hormone-related cancers.

3. ABBREVIATION LIST

AR: androgen receptor

ASR: age-standardized rate

AT: adipose tissue

BC: breast cancer

BMI: body mass index

CI: confidence intervals

EDC: endocrine disrupting chemical

Eeq: equivalent unit

EPA: Environment Protection Agency

ER: estrogen receptor

DDE: dichlorodiphenyldichloroethylene

DDT: dichlorodiphenyltrichloroethane

HCB: hexachlorobenzene

HCH: hexachlorocyclohexane

HDL: high-density lipoprotein

HPLC: high-performance liquid chromatography

IARC: International Agency for Research on Cancer

LOD: limit of detection

LDL: low density lipoprotein

MMP: matrix metalloproteinase

NHL: non-Hodgkin's lymphoma

npEDC: non-persistent endocrine disrupting chemical

NR: nuclear receptor

OCP: organochlorine pesticide

OR: odd ratio

PCB: polychlorinated biphenyl

POP: persistent organic pollutant

QT: chemotherapy

RPE: relative proliferative effect

RPP: relative proliferative potency

TEQ: toxic equivalent

TEXB: total effective xenoestrogen burden

TR: thyroid receptor

4. INTRODUCTION

4.1 Hormone-dependent cancer

The endocrine system is a network of glands and organs that produce and release hormones, which act as chemical messengers between different parts of the body. Each hormone targets a particular type of cells able to receive and respond to even very low doses of hormones, inducing different effects in these target organs and tissues such as growth and development, changes in the sexual function or reproduction. For this reason, homeostasis of hormonal synthesis and action is thoroughly regulated in the organisms (Neal 2016).

Hormone-dependent cancers are classically those of the reproductive tract, such as prostate and testis cancer in men, and breast, ovarian and endometrial cancer in women. In addition to the reproductive tract, thyroid is also a hormone-regulated organ (Carroll 1975) and, to a lesser extent, lung and liver cancer are also sensitive to sex steroid hormones (Stabile et al. 2002; Naugler et al. 2007).

Under physiological conditions, sex steroid hormones such as estrogens, androgens and progestins control cell proliferation and differentiation rates. When normal cells transform into tumor cells, they can retain or not their features. Hence, while some tumors are still controlled by hormones, others are no longer regulated by them. In women, it is estimated that 65% of total breast cancer (BC) are hormone-dependent, being more frequent during the postmenopausal period (DeSantis et al. 2014). Although the underlying factors for the development of hormone-dependent cancers are not fully understood, sex steroid hormones are considered the primary modulators of the normal development and maintenance of these organs, as well as of the malignant growth (Herington et al. 2010). This PhD thesis focuses on the role of hormone-mimicking chemicals in BC development, because the underlying estrogen-dependent mechanisms involved in the development of BC are the best understood among these hormone-sensitive cancers.

4.2 Breast cancer in numbers

Excluding skin cancers, BC is the most common cancer diagnosed among women in both developed and developing regions, with an estimated amount of 1.38 million new cancer cases (22.9% of all cancers) diagnosed worldwide in 2008. The age-standardized rate of incidence (ASR per 100.000 person-years) was 39.0 and the cumulative risk was 4.1% in 2008 (Ferlay et al. 2010), being the main cause of over half a million deaths (13.7% of all cancers) worldwide in 2008. The ASR of mortality was 12.5 and the cumulative risk was 1.3% (Ferlay et al. 2010)

(Table 1). By continents, North America has the highest incidence rate while Asia and Africa have the lowest incidence. Europe is at the middle with almost half a million new cases diagnosed in 2012 (28.8% of all cancers) (ASR of 94.2), being the leading cancer in terms of mortality in most European countries (Ferlay et al. 2013).

	Both sexes				Male				Female			
Cancer site	Cases	(%)	ASR (world)	Cum. Risk (0–74)	Cases	(%)	ASR (world)	Cum. Risk (0–74)	Cases	(%)	ASR (world)	Cum. Risk (0–74)
Lip, oral cavity	263	2.1	3.9	0.4	170	2.6	5.3	0.6	92	1.5	2.6	0.3
Nasopharynx	84	0.7	1.2	0.1	57	0.9	1.7	0.2	26	0.4	0.8	0.1
Other pharynx	135	1.1	2.0	0.2	107	1.6	3.4	0.4	27	0.4	0.8	0.1
Oesophagus	482	3.8	7.0	0.9	326	4.9	10.2	1.3	155	2.6	4.2	0.5
Stomach	989	7.8	14.1	1.7	640	9.6	19.8	2.4	349	5.8	9.1	1.0
Colorectum	1233	9.7	17.3	2.0	663	10.0	20.4	2.3	570	9.4	14.6	1.6
Liver	748	5.9	10.8	1.2	522	7.9	16.0	1.8	225	3.7	6.0	0.7
Gallbladder	145	1.1	2.0	0.2	58	0.9	1.8	0.2	86	1.4	2.2	0.3
Pancreas	277	2.2	3.9	0.4	144	2.2	4.4	0.5	133	2.2	3.3	0.4
Larynx	151	1.2	2.3	0.3	130	2.0	4.1	0.5	21	0.3	0.6	0.1
Lung	1608	12.7	23.0	2.8	1095	16.5	34.0	4.1	513	8.5	13.5	1.6
Melanoma of skin	197	1.6	2.8	0.3	101	1.5	3.1	0.3	96	1.6	2.6	0.3
Kaposi sarcoma	34	0.3	0.5	0.0	22	0.3	0.6	0.1	12	0.2	0.3	0.0
Breast	1383	10.9	39.0	4.1					1383	22.9	39.0	4.1
Cervix uteri	529	4.2	15.2	1.6					529	8.8	15.2	1.6
Corpus uteri	287	2.3	8.2	1.0					287	4.8	8.2	1.0
Ovary	225	1.8	6.3	0.7					225	3.7	6.3	0.7
Prostate	913	7.2	28.5	3.5	913	13.8	28.5	3.5				
Testis	52	0.4	1.5	0.1	52	0.8	1.5	0.1				
Kidney	271	2.1	3.9	0.5	167	2.5	5.2	0.6	103	1.7	2.8	0.3
Bladder	386	3.0	5.3	0.6	297	4.5	9.1	1.0	89	1.5	2.2	0.2
Brain, nervous system	238	1.9	3.5	0.3	127	1.9	3.9	0.4	110	1.8	3.2	0.3
Thyroid	212	1.7	3.1	0.3	49	0.7	1.5	0.2	163	2.7	4.7	0.5
Hodgkin lymphoma	67	0.5	1.0	0.1	40	0.6	1.2	0.1	27	0.4	0.8	0.1
Non-Hodgkin lymphoma	355	2.8	5.1	0.5	199	3.0	6.1	0.6	156	2.6	4.2	0.4
Multiple myeloma	102	0.8	1.5	0.2	54	0.8	1.7	0.2	47	0.8	1.3	0.1
Leukemia	351	2.8	5.1	0.5	195	2.9	5.9	0.6	155	2.6	4.3	0.4
All cancers excl. non-melanoma skin cancer	12677	100.0	181.8	18.7	6639	100.0	204.4	21.2	6038	100.0	164.9	16.5

Table 1. Worldwide incidence of cancer, 2008. Estimated new cases (thousands), age-standarized rates (ASR per 100,000) and cumulative risks to age 75 (percent) by sex and cancer site worldwide *(Ferlay J et al. 2010).*

The factors that contribute to the international variation in incidence rates largely stem from differences in reproductive and hormonal factors and the availability of early detection programs (Jemal et al. 2011). However, differential exposure rates to environmental factors, which are known to play an important role in the pathogenesis of the disease, may also contribute to this international variation (Soto and Sonnenschein 2010; Soto and Sonnenschein 2015). Although Spain in considered one of the European countries with lowest incidence of BC, more than 25.000 new cases were diagnosed in 2012, and 6,000 deaths were recorded in the

twelve Spanish cancer registries (Ferlay et al. 2013). In Spain, Huelva is the Spanish province with the highest incidence rate, while Granada has a low-middle incidence rate in comparison with other Spanish provinces. In 1985, a cancer registry was created in Granada. It has registered 1.337 new cases (ASR of 83.0) between 2009-2011.

In the Tunisian population, from which this PhD is partly focused, BC represents 33% of female cancers in Tunisia, with approximately 1,600 new cases per year. Although BC is less frequent in Tunisia than in European countries (Maalej et al. 2008), the crude incidence of diagnosed BCs increased from 25.5 cases/100.000 women in 1995–1998 (RCNT 1998) to 32.3 in 2004–2006 (RCNT 2006).

In addition to the crude numbers, the incidence and mortality trends are also useful to identify new putative risk factors and to evaluate cancer-control strategies. Despite the fact that BC mortality has decreased since 1993, it has been published that BC incidence is still increasing in Granada, with an annual increase of 2.5% (RCG 2014) and a recent recession in the whole country that could be related to the saturation of the screening programmes (López-Abente et al. 2014).

4.3 Breast cancer etiology: more than a genetic instability

Despite the fact that the etiology of BC is still largely unknown, there are consistent epidemiological data that have identified risk factors, crucial in the prevention and estimation of individual risk. In this regard, a person known or suspected to be at increased risk of BC may warrant surveillance based on clinical examination and imaging studies at specific intervals (Mahoney et al. 2008). However, it is worth mentioning that the majority of women with BC do not have any identifiable risk factor (Lacey et al. 2009).

Well-established risk factors for BC can be grouped into (1) non-modifiable risk factors (sex, age, ethnicity, genetic factors, family history, menarche), (2) modifiable risk factors (BMI, reproductive history, diet, physical activity, smoking habits, exogenous estrogen consumption or alcohol] and (3) potentially modifiable risk factors (age at first pregnancy, lactation or menopausal age) (Mahoney et al. 2008) (Table 2).

Considering the non-modifiable risk factors, it is known that BC is a rare event in males, with an incidence ratio of 1 in 100 in comparison with women (Ahmedin Jemal et al. 2008). Together with the sex, age is considered the main risk factor to develop BC, doubling the risk every 10 years until menopause, then the risk slows down (Vogel 2008). Studies carried out in

the US revealed that non-Hispanic white women have the highest incidence rate and Asian American and Hispanic women, the lowest (Ma and Jemal 2013).

Non-modifiable	Modifiable
Female gender	Body mass index
Age	Reproductive history
Genetic changes (mutations, BRCA,…)	Diet
Family history of breast cancer	Physical activity
Race / Ethnicity	Smoking habits
Density	Hormone-replacement therapy
Precocious Menarche	Oral contraceptives
Potentially modifiable	Alcohol
No. Pregnancies	
Cumulative duration of lactation	
Late menopause	

Table 2. Etiology of breast cancer. Best known risk factors for breast cancer stratified by the plausibility to be modified.

Regarding menarche and menopausal age, it is known that estrogenic stimuli lead to a higher risk of developing the disease (Kelsey et al. 1993). In this respect, precocious menarche has been associated with higher estradiol levels in the adolescent period (Bernstein 2002), and late menopause has been associated with higher risk of BC (Travis et al. 2003). In fact, it has been hypothesized that increased numbers of regular ovulatory menstrual cycles increase the risk of BC (Bernstein 2002), which is mainly regulated by sex hormones. Parity (Singletary et al. 2003) and longer cumulative duration of lactation, characterized for the critical influence of female hormones, have also been associated with a decreased risk for BC (Collaborative Group on Hormonal Factors in Breast Cancer 2002), while the consumption of oral contraceptives (Kahlenborn et al. 2006) or hormone replacement therapy has been suggested to increase BC risk (Million Women Study Collaborators 2003).

Concerning the inheritance of BC, most of cases are spontaneous and no familiar association can be found. However, it is known that the existence of family history increases the relative risk for the development of BC (Conzen 2008). Moreover, scientific research showed the

extremely high frequency of mutations in specific genes (BRCA1 and BRCA2) in breast tumors, and therefore, much effort has been made for a better understanding of the role of genetic instability in the development of BC (Miki et al. 1994).

However, in spite of the huge amount of genetic mutations in BC identified during the last decades, a vast array of studies has revealed that there are many environmental factors that can be modified for prevention purposes. In this sense, it is known that obesity (Lahmann et al. 2004), fatty diet (Sieri et al. 2008) and consumption of alcohol (Tjønneland et al. 2007) and tobacco (Baron et al. 1996) increase the risk of BC, while regular physical activity reduces the risk (Bernstein 2008).

In spite of that, the mentioned increase in the incidence of BC during the past 50 years cannot be accounted for by the introduction of screening mammography or by known risk factors (Soto and Sonnenschein 2015). It has been hypothesized that this significant increase in the incidence of BC in the industrialized world may be partially due to exposure to hormonally active chemicals, particularly xenoestrogens (Davis et al. 1993).

4.4 The endocrine disrupting hypothesis

From the late 1990s, a number of definitions of what is an endocrine disrupting chemical (EDC) have been coined by national and international government agencies. The first definition of what constitutes an endocrine disrupting chemical was published in the proceedings of a workshop organized by the United States Environment Protection Agency (EPA) (Kavlock et al. 1996):

"(...) an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes."

Despite this definition, in 1996 the term *"adverse"* was included, introducing a problematic issue for the first time. Since then, a group of keywords such as *"intact organisms"* or *"(sub)populations of organisms"* have been added to the original definition due to the increasing understanding of the underlying mechanisms by which an EDC can exert its effects, so the original idea of ECDs exerting actions primarily through nuclear hormone receptors such as estrogen (ERs), androgen (ARs) or thyroid receptors (TRs) has evolved to a much broader mechanism that also acts via non-nuclear steroid hormone receptors (e.g. membrane ERs), non-steroid receptors (e.g. neurotransmitter receptors), enzymatic pathways involved in steroid

biosynthesis and/or metabolism, and numerous other mechanisms that converge upon endocrine and reproductive systems (Diamanti-Kandarakis et al. 2009).

The working definition of an EDC adopted by the International Programme for Chemical Safety (IPCS - which involves WHO, UNEP and ILO), together with Japanese, USA, Canadian, OECD and European Union experts is:

"An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations."

4.4.1 Classification of EDCs. The problem of the chemical structure

There are growing concerns about the number of synthetic chemicals able to interfere with the hormone system. Traditionally, EDCs were identified due to their similarity with the chemical structure of the endogenous hormones, and therefore, with their ability to interfere with them and/or their receptors. Currently, the group of molecules identified as EDCs is highly heterogenous and may not appear to share any structural similarity. However, the whole group of demonstrated or suspected EDCs are molecules with small molecular mass (<1000 Daltons), and usually containing halogen group substitutions by chlorine and bromine, with a phenolic ring that is thought to mimic natural steroid hormones and therefore, these compounds are able to interact with steroid hormone receptors (Diamanti-Kandarakis et al. 2009).

Resistance of EDCs to physical, chemical and biological degradation as well as their degree of liposolubility are useful predictors of the environmental and biological kinetics of these compounds. In this sense, EDCs have been traditionally divided into "persistent organic pollutants" (POPs) and "non-persistent EDCs" (npEDCs).

The first group comprises a wide variety of organochlorine pesticides (OCPs) [including dichlorodiphenyltrichloroethane (DDT) and its metabolite dichlorodiphenyldichloroethylene (DDE), hexachlorobenzene (HCB), and γ- hexachlorocyclohexane (γ-HCH)]; polychlorinated biphenyls (PCBs), dioxins, furans and polybrominated diphenyl ethers (PBDEs) used as flame retardants. However, POPs can be included in a wider group of compounds called "persistent toxic substances" that also includes some heavy metals (e.g. mercury) (Porta et al. 2008a). These persistent substances were designed to have long half-lives to persist in soil and, therefore, they are very slowly metabolized in living organisms, and they can even be broken down into more toxic compounds than the parent molecule. Due to their persistence and lipophilicity, POPs tend to bioaccumulate and biomagnify in the food chain, resulting in

considerable exposure of living organisms (Frenich et al. 2000; Botella et al. 2004; Cerrillo et al. 2006; Fernandez et al. 2008; Lopez-Espinosa et al. 2008; CDC 2009; Arrebola et al. 2013a; Schettgen et al. 2015) even decades after these compounds were banned. In fact, since the early 1970s, most countries have banned or severely restricted the production, handling, and disposal of POPs due to the consistent evidence of their clinical effects at doses traditionally considered safe, including reproductive disorders, teratogenicity or carcinogenicity (Olea et al. 2001a, b; Porta et al. 2008b).

In the other hand, the npEDC group includes bisphenol A (BPA), parabens (methyl-paraben, ethyl-paraben, propyl-paraben and butyl-paraben), phthalates or UV-filters. They are less liposoluble than POPs and therefore, they are proned to be metabolized and excreted via the urine (Frederiksen et al. 2007; Søeborg et al. 2014) instead of accumulating in the adipose tissue (AT). Due to the fact that the clinical adverse effects of the npEDC group have been less studied, there is a lack of international ban in their production, handling, and disposal [with the exception of BPA which has been by the European Commission (2011/8/EU) (The European Commission 2011)], and control is limited to few national laws in most developed countries. For instance, on 21 March 2011, Denmark notified the European Commission the ban on propylparaben, butylparaben, their isoforms and salts in cosmetics for children on the grounds of reproductive toxicity.

This PhD thesis mainly focuses on EDCs from the POP group, including HCB, HCH, *p,p'*-DDE and PCBs (Figure 1). HCB, with a half-live of up to 23 years in soil, started to be used as a fungicide since 1945 to slow down fungal growth. Since then, it has been used in different crops. In 1971 the use of HCB as fungicide was banned in the US, but it is still used in the chloride solvent and pesticide production (ATSDR 2015). Currently, HCB can be mostly found in treated and background soils, sediments, and oceans. It can also be found in air, surface water, and groundwater due to the disposal of HCB products into these environmental compartments and as a byproduct of other processes. Moreover, HCB can also be detected in fish and food products. In fact, it has been estimated that fish coming from heavily polluted areas can contain levels of HCB greater than 100 ng/g tissue (ATSDR 2015). Current evidence suggests that HCB may interfere with ovarian function, fertility, seminal vesicle weight, as well as with children disorders such as mental and physical development or cryptorchidism (Reed et al. 2007). However, the role of HCB in BC is still unclear. Although some researchers have detected higher levels of HCB in BC patients than in controls (Mussalo-Rauhamaa et al. 1990; Charlier et al. 2003; Charlier et al. 2004), others have found no association (Tongzhang Zheng et al. 1999).

Lindane is the most used organochlorine pesticide from those included in this PhD thesis. It is the gamma isomer of HCH (γ-HCH), accounting for 15% of the total HCH. In fact, it is estimated that each tonne of lindane generates between 8 and 12 tonnes of other HCH waste isomers (e.g. α-HCH and β-HCH) (Bodenstein 1972). Despite its use is now banned in Western countries, lindane was extensively used as insecticide some decades ago. Based on the experience of our research group in the analysis of human exposure to lindane in Southern Spain, HCH is a usual residue in human tissues and fluids (Campoy et al. 2001; Botella et al. 2004; Carreño et al. 2007; Lopez-Espinosa et al. 2007). Regarding the health related effects of HCH, *in vitro* studies have demonstrated estrogenic activity of HCH in BC cell lines (Steinmetz et al. 1996). Furthermore, exposure to HCH has been positively associated with cryptorchidism and hypospadias in children (Fernandez et al. 2007a), and with obesity in adults (Arrebola et al. 2014). Moreover, exposure to HCH was found to be positively associated with increased BC risk (Ibarluzea et al. 2004) and non-Hodgkin's lymphoma (NHL) (Rafnsson 2006).

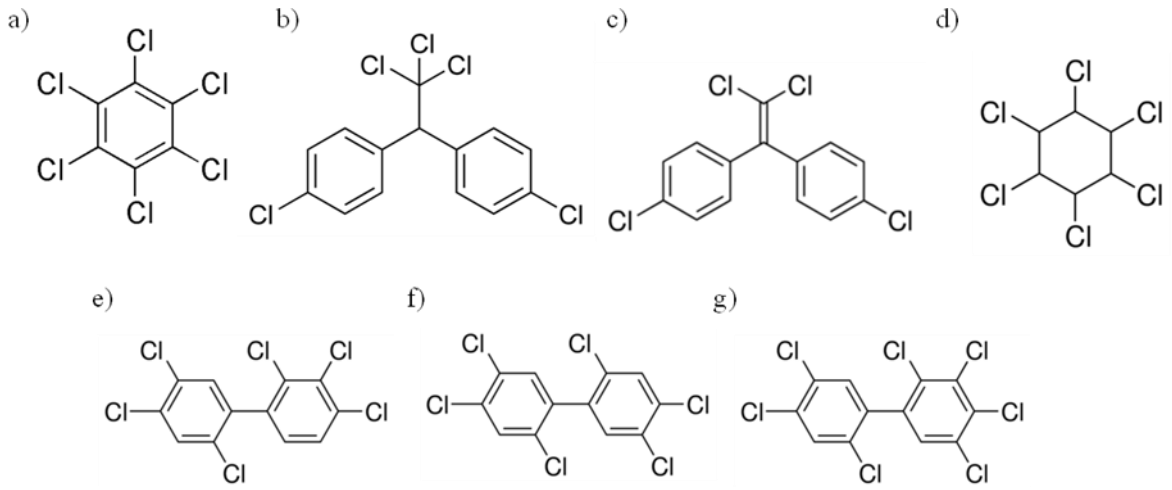


Figure 1. Chemical structure of POPs included in this PhD thesis: a) hexachlorobenzene (HCB); b) dichlorodiphenyltrichloroethane (DDT), c) dichlorodiphenyldichloroethylene (DDE); d) hexachlorocyclohexane (HCH); e) polychlorinated biphenyl (PCB)-138; f) PCB-153; g) PCB-180

DDT and its metabolite DDE have been extensively studied in relation to adverse human health effects. DDT is a pesticide widely used to control insects on agricultural crops and vectors carrying diseases like malaria or typhus in the past (ATSDR 2002). Although DDT use for agricultural purposes is banned in most countries, it is still used for vector control in a few countries, particularly in Africa. Although DDT was banned some decades ago, it can last in the soil for a very long time, potentially for hundreds of years. Although considerable controversy still exists regarding health effects associated to DDT/DDE exposure, overall epidemiological evidence suggests that positive associations with reproductive effects are usually found in areas where DDT was used for vector control purposes (Ayotte et al. 2001; Aneck-Hahn et al. 2007),

while either positive or no associations are found when studies are conducted in low exposed populations (Hauser et al. 2003; Charlier et al. 2004; Charlier and Foidart 2005). Similarly, epidemiological studies focused on BC have found positive (Wolff et al. 1993; Charlier et al. 2004) and no association with *p,p'*-DDE exposure (Toingzhang Zheng et al. 1999; Aronson et al. 2000; Wolff et al. 2000).

PCBs are a class of aromatic compounds comprising 209 congeners, which were widely used as dielectric fluid in capacitors and transformers, as well as in building materials. Although PCB production was banned in most countries by the 1980s, PCBs are still found in atmosphere, soil, rivers, lakes, fish, wildlife animals and humans (Safe 1994; Zhang et al. 2015). Despite exposure to these chemicals in the general population have decreased since they were banned, almost the entire population have detectable levels of some of them (Fernandez et al. 2008; Arrebola et al. 2012a). The role of PCBs in breast carcinogenesis is associated to their potential to induce estrogenic and anti-estrogenic effects in human breast cells (Bonefeld-Jørgensen et al. 2001), as well as to induce cytochrome P450 enzymes (Moysich et al. 1999). In fact, P450-metabolized PCBs result in more estrogenic compounds (Soto et al. 1995). In addition to BC, PCBs are suspected to be related to low birth weight (Rylander et al. 1998; Govarts et al. 2012) and neurodevelopmental disorders in children (Caspersen et al. 2016), among other health effects.

4.4.2 Sources of exposure to persistent EDCs

Despite the use of POPs is almost completely restricted in Western countries, severe human exposure to POPs is suspected in those countries where these chemicals are still in use today, such as DDT in malaria-affected countries (Whitworth et al. 2014), and in some heavily contaminated areas where old stockpiles have been poorly managed, as it has happened in some populated areas of Armenia (Dvorská et al. 2012), Mexico (Trejo-Acevedo et al. 2013), Bolivia (Mercado et al. 2013) and in some African countries like Tunisia (Dasgupta et al. 2010) (Figure 2). Sabiñánigo, a municipality located in Northern Spain, has also been recently pointed out as a heavily contaminated area, with a huge stockpile of technical HCH that has been poorly managed in the last decades (Fernández et al. 2013). Therefore, exposure to these chemicals in hot spots should be an issue of public health considering its magnitude in developing countries (Trejo-Acevedo et al. 2009).

In addition to these hot-spot areas, global population is suspected to be primarily exposed to POPs through the diet, rather than through inhalation, given the bioaccumulation pattern of these chemical in the food chain (Porta et al. 2008b). In fact, many studies conducted in

different populations have identified that diet, and especially fatty food is a crucial source of exposure for the general population (McGraw and Waller 2009; Gasull et al. 2010; Arrebola et al. 2012b). Therefore, high-fat diet consumers should be also considered a subgroup of population highly exposed to POPs. Hence, the general population is mainly exposed to POPs through the dietary intake, which in combination with the bioaccumulation pattern of these chemicals, leads to a long-term exposure to POPs. For example, salmon, an oil-rich fish with a life-cycle of approximately 3-8 years, contains 2.9 ng/g tissue HCB, 21 ng/g DDT, or 2.4 toxicity equivalents (TEQ) pg/g PCBs (Berntssen et al. 2010). Dairy products and egg consumption have also been identified as important sources of exposure to POPs due to their high fat content (Agudo et al. 2009). Moreover, residues of POPs have been found in fruit, vegetables and cereals that have come into contact with contaminated soils (Tao et al. 2005), although it should be expected from areas suspected of a recent use of these pesticides. Therefore, remedial strategies should focus on reducing POP into the food chain, especially in hot spot areas (Černá et al. 2012).

Figure 2. Current state and geographical distribution of obsolete stockpiles in Tunisia. National Agency for Waste Managment (ANGed). Department of Agriculture and Environment. Republic of Tunisia

4.4.3 Mechanisms of action in endocrine disruption

In addition to the identification of human diseases related to exposure to EDCs, much effort has been made in the last years to understand the mechanisms of action of these compounds, as well as to understand how different compounds without similar chemical structures can exert similar

physiological effects. In this regard, some of the properties of EDCs can make the study of these mechanisms more difficult (Olea et al. 2002), including:

- EDCs have a very low potential of action in comparison to natural hormones.
- The variety on the nature and chemical structure of EDCs makes difficult to identify potential compounds and their sources of exposure.
- The possibility that the combined effect of several EDCs may be crucial to exert a hormonal effect, making measurements even more difficult due to the lack of standardized methods to test these effects.
- The uncertainty about (1) the specific effect that each EDCs may exert on each target organ, the time-point of exposure or (3) the levels of endogenous hormones existing simultaneously.

There are three main pathways by which a xenobiotic can disrupt the hormone system (Figure 3). Although, theoretically, EDCs may affect every possible hormonal pathway, their interactions with nuclear hormone receptors (NRs), especially ERα, ERβ, is by far the most studied mechanism of endocrine disruption (Rüegg et al. 2009). These NRs can bind to DNA and modify the expression of down-stream genes, and can therefore act as orchestrators in the development, physiology and disease. However, EDCs also act outside the cell nucleus interacting with cell membrane-bound receptors, therefore, affecting a variety of well-known signalling cascade proteins (Schug et al. 2013). There is also strong evidence showing that EDCs can also disrupt the hormone system in an indirect way, through the modification of biosynthesis, metabolism and/or hormone excretion (Rüegg et al. 2009). In addition to that, there are some studies giving EDCs a putative role in other physiological mechanism, such as interfering with the epigenetic remodelling of DNA (Anway et al. 2005; Vilahur et al. 2014a) or modifying the activity of hormone receptors (Jansen et al. 2004).

4.4.4 Strategies for the evaluation of the endocrine activity of synthetic chemicals

This wide variety of mechanisms of action of EDCs significantly hampers the evaluation of the health effects associated to human exposure to endocrine disruptors. The first step aimed to clarify the adverse effect of this exposure should be to determine the endocrine disrupting properties for suspected compounds. In this regard, there are a variety of validated *in vitro* and *in vivo* assays available to test (anti-) estrogenicity (Soto et al. 1995; Takatori et al. 2003; Kuruto-Niwa et al. 2005), (anti-) androgenicity (Térouanne et al. 2000; Freyberger et al. 2010) and thyroid disruption (Santini et al. 2003). However, due to the interest in the estrogenic

effects of EDCs in this thesis, a more comprehensive description of the strategies for testing xenoestrogenic compounds has been provided.

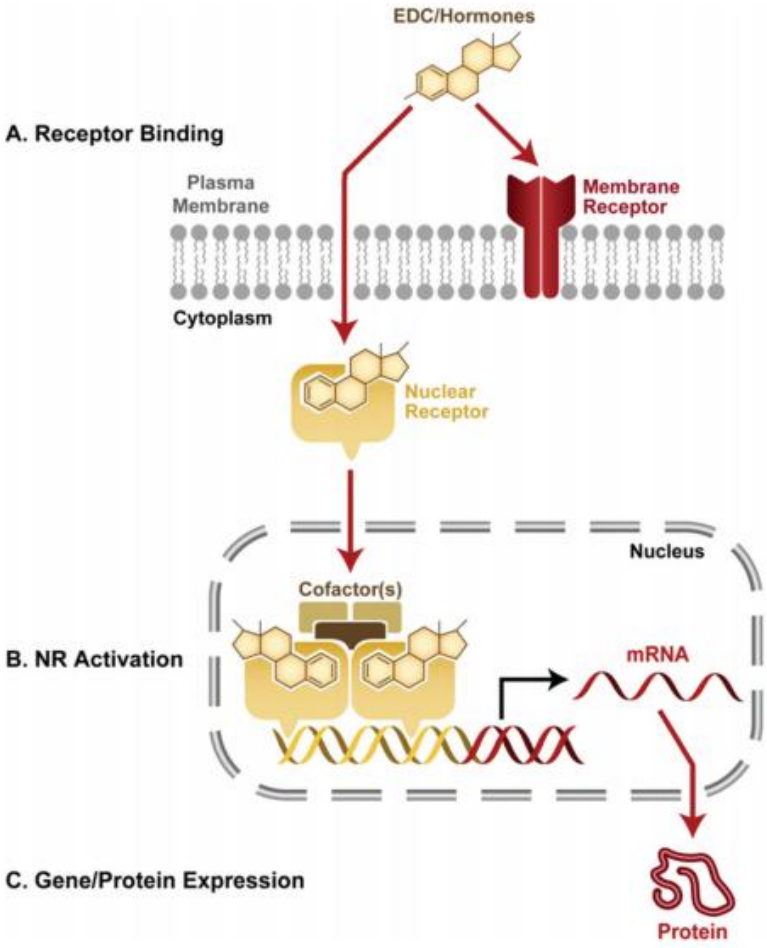


Figure 3. Mechanisms of action of endocrine disrupting chemicals (EDCs). Best known EDC mechanisms of action include extracellular binding to membrane receptor (A), interactions with nuclear receptors (B) and modification of gene and/or protein expression profile *(Schug TT et al. 2013).*

A number of *in vitro* assays have been reported for testing xenoestrogenic properties of suspected compounds. They are based on known mechanisms of action of the endogenous hormones, and include cell proliferation and gene expression assays in mammalian cells and yeast, competitive ligand binding assays using globulins and receptors or measurements of the enzymatic activity involved in steroid synthesis [reviewed in (Zacharewski 1998)]. However, in spite of this wide variety of strategies for testing xenoestrogenic properties of synthetic chemicals, by far most of the studies carried out to identify endocrine disrupting activity are based on the slightly modified version (Villalobos et al. 1995) of the cell proliferation assay called "E-SCREEN assay" (Soto et al. 1995). This cell-culture assay is based on the

proliferation effect exerted by an estrogen-like substance in a human BC estrogen-sensitive MCF-7 cell line. The E-SCREEN assay was developed following three premises: (i) factors in human serum inhibit the proliferation of MCF-7 cells, (ii) estrogens induce cell proliferation by negating this inhibitory effect, and (iii) non-estrogenic steroids and growth factors do not neutralize the inhibitory signal present in human serum. The simplicity of the methodology has led the E-SCREEN to be used in thousands of studies. Briefly, the E-SCREEN assay work as follows: a similar number of MCF-7 cells are seeded in each well, they are allowed to attach for 24 hours, and then the seeding medium is replaced by 10% CDHS-supplemented phenol red-free DME. A range of concentrations of the compound being tested should be added, and the assay is stopped after 144 hours by removing the medium from wells, fixing the cells, and staining them with sulforhodamine-B. Finally, bound dye is solubilized, transferred to a new well and colorimetrically measured. The estrogenic activity of xenobiotics is then assessed as follows: first, the ratio between the cell yield obtained and the proliferation of hormone-free control cells (negative control) is calculated. Then, results are expressed as proliferative effect (PE) [MCF-7 cell proliferation (-fold over control)]. Finally, the dose resulting in half-maximal MCF-7 cell proliferation (EC50 value) is calculated for each compound.

Following this methodology, a wide variety of synthetic compounds have been identified as exerting xenoestrogenic properties, including some POPs (Soto et al. 1995; Rasmussen et al. 2003), and npEDCs (Okubo et al. 2001; Bonefeld-Jorgensen et al. 2007) (Table 3).

Compound	RPE, %	RPP, %
Estradiol	100	100
***o,p'*-DDT**	96	0.016
***p,p'*-DDE**	92	0.008
HCB	-	-
β-HCH	61	0.04
PCB-138	-	-
PCB-153	-	-
PCB-180	-	-

Abbreviations: RPE, relative proliferative effect; RPP, relative proliferative potency

Table 3. Estrogenic response of studied POPs, using the E-SCREEN assay [modified from (Rasmussen et al. 2003)]

In addition to this competition with nuclear ER receptors, it has also been suggested that POPs may induce estrogenicity by alternative mechanisms, for example, increasing local biosynthesis

of estrogens by inducing aromatase and sulfatase pathways (Muñoz-de-Toro et al. 2006) or by reducing adiponectin levels (Duggan et al. 2010). Therefore, although POPs with xenoestrogenic potential frequently show a 1000-fold lower affinity for ER in comparison to estradiol (Lemaire et al. 2006), they might induce the production of natural estrogens with the subsequent activation of ERs (Pinzone et al. 2004).

4.4.5 EDCs: Single vs. combined effects

In addition to the importance of the identification of new synthetic chemicals with endocrine disrupting activity, much effort has been made in the last decades to assess human exposure to known EDCs as well as to determine their role in human health. In this regard, the majority of research groups have focused on the associations between levels of specific compounds and different diseases including neurodevelopment (Puertas et al. 2010; Freire et al. 2011; Forns et al. 2012; Shelton et al. 2014), fertility (Bay et al. 2006; Lassen et al. 2014), cancer (Boada et al. 2016; Pi et al. 2016) or metabolic disorders including obesity (Dirinck et al. 2011; Arrebola et al. 2014), diabetes (Airaksinen et al. 2011; Arrebola et al. 2013b) or hypertension (Arrebola et al. 2015). However, considering that (1) the endocrine potency is specific for each EDC, (2) human beings are globally exposed to complex mixtures of EDCs, with high inter-individual variability, and (3) EDCs can act cumulatively but also exerting synergistic or antagonistic effects, it has been suggested the need for assessing health effects from a mixture perspective (UNEP/WHO 2012). In fact, it has been pointed out that the determinant factor for BC risk is the mixture of different EDCs instead of one single EDCs (Boada et al. 2012). Hence, measuring the total estrogenic burden due to environmental contaminants present in a biological sample may be more meaningful than assessing exposure by single-chemical levels of known xenoestrogens (Soto et al. 1995).

In this regard, our research group developed a new tool to assess the total xenoestrogenic activity (TEXB) from the specific mixture of EDCs stored in each biological sample, which is based on the estrogenicity exerted in the E-SCREEN assay (that measures estrogen-dependent proliferative rate in MCF-7 BC cells) by lipophilic synthetic compounds (including POPs) previously separated from the endogenous hormones (Fernández et al. 2004). Hence, this new tool has been standardized for measurements in AT (Fernández et al. 2004) and in placentas (Lopez-Espinosa et al. 2009). Briefly, the biological sample is extracted following a tissue-specific validated method and the components of the residue obtained are then divided in two fractions by high-performance liquid chromatography (HPLC). The HPLC method was developed to allow the separation of natural estrogens (beta-fraction, eluted from 13-30 min) from more lipophilic xenoestrogens (alpha-fraction, eluted from 0-11 min) without their destruction. After drying the fractions under a nitrogen stream, alpha and beta-fractions are

dried and resuspended in charcoal-dextran serum and tested in the E-SCREEN bioassay for estrogenicity according to the originally described technique (Soto et al. 1995) with slight modifications (Villalobos et al. 1995). Each sample is assayed in triplicate with a negative (vehicle) and positive (estradiol) control in each plate. The plate efficiency of fractions is referred to the maximum effect obtained with estradiol and transformed into estradiol equivalent units (Eeq) by reading from a dose–response curve prepared using estradiol (concentration range from 0.1 pM to 10 nM). Results are expressed as total effective xenoestrogen burden (TEXB-alpha and TEXB-beta) in Eeq per gram of lipid (Fernández et al. 2004) (Figure 4).

Since the development in 2004 of the biomarker used to determine the combined or mixed effect of xenoestrogenic chemicals, it has been satisfactorily applied to a number of studies aimed at assessing the health effects of the exposure to environmental xenoestrogens in the last decade. Thus, TEXB burden in AT has been related to a higher risk of BC (Ibarluzea et al. 2004), especially in the leaner postmenopausal subgroup, and TEXB burden in placenta was positively associated with risk of cryptorchidism and hypospadias (Fernandez et al. 2007a), low birth weight (Vilahur et al. 2013) and impairment in neuropsychological development (Vilahur et al. 2014b) in newborns and children. These results, obtained from different populations and from two different biological matrices and for different diseases, may reflect the usefulness of the use of biomarkers of EDCs combined effect in human tissues.

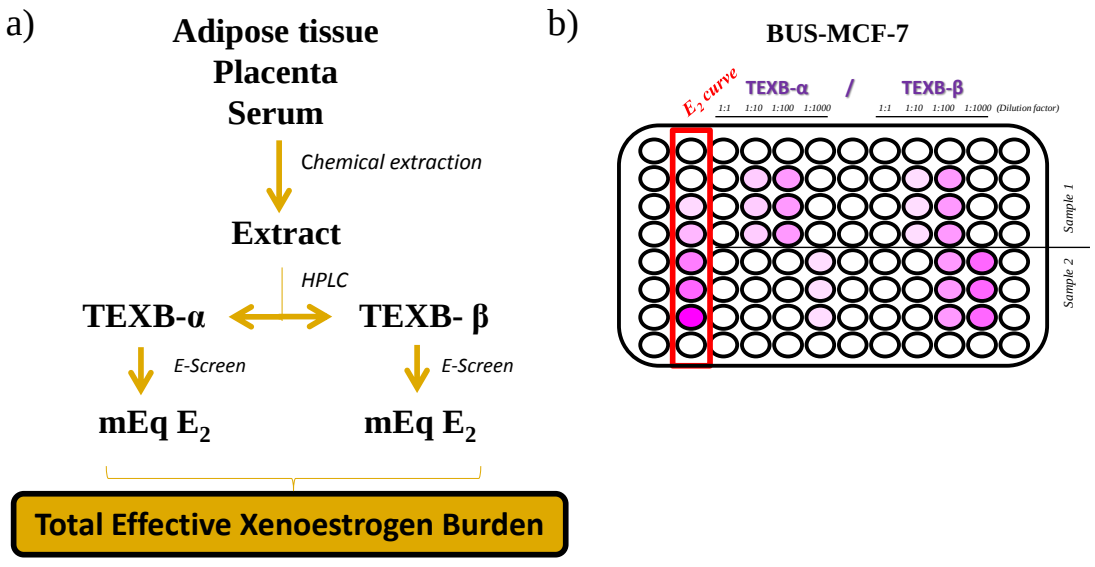


Figure 4. Determination of the Total Effective Xenoestrogen Burden (TEXB). A) Schematic procedure for measuring the Total Effective Xenoestrogen Burden (TEXB). The method includes a chemical extraction followed by fractionation by HPLC and finally the residue is tested in the E-SCREEN assay. B) Example of an E-SCREEN assay for serum extracts.

4.4.6 Biomarkers of human exposure to EDCs in breast cancer patients

The presence of EDCs in human tissues and fluids has been documented by many studies in the last decades (CDC 2009). While the urine is currently considered the most adequate biological matrix for the measurement of the levels of exposure to npEDCs, due to their water-soluble properties that allow them to be excreted via the urine (Søeborg et al. 2014), the estimation of internal burden of POPs is more controversial.

As mentioned above, POPs are a group of chemicals characterized by being highly lipophilic and highly resistant to physical, chemical and biological degradation (Olea et al. 2002). These properties lead to their bioaccumulation in fat compartments, and therefore AT is acknowledged to be the main repository of POPs in the body, accounting for all routes and sources of exposure and representing a stable and long-term reservoir of these compounds (Kohlmeier and Kohlmeier 1995).

However, since samples of AT can be obtained only from patients who undergo surgery, and samples from healthy people are usually difficult to obtain, most studies used blood serum for exposure assessment in the general population (Wolff et al. 1993; Høyer et al. 1998; Helzlsouer et al. 1999; Woodruff et al. 2011). Due to the extended use of serum for the determination of long-term exposure to POPs, the steady-state partitioning between serum and the AT is an important consideration (Rusiecki et al. 2005). In this regard, it was suggested that variation in the partitioning of chronically retained lipophilic xenobiotics between AT and serum may be related to variation in the lipid content of serum (Eyster et al. 1983; Guo et al. 1987).

In the 1980s-90s, it was argued that if lipid-adjusted concentrations in serum and in AT are highly correlated, then measurement in either of these two compartments may be considered a reliable biomarker of exposure (Mussalo-Rauhamaa 1991; Stellman et al. 1998; López-Carrillo et al. 1999; Rusiecki et al. 2005). However, although many authors have found a strong correlation between POP concentrations in serum and AT (López-Carrillo et al. 1999; Pauwels et al. 2000; Waliszewski et al. 2004; Whitcomb et al. 2005), it remains unclear whether POP concentrations in serum can accurately reflect the body burden of these chemicals in all situations (Mussalo-Rauhamaa 1991; Aronson et al. 2000; Wolff et al. 2000; Rusiecki et al. 2005; Arrebola et al. 2012a). In fact, it is possible that POP concentrations in these two matrices may be strongly correlated in some cases but not in others, given the fact that serum concentrations are influenced not only by current exposure but also by the recirculation of POPs from AT due to lipolysis (Crinnion 2009). Therefore, we should question whether the interpretation of serum measurements accurately reflects the AT concentrations of these

compounds, especially during the study of certain population groups suffering from severe physiological changes like BC. For example, most chemotherapy (QT) treatments are believed to reduce concentrations of serum lipid fractions [e.g. triglycerides, total cholesterol and low density lipoprotein (LDL)] in patients with BC (Ray et al. 2001; Shah et al. 2008). Furthermore, given the widespread administration of neoadjuvant treatments in BC treatment, it is relevant to assess whether they can act as confounders or effect modifiers in the potential association between POPs and BC risk.

4.4.7 State of art in the role of EDCs in breast cancer

The development of BC is known to be a multifactorial process but, considered separately, these factors cannot explain the magnitude of the problem. It has been estimated that population-attributable risk of BC risks for the well-established risk factors such as late first birth, high income or family history of BC, is only about 41% (Madigan et al. 1995). Due to the crucial role of long-term high levels of estrogens in the etiology of BC (Key et al. 2002), the potential contribution to carcinogenesis of long-term storage of xenobiotics with estrogenic properties aroused public concern some decades ago . In this regard, it has been shown that incidence rates for ER+ tumors are increasing, despite the global decrease of BC incidence (Ma and Jemal 2013).

Experimental studies revealed increased breast tumor growth and metastasis (Zou and Matsumura 2003; Wong and Matsumura 2007; García et al. 2010; Pontillo et al. 2013; Pestana et al. 2015), and increased angiogenesis (Pontillo et al. 2015) in cancer cell lines and *in vivo* models after exposure to POPs. In addition, there is growing evidence that suggests that these chemicals may also cause cancer via other mechanisms of action, including disruption of the epigenomic landscape (Knower et al. 2014), the induction of enzymes that produce genotoxic intermediates and DNA adducts (Yáñez et al. 2004), and an increase in reactive oxygen and nitrogen species through the induction of cytochrome P450 or mitochondrial changes (Khodarahmi and Azadbakht 2014). In general terms, these experimental studies have revealed that the breast is particularly sensitive to carcinogenic insult during morphogenesis and remodelling [reviewed in: (Fenton 2006)].

In line with this carcinogenic hypothesis for POPs, numerous epidemiologic investigations have been conducted over the last decade but efforts to show associations between the body burden of individual organochlorines and BC have yielded contradictory results. Some studies have showed a strong positive association (Falck Jr et al. 1991; Wolff et al. 1993; Djordjevic et al. 1994; Romieu et al. 2000; Boada et al. 2012; Cohn et al. 2015) while others showed no association [reviewed in: (Snedeker 2001)] but suggested that POPs may contribute to a worse

prognosis. Several factors have been described to explain these discrepancies such as the study design or the target population used, with highly varied historical and current exposure levels to POPs, different ethnicities, age groups and dietary patterns. In addition to that, the biological sample used for the assessment of the POP burden has also been postulated as a potential confounding factor during the evaluation of the role of EDCs in BC risk. In this respect, as we already mentioned, AT is the primary long-term repository for POPs in the body, whereas serum burden is affected by (1) the steady-state partitioning between AT and serum, and (2) current exposure from air and diet (Crinnion 2009). The limited statistical power is another potential confounding factor that may preclude us to find more consistent data. In this regard, the pooled analysis of prospective studies linking organochlorine and BC could rely on only a few thousand women with BC and some thousands of healthy women. In contrast, the number of women included to demonstrate the association between BC and other factors such as hormone replacement therapy exceeded 150.000 women (Collaborative Group on Hormonal Factors in Breast Cancer 1997). Therefore, in view of the limited statistical power of the studies that assess the role of POPs in BC, Kortenkamp A. (2006) reported that it is not very likely that the contribution of an individual factor to BC could be demonstrated. However, this does not prove absence of risks and the conclusion that can be drawn is that the data published so far are inconclusive.

Another relevant confounding factor is the measurements of individual chemicals. In this regard, it has been highlighted that the development of biomarkers that integrate the effects of multiple exposures on the same biological pathway should be a priority for future research (Rudel et al. 2014). It is assumed that the biological effects exerted by environmental contaminants on human tissues, taken individually, clearly differ from the effects exerted by combinations or mixtures of contaminants. However, as mentioned before, most studies about environmental contaminants and BC risk focus on single-chemicals. It is obvious that EDCs do not act in isolation, but also there is a multitude of chemicals that share similar features and whose contribution to the internal xenoestrogenic burden cannot be ignored (Kortenkamp 2006). Complex interactions between chemicals, endogenous or exogenous hormones, and their natural ligands and receptors may alter the homeostasis of the estrogenic environment of breast tissue, leading to malignant transformation (Boada et al. 2012). Moreover, it has been reported that mixtures of EDCs at levels below their individual no-observed-effect level caused significant xenoestrogenic activities in the E-SCREEN assay (Silva et al. 2002) or in the rat uterotrophic assay (Tinwell and Ashby 2004). In this regard, it has been published that effects exerted by individual POPs on the viability of the human mammary epithelial cells (HMEC) clearly differed from those exerted by POP mixtures (at AT concentrations), inducing a strong upregulation of genes involved in cellular transformation (Valerón et al. 2009). Similar results

were obtained by Aube M et al. (2011) in MCF-7 BC cell line, showing that low concentrations of a mixture of DDT analogues, aldrin, dieldrin, β-HCH and toxaphene increased the proliferation of MCF-7 cells.

In addition to *in vitro* data, population-based evidence on the role of POP mixtures in relation to BC is still scarce with only two studies available (Ibarluzea et al. 2004; Boada et al. 2012). Both epidemiological studies have revealed the importance of mixtures of chemicals rather than the single chemicals. In a recent study carried out in the Canary Islands (Spain), Boada et al. (2012) identified different profiles of organochlorine pesticide mixtures in healthy women and in those who have developed BC, and found that the mixture of aldrin / *p,p'*-DDE / *p,p'*-DDD may play a relevant role in BC development. They also reported that the POP mixtures identified in BC patients exerted an anti-androgenic effect in addition to the observed estrogenic activity and effect on cell viability (Rivero et al. 2015). However, the main effect of POPs on BC seems to be due to their proven estrogenic effect. In this regard, our research group has demonstrated that the total effective xenoestrogen burden (TEXB-alpha) measured in the AT was positively associated with the risk of BC in a hospital-based case-control study conducted between 1996 and 1998 in Southern Spain (Ibarluzea et al. 2004). This study included 198 newly diagnosed breast cancer patients and 260 controls matched by age and hospital that were recruited from women undergoing non-cancer-related surgery. Although we were unable to detect a statistically significant relationship between cancer risk and TEXB-alpha in the study population as a whole, stratified analysis by BMI index allowed us to find that in leaner women (BMI < median), those with highest levels of estradiol equivalent in the alpha-fraction (>197.51 pMEeq/g lipid; fourth quartile) had a 2.4-fold significantly greater risk of BC than those with the lowest levels (≤0.25 pMEeq/g lipid; first quartile). When the estrogenicity of the beta-fraction was included in the model, the leaner women with the highest levels in the alpha-fraction showed an even greater risk (OR: 3.42; 95% CI1.22–9.59). Among the leaner postmenopausal women, the risk for those in the highest tertile of TEXB-alpha increased to 5.67 (95% CI 1.59–20.21) (Ibarluzea et al. 2004). Moreover, TEXB-alpha levels were positively associated with age, family history of BC, lactation experience and smoking. This shows that this biomarker of combined effect takes into account environmental, dietary, lifestyle, genetic and reproductive factors, which are not systematically measured across studies (Fernandez et al. 2007b). However, although TEXB-alpha fraction includes organochlorine pesticides, PCBs and halogenated bisphenols and alkylphenols, no correlation was found between the concentration of any single chemical and the estrogenicity determined in the bioassay (Fernández et al. 2004). This may be due to the fact that the estrogenic effects depicted in the E-SCREEN bioassay are a consequence of the combined effect of several organohalogens or that the proliferative effect is due to other chemicals not measured.

5. HYPOTHESIS AND OBJECTIVES

There is an intriguing plausibility and experimental evidence that support a potential role of long-term exposure to low-doses of POPs in BC development. However, there are currently more than 50 studies published on this issue, and a considerable body of epidemiological data has reported no association between persistent EDC exposure (POPs) and higher risk of BC. The discrepancies between studies may be the result of different methodological aspects, including study designs, target populations, or exposure assessment. In this regard, serum is the preferred matrix in most epidemiological studies because it can be easily sampled and in sufficient amounts. In addition to this, it is commonly assumed that, under certain conditions of equilibrium, serum POP burden is a good indicator of the POP concentrations stored in the AT. However, it has been described that serum POP burden can be affected by changes in fat metabolism and by current exposures from air and diet. Therefore, assessment of the biological meaning of serum POP burden is paramount in the evaluation of BC risk, especially because BC patients often present significant metabolic changes that may imbalance the steady-state partitioning between AT and serum.

The majority of studies carried out in Europe and US found little or no association between POPs and BC, supporting the dominant view that these EDCs are not causally related to BC. Despite the fact that the number of studies addressing the link between exposure to POPs and BC risk in developing countries is still very limited, most of them have found positive associations between some POPs and BC risk, for example in India and Mexico. Interestingly, the level of human exposure to POPs is higher in the developing world than in developed countries. Therefore, it would be interesting to investigate the magnitude of the exposure to EDCs and their role in BC risk in women from Tunisia, one of the most developed African countries with a recent use of OCPs in agriculture practices and a similar level of use of IT-devices to European and American countries with the generation of large amounts of electronic waste. In addition to that, available information regarding human exposure to non-persistent EDCs, including BPA, BP-3 or parabens, in that country is even scarcer.

Today, it is generally accepted that the hormone-disrupting potential exerted by individual chemicals differ from the effects resulting from combinations or mixtures of chemicals. In fact, complex mixtures of chemicals showed null effect when the chemicals were tested individually for hormonal activity at the concentration found in blood or AT, but the mixtures showed significant xenoestrogenic activity in the appropriate bioassays. In this respect, our research group demonstrated that the total effective xenoestrogen burden (TEXB-α) measured in the extract of AT obtained from BC patients was positively associated with the risk of BC. Unfortunately, difficulties associated to the invasive procedures required to isolate AT hampered further studies to corroborate our findings. Nevertheless, it seems clear that new epidemiological studies focused in the assessment of a few POPs and their contribution to risk

of BC should be based on new tools able to assess the combined contribution of the internal burden of EDCs. Therefore, in order to corroborate our previous findings by using a more accessible matrix and a biomarker of combined effect, we hypothesize that the TEXB assessed in chemical extracts from serum may also be of utility for exposure assessment in a case control study investigating the risk of BC.

Hence, the main hypothesis of this PhD thesis is:

Breast cancer disease, closely linked to the levels of circulating endogenous estrogens, may also be related to the levels of circulating xenoestrogens. In the exposure assessment process of BC patients, the information provided by serum POP concentrations in comparison with the well-established AT levels, as the preferable biomarker of long-term exposure, may have a different biological meaning. Finally, the target population in which the association of POP exposure and breast carcinogenesis is explored may also have an impact on the strength of such association.

The specific objectives set out in this PhD thesis are to:

1. Assess the differences between the biological meaning of the two most widely used biological matrices –AT and serum- in the study of the role of POPs in BC pathogenesis, as well as to identify potential modifiers of serum POP burden in relation to those affecting the AT burden in a cohort of newly diagnosed BC patients.

2. Evaluate the magnitude of the exposure to persistent and non-persistent EDCs and identify predictors of the exposure in a subset of healthy women from Tunisia.

3. Estimate the association between levels of single POPs and the risk of BC in a Tunisian population.

4. Investigate whether the combined effect of xenoestrogens circulating in the blood is associated with the risk of BC in a population-based multicase-control study in Spain

6. MATERIAL AND METHODS

6.1 Study population

Objective 1 was addressed using a cross-sectional study. A total of 103 newly diagnosed BC patients were recruited at San Cecilio University Hospital in the city of Granada (Southern Spain) between January 2012 and June 2014. Out of 204 eligible patients with newly diagnosed BC, 33 (16.2%) refused to participate in this study. Among the remaining 171 participants, 68 (39.8%) were excluded due to an inadequate biological sample volume. Therefore, the final study population comprised 103 BC patients. No statistically significant differences in age, BMI, educational level, or histopathological grade were found between included and excluded volunteers. All patients signed their informed consent to participate in the study, which was approved by the Ethics Committee of Granada (*Comité de Ética de la Investigación Biomédica de la Provincia de Granada*)

Objective number 2 was explored using a subset of healthy Tunisian women at either the Salah Azaiz Hospital (Tunis state) or the Ariana Hospital (Ariana state) who were in those hospitals for non-disease-related reasons (women accompanying patients, hospital staff or blood donors) between May and October 2012. Out of the 77 women invited to participate as controls, 56 (70%) were finally enrolled in the study. Inclusion criteria were the following: age over 18 years, able to read and understand French, no alcohol consumption, absence of hormone-related disease or cancer, no hormonal therapy, and residence in the study area for at least 10 years. All women signed an informed consent form to participate in the study, which was approved by the Ethics Committee of the corresponding hospital.

The association between levels of single POPs and the risk of BC in a "hot-spot" population (objective number 3) was estimated in a case-control study carried out in Tunisia. The study population was recruited between May and October 2012 from among patients attending the two main specialist cancer centers in the country, Salah Azaiz Hospital and Ariana Hospital, which are both in the Grand Tunis metropolitan area (Northern Tunisia). Cases were women with BC admitted to hospital for mastectomy, tumorectomy (Salah Azaiz Hospital), or chemotherapy (Cancer Center of Ariana). Out of the 96 eligible cases, 69 (72%) were finally included and provided signed consent and a blood sample. Patients were included if they were aged 18 years or over and able to give informed consent and complete a questionnaire and excluded if they had a previous history of cancer or evidenced distant metastasis at diagnosis. The subset of healthy Tunisian women from the objective number 2 served as control group. Biological samples were collected before surgery or chemotherapy. No significant differences were found between included and excluded participants in age, marital status, or occupational class. All participants signed their informed consent to participate in the study, which was approved by the ethics committees of the hospitals.

Finally, the objective number 4 was tackled as part of a wider research project designed to identify environmental, personal, and genetic factors related to five common cancers, including breast, prostate, colorectal, stomach, and chronic lymphocytic leukemia. MCC-Spain (http://www.mccspain.org) is a population-based multicase-control study conducted in 12 Spanish provinces between 2008 and 2013 (Figure 5). The study recruited 6,082 histologically confirmed incident cancer cases aged 20–85 years, including 1,750 BCs, 1,115 prostate cancers, 2,171 colorectal cancers, 492 gastro-oesophageal cancers, and 554 cases of leukemia, as well as a single set of 4,101 population-based controls frequency matched to cases by province, sex, and 5-year age interval. The overall response rates were 70% among cases and 53% among controls. All participants completed computer-assisted personal interviews on sociodemographic factors, self-reported anthropometric data, lifestyle, reproductive history, hormonal factors, medications, and personal and family medical history. Blood samples were collected from 76% of participants. The study was approved by the ethics committees of the participating institutions. Written informed consent was obtained from each participant. For this study, we randomly selected 204 BC cases among those who agreed to donate blood samples in the provinces of Madrid, Barcelona, Navarra, and Cantabria and female controls frequency-matched to cases by province, 5-year age interval, and 2-unit category of BMI.

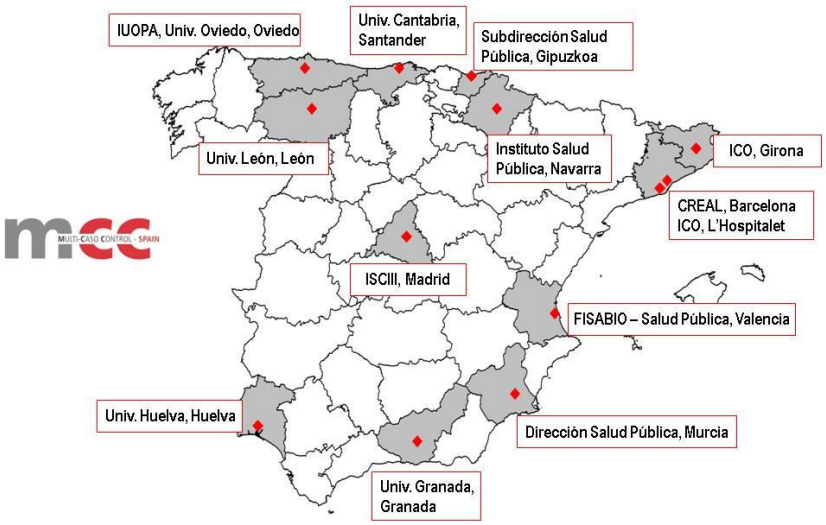


Figure 5. Multi-case-control study (MCC-Spain). Participating institutions in this multi-center study (mccspain.org).

6.2 Covariates

Socio-demographic data, including age, residence, occupation, and educational level, were gathered from a questionnaire completed by each participant before surgery in a face-to-face interview with a trained interviewer during the hospital stay. The height and weight of the participants were recorded, calculating their BMI as weight/height squared (kg/m^2). In the studies conducted in Tunisia, participants were grouped according to their place of residence into women living in Northern Tunisia and women living in Southern Tunisia. The division line passed through the provinces of Sousse, Kairouan and Kasserine.

Clinical and reproductive data was also gathered from the clinical records of the hospital. The number of pregnancies was recorded as a discrete variable (multiparous/nulliparous). Accumulated breastfeeding time (months) were recorded as a continuous variable. Age of menarche was also recorded. Menopausal status and hormone replacement status were considered as dichotomous variables (pre/post; yes/no, respectively).

A dietary section was also included in the studies carried out in Tunisia. Information regarding consumption habits and frequencies of main food groups (meat, milk, dairy products, vegetables and cereals) was recorded. Since bread is eaten on a daily basis in our population, questions regarding cereal consumption only referred to pasta, rice and couscous consumption.

6.3 Sample extraction

Approximately 10 g of breast AT and 10 mL of blood were collected during surgery under fasting conditions. Blood samples were immediately centrifuged for 5 min at 2500 rpm and 4ºC to separate the serum. Both serum and AT samples were immediately coded and stored at −80 º C until chemical analysis.

A chemical extraction procedure was performed to isolate the selected POPs from the AT and serum samples. Briefly, 150-200 mg of AT were extracted using n-hexane, and the solution was then purified through 200 mg alumina in a glass column, as previously described (Martínez-Vidal et al. 2002; Moreno-Frías et al. 2004). Depending on the study, two or three milliliters of serum were extracted with methanol and hexane/ethyl ether (1:1 v/v), resuspended in n-hexane, and passed through a Bond Elut-PCB cartridge previously conditioned with 1.5 mL hexane (Turci et al. 2010). For both matrices, *p,p´*-dichlorobenzophenone was used as internal standard.

Dried AT and serum extracts were fractionated by HPLC and reconstituted in 200 µL hexane, as described elsewhere (Rivas et al. 2001). This semipreparative HPLC method was developed to efficiently separate organohalogenated lipophilic xenoestrogens (alpha fraction) from

endogenous hormones and more polar xenoestrogens (beta fraction) with a high recovery rate, using a normal-phase column that separated compounds according to their polarity.

Exposure assessment to BPA, parabens and benzophenones was performed in single spot urine samples collected in polypropylene containers pretested to ensure that they did not contain or leach any of the compounds under study. All urine samples were frozen at -20 °C until they were shipped on dry ice (-70 °C) to San Cecilio University Hospital, in Granada (Spain), where they were analyzed. The applied methodology for the analysis of BPA, parabens (MP, EP, PP and BP) and BP-type UV filters (BP-1, BP-2, BP-3, BP-6, BP-8 and 4-OH-BP) was performed by dispersive liquid–liquid microextraction (DLLME) and ultra-high performance liquid chromatography with tandem mass spectrometry detection (UHPLC-MS/MS), as described in a previous study (Vela-Soria et al. 2014) with slight modifications.

6.4 Quantification of the total effective xenoestrogen burden and chemical analysis

After HPLC fractionation, duplicated dry alpha and beta HPLC fractions were resuspended in phenol red-free medium, supplemented with 2.5 mL of charcoal-dextran fetal bovine serum, and tested in the E-Screen bioassay for estrogenicity (Soto et al. 1994). The combined estrogenic activity of each fraction was analyzed from its proliferative effect on MCF-7 human BC cells. Each sample was assayed in triplicate with negative (steroid-free) and positive (estradiol-treated) controls in each culture plate. The proliferative effects of alpha and beta fractions were calculated as the difference in MCF-7 cell proliferation between the fraction extract and the steroid-free control, divided by the highest difference in proliferation between the estradiol-treated and steroid-free control cells. These relative proliferative effects were transformed into estradiol equivalent units by reading from a sigmoidal dose-response curve prepared with estradiol at concentrations of 0.1–1000 pM, and they were expressed as TEXB values of alpha (TEXB-α) and beta (TEXB-β) fractions in Eeq picomolar per milliliter of serum (Fernández et al. 2004). Thus, TEXB-α can be regarded as a biomarker of the combined estrogenic effect of mixtures of organohalogenated lipophilic xenoestrogens, whereas TEXB-β represents the combined estrogenic activity of endogenous hormones and more polar xenoestrogens.

Residues of *p,p'*-DDE, HCB, PCBs (congeners -138, -153, and -180), β-HCH, α-endosulfan, endosulfan ether, heptachlor and oxychlordane were quantified by high-resolution gas chromatography with micro-electron capture detection, using a VARIAN CP-3800 chromatograph equipped with an electron capture 63Ni detector (GC-ECD, Walnut Creek, CA, US).

Procedural laboratory blanks with solvents alone were tested and always yielded a negative result. Laboratory fortified matrix samples at different concentrations were used for quality

control. Inter- and intra-day variabilities were calculated by analyzing fortified samples within the same day (repeatability) and on different days (intermediate precision), respectively, always yielding values < 20%. Recovery of the POPs from serum was studied to assess the extraction efficiency of the method, spiking 10 blank samples with target analytes at an intermediate point on the calibration curve and processing them as described above; recovery rates ranged from 90 to 98%.

6.5 Determination of lipid content

Total lipid content was quantified gravimetrically in AT samples using a previously reported method (Rivas et al. 2001) consisting of a homogenization step of 150-200 mg AT with 5 mL of chloroform: methanol: hydrochloric acid (20:10:0.1) and acidification with 0.1 N hydrochloric acid before collecting and weighing the organic phase. For serum samples, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglyceride concentrations were enzymatically quantified in 10 μL of serum from each participant, and the total lipid content was calculated using the short formula of Phillips et al. (1989). POP concentrations were expressed on both wet basis (ng/g AT or ng/mL serum) and lipid basis (ng/g lipid, in both matrices).

6.6 Limits of detection

The limit of detection (LOD) for TEXB-α and TEXB-β was 0.1 Eeq pM/mL, which corresponded to the minimum concentration needed to produce a significantly different proliferative effect from that observed in steroid-free control cells. For 6.3% and 3.7% of participants with TEXB-α and TEXB-β determinations below the LOD, a level equal to the LOD divided by the square root of 2 was imputed. TEXB-α and TEXB-β levels could not be determined in 44.2% and 35.1% of serum samples, respectively, because MCF-7 cells treated with their extracts grew less than steroid-free control cells, which hampered reading the proliferative effect in the estradiol dose-response curve.

The LOD for the levels of the residues obtained in the chemical analyses was determined as the smallest amount of the analyte that gave a signal-to-noise ratio ≥ 3, and was set at 0.05 ng/mL for each POP analyte and 0.2 ng/mL for each npEDC compounds. Concentrations below the LOD were assigned a random value between zero and the LOD, which was calculated using the random numbers function of SPSS. The multivariable analyses were repeated twice, considering concentrations <LOD as one half of the LOD or as the square root of one half of the LOD, finding no discrepancy with the associations reported.

6.7 Statistical analyses

Variables were described using means, standard deviations and medians and 25th – 75th percentiles (quantitative variables), and frequencies (categorical variables). Because ANOVA assumptions were not always fulfilled, the Mann-Whitney U-test was used to compare continuous variables and Fisher's exact test for categorical variables between characteristics of non-participants and participants, where appropriate. The Mann Whitney U-test and Fisher´s exact test were applied to assess differences between characteristics of non-participants and participants. Both wet-and lipid-basis AT:serum ratios were calculated by dividing each AT concentration by each serum concentration. Spearman's test was used to evaluate linear monotonic correlations between different POPs in the same matrix and between the same POPs in the two different matrices.

Potential predictors of log-transformed POP concentrations in serum and AT were assessed using bivariate and multivariate linear regression analyses. First, we created bivariate models with each potential predictor of POP concentrations, and the associations were then verified in multivariate models, using a backward stepwise selection technique. Once the models were created, the collinearity between independent variables, linearity of quantitative independent variables, and homoscedasticity were assessed for the model diagnostics. Given the log-transformation of POP concentrations, β coefficients are also presented as exp(β). Models in the first objective were tested in two different scenarios: a) expressing POP concentrations on wet basis with adjustment for total serum lipids; and b) expressing POP concentrations on lipid basis without adjustment for total serum lipids. There were no relevant differences between the multivariate models in the associations found; therefore, only the results for wet-basis concentrations are reported in the tables.

The relationship between POP serum levels and BC risk was assessed by using unconditional logistic regression analyses, entering POP concentrations as continuous variables. In addition, the associations found were further explored by entering POP concentrations in the models as tertiles, with the exception of β-HCH and α-endosulfan, which were entered as dichotomous variables (<LOD/ ≥LOD) because of the low frequency of their detection. Bivariate models with individual POP concentrations as independent variable were created and then adjusted for variables whose inclusion produced changes of 10% in beta coefficients and for those described as relevant factors in the literature. Finally, a single adjusted model was created that included all covariates and POP concentrations significantly associated with BC risk in the previous adjusted models, i.e., β-HCH, heptachlor, and *p,p'*-DDE. Odds ratios (ORs) for the risk of BC with their corresponding 95% confidence intervals (95% CIs) were calculated and trends were evaluated with Mantel–Haenszel's chi-square test for linear trend. Wet-basis concentrations

were all entered as ng/mL with the exception of *p,p'*-DDE (ng/dL), while lipid-basis concentrations were all entered as mg/g lipid with the exception of *p,p'*-DDE (ng/g lipid). We considered that POP concentrations were significantly associated with the risk of BC when the 95% CIs of the OR in the adjusted models did not overlap the null value (1).

Associations between serum TEXB levels and the risk of BC were explored by using logistic regression models. Participants were grouped into tertiles of serum TEXB-α and TEXB-β levels based on their distributions among controls. Odds ratios for BC and 95% CIs comparing the second and third tertiles with the first tertile of serum TEXB-α and TEXB-β were estimated using logistic regression models. We also estimated the OR for women with undetermined estrogenicity in the bioassay compared with all other women with determined estrogenicity. Tests for linear risk trend across serum TEXB-α and TEXB-β tertiles were performed by including an ordinal variable with the median level of each tertile among controls in logistic regression models. To further explore the shape of the dose-response relations of serum TEXB-α and TEXB-β levels with BC risk, we used restricted quadratic splines for log-transformed TEXB-α and TEXB-β levels with knots at the 10th, 50th, and 95th percentiles of their control distributions (the first knot was set at the 10th percentile to exceed levels below the limit of detection) (Greenland 1995). We also estimated odds ratios for BC comparing tertiles of specific organohalogenated compounds (PCB-138, PCB-153, PCB-180, HCB, and *p,p'*-DDE) based on their control distributions. Logistic regression models were fitted with increasing degrees of adjustment. The first model adjusted for variables used in frequency matching, such as province (Madrid, Barcelona, Navarra, or Cantabria), age (continuous), and BMI (continuous), as well as for education level (primary or less, high school, or college) and serum total lipid levels (continuous). The second model further adjusted for BC risk factors, including smoking status (never, former, or current), number of births (nulliparous, 1–2, or ≥3), age at first birth (continuous), menopausal status (premenopausal or postmenopausal), use of hormone replacement therapy (never or ever), previous breast biopsy (no or yes), and family history of BC (no, second-degree relative, or first-degree relative). Finally, the third model mutually adjusted serum TEXB-α and TEXB-β levels for each other. Effect modifications were contrasted by including interaction terms of serum TEXB-α and TEXB-β tertiles with each of the above covariates in logistic regression models.

The significance level was set at p=0.05. Analyses were performed using Stata, version 13.1 (Stata Corp., College Station, Texas); R statistical computing environment, version 2.15 and 3.0 (R Foundation for Statistical Computing, Vienna, Austria); and SPSS 20.0 (IBM, Chicago, IL).

7. RESULTS AND DISCUSSION

7.1. Objective 1. Assess the differences between the biological meanings of the two most widely used biological matrices –adipose tissue and serum- in the study of the role of POPs in breast cancer pathogenesis, as well as to identify potential modifiers of serum POP burden in relation to those affecting the adipose tissue burden in a cohort of newly diagnosed breast cancer patients.

SERUM AND ADIPOSE TISSUE AS MATRICES FOR ASSESSMENT OF EXPOSURE TO PERSISTENT ORGANIC POLLUTANTS IN BREAST CANCER PATIENTS

Artacho-Cordón F*, Fernández-Rodríguez M, Garde C, Salamanca E, Iribarne-Durán LM, Torné P, Expósito J, Papay-Ramírez L, Fernández MF, Olea N, Arrebola JP*

Environmental Research; Impact factor 4.373; 1st Quartile; 1st Decile

ABSTRACT

The aim of this study was to assess differences between two biological matrices (serum and breast adipose tissue) in the evaluation of persistent organic pollutant (POP) exposure in breast cancer patients. The study population consisted of 103 women undergoing surgery for newly diagnosed breast carcinoma in a public hospital in Granada, Southern Spain. Independent variables were gathered from questionnaires and clinical records. POP concentrations were quantified in breast adipose tissue and serum samples. Spearman correlation tests were performed between pairs of POP concentrations and stepwise multivariate linear regression analyses were conducted to assess predictors of concentrations in the two matrices. *p,p'*-DDE showed the highest median concentration in both matrices (194.34 and 173.84 ng/g lipid in adipose tissue and serum, respectively). Median wet-basis adipose tissue:serum ratios ranged from 109.34 to 651.62, while lipid-basis ratios ranged from 0.88 to 4.34. In general, we found significant positive correlation coefficients between pairs of POPs in adipose tissue and in serum, which were always higher in adipose tissue. We found positive and statistically significant correlations between serum and adipose tissue concentrations of p,p´-DDE and HCB but not of PCBs. Age was positively associated with most POPs in adipose tissue and serum, while the BMI was positively associated with adipose tissue HCB concentrations and negatively associated with serum PCB-153 and PCB-138 concentrations. Recent weight loss was inversely associated with POP residues in adipose tissue and positively associated with POP residues in serum. Serum HCB and PCB-180 concentrations were lower in patients who had received preoperative chemotherapy. According to our results, serum and adipose tissue POP concentrations in breast cancer patients may be differentially affected by external predictors. Taken together, these findings indicate the need to take account of the individual POP(s) under study and the biological matrix used when relating internal POP exposure to breast cancer disease and to make a careful selection of covariates for adjusting the model.

Environmental Research 142 (2015) 633–643

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Serum and adipose tissue as matrices for assessment of exposure to persistent organic pollutants in breast cancer patients

F. Artacho-Cordón [a,c], M. Fernández-Rodríguez [a,c], C. Garde [a], E. Salamanca [a,c], L.M. Iribarne-Durán [a,c], P. Torné [a], J. Expósito [a,b], L. Papay-Ramírez [a], M.F. Fernández [a,c,d], N. Olea [a,c,d], J.P. Arrebola [a,b,c,d,*]

[a] Instituto de Investigación Biosanitaria ibs. GRANADA, Hospitales Universitarios de Granada, Spain
[b] Radiation Oncology Department, Virgen de las Nieves University Hospital, Granada, Spain
[c] Radiology and Physical Medicine Department, University of Granada, Spain
[d] CIBER en Epidemiología y Salud Pública (CIBERESP), Spain

ARTICLE INFO

Article history:
Received 11 June 2015
Received in revised form
14 August 2015
Accepted 18 August 2015

Keywords:
Organochlorine pesticides
Polychlorinated biphenyls
Adipose tissue
Serum
Breast cancer

ABSTRACT

The aim of this study was to assess differences between two biological matrices (serum and breast adipose tissue) in the evaluation of persistent organic pollutant (POP) exposure in breast cancer patients. The study population consisted of 103 women undergoing surgery for newly diagnosed breast carcinoma in a public hospital in Granada, Southern Spain. Independent variables were gathered from questionnaires and clinical records. POP concentrations were quantified in breast adipose tissue and serum samples. Spearman correlation tests were performed between pairs of POP concentrations and stepwise multivariable linear regression analyses were conducted to assess predictors of concentrations in the two matrices. *p,p′*- Dichlorodiphenyldichloroethylene (*p,p′*-DDE) showed the highest median concentration in both matrices (194.34 and 173.84 ng/g lipid in adipose tissue and serum, respectively). Median wet-basis adipose tissue:serum ratios ranged from 109.34 to 651.62, while lipid-basis ratios ranged from 0.88 to 4.34. In general, we found significant positive correlation coefficients between pairs of POPs in adipose tissue and in serum, which were always higher in adipose tissue. We found positive and statistically significant correlations between serum and adipose tissue concentrations of *p,p′*-DDE and hexachlorobenzene (HCB) but not of polychlorinated biphenyls (PCBs). Age was positively associated with most POPs in adipose tissue and serum, while the body mass index was positively associated with adipose tissue HCB concentrations and negatively associated with serum PCB-153 and PCB-138 concentrations. Recent weight loss was inversely associated with POP residues in adipose tissue and positively associated with POP residues in serum. Serum HCB and PCB-180 concentrations were lower in patients who had received preoperative chemotherapy. According to our results, serum and adipose tissue POP concentrations in breast cancer patients may be differentially affected by external predictors. Taken together, these findings indicate the need to take account of the individual POP(s) under study and the biological matrix used when relating internal POP exposure to breast cancer disease and to make a careful selection of covariates for adjusting the model.

1. Introduction

Persistent organic pollutants (POPs) are a wide group of highly lipophilic environmental pollutants that tend to accumulate and biomagnify in the food chain, resulting in the considerable exposure of living organisms (UNEP, 2003). POPs include organochlorine pesticides (OCPs), such as dichlorodiphenyltrichloroethane (DDT) and its metabolites (notably, *p,p′*-dichlorodiphenyldichloroethylene [*p,p′*-DDE]), hexachlorobenzene (HCB), and polychlorinated biphenyls (PCBs), among others. While DDT and HCB were primarily commercialized for vector control and agricultural purposes, PCBs were mainly used as fluid insulators in electrical transformers and capacitors. Since the early 1970s, most countries have banned or severely restricted the production, handling, and disposal of most POPs. This is due to their high environmental persistence and their proven or suspected adverse human health effects at doses traditionally considered safe, including reproductive disorders, endocrine disruption, and carcinogenicity (Arrebola et al., 2013; Bonefeld-Jorgensen, 2010; Fernandez et al., 2007a, 2007b; Gasull et al., 2010; Krüger et al., 2012; Lee et al., 2014).

* Corresponding author at: Instituto de Investigación Biosanitaria ibs. GRANADA, Hospitales Universitarios de Granada, University of Granada, 18071 Granada, Spain. Fax: +34 958 249953.
E-mail address: jparrebola@ugr.es (J.P. Arrebola).

http://dx.doi.org/10.1016/j.envres.2015.08.020
0013-9351/

Despite their prohibition, POPs are still commonly detected in air, water and soil, among other environmental media (Syed et al., 2013). Besides respiratory and dermal routes, diet is believed to be the main route of exposure to POPs in the general population (Gasull et al., 2010). Due to their lipophilicity, POPs tend to bioaccumulate in fat components, and adipose tissue is therefore acknowledged to be the main deposit of these contaminants, accounting for all routes and sources of exposure and representing a stable and long-term reservoir of these compounds (Kohlmeier and Kohlmeier, 1995).

Hormone homeostasis is crucial in diseases related to the endocrine system, including the majority of breast cancers. Thus, estrogen signaling and the estrogen receptor (ER) have been implicated in breast cancer progression, and most human breast cancers start out as estrogen dependent (Saha Roy and Vadlamudi, 2011). In fact, ER-alpha antagonism is widely used in the treatment of ER-alpha-positive breast cancer patients. In this regard, some *in vitro* studies have revealed that exposure to some POPs can interact with ERs and cause estrogen-related effects, such as breast cancer cell proliferation. The suspected mechanisms of action have not been fully elucidated, but *in vitro* studies have shown that numerous POPs can interact with estrogen and/or androgen receptors, exerting significant effects at very low doses (Andersen et al., 2002; Bonefeld-Jørgensen et al., 2001; Grünfeld and Bonefeld-Jorgensen, 2004; Soto et al., 1994). In fact, the estrogenic potency of most POPs is approximately six orders of magnitude lower than of estradiol (Soto et al., 1994). Nevertheless, some epidemiological evidence has emerged on the potential role of POP exposure in the etiology of breast cancer, with a wide range of studies reporting positive associations (Aronson et al., 2000; Arrebola et al., 2015, 2014a; Boada et al., 2012; Bonefeld-Jorgensen et al., 2011; Bonefeld-Jørgensen et al., 2014; Ibarluzea et al., 2004), although others found no or even negative associations (Gatto et al., 2007; Itoh et al., 2009; Xu et al., 2010). Key differences among these studies include not only the study design and target population but also the biological matrix used to estimate the exposure, with serum and adipose tissue being the most frequent.

Whereas it is viable to obtain breast adipose tissue from patients undergoing surgery, the difficulties in obtaining these samples from other populations means that blood serum has more frequently been used as a matrix for exposure assessment of the general population. However, although many authors have found a high correlation between POP concentrations in serum and adipose tissue (López-Carrillo et al., 1999; Pauwels et al., 2000; Waliszewski et al., 2004; Whitcomb et al., 2005), it remains unclear whether serum POP concentrations can accurately reflect the body burden of these chemicals in all situations (Aronson et al., 2000; Arrebola et al., 2012a; Mussalo-Rauhamaa, 1991; Rusiecki et al., 2005; Wolff et al., 2000). In fact, it is possible that POP concentrations in the two matrices may be strongly correlated in some cases but not in others, given that serum concentrations are influenced not only by current exposure but also by the recirculation of POPs from adipose tissue due to lipolysis (Crinnion, 2009).

The steady-state partitioning of POPs between serum and breast adipose tissue is an important consideration in attempts to predict adipose tissue concentrations from those found in serum (Rusiecki et al., 2005). Thus, it has been suggested that variations in the lipid content of serum can induce changes in the partitioning coefficient between adipose tissue and serum POP concentrations (Guo et al., 1987). In this regard, most chemotherapy (QT) treatments are believed to reduce concentrations of serum lipid fractions [e.g. triglycerides, total cholesterol and low density lipoprotein (LDL)] in patients with breast cancer (Ray et al., 2001; Shah et al., 2008). It is therefore of interest to assess whether the serum POP concentrations of these patients can always predict the total body burden. Furthermore, given the widespread application of neoadjuvant treatments in breast cancer, it is relevant to assess whether they can act as confounders or effect modifiers in the potential association between POPs and breast cancer risk.

The aim of this study was to assess differences between two biological matrices (serum and breast adipose tissue) in the evaluation of POP exposure in breast cancer patients.

2. Material and methods

2.1. Study population

Breast cancer patients were recruited between January 2012 and June 2014 among newly diagnosed women at San Cecilio University Hospital in the city of Granada (Southern Spain). Out of 204 eligible newly diagnosed breast cancer patients, 33 (16.2%) refused to participate in this study. Among the remaining 171 participants, 68 (39.8%) were excluded due to an inadequate biological sample volume. Therefore, the final study population comprised 103 breast cancer patients. No statistically significant differences in age, BMI, educational level, or histopathological grade were found between included and excluded volunteers (data not shown in tables). All patients signed their informed consent to participate in the study, which was approved by the Ethics Committee of Granada "Comité de Ética de la Investigación Biomédica de la Provincia de Granada".

2.2. Independent variables

Socio-demographic data, including age, residence, occupation, and educational level, were gathered from a questionnaire completed by each participant before surgery in a face-to-face interview with a trained interviewer during the hospital stay. Questionnaires and research procedures were validated in a previous study (Arrebola et al., 2009, 2010). The height and weight of the participants were recorded, calculating their body mass index (BMI) as weight/height squared (kg/m^2). Residence in the city of Granada or in its metropolitan area at the time of the surgery was considered "urban" and residence in other towns/villages was considered "rural". Goldthorpe proposed the following occupational classes in Spain: I, managers of companies with ≥ 10 employees, senior technical staff, and free professionals; II, managers of companies with < 10 employees and intermediate occupations; III, administrative personnel, financial management support professionals, self-employed professionals, supervisors of manual workers, and other skilled non-manual workers; IV, skilled and semi-skilled manual workers; and V, unskilled manual workers. A sixth group is formed by those working mainly as homemakers (Regidor, 2001). However, because of sample size limitations, we grouped subjects in two categories: non manual (classes I+II + III) and manual (classes IV + V + homemakers).

Clinical and reproductive data was also gathered from the clinical records of the hospital. The number of pregnancies was recorded as a discrete variable (multiparous/nulliparous). Accumulated breastfeeding time (months) were recorded as a continuous variable. Age of menarche was also recorded. Menopausal status and hormone replacement status were considered as dichotomous variables (pre/post; yes/no, respectively). Clinical data also included information on the neoadjuvant tumor treatment (yes/no), biological aggressiveness (G1/G2/G3), presence of estrogen receptors (negative/positive), tumor stage (0-IIB/IIIA-IIIB/IV), histopathological status (benign/malign), and molecular subtype (Luminal A/Luminal B/Her+/Triple Negative).

2.3. Sampling and chemical analysis

Approximately 10 g of breast adipose tissue and 10 mL of blood were collected during surgery under fasting conditions. Blood samples were immediately centrifuged for 5 min at 2500 rpm and 4 °C to separate the serum. Both serum and adipose tissue samples were immediately coded and stored at −80 °C until chemical analysis.

A chemical extraction procedure was performed to isolate the selected analytes from the adipose tissue and serum samples. Briefly, 150–200 mg of adipose tissue was extracted using n-hexane, and the solution was then purified through 200 mg alumina in a glass column, as previously described (Martínez-Vidal et al., 2002; Moreno-Frías et al., 2004). Two milliliters of serum were extracted with methanol and hexane/ethyl ether (1:1 v/v), resuspended in n-hexane, and passed through a Bond Elut-PCB cartridge previously conditioned with 1.5 mL hexane (Turci et al., 2010). For both matrices, *p,p′*-dichlorobenzophenone was used as internal standard.

Dried adipose tissue and serum extracts were fractionated by high-performance liquid chromatography (HPLC) and reconstituted in 200 μL hexane, as described elsewhere (Rivas et al., 2001). Residues of *p,p′*-DDE, HCB and PCBs (congeners -138, -153, and -180) were quantified by high-resolution gas chromatography with micro-electron capture detection, using a VARIAN CP-3800 chromatograph equipped with an electron capture 63Ni detector (GC-ECD, Walnut Creek, CA, US).

Procedural laboratory blanks with solvents alone were tested and always yielded a negative result. Laboratory fortified matrix samples at different concentrations were used for quality control. Inter- and intra-day variabilities were calculated by analyzing fortified samples within the same day (repeatability) and on different days (intermediate precision), respectively, always yielding values $< 20\%$. *p,p′*-dichlorobenzophenone was used as internal standard. Recovery of the POPs from serum was studied to assess the extraction efficiency of the method, spiking 10 blank samples with target analytes at an intermediate point on the calibration curve and processing them as described above; recovery rates ranged from 90% to 98%. The limit of detection (LOD) was determined as the smallest amount of the analyte that gave a signal-to-noise ratio ≥ 3 and was set at 0.05 ng/mL for each analyte. Concentrations below the LOD were assigned a random value between zero and the LOD.

Concentrations below the LOD were assigned a random value between zero and the LOD as recommended elsewhere (Antweiler and Taylor, 2008), which was calculated using the random numbers function of SPSS. The multivariable analyses were repeated twice, considering concentrations $<$ LOD as one half of the LOD or as the square root of one half of the LOD, finding no discrepancy with the associations reported in the present manuscript (data not shown in tables).

Total lipid content was quantified gravimetrically in adipose tissue samples using a previously reported method (Rivas et al., 2001) consisting of a homogenization step of 150–200 mg adipose tissue with 5 mL of chloroform: methanol: hydrochloric acid (20:10:0.1) and acidification with 0.1 N hydrochloric acid before collecting and weighing the organic phase. For serum samples, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglyceride concentrations were enzymatically quantified in 10 μL of serum from each participant using a Cobas 400 machine (Roche, Rotkreuz, Switzerland), and the total lipid content was calculated using the short formula of Phillips et al. (1989). POP concentrations were expressed on both wet basis (ng/g adipose tissue or ng/mL serum) and lipid basis (ng/g lipid, in both matrices). A double-blinded procedure was followed so that neither the chemical analysts nor statistical staff knew the identity or characteristics of any study subject.

2.4. Statistical analysis

Variables were described using medians and 25th–75th percentiles. Because ANOVA assumptions were not always fulfilled, the Mann–Whitney *U*-test was used to compare continuous variables and Fisher's exact test for categorical variables between characteristics of non-participants and participants, where appropriate. Both wet- and lipid-basis adipose tissue:serum ratios were calculated by dividing each adipose tissue concentration by each serum concentration Spearman's test was used to evaluate monotonic correlations between different POPs in the same matrix and between the same POPs in the two different matrices.

Potential predictors of log-transformed POP concentrations in serum and adipose tissue were assessed using bivariate and multivariable linear regression analyses. First, we created bivariate models with each potential predictor of POP concentrations, and the associations were then verified in multivariable models, using a backward stepwise selection technique. Once the models were created, the collinearity between independent variables, linearity of quantitative independent variables, and homoscedasticity were assessed for the model diagnostics. Given the log-transformation of POP concentrations, β coefficients are also presented as $\exp(\beta)$. Models were tested in two different scenarios: a) expressing POP concentrations on wet basis with adjustment for total serum lipids; and b) expressing POP concentrations on lipid basis without adjustment for total serum lipids. There were no relevant differences between the multivariable models in the associations found; therefore, only the results for wet-basis concentrations are reported in the tables. The significance level was set at $p=0.05$. R statistical computing environment 3.0 (http://www.r-project.org/) and SPSS Statistics 22.0 (IBM, Chicago, IL) were used for data analyses.

3. Results

3.1. Characteristics of the study population

The main characteristics of the study population are summarized in Table 1. The mean age ($\pm$ standard deviation [SD]) was 53.6 ($\pm$ 11.8) years. The majority of women (62.1%) were overweight/obese (BMI $>$ 25 kg/m^2) at the time of their diagnosis according to the World Health Organization classification (WHO, 2000), and 33 (32.0%) reported perceived weight loss during the year before the surgery. Main causes of weight loss in our population included psychological stress (18%), weight reduction diet (36%), and cancer treatment (18%) (data not shown in tables). Out of the 103 women, 31 (30.1%) resided in an urban area and 58 (56.3%) held a university degree, while 28 (27.2%) were classified as non-manual workers and 75 (72.8%) as manual workers. Post-menopausal status was reported by 42 women (40.8%), 90 patients (87.3%) had at least one child, and the mean accumulated breastfeeding time was 10.3 ($\pm$ 12.9) months. Out of 103 women, 13 (12.6%) were receiving hormonal replacement therapy and the mean age of menarche in the study population was 12.0 ($\pm$ 1.5) years. Preoperative QT treatment had been received by 42 (40.8%) of the women.

3.2. POP concentrations and lipid content in adipose tissue and serum

POP detection frequencies, concentrations, and adipose tissue:serum ratios are shown in Table 2. The detection frequency ranged from 97.1% (PCB-180) to 100% (HCB and *p,p′*-DDE) in adipose tissue

Table 1
Characteristics of the study population.

	N (%)	Mean (SD)	Median	P25–P75[a]
Age (year)	103 (100)	53.6 (11.8)	51	47.0–62.0
BMI (kg/m^2)	103 (100)	26.7 (4.4)	26.3	23.4–29.3
Underweight (BMI < 18.5)	0 (0.0)			
Normal range (18.5 < BMI < 25.0)	41 (39.8)	–	–	–
Overweight (25.0 < BMI < 30.0)	40 (38.8)	–	–	–
Obese (BMI > 30.0)	22 (21.4)	–	–	–
Residence				
Urban	31 (30.1)	–	–	–
Rural	72 (69.9)	–	–	–
Educational level				
University	58 (56.3)	–	–	–
Secondary school	30 (29.1)	–	–	–
Up to primary school	15 (14.6)	–	–	–
Occupational status				
Non-manual (I–III)	28 (27.2)	–	–	–
Manual (IV–V)	75 (72.8)	–	–	–
Occupational class				
Homemakers	46 (44.7)	–	–	–
Trade	14 (13.6)	–	–	–
Agriculture	8 (7.8)	–	–	–
Civil service	8 (7.8)	–	–	–
Others	27 (26.2)	–	–	–
Perceived weight loss[b]	33 (32.0)	–	–	–
Parity				
Primiparous/multiparous	90 (87.4)	–	–	–
Nulliparous	13 (12.6)	–	–	–
Accumulated breastfeeding time (months)[c]	103 (100)	10.3 (12.9)	6	1.25–12.0
Age of menarche (years)	103 (100)	12.3 (1.5)	12	11.0–14.0
Menopausal status				
Premenopausal	42 (40.8)	–	–	–
Postmenopausal	61 (59.2)	–	–	–
Hormone replacement therapy	13 (12.6)	–	–	–
Preoperative chemotherapy	42 (40.8)	–	–	–
Tumor stage				
0-IIB	81 (78.6)	–	–	–
IIIA-IIIB	17 (16.5)	–	–	–
IV	5 (4.9)	–	–	–
Histopathological grade				
Benign	3 (2.9)	–	–	–
Malign	100 (97.1)	–	–	–
Tumor molecular subtype				
Luminal A	31 (30.1)	–	–	–
Luminal B	49 (47.6)	–	–	–
Her+	9 (8.7)	–	–	–
Triple negative	14 (13.6)	–	–	–
Biological aggressiveness grade				
G1	46 (44.7)	–	–	–
G2	34 (33.0)	–	–	–
G3	23 (22.3)	–	–	–
Estrogen receptor				
Negative	24 (23.3)	–	–	–
Positive	79 (76.7)	–	–	–

SD: standard deviation; BMI: body mass index.

[a] 25th–75th percentiles.

[b] During the previous year.

[c] Includes all women. When considering breastfeeders alone, mean(SD) was 12.6 (13.2) months.

and from 65.0% (PCB-153) to 99.0% (*p,p′*-DDE) in serum. Among the selected POPs, *p,p′*-DDE showed the highest median concentration in both matrices (194.34 and 173.84 ng/g lipid in adipose tissue and serum, respectively). Median values of wet-basis adipose tissue:serum ratios ranged from 109.34 for *p,p′*-DDE to 651.62 for PCB-138, while lipid-basis ratios ranged from 0.88 for *p,p′*-DDE to 4.34 for PCB-138. Distribution of POP levels grouped by characteristics of the study population is shown in Supplementary Table 1.

Mean ± SD serum cholesterol, triglycerides, and total lipid concentrations were 2.0 ± 0.7, 1.0 ± 0.6 g/l, and 6.1 ± 1.3 g/l, respectively (data not shown in tables). The mean lipid content in adipose tissue was 77.9 ± 10.1%. Lipid concentrations in adipose tissue were lower in patients who received preoperative QT than in those who did not (74.96 ± 11.62 *versus* 79.93 ± 8.93%, respectively, $p=0.029$), but this difference was not statistically significant in serum (5.81 ± 1.19 *versus* 6.24 ± 1.40 g/L, respectively, $p=0.112$).

Table 3 displays Spearman correlation coefficients between pairs of POP concentrations in serum and adipose tissue. In general, we found significant positive correlation coefficients between pairs of POPs in adipose tissue and in serum, which were always higher in adipose tissue. The inter-matrix correlations revealed significant positive associations for HCB ($r=0.85$, $p<0.001$) and *p,p′*-DDE ($r=0.63$, $p<0.001$). The strength of the correlation for *p,p′*-DDE was lower in patients who received QT ($r=0.46$, $p=0.08$) than in those who did not ($r=0.83$, $p\leq 0.001$). No significant inter-matrix correlation was observed for any PCB congener in the global analysis or after stratifying by QT treatment. Non-linear associations were tested using quadratic and cubic splines, but no association was found for any POP (data not shown in tables). Correlation coefficients obtained when the POP burden was considered on wet basis were similar to those on lipid basis (data not shown in tables).

3.3. Predictors of POP concentrations in serum and adipose tissue

Tables 4 and 5 exhibit the results of bivariate and multivariable linear regression analyses of the predictors of adipose tissue and serum concentrations. Additionally, Supplementary Tables 2 and 3 exhibit comparisons between the full model (including all covariates) and the final model for each POP in each matrix.

In regard to adipose tissue samples, age was positively associated with the concentrations of every selected POP and BMI was positively associated with HCB concentrations. Lower p,p′-DDE concentrations were found in those who had a university degree than in those who did not. Lower HCB, *p,p′*-DDE, PCB-138, and PCB-153 concentrations were observed in those who reported weight loss during the year before surgery *versus* those who did not. HCB, PCB-138, and PCB-180 concentrations were inversely associated with accumulated breastfeeding time. Age of menarche and HCB concentrations were positively correlated. Finally, adipose tissue PCB-153 concentrations were negatively associated with the presence of estrogen receptors in the tumor (Tables 4 and 5).

In regard to serum POP concentrations, age was positively associated with concentrations of HCB, PCB-153, and PCB-180, observing a quadratic relationship with PCB-180. BMI was negatively associated with PCB-153 and PCB-138 concentrations, observing a quadratic relationship with the latter. HCB concentrations were lower in those who had a university degree in comparison to those who did not and in non-manual *versus* manual workers. A similar association was found between PCB-138 and occupational class. Higher PCB-138 concentrations were observed in those who reported weight loss during the year before surgery than in those who did not. HCB and PCB-153 concentrations were negatively associated with accumulated breastfeeding time. Higher PCB-180 concentrations were found in post- *versus* pre-menopausal women. *p,p′*-DDE concentrations were negatively associated with the degree of tumor aggressiveness in the multivariable model. Finally, lower serum HCB and PCB-180 concentrations were observed in patients who received preoperative QT than in those who did not (Tables 4 and 5).

When log-transformed serum concentrations of each POP were introduced into the adipose tissue models and *vice-versa*, serum HCB and *p,p′*-DDE concentrations were positively associated with

Table 2
Concentrations of selected POPs in serum and adipose tissue.

	Adipose tissue						Serum						Adipose tissue:serum ratio		
	N (%) > LOD	Mean	SD	Percentiles			*N* (%) > LOD	Mean	SD	Percentiles			Percentiles		
				25th	50th	75th				25th	50th	75th	25th	50th	75th
HCB	103 (100)						91 (88.0)								
Wet-basis[a]		119.40	206.49	15.33	56.73	153.96		0.46	0.62	0.09	0.20	0.57	108.24	233.32	541.08
Lipid-basis		156.25	263.44	19.35	67.38	194.62		73.31	92.54	14.30	39.59	87.20	0.92	1.73	3.88
***p,p′*-DDE**	103 (100)						102 (99.0)								
Wet-basis		303.83	421.07	64.90	156.20	356.29		2.60	4.63	0.38	1.19	2.81	54.02	109.38	464.31
Lipid-basis		396.63	573.85	85.26	194.34	444.86		431.86	761.50	65.11	173.84	520.42	0.41	0.88	3.88
PCB-138	102 (99.0)						80 (77.7)								
Wet-basis		88.66	109.56	40.41	60.51	96.31		0.39	1.25	0.05	0.12	0.23	196.05	651.62	1610.76
Lipid-basis		114.99	143.33	51.08	80.84	128.33		66.09	216.52	7.78	18.56	40.36	1.16	4.34	9.20
PCB-153	102 (99.0)						67 (65.0)								
Wet-basis		80.37	84.64	24.34	55.28	90.30		0.21	0.48	< LOD	0.10	0.18	227.32	605.93	1594.03
Lipid-basis		102.11	100.45	34.39	72.64	137.30		34.05	78.64	4.08	18.30	27.93	1.33	3.51	9.39
PCB-180	100 (97.1)						80 (77.7)								
Wet-basis		49.33	48.92	16.06	32.73	66.58		0.31	0.66	0.06	0.14	0.28	76.07	280.98	629.58
Lipid-basis		62.99	59.60	19.76	44.71	85.83		53.78	111.57	9.42	23.36	46.10	0.43	1.83	4.18

LOD: limit of detection; SD: standard deviation.

[a] Serum wet-basis refers to ng/mL and adipose tissue wet-basis refers to ng/g tissue.

Table 3
Correlation coefficients of adipose tissue versus serum concentration of selected POPs[a].

	HCB	*p,p′*-DDE	PCB-138	PCB-153	PCB-180	∑PCBs
Adipose tissue	–					
HCB	–					
***p,p′*-DDE**	0.48**	–				
PCB-138	0.43*	0.74**	–			
PCB-153	0.66**	0.70**	0.75**	–		
PCB-180	0.64**	0.70**	0.77**	0.97***	–	–
∑PCBs	0.64**	0.75**	0.85**	0.98***	0.98***	–
	HCB	***p,p′*-DDE**	**PCB-138**	**PCB-153**	**PCB-180**	**∑PCBs**
Serum	–					
HCB	–					
***p,p′*-DDE**	0.37**	–				
PCB-138	0.27*	0.60**	–			
PCB-153	0.25	0.65**	0.59**	–		
PCB-180	0.26*	0.59**	0.58**	0.69**	–	–
∑PCBs	0.28*	0.71**	0.89**	0.78**	0.81**	–
	HCB	***p,p′*-DDE**	**PCB-138**	**PCB-153**	**PCB-180**	**∑PCBs**
Adipose tissue versus serum						
Total (*N*=103)	0.85***	0.63***	0.09	0.31	−0.03	−0.04
Preoperative chemotherapy=No (*N*=61)	**0.74*****	**0.83*****	0.10	0.43	−0.15	−0.12
Preoperative chemotherapy=Yes (*N*=42)	**0.92*****	**0.46****	0.07	0.33	0.10	0.08

[a] Analyses were carried-out using lipid-basis concentrations.

. $p < 0.1$.

* $p \leq 0.05$.

** $p \leq 0.01$.

*** $p \leq 0.001$.

adipose tissue concentrations and *vice-versa*, but serum concentrations of PCB congeners were not associated with their adipose tissue concentrations (Supplementary Tables 4 and 5).

4. Discussion

The results of this study suggest that the serum burden might be a proxy value for the historical exposure in breast cancer patients reflected by the adipose tissue burden of some POPs, but not others. However, our study also identifies some variables that should be taken into account in the adjustment of statistical models in future studies on the effect of POPs on breast cancer risk, such as recent weight loss and neoadjuvant chemotherapy.

In the present study, a significant and positive inter-matrix correlation was found for HCB and *p,p′*-DDE, as previously reported (Archibeque-Engle et al., 1997; Arrebola et al., 2012a; Kanja et al., 1992; López-Carrillo et al., 1999; Mussalo-Rauhamaa, 1991; Pauwels et al., 2000; Rusiecki et al., 2005; Stellman et al., 1998; Whitcomb et al., 2005), but not for the PCBs. Results of the few studies on this issue in PCBs have been controversial, with some researchers reporting strong and positive correlations (Archibeque-Engle et al., 1997; Lv et al., 2015; Mussalo-Rauhamaa, 1991; Pauwels et al., 2000; Rusiecki et al., 2005; Whitcomb et al., 2005) but others finding weak or no correlations (Arrebola et al., 2012a; Stellman et al., 1998). Interestingly, the present results are in agreement with findings by our group in cancer-free adults from Bolivia, i.e., a positive correlation between serum and adipose tissue concentrations of organochlorine pesticides but not PCBs (Arrebola et al., 2012a).

Table 4
Predictors of log-transformed concentrations of selected POPs in adipose tissue and serum. Bivariate and multivariable linear regression analyses (I).

	HCB[φ]														
	Adipose tissue							Serum							
	Bivariate analysis				Multivariable analysis (R^2=0.602)				Bivariate analysis				Multivariable analysis (R^2=0.647)			
	exp (β)	CI (95%)			exp (β)	CI (95%)			exp (β)	CI (95%)			exp (β)	CI (95%)		
Total lipid content	1.01	0.99	1.04	.	1.00	0.98	1.02		1.21	1.04	1.42	*	1.06	0.93	1.21	
Age (years)	1.06	1.04	1.08	***	1.07	1.05	1.09	***	1.03	1.02	1.05	***	1.02	1.01	1.04	**
Residence=urban[a]	1.15	0.60	2.20		–	–	–		0.75	0.48	1.17		–	–	–	
Educational level=university[b]	0.56	0.31	1.01	.	–	–	–		0.47	0.32	0.71	***	0.54	0.37	0.79	**
Ocupational class=manual+homemaker[c]	1.17	0.59	2.30		–	–	–		1.73	1.10	2.72	*	1.54	1.02	2.32	*
BMI	1.11	1.04	1.19		1.06	1.01	1.11	*	1.05	1.01	1.11	*	–	–	–	
Perceived weight loss=yes[d]	0.65	0.34	1.23		0.60	0.39	0.93	*	1.10	0.70	1.74		–	–	–	
Preoperative chemotherapy=yes[e]	0.79	0.42	1.47		–	–	–		0.64	0.42	0.97	*	0.56	0.39	0.79	**
Parity=primiparous/ multiparous[f]	0.89	0.33	2.36		–	–	–		0.88	0.45	1.74		–	–	–	
Accumulated breastfeeding time (months)	1.00	0.97	1.02		0.98	0.96	0.99	**	0.99	0.98	1.01		0.98	0.96	0.99	**
Age of menarche (years)	1.16	0.98	1.37	.	1.13	0.99	1.29	.	1.03	0.90	1.17					
Menopausal status=yes[g]	2.71	2.65	2.78	*	–	–	–		1.79	1.20	2.68	**	–	–	–	
Hormone replacement therapy=yes[h]	1.43	0.65	3.15		–	–	–		1.23	0.66	2.30					
Biological aggressiveness grade																
G2	0.57	0.29	1.12		–	–	–		0.73	0.45	1.18		–	–	–	
G3	0.47	0.21	1.04	.	–	–	–		0.64	0.38	1.10		–	–	–	
Estrogen receptor=positive[i]	1.79	0.87	3.69		–	–	–		1.98	1.20	3.27	**	–	–	–	
	p,p'-DDE[φ]															
	Adipose tissue							Serum							
	Bivariate analysis				Multivariable analysis (R^2=0.106)				Bivariate analysis				Multivariable analysis (R^2=0.271)			
	exp (β)	CI (95%)			exp (β)	CI (95%)			exp (β)	CI (95%)			exp (β)	CI (95%)		
Total lipid content	1.00	0.98	1.03		1.00	0.98	1.02		1.18	0.99	1.41	.	1.21	0.99	1.48	.
Age (years)	1.03	1.01	1.05	**	1.03	1.00	1.05	*	1.02	1.00	1.04	.	–	–	–	
Residence=urban[a]	0.81	0.47	1.39		–	–	–		0.77	0.47	1.28		–	–	–	
Educational level=university[b]	0.58	0.35	0.93	*	0.66	0.40	1.08	.	0.67	0.42	1.07	.	–	–	–	
Ocupational class=manual+homemaker[c]	1.32	0.75	2.31		–	–	–		1.37	0.82	2.32		–	–	–	
BMI	1.01	0.96	1.07		–	–	–		1.01	0.96	1.07		–	–	–	
Perceived weight loss=Yes[d]	0.70	0.41	1.20		0.60	0.36	1.00	*	1.42	0.86	2.34		–	–	–	
Preoperative chemotherapy=yes[e]	1.10	0.65	1.84		–	–	–		0.83	0.52	1.33		–	–	–	
Parity=primiparous/ multiparous[f]	1.04	0.87	1.24		–	–	–		1.10	0.49	2.47		–	–	–	
Accumulated breastfeeding time (months)	1.01	0.99	1.03		–	–	–		1.01	0.99	1.03		–	–	–	
Age of menarche (years)	1.06	0.90	1.23		–	–	–		0.89	0.75	1.06		–	–	–	
Menopausal status=yes[g]	1.33	0.81	2.18		–	–	–		1.45	0.91	2.31		–	–	–	
Hormone replacement therapy=yes[h]	0.70	0.35	1.42		–	–	–		0.76	0.34	1.69		–	–	–	
Biological aggressiveness grade																
G2	0.86	0.48	1.57		–	–	–		0.52	0.29	0.94	*	0.52	0.29	0.94	*
G3	0.97	0.48	1.99		–	–	–		0.49	0.26	0.94	*	0.49	0.26	0.94	*
Estrogen receptor=positive[i]	0.83	0.44	1.57		–	–	–		1.58	0.85	2.92		–	–	–	

BMI=body mass index; n.i.=not included.

[φ] Models adjusted by total lipids.
[a] Ref. cat=rural.
[b] Ref. cat=up to primary school.
[c] Ref. cat=non-manual.
[d] Ref. cat=no.
[e] Ref. cat=no.
[f] Ref. cat=nulliparous.
[g] Ref. cat=no.
[h] Ref. cat=no.
[i] Ref. cat=negative.
. $p<0.1$.
* $p<0.05$.
** $p<0.01$.
*** $p<0.001$.

Lipid-basis standardization of adipose tissue and serum POP concentrations is the most widely-used approach and therefore the most appropriate method for comparison with other studies. In this regard, lipid-basis adipose tissue:serum ratios ranged from 1 to 4 in our study, similar to previous findings in cancer patients (López-Carrillo et al., 1999; Rusiecki et al., 2005) and medium-high in comparison to ranges reported in non-cancer populations (Arrebola et al., 2012a, 2012b; Mussalo-Rauhamaa, 1991; Patterson et al., 1988; Whitcomb et al., 2005). López-Carrillo et al. (1999) compared the adipose tissue:serum *p,p′*-DDE ratio between women with breast cancer patients and those with benign breast disease and concluded that breast cancer per se has little impact on the adipose tissue:serum balance of the POP burden. Previous research showed that pollutants with higher molecular weight have higher 1-octanol-water partition coefficients in animal species (Kanazawa, 1982). Therefore, it might be hypothesized that molecular weight may also contribute to the partition coefficients in humans. In this regard, it is plausible that heavier pollutants, such as the three studied PCBs (molecular weight: 360–395 g/mol) are more prone to accumulate in adipose tissue in comparison to HCB or *p,p′*-DDE (molecular weights: 284.8 and 318.0 g/mol, respectively). Indeed, in our study, adipose tissue PCBs showed (1) lower inter-matrix linear correlation coefficients and (2) higher lipid-adjusted levels of PCBs in comparison to serum concentrations, and this was not observed for HCB or *p,p′*-DDE.

Among the predictors of the POP concentrations studied, we observed a positive relationship between age and the concentration of most of the POPs in both serum and adipose tissue samples, in concordance with the majority of previous studies (Agudo et al., 2009; Glynn et al., 2003; Ibarluzea et al., 2011; Laden et al., 1999; Vaclavik et al., 2006). A greater POP accumulation is usually expected in older people due to their longer bioaccumulation and/or because they were born in a more heavily polluted environment, i.e., a cohort effect (Ahlborg et al., 1995).

The BMI was positively associated with adipose tissue HCB concentrations but inversely associated with serum PCB (-138 and -153) concentrations. Published results have not been consistent, with reports of positive, inverse, and non-linear relationships between the BMI and POP concentrations, probably due to differences in study designs, target populations, and the POPs under study (Arrebola et al., 2012c, 2014b; Bräuner et al., 2012; Vaclavik et al., 2006). BMI might act as a surrogate of dietary exposure, given that participants with higher BMI values are more likely to have a greater food intake and therefore an increased dietary POP exposure. On the contrary, many POPs are also suspected of acting as obesogens, i.e., capable of altering lipid accumulation and adipogenesis (Grün and Blumberg, 2007) and promoting obesity and obesity-related disorders (Arrebola et al., 2014b; Sharpe and Drake, 2013).

Lower serum HCB concentrations were observed in samples from women with higher educational level and higher serum PCB-138 and adipose tissue *p,p′*-DDE concentrations and lower serum HCB concentrations were detected in those from non-manual *versus* manual workers. Educational level and occupational class are complex indicators that can involve lifestyle patterns, dietary habits, and occupational exposure (Glynn et al., 2003); hence, their implications for exposure levels may vary among different countries and study populations (Arrebola et al., 2014b; de Basea et al., 2011; Ibarluzea et al., 2011).

Accumulated breastfeeding time was negatively associated with adipose tissue HCB, PCB-138, and PCB-180 concentrations and with serum HCB and PCB-153 concentrations. Lactation was found to be a major route of POP excretion in cross-sectional and follow-up studies (Ibarluzea et al., 2011; Laden et al., 1999; Weldon et al., 2010). These lipophilic chemicals tend to accumulate in human milk and are therefore released during breast feeding.

Weight loss was negatively associated with the majority of POPs in adipose tissue and was positively associated with serum PCB-138 concentrations alone. Although the mechanisms involved are not fully understood, the negative influence of weight loss on adipose tissue POP concentrations may be related to their release during fat mobilization, enhancing the exchange of POPs between the different body compartments and therefore their elimination (De Roos et al., 2012; Kim et al., 2010). In fact, this mechanism may underlie the reported increase in serum POP concentrations for up to 12 months after drastic weight loss from bariatric surgery (Chevrier et al., 2000; Imbeault et al., 2002; Kim et al., 2010; Pelletier et al., 2002).

We found a negative association between the degree of biological aggressiveness and serum *p,p′*-DDE concentrations in both bivariate and multivariable analyses and also between ER-positive status and adipose tissue PCB-153 concentrations. The biological aggressiveness and estrogen receptor (ER) status of the tumor are two of the most clinically relevant prognostic factors in breast cancer patients but have been poorly studied as potential predictors of the POP burden, perhaps because they have been considered consequences rather than causes of POP exposure. Thus, estrogen receptor presence in breast tumor has been associated with *p,p′*-DDE concentrations in breast adipose tissue (Dewailly et al., 1997). However, it is plausible that the biology of the tumor may modify the distribution of fats and therefore the stability of POPs. In this context, increased adipose tissue levels of leptin, a hormone that enhances fat metabolism, have been reported in patients with higher TNM staging (Tessitore et al., 2000). Hence, the tumor itself may hasten fat remodeling and affect the steady-state partitioning of POPs between body compartments.

Our data on the contribution of neo-adjuvant treatments to POP concentrations in each biological matrix are of special interest, because these are generally undergone before breast cancer surgery, hampering the collection of pre-treatment biological samples (Thompson and Moulder-Thompson, 2012). Serum HCB and PCB-180 concentrations were lower in the women who had received preoperative QT (41% of the total series), but no differences were observed in any adipose tissue POP concentration. Preoperative QT was also related to a reduction in the strength of the bivariate correlation between *p,p′*-DDE in serum and adipose tissue samples. These findings are consistent with the report by Baris et al. (2000) of significantly reduced serum *p,p′*-DDE, PCB-138 and PCB-153 concentrations after QT, In contrast, Gammon et al. (1996) described increased serum *p,p′*-DDE and PCBs concentrations after adjuvant treatment, although they considered radiotherapy and hormone therapy as well as QT in the treatment group, which only contained seven patients. Patients undergoing QT usually evidence severe side-effects related to major metabolic changes, including an alteration of serum-lipid fractions (Ray et al., 2001). Thus, tamoxifen therapy was found to significantly reduce fasting plasma levels of total cholesterol and low- and high-density lipoproteins (Love et al., 1990), which may affect the partitioning coefficient between adipose tissue and serum POP concentrations (Guo et al., 1987).

Our study has several shortcomings. The sample size was relatively limited, which may have precluded the detection of a wider range of associations. Our findings on some differences between the predictors of serum and adipose tissue POP concentrations need to be confirmed in wider studies that take account of other potential predictors, such as dietary habits or lifestyle patterns. Moreover, given the cross-sectional design of this study, we cannot completely rule out the presence of reverse-causality in the associations found. This possibility is more relevant for serum POP concentrations, which are more highly influenced by recent exposures, than for adipose tissue concentrations. In addition, our utilization of a backward stepwise method

Table 5
Predictors of log-transformed concentrations of selected POPs in adipose tissue and serum. Bivariate and multivariable linear regression analyses (II).

	PCB-138[♥]													
	Adipose tissue							Serum						
	Bivariate analysis			Multivariable analysis (R^2=0.263)				Bivariate analysis			Multivariable analysis (R^2=0.130)			
	exp (β)	CI (95%)		exp (β)	CI (95%)			exp (β)	CI (95%)		exp (β)	CI (95%)		
Total lipid content	1.00	0.99	1.02	1.00	0.99	1.02	***	1.16	0.98	1.37	1.13	0.96	1.33	
Age (years)	1.01	1.00	1.02 .	1.03	1.01	1.04	***	1.01	0.99	1.03	–	–	–	
Residence=urban[a]	1.07	0.75	1.51	–	–	–		1.17	0.71	1.91	–	–	–	
Educational level=university[b]	1.10	0.79	1.52	–	–	–		1.26	0.79	1.99	–	–	–	
Occupational class=manual+homemaker[c]	0.89	0.62	1.27	–	–	–		0.58	0.35	0.95 *	0.49	0.30	0.79	**
BMI	1.01	0.97	1.04	–	–	–		1.04	0.98	1.11	1.06	1.00	1.12	
$(BMI)^2$	*n.i.*	*n.i.*	*n.i.*	–	–	–		0.99	0.99	1.00	0.99	0.99	1.00	
Perceived weight loss=Yes[d]	0.60	0.43	0.85 **	0.58	0.43	0.79	***	1.79	1.11	2.89 *	1.77	1.11	2.83	*
Preoperative chemotherapy=yes[e]	0.86	0.63	1.19	–	–	–		0.94	0.59	1.51	–	–	–	
Parity=primiparous/multiparous[f]	0.76	0.46	1.26	–	–	–		0.76	0.36	1.57	–	–	–	
Accumulated breastfeeding time (months)	0.99	0.98	1.00 *	0.98	0.97	0.99	***	1.00	0.98	1.02	–	–	–	
Age of menarche (years)	1.01	0.91	1.11	–	–	–		0.92	0.79	1.07	–	–	–	
Menopausal status=yes[g]	1.48	1.09	2.01 *	–	–	–		0.99	0.63	1.57	–	–	–	
Hormone replacement therapy=yes[h]	1.24	0.79	1.94	–	–	–		1.53	0.77	3.04	–	–	–	
Biological aggressiveness grade														
G2	1.04	0.72	1.51	–	–	–		0.97	0.55	1.70	–	–	–	
G3	0.69	0.44	1.08	–	–	–		0.68	0.37	1.27	–	–	–	
Estrogen receptor=positive[i]	0.90	0.60	1.34	–	–	–		1.38	0.77	2.45	–	–	–	

	PCB−153[♥]													
	Adipose tissue							Serum						
	Bivariate analysis			Multivariable analysis (R^2=0.380)				Bivariate analysis			Multivariable analysis (R^2=0.281)			
	exp (β)	CI (95%)		exp (β)	CI (95%)			exp (β)	CI (95%)		exp (β)	CI (95%)		
Total lipid content	1.01	0.99	1.03	1.01	1.00	1.03	***	1.11	0.96	1.29	1.08	0.94	1.24	
Age (years)	1.03	1.02	1.05 ***	1.04	1.03	1.06	***	1.02	1.00	1.03 .	1.02	1.00	1.04	*
Residence=urban[a]	1.39	0.93	2.09	–	–	–		1.24	0.80	1.93	–	–	–	
Educational level=university[b]	0.89	0.61	1.31	–	–	–		0.94	0.61	1.43	–	–	–	
Occupational class=manual+homemaker[c]	0.80	0.52	1.22	–	–	–		0.93	0.58	1.48	–	–	–	
BMI	1.01	0.97	1.06	–	–	–		0.98	0.93	1.02	0.94	0.90	0.98	*
Perceived weight loss=yes[d]	0.83	0.55	1.25	0.69	0.47	1.01 .		1.19	0.76	1.85	–	–	–	
Preoperative chemotherapy=yes[e]	0.75	0.51	1.11	–	–	–		1.22	0.81	1.84	–	–	–	
Parity=primiparous/multiparous[f]	0.83	0.45	1.54	–	–	–		0.92	0.67	1.27	–	–	–	
Accumulated breastfeeding time (months)	1.00	0.98	1.01	–	–	–		1.01	1.00	1.03 .	0.98	0.93	1.02	
(Accumulated breastfeeding time (months))^2	*n.i.*	*n.i.*	*n.i.*	–	–	–		*n.i.*	*n.i.*	*n.i.*	1.00	1.00	1.00	.
Age of menarche (years)								1.06	0.93	1.22	–	–	–	
Menopausal status=yes[g]	1.52	1.05	2.20 *	–	–	–		1.33	0.88	2.00	–	–	–	
Hormone replacement therapy=yes[h]								0.80	0.44	1.46	–	–	–	
Biological aggressiveness grade														
G2	0.92	0.59	1.44	–	–	–		0.84	0.50	1.41	–	–	–	
G3	0.98	0.57	1.67	–	–	–		0.73	0.41	1.30	–	–	–	
Estrogen receptor=positive[i]	0.76	0.47	1.23	0.59	0.38	0.94 *		1.24	0.70	2.22	–	–	–	

	PCB-180[♥]													
	Adipose tissue							Serum						
	Bivariate analysis			Multivariable analysis (R^2=0.411)				Bivariate analysis			Multivariable analysis (R^2=0.489)			
	exp (β)	CI (95%)		exp (β)	CI (95%)			exp (β)	CI (95%)		exp (β)	CI (95%)		
Total lipid content	1.01	0.99	1.03	1.01	1.00	1.03	***	1.09	0.96	1.25	1.11	0.97	1.25	
Age (years)	1.03	1.02	1.05 ***	1.04	1.03	1.06	***	1.01	0.99	1.03	0.99	0.97	1.01	
(Age)^2	*n.i.*	*n.i.*	*n.i.*	*n.i.*	*n.i.*	*n.i.*		1.00	1.00	1.00	1.01	1.00	1.02	*
Residence=urban[a]	1.39	0.91	2.13	–	–	–		1.07	0.74	1.55	–	–	–	

Table 5 (*continued*)

	PCB-138[φ]											
	Adipose tissue						Serum					
	Bivariate analysis			Multivariable analysis (R^2=0.263)			Bivariate analysis			Multivariable analysis (R^2=0.130)		
	exp (β)	CI (95%)		exp (β)	CI (95%)		exp (β)	CI (95%)		exp (β)	CI (95%)	
Educational level=university[b]	0.92	0.61	1.38	–	–	–	1.23	0.87	1.73	–	–	–
Occupational class=manual+homemaker[c]	1.01	0.99	1.03	–	–	–	0.86	0.58	1.27	–	–	–
BMI	1.02	0.97	1.06	–	–	–	0.99	0.96	1.04	–	–	–
Perceived weight loss=yes[d]	0.78	0.51	1.20	–	–	–	1.09	0.75	1.59	–	–	–
Preoperative chemotherapy=yes[e]	0.64	0.43	0.96 *	–	–	–	0.68	0.49	0.95 *	0.65	0.46	0.93 *
Parity=primiparous/multiparous[f]	0.90	0.47	1.71	–	–	–	0.63	0.37	1.08	–	–	–
Accumulated breastfeeding time (months)	0.99	0.98	1.01	0.98	0.97	1.00 *	1.00	0.99	1.02	–	–	–
Age of menarche (years)	1.03	0.91	1.16	–	–	–	1.01	0.90	1.13	–	–	–
Menopausal status=yes[g]	1.49	1.00	2.21	–	–	–	1.50	1.07	2.10 *	1.66	1.12	2.46 *
Hormone replacement therapy=yes[h]	1.12	0.64	1.95	–	–	–	0.87	0.49	1.53	–	–	–
Biological aggressiveness grade												
G2	0.91	0.57	1.44	–	–	–	0.95	0.60	1.51	–	–	–
G3	0.94	0.54	1.63	–	–	–	0.88	0.53	1.47	–	–	–
Estrogen receptor=positive[i]	0.81	0.44	1.52	–	–	–	1.22	0.68	2.19	–	–	–

BMI=body mass index; n.i.=not included.

[φ] Models adjusted by total lipids.

· $p<0.1$.

* $p<0.05$.

** $p<0.01$.

*** $p<0.001$.

[a] Ref. cat=rural.

[b] Ref. cat=up to primary school.

[c] Ref. cat=non-manual.

[d] Ref. cat=no.

[e] Ref. cat=no.

[f] Ref. cat=nulliparous.

[g] Ref. cat=no.

[h] Ref. cat=no.

[i] Ref. Cat=negative.

to select variables for the statistical analyses means that we are unable to rule out chance findings resulting from the order in which they were entered in the models. However, the fact that many associations were reproduced in different POPs may support the robustness of our analyses. Finally, some of the associations found with BMI, occupational class, and educational level, among other variables, may in part be explained by dietary factors, which were not considered in our analysis. Therefore, our study highlights novel results that need to be confirmed by larger studies due to the difficulties during sampling collection. Future studies should also: (i) consider specific adjustment variables, such as recent weight loss or neoadjuvant chemotherapy treatment; and (ii) take into account that serum and adipose tissue might not have the same biological meaning for specific POPs (e.g. PCBs) and under certain physiological conditions. Finally, given that humans are commonly exposed to complex mixtures of environmental pollutants that might have synergic/antagonistic effects, there is a need for biomarkers of exposure to multiple chemicals, specifically those with common mechanisms of action (Fernández et al., 2004; Ibarluzea et al., 2004).

The role of POP exposure in the development and prognosis of breast cancer remains unclear (Salehi et al., 2008). Our results reveal that serum and adipose tissue POP concentrations in breast cancer patients may be differentially influenced by external predictors and that QT modifies the serum concentration of certain POPs. These findings underline the importance of taking into account the individual POP(s) under study and the biological matrix used when assessing internal POP exposure, especially in relation to health outcomes; the results also highlight the need to make a careful selection of covariates for adjusting the model.

Competing interests

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

Funding sources

This study was supported by the Andalusian Regional Government (PI-0513-2012). F. Artacho-Cordón has a research Grant from the Spanish Ministry of Education (AP2012-2524), and Dr J.P. Arrebola has a postdoctoral contract from the Andalusian Council of Health (RH-0092-2013).

The study was approved by the Ethics Committee of Granada "Comité de Ética de la Investigación Biomédica de la provincia de Granada", which is affiliated to the Public Health System of Andalusia.

Acknowledgments

The authors are indebted to all the participants and staff from the San Cecilio University Hospital, without whom this study

would have been impossible. Authors are also grateful to Richard Davies for editorial assistance.

Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2015.08.020.

References

Agudo, A., Goni, F., Etxeandia, A., Vives, A., Millán, E., López, R., Amiano, P., Ardanaz, E., Barricarte, A., Chirlaque, M.D., 2009. Polychlorinated biphenyls in Spanish adults: determinants of serum concentrations. Environ Res. 109, 620–628.

Ahlborg, U.G., Lipworth, L., Titus-Ernstoff, L., Hsieh, C.-C., Hanberg, A., Baron, J., Trichopoulos, D., Adami, H.-O., 1995. Organochlorine compounds in relation to breast cancer, endometrial cancer, and endometriosis: an assessment of the biological and epidemiological evidence. CRC Crit. Rev. Toxicol. 25, 463–531.

Andersen, H.R., Vinggaard, A.M., Rasmussen, T.H., Gjermandsen, I.M., Bonefeld-Jørgensen, E.C., 2002. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. Toxicol. Appl. Pharmacol. 179, 1–12.

Antweiler, R.C., Taylor, H.E., 2008. Evaluation of statistical treatments of left-censored environmental data using coincident uncensored data sets: I. Summary statistics. Environ. Sci. Technol. 42, 3732–3738.

Archibeque-Engle, S.L., Tessari, J.D., Winn, D.T., Keefe, T.J., Nett, T.M., Zheng, T., 1997. Comparison of organochlorine pesticide and polychlorinated biphenyl residues in human breast adipose tissue and serum. J. Toxicol. Environ. Health 52, 285–293.

Aronson, K.J., Miller, A.B., Woolcott, C.G., Sterns, E.E., McCready, D.R., Lickley, L.A., Fish, E.B., Hiraki, G.Y., Holloway, C., Ross, T., 2000. Breast adipose tissue concentrations of polychlorinated biphenyls and other organochlorines and breast cancer risk. Cancer Epidemiol. Biomark. Prev. 9, 55–63.

Arrebola, J., Cuellar, M., Claure, E., Quevedo, M., Antelo, S., Mutch, E., Ramirez, E., Fernandez, M., Olea, N., Mercado, L., 2012a. Concentrations of organochlorine pesticides and polychlorinated biphenyls in human serum and adipose tissue from Bolivia. Environ. Res. 112, 40–47.

Arrebola, J., Martin-Olmedo, P., Fernandez, M., Sanchez-Cantalejo, E., Jimenez-Rios, J., Torne, P., Porta, M., Olea, N., 2009. Predictors of concentrations of hexachlorobenzene in human adipose tissue: a multivariate analysis by gender in Southern Spain. Environ. Int. 35, 27–32.

Arrebola, J., Mutch, E., Cuellar, M., Quevedo, M., Claure, E., Mejía, L., Fernández-Rodríguez, M., Freire, C., Olea, N., Mercado, L., 2012b. Factors influencing combined exposure to three indicator polychlorinated biphenyls in an adult cohort from Bolivia. Environ. Res. 116, 17–25.

Arrebola, J., Mutch, E., Rivero, M., Choque, A., Silvestre, S., Olea, N., Ocaña-Riola, R., Mercado, L., 2012c. Contribution of sociodemographic characteristics, occupation, diet and lifestyle to DDT and DDE concentrations in serum and adipose tissue from a Bolivian cohort. Environ. Int. 38, 54–61.

Arrebola, J.P., Belhassen, H., Artacho-Cordón, F., Ghali, R., Ghorbel, H., Boussen, H., Perez-Carrascosa, F.M., Expósito, J., Hedhili, A., Olea, N., 2015. Risk of female breast cancer and serum concentrations of organochlorine pesticides and polychlorinated biphenyls: a case – control study in Tunisia. Sci. Total Environ. 520, 106–113.

Arrebola, J.P., Fernández, M.F., Martín-Olmedo, P., Molina-Molina, J.M., Sánchez-Pérez, M.J., Sánchez-Cantalejo, E., Molina-Portillo, E., Expósito, J., Bonde, J.P., Olea, N., 2014a. Adipose tissue concentrations of persistent organic pollutants and total cancer risk in an adult cohort from Southern Spain: preliminary data from year 9 of the follow-up. Sci. Total Environ. 500–501, 243–249.

Arrebola, J.P., Fernandez, M.F., Porta, M., Rosell, J., de la Ossa, R.M., Olea, N., Martin-Olmedo, P., 2010. Multivariate models to predict human adipose tissue PCB concentrations in Southern Spain. Environ. Int. 36, 705–713.

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

Arrebola, J.P., Pumarega, J., Gasull, M., Fernandez, M.F., Martin-Olmedo, P., Molina-Molina, J.M., Fernández-Rodríguez, M., Porta, M., Olea, N., 2013. Adipose tissue concentrations of persistent organic pollutants and prevalence of type 2 diabetes in adults from Southern Spain. Environ. Res. 122, 31–37.

Baris, D., Kwak, L.W., Rothman, N., Wilson, W., Manns, A., Tarone, R.E., Hartge, P., 2000. Blood levels of organochlorines before and after chemotherapy among non-Hodgkin's lymphoma patients. Cancer Epidemiol. Biomark. Prev. 9, 193–197.

Boada, L.D., Zumbado, M., Henríquez-Hernández, L.A., Almeida-González, M., Álvarez-León, E.E., Serra-Majem, L., Luzardo, O.P., 2012. Complex organochlorine pesticide mixtures as determinant factor for breast cancer risk: a population-based case-control study in the Canary Islands (Spain). Environ. Health 11, 6–7.

Bonefeld-Jorgensen, E., 2010. Biomonitoring in Greenland: human biomarkers of exposure and effects – a short review. Rural Remote Health 10, 1362.

Bonefeld-Jørgensen, E.C., Andersen, H.R., Rasmussen, T.H., Vinggaard, A.M., 2001. Effect of highly bioaccumulated polychlorinated biphenyl congeners on estrogen and androgen receptor activity. Toxicology 158, 141–153.

Bonefeld-Jorgensen, E.C., Long, M., Bossi, R., Ayotte, P., Asmund, G., Kruger, T., Ghisari, M., Mulvad, G., Kern, P., Nzulumiki, P., 2011. Perfluorinated compounds are related to breast cancer risk in Greenlandic Inuit: a case control study. Environ. Health 10, 88.

Bonefeld-Jørgensen, E.C., Long, M., Fredslund, S.O., Bossi, R., Olsen, J., 2014. Breast cancer risk after exposure to perfluorinated compounds in Danish women: a case–control study nested in the Danish National Birth Cohort. Cancer Causes & Control. 25, 1439–1448.

Bräuner, E.V., Raaschou-Nielsen, O., Gaudreau, E., Leblanc, A., Tjønneland, A., Overvad, K., Sørensen, M., 2012. Predictors of adipose tissue concentrations of organochlorine pesticides in a general Danish population. J. Expo. Sci. Environ. Epidemiol. 22, 52–59.

Crinnion, W.J., 2009. Chlorinated pesticides: threats to health and importance of detection. Altern. Med. Rev.: J. Clin. Ther. 14, 347–359.

Chevrier, J., Dewailly, E., Ayotte, P., Mauriege, P., Despres, J., Tremblay, A., 2000. Body weight loss increases plasma and adipose tissue concentrations of potentially toxic pollutants in obese individuals. Int. J. Obes. Relat. Metab. Disord.: J. Int. Assoc. Study Obes. 24, 1272–1278.

de Basea, M.B., Porta, M., Alguacil, J., Puigdomènech, E., Gasull, M., Garrido, J.A., López, T., 2011. Relationships between occupational history and serum concentrations of organochlorine compounds in exocrine pancreatic cancer. Occup. Environ. Med. 68, 332–338.

De Roos, A.J., Ulrich, C.M., Sjodin, A., McTiernan, A., 2012. Adiposity, body composition, and weight change in relation to organochlorine pollutant plasma concentrations. J. Expo. Sci. Environ. Epidemiol. 22, 617–624.

Dewailly, É., Ayotte, P., Dodin, S., 1997. Could the rising levels of estrogen receptor in breast cancer be due to estrogenic pollutants? J. Natl. Cancer Inst. 89, 888–889.

Fernandez, M.F., Olmos, B., Granada, A., López-Espinosa, M.J., Molina-Molina, J.-M., Fernandez, J.M., Cruz, M., Olea-Serrano, F., Olea, N., 2007a. Human exposure to endocrine-disrupting chemicals and prenatal risk factors for cryptorchidism and hypospadias: a nested case-control study. Environ Health Perspect. 115, 8–14.

Fernández, M.F., Rivas, A., Olea-Serrano, F., Cerrillo, I., Molina-Molina, J.M., Araque, P., Martínez-Vidal, J.L., Olea, N., 2004. Assessment of total effective xenoestrogen burden in adipose tissue and identification of chemicals responsible for the combined estrogenic effect. Anal. Bioanal. Chem. 379, 163–170.

Fernandez, M.F., Santa-Marina, L., Ibarluzea, J.M., Exposito, J., Aurrekoetxea, J.J., Torne, P., Laguna, J., Rueda, A.I., Pedraza, V., Olea, N., 2007b. Analysis of population characteristics related to the total effective xenoestrogen burden: a biomarker of xenoestrogen exposure in breast cancer. Eur. J. Cancer 43, 1290–1299.

Gammon, M.D., Wolff, M.S., Neugut, A.I., Terry, M.B., Britton, J.A., Greenebaum, E., Hibshoosh, H., Levin, B., Wang, Q., Santella, R.M., 1996. Treatment for breast cancer and blood levels of chlorinated hydrocarbons. Cancer Epidemiol. Biomark. Prev. 5, 467–471.

Gasull, M., Porta, M., Pumarega, J., Vioque, J., de Basea, M.B., Puigdomènech, E., Morales, E., Grimalt, J.O., Malats, N., 2010. The relative influence of diet and serum concentrations of organochlorine compounds on K-< i > ras < /i > mutations in exocrine pancreatic cancer. Chemosphere 79, 686–697.

Gatto, N., Longnecker, M., Press, M., Sullivan-Halley, J., McKean-Cowdin, R., Bernstein, L., 2007. Serum organochlorines and breast cancer: a case–control study among African-American women. Cancer Causes Control 18, 29–39.

Glynn, A.W., Granath, F., Aune, M., Atuma, S., Darnerud, P.O., Bjerselius, R., Vainio, H., Weiderpass, E., 2003. Organochlorines in Swedish women: determinants of serum concentrations. Environ. Health Perspect. 111, 349.

Grün, F., Blumberg, B., 2007. Perturbed nuclear receptor signaling by environmental obesogens as emerging factors in the obesity crisis. Rev. Endocr. Metab. Disord. 8, 161–171.

Grünfeld, H., Bonefeld-Jorgensen, E., 2004. Effect of in vitro estrogenic pesticides on human oestrogen receptor α and β mRNA levels. Toxicol. Lett. 151, 467–480.

Guo, Y.L., Emmett, E.A., Pellizzari, E.D., Rohde, C.A., 1987. Influence of serum cholesterol and albumin on partitioning of PCB congeners between human serum and adipose tissue. Toxicol. Appl. Pharmacol. 87, 48–56.

Ibarluzea, J., Alvarez-Pedrerol, M., Guxens, M., Marina, L.S., Basterrechea, M., Lertxundi, A., Etxeandia, A., Goni, F., Vioque, J., Ballester, F., 2011. Sociodemographic, reproductive and dietary predictors of organochlorine compounds levels in pregnant women in Spain. Chemosphere 82, 114–120.

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

Imbeault, P., Tremblay, A., Simoneau, J.-A., Joanisse, D.R., 2002. Weight loss-induced rise in plasma pollutant is associated with reduced skeletal muscle oxidative capacity. Am. J. Physiol. Endocrinol. Metab. 282, E574–E579.

Itoh, H., Iwasaki, M., Hanaoka, T., Kasuga, Y., Yokoyama, S., Onuma, H., Nishimura, H., Kusama, R., Tsugane, S., 2009. Serum organochlorines and breast cancer risk in Japanese women: a case-control study. Cancer Causes Control 20, 567–580.

Kanazawa, J., 1982. Relationship between the molecular weights of pesticides and their bioconcentration factors by fish. Experientia 38, 1045–1046.

Kanja, L.W., Skaare, J.U., Ojwang, S., Maitai, C., 1992. A comparison of organochlorine pesticide residues in maternal adipose tissue, maternal blood, cord blood, and human milk from mother/infant pairs. Arch. Environ. Contam.

Toxicol. 22, 21–24.
Kim, M.-J., Marchand, P., Henegar, C., Antignac, J.-P., Alili, R., Poitou, C., Bouillot, J.-L., Basdevant, A., Le Bizec, B., Barouki, R., 2010. Fate and complex pathogenic effects of dioxins and polychlorinated biphenyls in obese subjects before and after drastic weight loss. Environ. Health Perspect. 119, 377–383.
Kohlmeier, L., Kohlmeier, M., 1995. Adipose tissue as a medium for epidemiologic exposure assessment. Environ. Health Perspect. 103, 99.
Krüger, T., Long, M., Ghisari, M., Bonefeld-Jørgensen, E.C., 2012. The combined effect of persistent organic pollutants in the serum POP mixture in Greenlandic Inuit: xenoestrogenic, xenoandrogenic and dioxin-like transactivities. Biomarkers 17, 692–705.
Laden, F., Neas, L.M., Spiegelman, D., Hankinson, S.E., Willett, W.C., Ireland, K., Wolff, M.S., Hunter, D.J., 1999. Predictors of plasma concentrations of DDE and PCBs in a group of US women. Environ. Health Perspect. 107, 75.
Lee, D.-H., Porta, M., Jacobs Jr, D.R., Vandenberg, L.N., 2014. Chlorinated persistent organic pollutants, obesity, and type 2 diabetes. Endocr. Rev. 35, 557–601.
López-Carrillo, L., Torres-Sánchez, L., López-Cervantes, M. a, Blair, A., Cebrián, M.E., Uribe, M., 1999. The adipose tissue to serum dichlorodiphenyldichloroethane (DDE) ratio: some methodological considerations. Environ. Res. 81, 142–145.
Love, R.R., Newcomb, P.A., Wiebe, D.A., Surawicz, T.S., Jordan, V.C., Carbone, P.P., DeMets, D.L., 1990. Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. J. Natl. Cancer Inst. 82, 1327–1332.
Lv, Q.-X., Wang, W., Li, X.-H., Yu, L., Zhang, Y., Tian, Y., 2015. Polychlorinated biphenyls and polybrominated biphenyl ethers in adipose tissue and matched serum from an E-waste recycling area (Wenling, China). Environ. Pollut. 199, 219–226.
Martínez-Vidal, J., Moreno-Frías, M., Frenich, G.A., Olea-Serrano, F., Olea, N., 2002. Determination of endocrine-disrupting pesticides and polychlorinated biphenyls in human serum by GC–ECD and GC–MS–MS and evaluation of contributions to the uncertainty of the results. Anal. Bioanal. Chem. 372, 766–775.
Moreno-Frías, M., Jiménez-Torres, M., Frenich, A.G., Martínez Vidal, J.L., Olea-Serrano, F., Olea, N., 2004. Determination of organochlorine compounds in human biological samples by GC-MS/MS. Biomed. Chromatogr. 18, 102–111.
Mussalo-Rauhamaa, H., 1991. Partitioning and levels of neutral organochlorine compounds in human serum, blood cells, and adipose and liver tissue. Sci. Total Environ. 103, 159–175.
Patterson Jr, D., Needham, L., Pirkle, J., Roberts, D., Bagby, J., Garrett, W., Andrews Jr, J., Falk, H., Bernert, J., Sampson, E., 1988. Correlation between serum and adipose tissue levels of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin in 50 persons from Missouri. Arch. Environ. Contam. Toxicol. 17, 139–143.
Pauwels, A., Covaci, A., Weyler, J., Delbeke, L., Dhont, M., De Sutter, P., D'Hooghe, T., Schepens, P., 2000. Comparison of persistent organic pollutant residues in serum and adipose tissue in a female population in Belgium, 1996–1998. Arch. Environ. Contam. Toxicol. 39, 265–270.
Pelletier, C., Doucet, E., Imbeault, P., Tremblay, A., 2002. Associations between weight loss-induced changes in plasma organochlorine concentrations, serum T3 concentration, and resting metabolic rate. Toxicol. Sci. 67, 46–51.
Phillips, D.L., Pirkle, J.L., Burse, V.W., Bernert Jr, J.T., Henderson, L.O., Needham, L.L., 1989. Chlorinated hydrocarbon levels in human serum: effects of fasting and feeding. Arch. Environ. Contam. Toxicol. 18, 495–500.
Ray, A., Jain, D., Yadav, R., Naik, S., Sharma, S., Bahadur, A., Sharma, B., 2001. Effect of cancer treatment modalities on serum lipids and lipoproteins among women with carcinoma of the breast. Indian J. Physiol. Pharmacol. 45, 337–344.
Regidor, E., 2001. The Goldthorpe social class classification: framework of reference for the proposal for the measure of social class by the working group of the Spanish Epidemiological Society. Rev. Española Salud Pública 75, 13–72.
Rivas, A., Fernandez, M.F., Cerrillo, I., Ibarluzea, J., Olea-Serrano, M.F., Pedraza, V., Olea, N., 2001. Human exposure to endocrine disrupters: standardisation of a marker of estrogenic exposure in adipose tissue. APMIS 109, S189–S202.
Rusiecki, J.A., Matthews, A., Sturgeon, S., Sinha, R., Pellizzari, E., Zheng, T., Baris, D., 2005. A correlation study of organochlorine levels in serum, breast adipose tissue, and gluteal adipose tissue among breast cancer cases in India. Cancer Epidemiol. Biomark. Prev. 14, 1113–1124.
Saha Roy, S., Vadlamudi, R.K., 2011. Role of estrogen receptor signaling in breast cancer metastasis. Int. J. Breast Cancer 2012, 1–8.
Salehi, F., Turner, M.C., Phillips, K.P., Wigle, D.T., Krewski, D., Aronson, K.J., 2008. Review of the etiology of breast cancer with special attention to organochlorines as potential endocrine disruptors. J. Toxicol. Environ. Health B 11, 276–300.
Shah, F.D., Shukla, S.N., Shah, P.M., Patel, H.R., Patel, P.S., 2008. Significance of alterations in plasma lipid profile levels in breast cancer. Integr. Cancer Ther. 7, 33–41.
Sharpe, R.M., Drake, A.J., 2013. Obesogens and obesity – an alternative view? Obesity 21, 1081–1083.
Soto, A.M., Chung, K.L., Sonnenschein, C., 1994. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environ. Health Perspect. 102, 380.
Stellman, S.D., Djordjevic, M.V., Muscat, J.E., Gong, L., Bernstein, D., Citron, M.L., White, A., Kemeny, M., Busch, E., Nafziger, A.N., 1998. Relative abundance of organochlorine pesticides and polychlorinated biphenyls in adipose tissue and serum of women in Long Island, New York. Cancer Epidemiol. Biomark. Prev. 7, 489–496.
Syed, J.H., Malik, R.N., Liu, D., Xu, Y., Wang, Y., Li, J., Zhang, G., Jones, K.C., 2013. Organochlorine pesticides in air and soil and estimated air–soil exchange in Punjab, Pakistan. Sci. Total Environ. 444, 491–497.
Tessitore, L., Vizio, B., Jenkins, O., De Stefano, I., Ritossa, C., Argiles, J., Benedetto, C., Mussa, A., 2000. Leptin expression in colorectal and breast cancer patients. Int. J. Mol. Med. 5, 421–427.
Thompson, A.M., Moulder-Thompson, S.L., 2012. Neoadjuvant treatment of breast cancer. Ann. Oncol. 23, x231–x236.
Turci, R., Balducci, C., Brambilla, G., Colosio, C., Imbriani, M., Mantovani, A., Vellere, F., Minoia, C., 2010. A simple and fast method for the determination of selected organohalogenated compounds in serum samples from the general population. Toxicol. Lett. 192, 66–71.
UNEP, Regionally Based Assessmentof Persistent Toxic Substances. Mediterranean Regional Report. United Nations Environment Programme on Chemicals. United Nations Environment Programme on Chemicals. Available from: ⟨iwlearn.net/iw-projects/14/reports/mediterranean-report⟩. 2003.
Vaclavik, E., Tjonneland, A., Stripp, C., Overvad, K., Philippe Weber, J., Raaschou-Nielsen, O., 2006. Organochlorines in Danish women: predictors of adipose tissue concentrations. Environ. Res. 100, 362–370.
Waliszewski, S., Carvajal, O., Infanzon, R., Trujillo, P., Hart, M., 2004. Copartition ratios of persistent organochlorine pesticides between human adipose tissue and blood serum lipids. Bull. Environ. Contam. Toxicol. 73, 732–738.
Weldon, R.H., Webster, M., Harley, K.G., Bradman, A., Fenster, L., Davis, M.D., Hubbard, A., Barr, D.B., Holland, N., Eskenazi, B., 2010. Serum persistent organic pollutants and duration of lactation among Mexican-American women. J. Environ. Public Health 2010, 1–11.
Whitcomb, B.W., Schisterman, E.F., Buck, G.M., Weiner, J.M., Greizerstein, H., Kostyniak, P.J., 2005. Relative concentrations of organochlorines in adipose tissue and serum among reproductive age women. Environ. Toxicol. Pharmacol. 19, 203–213.
WHO, 2000. Obesity: Preventing and Managing the Global Epidemic. World Health Organization, Geneva, Switzerland.
Wolff, M.S., Zeleniuch-Jacquotte, A., Dubin, N., Toniolo, P., 2000. Risk of breast cancer and organochlorine exposure. Cancer Epidemiol. Biomark. Prev. 9, 271–277.
Xu, X., Dailey, A.B., Talbott, E.O., Ilacqua, V.A., Kearney, G., Asal, N.R., 2010. Associations of serum concentrations of organochlorine pesticides with breast cancer and prostate cancer in US adults. Environ. Health Perspect. 118, 60–66.

7.2. Objective 2. Evaluate the magnitude of the exposure to persistent and non-persistent EDCs and identify predictors of the exposure in a subset of healthy women from Tunisia.

SERUM LEVELS OF PERSISTENT ORGANIC POLLUTANTS AND PREDICTORS OF EXPOSURE IN TUNISIAN WOMEN

Artacho-Cordón F, *Belhassen H, Arrebola JP,Ghali R, Amira D, Jiménez-Díaz I, Pérez-Lobato R, Boussen H, Hedili A, Olea N*

Science of the Total Environment; Impact factor 4.099; 1st Quartile; 1st Decile

ABSTRACT

Introduction: In spite of the international consensus on the human health risks associated with exposure to persistent organic pollutants (POPs), the Tunisian population is suspected to have been inadvertently exposed to POPs over the last decades.

Objectives: The aim of this study was to evaluate POP concentrations in the serum of a subset of 54 Tunisian women and to identify some socio-demographic and dietary predictors of exposure to POPs.

Results: Of the ten POPs analyzed, three polychlorinated biphenyl (PCB) congeners (-138, -153 and -180), and two organochlorine pesticides (OCPs), HCB and *p,p'*-DDE, showed frequencies ranging from 98 to 100%. Serum median concentrations of PCB congeners (-138, -153 PCB-180) were 26.08, 119.1 and 29.84 ng/g lipid, respectively, and median concentrations of HCB and *p,p'*-DDE were 19.98 and 127.59 ng/g lipid, respectively. Age was positively correlated with serum levels of selected POPs. Women living in northern Tunisia showed higher serum levels of all PCBs. Working outside home and cereal consumption were positively associated to serum levels of *p,p'*-DDE. The duration of the lactation was also related to lower serum levels of *p,p'*-DDE and HCB.

Conclusion: The levels of exposure to POPs found warrant a biomonitoring program in order to identify routes of exposure and population groups at higher risk. This program will help to establish prevention policies and to determine the association between exposure to POPs and chronic diseases.

Science of the Total Environment 511 (2015) 530–534

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Serum levels of persistent organic pollutants and predictors of exposure in Tunisian women

F. Artacho-Cordón [a,b,*], H. Belhassen [c], J.P. Arrebola [b,d,e], R. Ghali [c], D. Amira [c], I. Jiménez-Díaz [b], R. Pérez-Lobato [b], H.Boussen [f], A.Hedili [c], N. Olea [a,b,d]

[a] Radiology and Physical Medicine Department, University of Granada, Granada, Spain
[b] Instituto de Investigación Biosanitaria IBS.Granada, Hospitales Universitarios de Granada/Universidad de Granada, Granada, Spain
[c] Laboratory of Toxicology and Environment (LR12SP07), Center of Urgent Medical Assistance of Tunis, Tunis, Tunisia
[d] CIBER en Epidemiología y Salud Pública (CIBERESP), Spain
[e] Radiation Oncology Department, Virgen de las Nieves University Hospital, Granada, Spain
[f] Cancer Center of Ariana, Ariana, Tunisia

HIGHLIGHTS

- HCB, p,p′-DDE and PCBs were detected in most women of this study.
- Women living in North Tunisia showed higher levels of PCBs that in the South.
- Higher *p,p′*-DDE levels in cereal consumers suggest a recent use of this pesticide.

ARTICLE INFO

Article history:
Received 9 October 2014
Received in revised form 4 December 2014
Accepted 28 December 2014
Available online xxxx

Editor: Adrian Covaci

Keywords:
Persistent organic pollutant
Organochlorine pesticides
Polychlorinated biphenyls
Exposure predictors
Serum
Tunisia

ABSTRACT

Introduction: In spite of the international consensus on the human health risks associated with exposure to persistent organic pollutants (POPs), the Tunisian population is suspected to have been inadvertently exposed to POPs over the last decades.
Objectives: The aim of this study was to evaluate POP concentrations in the serum of a subset of 54 Tunisian women and to identify some socio-demographic and dietary predictors of exposure to POPs.
Results: Of the ten POPs analyzed, three polychlorinated biphenyl (PCB) congeners (-138, -153 and -180), and two organochlorine pesticides (OCPs), HCB and *p,p′*-DDE, showed frequencies ranging from 98 to 100%. Serum median concentrations of PCB congeners (-138, -153 PCB-180) were 26.08, 119.1 and 29.84 ng/g lipid, respectively, and median concentrations of HCB and *p,p′*-DDE were 19.98 and 127.59 ng/g lipid, respectively. Age was positively correlated with serum levels of selected POPs. Women living in northern Tunisia showed higher serum levels of all PCBs. Working outside home and cereal consumption were positively associated to serum levels of *p,p′*-DDE. The duration of the lactation was also related to lower serum levels of *p,p′*-DDE and HCB.
Conclusion: The levels of exposure to POPs found warrant a biomonitoring program in order to identify routes of exposure and population groups at higher risk. This program will help to establish prevention policies and to determine the association between exposure to POPs and chronic diseases.

1. Introduction

Persistent organic pollutants (POPs) such as dichlorodiphenyltrichloroethylene (DDT) and its metabolites (notably, *p,p′*-dichlorodiphenyl-dichloroethylene [*p,p′*-DDE]), hexachlorobenzene (HCB), and polychlorinated biphenyls (PCBs) are a group of chemicals that have been banned in most of the Mediterranean countries. Despite this ban, a vast array of studies has shown the persistence and liposolubility of these chemicals, as well as their tendency to bioaccumulate and biomagnify in the food chain (Porta et al., 2008) and therefore to reach human population (Kelly et al., 2004). Human exposure has been associated to adverse clinical effects at doses traditionally considered safe, including reproductive disorders, endocrine disruption, and carcinogenicity (Qing Li et al., 2006).

Most POPs were used until the 1980s for insect control, sanitary purposes as well as in agriculture and industry (APEK, 2005). However, in Tunisia there are still hundreds of obsolete pesticide stockpiles, and a large number of power transformers containing PCBs are still in use or

* Corresponding author at: Radiology and Physical Medicine Department, University of Granada, Spain, 18071 Granada, Spain.
E-mail address: fartacho@ugr.es (F. Artacho-Cordón).

http://dx.doi.org/10.1016/j.scitotenv.2014.12.093
0048-9697/

stored in inappropriate conditions (APEK, 2005; Dasgupta et al., 2010), particularly in the north of the country (ANGed, 2008). This scenario suggests that the Tunisian population may have been inadvertently exposed to POPs as reported by two studies (Ben Hassine et al., 2013; Ennaceur and Driss, 2010), which have paid little attention to the identification of exposure routes. Therefore, included in a larger project, the aim of this study was to evaluate POP concentrations in the serum of a subset of female volunteers from Tunisia and to identify some socio-demographic and dietary predictors of exposure to POPs in order to set the basis for a large-scale biomonitoring study in the country.

2. Material and methods

2.1. Study population

The present study was conducted between May and October 2012 and included a subset of 54 Tunisian women at either the Salah Azaiz Hospital (Tunis) or the Ariana Hospital (Ariana) who were in those hospitals for non-disease-related reasons (women accompanying patients, hospital staff or blood donors). Inclusion criteria were the following: age over 18 years, able to read and understand French, no alcohol consumption, absence of hormone-related disease or cancer, no hormonal therapy, and residence in the study area for at least 10 years. All women signed an informed consent form to participate in the study, which was approved by the Ethics Committee of the corresponding hospital.

2.2. Sampling and chemical analysis

Two milliliters of serum were collected from each woman under 12-h fasting conditions and extracted using a method described elsewhere (Turci et al., 2010) with slight modifications (Musaiger, 1993). The extracts were reconstituted in hexane for chemical analysis. A group of POPs (*p,p′*-DDE, HCB, β-HCH, endosulfan α, heptachlor, endosulfan ether and oxychlordane) and PCBs (congeners 138, 153, and 180) were quantified by high-resolution gas chromatography with a fused silica capillary BR-5 ms column (30 m × 0.25 mm, df = 0.25 μm; Bruker, Germany) and a ^{63}Ni micro-electron capture detection (Varian CP-3800 GC-ECD, Walnut Creek, CA, US).

Procedural blanks with solvents alone were analyzed and always yielded a negative result. Laboratory fortified matrix samples at different concentrations were used for quality control. Inter- and intra-day variabilities were calculated by analyzing fortified samples within the same day (repeatability) and in different days (intermediate precision), respectively, always yielding a result <20%. *p,p′*-dichlorobenzophenone was used as internal standard. In order to assess the extraction efficiency of the method, the recovery of the POPs was measured by spiking 10 blank samples with the target analytes at an intermediate point on the calibration curve and processing them as described above. Recovery rates ranged from 91 to 97%.

Limits of detection (LODs) were set at 0.05 ppb for each analyte. A random value between zero and the LOD was assigned to those concentrations below the LOD. POP concentrations were expressed on a wet weight basis (as nanogram per mL) and a lipid weight basis (as nanogram per gram of lipid).

2.3. Independent variables

Socio-demographic characteristics, reproductive history and fertility, diet, tobacco and alcohol consumption were recorded using an ad-hoc questionnaire. Occupational class was gathered and classified into outside workers and homemakers. Socioeconomic status of the family unit was categorized into three groups: <200 €/month, 200–500 €/month, and >500 €/month. Participants were grouped according to their place of residence into women living in Northern Tunisia and women living in Southern Tunisia. The division line passed through the provinces of Sousse, Kairouan and Kasserine. A dietary section with information regarding consumption habits and frequencies of main food groups (meat, milk, dairy products, vegetables and cereals) was also included. Since bread is eaten on a daily basis in our population, questions regarding cereal consumption only referred to pasta, rice and couscous consumption.

2.4. Statistical analysis

Due to their non-normal distribution, all POP concentrations were log-transformed. Variables are described using medians and interquartile ranges. Potential predictors of serum concentrations of POPs were assessed using multiple linear regression models, which were estimated using a backward stepwise technique. Once the models were created, colinearity between independent variables, linearity of the quantitative independent variables and homoscedasticity were calculated. Due to log-transformation of POP concentrations, β coefficients are also presented as exp(β). The significance level was set at $p = 0.05$. R statistical computing environment v3.0 (http://www.r-project.org/) was used for data analyses.

3. Results and discussion

Characteristics and dietary habits of the study population at enrollment are presented in Table 1.

Table 1
Characteristics of study population (n = 54).

	N (%)	Median	IQR
Baseline characteristics			
Age (years)		43.5	38.0–49.7
BMI (kg/m^2)		26.08	23.9–28.5
Occupational class			
Homemaker	15 (27.8)		
Outside workers	39 (72.2)		
Socioeconomic status			
Low (<200 €/month)	2 (3.7)		
Medium (200–500 €/month)	49 (90.7)		
High (>500 €/month)	3 (5.7)		
Location			
Northern Tunisia	45 (83.3)		
Southern Tunisia	9 (16.7)		
Marital status			
Single	8 (14.8)		
Married	46 (85.2)		
Lifestyle			
Smoking			
Yes	2 (3.7)		
No	52 (96.3)		
Alcohol			
Yes	0 (0.0)		
No	54 (100.0)		
Reproductive history			
Menarche age (years)		13.0	12.0–13.0
Age at first birth (years)		26.5	22.3–30.0
Lactation (months)		12.0	5.3–36.0
No. children		2.0	1.0–3.0
Menopausal status			
Pre-menopausic	40 (74.1)		
Post-menopausic	14 (25.9)		
Parity			
Nulliparous	8 (14.8)		
Primiparous	8 (14.8)		
Multiparous	38 (70.4)		
Diet			
Vegetables consumption (≥1 serving/week)			
Yes	53 (98.2)		
No	1 (1.8)		
Dairy products consumption (time per week)		7.0	6.0–7.0
Milk consumption (cup/day)		1.0	1.0–2.0
Meat consumption (times per week)		6.0	3.0–7.0
Cereals consumption (time per week)		4.0	2.0–5.0

IQR: interquartile range; BMI: body mass index.

3.1. Concentrations of selected POPs

Frequencies of detection and concentrations of the selected POPs are shown in Table 2. All analyzed samples were positive for one or more POP residues. The concentrations of HCB, *p,p′*-DDE and PCB congeners-138, -153 and -180 were found above the LOD in almost all samples. Median concentrations of *p,p′*-DDE and HCB were 127.59 ng/g lipid and 19.98 ng/g lipid. The median concentrations of PCBs were 26.08 ng/g lipid, 111.99 ng/g lipid and 29.84 ng/g lipid for PCB congeners-138, -153 and -180, respectively.

As a result of the worldwide ban on the use of POPs, biomonitoring programs have revealed a downward trend in the concentrations found in humans (Kim and Yoon, 2014; Nøst et al., 2013). However, previous studies have reported increased environmental levels of POPs like OCPs and PCBs (Gioia et al., 2014) in different African countries, which have resulted in increased levels in humans (Luzardo et al., 2014). Concerning Tunisia, limited information is available regarding the past use of POPs. Although the Tunisian government banned the use and import of most POPs in the 1980s (APEK, 2005), 89% of obsolete pesticides are still stored in damaged packages in Tunisia (Dasgupta et al., 2010), and illegal import of pesticides has been admitted (APEK, 2005). The levels of HCB and *p,p′*-DDE in the study population were of a similar magnitude to those previously observed in Tunisia (Ennaceur and Driss, 2010) and in the Bizerte region population (Ben Hassine et al., 2013). In comparison with other Mediterranean countries such as Egypt (Ahmed et al., 2002), Italy (Bergonzi et al., 2009), Spain (Llop et al., 2010) or Greece (Vafeiadi et al., 2014), the levels of HCB and *p,p′*-DDE were also similar as well as in other developing countries such as Brazil (Sarcinelli et al., 2003) and Bolivia (Arrebola et al., 2012a). Moreover, Tunisia is undergoing a rapid transformation in the IT field and is importing a considerable amount of PCB-containing equipment (World Bank, 2010). Indeed, the levels of PCBs in our study reached those reported for developed, more industrialized Mediterranean countries such as Italy (Bergonzi et al., 2009) and Spain (Llop et al., 2010), with levels being even higher for PCB-153.

3.2. Multivariable analyses of predictors of POP exposure

The results from the unadjusted and global stepwise multiple linear regression analyses are shown in Table 3. The models are shown for wet-basis concentrations and adjusted for total lipids (Phillips et al., 1989).

3.2.1. Socio-demographic and reproductive history predictors

As expected, age was positively associated with all the serum levels of *p,p′*-DDE, HCB and ΣPCBs. This finding is in agreement with previous studies because age is one of the most significant factors affecting POP burden in human serum (Agudo et al., 2009; Ben Hassine et al., 2013; Cerrillo et al., 2006; Ibarluzea et al., 2011; Laden et al., 1999). A two-sided phenomenon that includes a lifespan bioaccumulation effect and a birth cohort effect has traditionally been used to explain this association (Laden et al., 1999). Older women had a higher risk of exposure to high levels of POPs because they were alive when POPs were widely used in Tunisia and in addition they have had longer periods of time to accumulate the residues in their bodies.

Although a previous study in a Tunisian female population reported no significant differences in the serum levels of POPs between different regions (Ennaceur and Driss, 2010) we have found a south–north gradient. As expected, these geographical differences in the serum levels of POPs corresponded with the particular distribution of all publicly-run POP storage facilities in the country (ANGed, 2008). i.e., the number of obsolete pesticide stockpiles is higher in the northern regions than in the south. Data derived from the linear regression analyses (Table 3) indicate that women living in Southern Tunisia showed lower serum levels of PCB-138 [exp(β): 0.86, 95% CI: 0.72–1.00)], -153 [exp(β): 0.81, 95% CI: 0.70–0.93] and -180 [exp(β): 0.77, 95% CI: 0.65–0.93] than women in Northern Tunisia. Tunisian government reports, which have estimated that up to 1047 PCB-contaminated transformers and up to 631 tons of PCB contaminated oil processors are spread across Tunisia with the highest concentrations in Greater Tunis and Bizerte Governorates (Northern Tunisia) (World Bank, 2010), may help explain the north–south differences. Moreover, other factors may be also involved, including the fact that Northern Tunisia is more industrialized than the south (Verdier-Chouchane et al., 2011). In this regard, a recent study in South Portugal has reported the highest PCB-153 concentrations in women living close to industrial areas (Lopes et al., 2014). Similar findings were reported by other cohorts, supporting the fact that people living near industrial areas and contaminated buildings may have increased PCB burden (Arrebola et al., 2012b).

Table 2
Concentrations of selected OCPs and PCBs.

		>LOD (%)	Mean	SD	Percentiles		
					25th	50th	75th
Organochlorine pesticides							
HCB	ng/mL	100.00	0.14	0.06	0.09	0.12	0.16
	ng/g lipid		23.91	10.02	18.25	19.98	28.22
p,p′-DDE	ng/mL	98.10	1.07	1.18	0.38	0.60	1.53
	ng/g lipid		215.05	214.72	66.02	127.59	276.93
β-HCH	ng/mL	33.30	<LOD	<LOD	<LOD	<LOD	0.05
	ng/g lipid		–	–	–	–	9.51
Endosulfan α	ng/mL	33.30	<LOD	<LOD	<LOD	<LOD	0.05
	ng/g lipid		–	–	–	–	7.92
Endosulfan ether	ng/mL	0.00	<LOD	<LOD	<LOD	<LOD	<LOD
	ng/g lipid		–	–	–	–	–
Heptachlor	ng/mL	79.60	0.09	0.06	0.05	0.08	0.11
	ng/g lipid		14.88	14.86	6.36	12.39	16.33
Oxychlordane	ng/mL	3.70	<LOD	<LOD	<LOD	<LOD	<LOD
	ng/g lipid		–	–	–	–	–
Polychlorinated biphenyls							
PCB 138	ng/mL	100.00	0.17	0.08	0.13	0.15	0.18
	ng/g lipid		28.59	16.64	22.26	26.08	31.33
PCB 153	ng/mL	100.00	0.68	0.21	0.54	0.62	0.76
	ng/g lipid		119.07	35.96	88.90	111.99	149.57
PCB 180	ng/mL	98.10	0.18	0.06	0.14	0.17	0.23
	ng/g lipid		31.74	11.00	22.76	29.84	40.44

LOD: limit of detection (0.05 ng/mL).

Table 3
Crude and global multiple linear regression model for selected POPs.[#]

Ln *p,p′*-DDE (ng/mL)

	Unadjusted				Global ($R^2 = 0.49$)			
	β	exp(β)	expCI	(95%)	β	exp(β)	expCI	(95%)
(Intercept)	−3.039	0.048	0.003	0.679*	−3.887	0.021	0.006	0.071*
Age (years)	0.062	1.064	1.023	1.107*	0.065	1.067	1.041	1.093*
Occupational class = outside worker[§]	0.943	2.569	1.094	6.030*	0.750	2.117	1.300	3.447*
Lactation (months)	−0.007	0.993	0.981	1.006	−0.014	0.986	0.978	0.995*
Cereals consumption	0.141	1.152	0.989	1.342·	0.129	1.138	1.024	1.264*

Ln HCB (ng/mL)

	Unadjusted				Global ($R^2 = 0.34$)			
	β	exp(β)	expCI	(95%)	β	exp(β)	expCI	(95%)
(Intercept)	−3.702	0.025	0.008	0.077*	−3.008	0.049	0.028	0.087*
Age (years)	0.034	1.035	1.017	1.052*	0.024	1.024	1.010	1.038*
Lactation (months)	−0.004	0.996	0.991	1.002	−0.005	0.995	0.991	0.999*

Ln PCB-138 (ng/mL)

	Unadjusted				Global ($R^2 = 0.39$)			
	β	exp(β)	expCI	(95%)	β	exp(β)	expCI	(95%)
(Intercept)	−2.415	0.089	0.042	0.189*	−2.759	0.063	0.041	0.099*
Age (years)	0.017	1.017	1.006	1.029*	0.022	1.022	1.013	1.031*
Location = South Tunisia[†]	−0.130	0.878	0.697	1.106	−0.168	0.855	0.715	0.999*
Lactation (months)	−0.005	0.995	0.992	0.999*	−0.006	0.994	0.991	0.997*

Ln PCB-153 (ng/mL)

	Unadjusted				Global ($R^2 = 0.20$)			
	β	exp(β)	expCI	(95%)	β	exp(β)	expCI	(95%)
(Intercept)	−0.355	0.701	0.323	1.524	−0.744	0.475	0.334	0.675*
Age (years)	0.005	1.005	0.993	1.016	0.009	1.009	1.001	1.017*
Location = South Tunisia[†]	−0.227	0.797	0.628	1.012·	−0.217	0.805	0.695	0.932*

Ln PCB-180 (ng/mL)

	Unadjusted				Global ($R^2 = 0.20$)			
	β	exp(β)	expCI	(95%)	β	exp(β)	expCI	(95%)
(Intercept)	−2.121	0.120	0.040	0.358*	−2.161	0.115	0.075	0.176*
Age (years)	0.015	1.015	0.999	1.032·	0.011	1.011	1.002	1.021*
Location = South Tunisia[†]	−0.183	0.833	0.594	1.166	−0.257	0.773	0.646	0.925*

Ln ΣPCBs (ng/mL)

	Unadjusted				Global ($R^2 = 0.36$)			
	β	exp(β)	expCI	(95%)	β	exp(β)	expCI	(95%)
(Intercept)	−0.108	0.897	0.473	1.702	−0.372	0.689	0.511	0.929
Age (years)	0.008	1.008	0.998	1.017	0.010	1.010	1.003	1.017*
Location = South Tunisia[†]	−0.205	0.814	0.669	0.992*	−0.225	0.798	0.716	0.891*

[#] All multivariable analyses were adjusted by total serum lipids.
[§] Reference category: homemaker.
[†] Reference category: Northern Tunisia; $p < 0.1$, *$p < 0.05$.

Regarding occupational class, we found that outside workers showed higher serum levels of *p,p′*-DDE than homeworkers [exp(β): 2.12, 95% CI: 1.30–3.45], suggesting occupational exposure to POPs, as previously noted for agricultural workers (Mercado et al., 2013).

The duration of lactation was found to be a predictor of exposure to POP. It was inversely associated with serum levels of residues from agricultural and industrial origins, e.g., *p,p′*-DDE [exp(β): 0.99, 95% CI: 0.98–1.00], HCB [exp(β): 0.99, 95% CI: 0.99–1.00] and PCB-138 [exp(β): 0.99, 95% CI: 0.99–1.00]. This inverse association was suggested previously by some Spanish cohorts (Cerrillo et al., 2006; Ibarluzea et al., 2011; Llop et al., 2010) and other populations (Laden et al., 1999). Because lactation is believed to be a major route for excretion of *p,p′*-DDE and PCBs (Laden et al., 1999) due to their lipophilicity and the high fat content of breast milk, caution should be taken to reduce children exposure.

3.2.2. Dietary predictors

Regarding the contribution of the diet to POP burden, our data showed that cereal consumption was positively associated with serum levels of *p,p′*-DDE [exp(β): 1.14, 95% CI: 1.02–1.26]. This association was also reported by previous studies in Spain (Llop et al., 2010). In this respect, the Tunisian government has confirmed illegal import of some banned POPs from neighboring countries where they are not banned (APEK, 2005). Although this report does not specifically mention DDT, it seems plausible that cereal crops might be highly polluted with these illegal pesticides and therefore, contribute to human exposure to *p,p′*-DDE.

Several reasons could explain the limited associations found between diet and POP levels in Tunisian women, including diet changes and differences in the concentration of POPs in the same food item depending on when and where it is consumed. In addition, the fact that serum concentrations of *p,p′*-DDE, HCB and PCBs were not strongly associated with specific food groups seems coherent with a low-dose exposure to these OCs through a variety of foods, as reported by previous studies, reflecting the need for more accurate recording of dietary habits (Gasull et al., 2010). Moreover, the limited sample size of our study might prevent us to find subtle associations between dietary habits and POP exposure.

4. Conclusion

This is the first study that assesses routes of exposure to POPs in a Tunisian population. Due to the fact that this study is a pilot research, our results cannot be extrapolated to the general population and need to be supported by further studies. However, the levels of exposure to POPs and the significant levels of PCB-153 found would warrant the design of biomonitoring programs in Tunisia. These programs would help identify population groups at risk of exposure, to characterize the different routes of exposure and to design prevention programs. Environmental health policies and strategies in Northern and Southern Mediterranean countries should be, at least, complementary. However, since African countries have financial limitations to develop these programs, Northern Mediterranean countries must be committed to collaborate with the developing countries to carry out studies aimed at the general population.

Competing interests

The authors declare no conflict of interest.

Acknowledgments

The authors are indebted to all the participants, without whom this work would not have been possible. The authors are also grateful to Esther Moreno for her editorial assistance. This study was supported in part by research grants from CIBER de Epidemiología y Salud Publica and Junta de Andalucía (SAS 01/264, P09-CTS-5488, SAS PI-0675-2010, PI-0513/2012 and EF-0708-2013). F. Artacho-Cordón has a fellowship from the Spanish Ministry of Education (FPU12/02524). H. Belhassen was granted a scholarship by the Ministry of Higher Education and Scientific Research of Tunisia. Dr J.P. Arrebola currently receives a postdoctoral fellowship from the Consejería de Igualdad, Salud y Políticas Sociales, Junta de Andalucía, Spain (RH-0092-2013). Dr I. Jiménez-Díaz currently receives a postdoctoral research contract (Sara Borrell Program, grant no. CD012/00462) from the Instituto de Salud Carlos III.

References

Agudo, A., Goñi, F., Etxeandia, A., Vives, A., Millán, E., López, R., et al., 2009. Polychlorinated biphenyls in Spanish adults: determinants of serum concentrations. Environ. Res. 109, 620–628.

Ahmed, M.T., Loutfy, N., El Shiekh, E., 2002. Residue levels of DDE and PCBs in the blood serum of women in the Port Said region of Egypt. J. Hazard. Mater. 89, 41–48.

ANGed, 2008. Annexes. Environmental and Social Impact Study of the Program APPS-Tunisia. National Waste Management Agency.

APEK, 2005. Tunisia Country Situation Report. The International POPs Elimination Project, Tunisia.

Arrebola, J.P., Cuellar, M., Claure, E., Quevedo, M., Antelo, S.R., Mutch, E., et al., 2012a. Concentrations of organochlorine pesticides and polychlorinated biphenyls in human serum and adipose tissue from Bolivia. Environ. Res. 112, 40–47.

Arrebola, J.P., Mutch, E., Cuellar, M., Quevedo, M., Claure, E., Mejía, L.M., et al., 2012b. Factors influencing combined exposure to three indicator polychlorinated biphenyls in an adult cohort from Bolivia. Environ. Res. 116, 17–25.

Ben Hassine, S., Hammami, B., Ben Ameur, W., Megdiche, Y., Barhoumi, B., Abidi, R., et al., 2013. Concentrations of organochlorine pesticides and polychlorinated biphenyls in human serum and their relation with age, gender, and BMI for the general population of Bizerte, Tunisia. Environ. Sci. Pollut. Res. 1–11.

Bergonzi, R., Specchia, C., Dinolfo, M., Tomasi, C., De Palma, G., Frusca, T., et al., 2009. Distribution of persistent organochlorine pollutants in maternal and foetal tissues: data from an Italian polluted urban area. Chemosphere 76, 747–754.

Cerrillo, I., Olea-Serrano, M., Ibarluzea, J., Exposito, J., Torne, P., Laguna, J., et al., 2006. Environmental and lifestyle factors for organochlorine exposure among women living in southern Spain. Chemosphere 62, 1917–1924.

Dasgupta, S., Meisner, C., Wheeler, D., 2010. Stockpiles of obsolete pesticides and cleanup priorities: a methodology and application for Tunisia. J. Environ. Manag. 91, 824–830.

Ennaceur, S., Driss, M.R., 2010. Serum organochlorine pesticide and polychlorinated biphenyl levels measured in delivering women from different locations in Tunisia. Int. J. Environ. Anal. Chem. 90, 821–828.

Gasull, M., Porta, M., Pumarega, J., Vioque, J., de Basea, M.B., Puigdomènech, E., et al., 2010. The relative influence of diet and serum concentrations of organochlorine compounds on K-ras mutations in exocrine pancreatic cancer. Chemosphere 79, 686–697.

Gioia, R., Akindele, A.J., Adebusoye, S.A., Asante, K.A., Tanabe, S., Buekens, A., et al., 2014. Polychlorinated biphenyls (PCBs) in Africa: a review of environmental levels. Environ. Sci. Pollut. Res. 21, 6278–6289.

Ibarluzea, J., Alvarez-Pedrerol, M., Guxens, M., Marina, L.S., Basterrechea, M., Lertxundi, A., et al., 2011. Sociodemographic, reproductive and dietary predictors of organochlorine compounds levels in pregnant women in Spain. Chemosphere 82, 114–120.

Kelly, B.C., Gobas, F.A.P.C., McLachlan, M.S., 2004. Intestinal absorption and biomagnification of organic contaminants in fish, wildlife, and humans. Environ. Toxicol. Chem. 23, 2324–2336.

Kim, S.-K., Yoon, J., 2014. Chronological trends of emission, environmental level and human exposure of POPs over the last 10 years (1999–2010) in Korea: implication to science and policy. Sci. Total Environ. 470, 1346–1361.

Laden, F., Neas, L.M., Spiegelman, D., Hankinson, S.E., Willett, W.C., Ireland, K., et al., 1999. Predictors of plasma concentrations of DDE and PCBs in a group of US women. Environ. Health Perspect. 107, 75.

Llop, S., Ballester, F., Vizcaino, E., Murcia, M., Lopez-Espinosa, M.-J., Rebagliato, M., et al., 2010. Concentrations and determinants of organochlorine levels among pregnant women in eastern Spain. Sci. Total Environ. 408, 5758–5767.

Lopes, B., Arrebola, J., Serafim, A., Company, R., Rosa, J., Olea, N., 2014. Polychlorinated biphenyls (PCBs) and p, p′-dichlorodiphenyldichloroethylene (DDE) concentrations in maternal and umbilical cord serum in a human cohort from South Portugal. Chemosphere 114, 291–302.

Luzardo, O.P., Boada, L.D., Carranza, C., Ruiz-Suárez, N., Henríquez-Hernández, L.A., Valerón, P.F., et al., 2014. Socioeconomic development as a determinant of the levels of organochlorine pesticides and PCBs in the inhabitants of Western and Central African countries. Sci. Total Environ. 497, 97–105.

Mercado, L.A., Freille, S.M., Vaca-Pereira, J.S., Cuellar, M., Flores, L., Mutch, E., et al., 2013. Serum concentrations of p, p′-dichlorodiphenyltrichloroethane (p, p′-DDE) in a sample of agricultural workers from Bolivia. Chemosphere 91, 1381–1385.

Musaiger, A.O., 1993. Socio-cultural and economic factors affecting food consumption patterns in the Arab countries. J. R. Soc. Promot. Health 113, 68–74.

Nøst, T.H., Breivik, K., Fuskevåg, O.-M., Nieboer, E., Odland, J.Ø., Sandanger, T.M., 2013. Persistent organic pollutants in Norwegian men from 1979 to 2007: intraindividual changes, age–period–cohort effects, and model predictions. Environ. Health Perspect. 121, 1292.

Phillips, D.L., Pirkle, J.L., Burse, V.W., Bernert Jr., J.T., Henderson, L.O., Needham, L.L., 1989. Chlorinated hydrocarbon levels in human serum: effects of fasting and feeding. Arch. Environ. Contam. Toxicol. 18, 495–500.

Porta, M., Puigdomènech, E., Ballester, F., Selva, J., Ribas-Fitó, N., Llop, S., et al., 2008. Monitoring concentrations of persistent organic pollutants in the general population: the international experience. Environ. Int. 34, 546–561.

Qing Li, Q., Loganath, A., Seng Chong, Y., Tan, J., Philip, Obbard J., 2006. Persistent organic pollutants and adverse health effects in humans. J. Toxicol. Environ. Health A 69, 1987–2005.

Sarcinelli, P.N., Pereira, A.C.S., Mesquita, S.A., Oliveira-Silva, J.J., Meyer, A., Menezes, M.A., et al., 2003. Dietary and reproductive determinants of plasma organochlorine levels in pregnant women in Rio de Janeiro. Environ. Res. 91, 143–150.

Turci, R., Balducci, C., Brambilla, G., Colosio, C., Imbriani, M., Mantovani, A., et al., 2010. A simple and fast method for the determination of selected organohalogenated compounds in serum samples from the general population. Toxicol. Lett. 192, 66–71.

Vafeiadi, M., Vrijheid, M., Fthenou, E., Chalkiadaki, G., Rantakokko, P., Kiviranta, H., et al., 2014. Persistent organic pollutants exposure during pregnancy, maternal gestational weight gain, and birth outcomes in the mother–child cohort in Crete, Greece (RHEA study). Environ. Int. 64, 116–123.

Verdier-Chouchane, A., Obayashi, N., Castel, V., 2011. The Revolution in Tunisia: Economic Challenges and Prospects. African Development Bank.

World Bank, 2010. Tunisia — Demonstrating and Promoting Best Techniques and Practices for Managing Healthcare Waste and Polychlorinated Biphenyls (PCBs) Project (English). National Waste Management Agency, Washington, DC.

7.2. Objective 2. Evaluate the magnitude of the exposure to persistent and non-persistent EDCs and identify predictors of the exposure in a subset of healthy women from Tunisia.

URINARY LEVELS OF BISPHENOL A, BENZOPHENONES AND PARABENS IN TUNISIAN WOMEN: A PILOT STUDY

*Jiménez-Díaz I, **Artacho-Cordón F**, Vela-Soria F, Belhassen H, Arrebola J.P, Fernández M.F, Ghali R, Hedhili A, Olea N.*

Science of the Total Environment; Impact factor 4.099; 1st Quartile; 1st Decile

ABSTRACT

Bisphenol A (BPA), benzophenones and parabens are commonly used in the production of polycarbonate plastics, as UV-filters and as antimicrobial preservatives, respectively, and they are thought to exhibit endocrine disrupting properties. Exposure to these compounds remains poorly characterized in developing countries, despite the fact that certain behaviors related to westernization have the potential to influence exposure.

The aim of this pilot study was to measure urinary concentrations of BPA, six different benzophenones and four parabens in 34 Tunisian women. In addition, we identified some socio-demographic and dietary predictors of exposure to these compounds. Chemical analyses were carried out by dispersive liquid–liquid microextraction (DLLME) and ultra-high performance liquid chromatography with tandem mass spectrometry detection (UHPLC-MS/MS). Detection frequencies of methylparaben (MP), ethylparaben (EP) and propylparaben (PP) ranged between 67.6 and 94.1%. Butylparaben (BP) was found in 38.2% of the analyzed samples; BPA in 64.7%; and benzophenone-1 (BP-1) and benzophenone-3 (BP-3) were detected in 91.2 and 64.7% of the analyzed samples, respectively. Urinary geometric mean concentrations of MP, EP, PP, and BP were 30.1, 1.4, 2.0 and 0.5 ng mL− 1, respectively. Geometric mean concentrations of BPA, BP-1, and BP-3 were 0.4, 1.3 and 1.1 ng mL− 1, respectively.

Our results suggest that Tunisian women are widely exposed to BPA, parabens and some benzophenones. Further studies on the general Tunisian population are needed in order to assess the levels of exposure to these compounds and to identify sources of exposure and population groups at higher risk.

Science of the Total Environment 562 (2016) 81–88

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Urinary levels of bisphenol A, benzophenones and parabens in Tunisian women: A pilot study

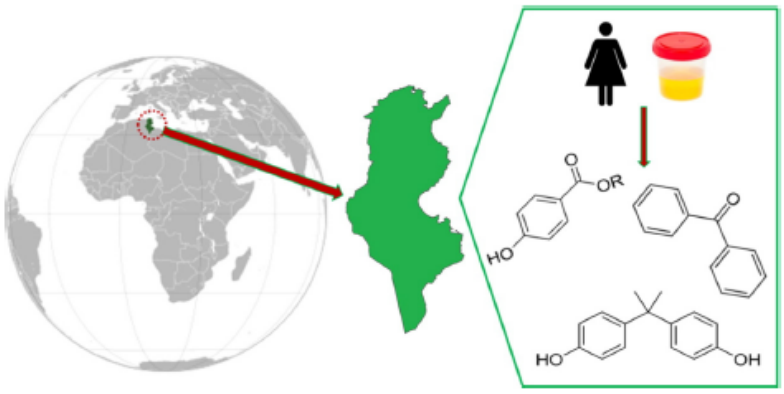
I. Jiménez-Díaz [a,b,*], F. Artacho-Cordón [a,c], F. Vela-Soria [a], H. Belhassen [d], J.P. Arrebola [a,b,e], M.F. Fernández [a,b,c], R. Ghali [d], A. Hedhili [d], N. Olea [a,b,c]

[a] Instituto de Investigación Biosanitaria ibs.GRANADA, Granada E-18012, Spain
[b] CIBER Epidemiology and Public Health (CIBERESP), Granada E-18071, Spain
[c] Radiology and Physical Medicine Department, University of Granada, Spain
[d] Center of Urgent Medical Assistance of Tunis, Laboratory of Toxicology and Environment (LR12SP07), Tunis, Tunisia
[e] Radiation Oncology Department, Virgen de las Nieves University Hospital, Granada, Spain

HIGHLIGHTS

- BPA, benzophenones and parabens were analyzed in urine from Tunisian women.
- All analyzed samples were positive for at least one EDC residue.
- Women were widely exposed to BPA, parabens and some benzophenones.
- Methylparaben (94.1%) and benzophenone-1 (91.2%) were highly detected.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 29 January 2016
Received in revised form 13 March 2016
Accepted 25 March 2016
Available online xxxx

Editor: Adrian Covaci

Keywords:
Endocrine disrupting chemicals
Bisphenol A
Parabens
Benzophenones
Urine
Tunisian women

ABSTRACT

Bisphenol A (BPA), benzophenones and parabens are commonly used in the production of polycarbonate plastics, as UV-filters and as antimicrobial preservatives, respectively, and they are thought to exhibit endocrine disrupting properties. Exposure to these compounds remains poorly characterized in developing countries, despite the fact that certain behaviors related to westernization have the potential to influence exposure.
The aim of this pilot study was to measure urinary concentrations of BPA, six different benzophenones and four parabens in 34 Tunisian women. In addition, we identified some socio-demographic and dietary predictors of exposure to these compounds. Chemical analyses were carried out by dispersive liquid–liquid microextraction (DLLME) and ultra-high performance liquid chromatography with tandem mass spectrometry detection (UHPLC-MS/MS). Detection frequencies of methylparaben (MP), ethylparaben (EP) and propylparaben (PP) ranged between 67.6 and 94.1%. Butylparaben (BP) was found in 38.2% of the analyzed samples; BPA in 64.7%; and benzophenone-1 (BP-1) and benzophenone-3 (BP-3) were detected in 91.2 and 64.7% of the analyzed samples, respectively. Urinary geometric mean concentrations of MP, EP, PP, and BP were 30.1, 1.4, 2.0 and 0.5 ng mL^{-1}, respectively. Geometric mean concentrations of BPA, BP-1, and BP-3 were 0.4, 1.3 and 1.1 ng mL^{-1}, respectively.

* Corresponding author at: Instituto de Investigación Biosanitaria ibs.GRANADA, Granada E-18012, Spain.
E-mail address: ijdiaz@ugr.es (I. Jiménez-Díaz).

http://dx.doi.org/10.1016/j.scitotenv.2016.03.203

Our results suggest that Tunisian women are widely exposed to BPA, parabens and some benzophenones. Further studies on the general Tunisian population are needed in order to assess the levels of exposure to these compounds and to identify sources of exposure and population groups at higher risk.

1. Introduction

The last century has witnessed a significant development in science and technology that has allowed the emergence of a large number of manufactured products with a wide range of applications. Nevertheless, this development has resulted in an increased exposure of the general population to a wide variety of xenobiotics that can cause adverse health effects. Among these compounds, endocrine disrupting chemicals (EDCs) have become of special concern in the last decades.

The term EDC covers a wide range of synthetic and natural substances that can interfere with the normal hormone function in wildlife and humans (Sonnenschein and Soto, 1998). In addition to some naturally occurring compounds such as lignans, coumestans, isoflavones, mycotoxins, and phytoestrogens, numerous synthetic chemicals such as bisphenol A (BPA), benzophenones, and parabens, have been involved in endocrine disruption (Darbre, 2015).

BPA is a well-known EDC (Schecter et al., 2010; Sharpe and Drake, 2010; Vandenberg et al., 2010) with an estimated global production of 5.5 million metric tons in 2011 (Bailin et al., 2008). BPA is used primarily in the production of polycarbonate plastics used in a variety of common products including reusable beverage containers, infant feeding bottles, digital media, electrical and electronic equipment, automobiles and sports safety equipment, among other uses (Geens et al., 2012). In vitro studies have shown the ability of BPA to disrupt epigenetic regulation of developmentally relevant genes, induce proliferation of human prostate cancer cells and inhibit the synthesis of testosterone (Vandenberg et al., 2010; Molina-Molina et al., 2013; Susiarjo et al., 2013). In addition, some epidemiological studies suggest that environmental exposure to BPA is associated with behavioral and reproductive abnormalities, as well as chronic diseases, especially when exposure occurs during critical windows of development (Lang et al., 2008; Braun et al., 2011b; Meeker et al., 2011; Pérez-Lobato et al., 2016; Fernández et al., 2016).

Benzophenone (BP)-type UV filters comprise approximately 29 compounds (Yang et al., 2013) including BP-1 to BP-12 as well as others such as 4-hydroxybenzophenone (4-OH-BP) and 2-hydroxybenzophenone (2-OH-BP). Benzophenones are widely used as UV-filters in sunscreen formulations, and in the production of plastics and food-packaging materials. They are also used as photo-initiators in UV-cured inks and adhesives. Despite the widespread use of BPs some of these compounds have been related to feminized sexual behavior and increased uterine weight in exposed mice and rats (Schlumpf et al., 2010; Krause et al., 2012).

Parabens (alkyl esters of the *p*-hydroxybenzoic acid) are widely used as antimicrobial preservatives, especially against mold and yeast, in cosmetics, pharmaceuticals, and in food and beverages. Methylparaben (MP), ethylparaben (EP), propylparaben (PP) and butylparaben (BP) are the most commonly used parabens. The widespread use of parabens as preservatives arises from their low toxicity, broad inertness, worldwide regulatory acceptance and low cost (Soni et al., 2005). However, in recent years there has been a growing concern about human exposure to parabens and several in vitro and in vivo studies have reported estrogenic (Darbre and Harvey, 2008; Charles and Darbre, 2013) and antiandrogenic activity of parabens (Oishi, 2002; Chen et al., 2007), in addition to decreased semen quality and testosterone levels in paraben-exposed male rodents (Soni et al., 2005).

Exposure to BPA, parabens and benzophenones may occur through ingestion, inhalation and dermal absorption (Hayden et al., 2005; El Hussein et al., 2007; Vandenberg et al., 2007). These compounds may conjugate to β-D-glucuronide and sulfate, which reduces their bioactivity and facilitates urinary excretion. Following excretion, the parent compounds can be measured in urine and have been shown to be valid biomarkers of exposure (Ye et al., 2006).

It is important to understand the profiles of environmental exposure to BPA, parabens and benzophenones in vulnerable populations, especially in developing countries where exposures remain largely uncharacterized. In this context, the main goal of this pilot study was to investigate urinary levels of BPA, four parabens (MP, EP, PP and BP) and six benzophenones (BP-1, BP-2, BP-3, BP-6, BP-8 and 4-OH-BP) in female volunteers from Tunisia. In addition, we aimed at identifying some socio-demographic and dietary determinants of exposure in order to set the basis for a large-scale biomonitoring study in Tunisia.

2. Material and methods

2.1. Study population

The participants of this pilot study were recruited from a control population that was used in the context of a broader research project with the basic aim of assessing environmental determinants of health (Artacho-Cordón et al., 2015; Arrebola et al., 2015; Belhassen et al., 2015). From May to October 2012, healthy Tunisian women from the Salah Azaiz Hospital (Tunis) and the Ariana Hospital (Ariana) were selected at random from a healthy population of women that included hospital visitors, hospital staff and blood donors, aged 18 years or older. Inclusion criteria included being able to give informed consent, to read and understand French and complete a questionnaire, and residence in the study area for at least 10 years. Exclusion criteria included the presence of any hormone-related disease and hormonal therapy. Of the 77 eligible women, 56 agreed to participate. 41 of the 56 women provided a sufficient amount of urine. Since those 41 urine samples were also used for other studies, only 34 samples were finally available for the present study. All of the participants underwent a face-to-face interview that covered socio-demographic factors, a full medical and reproductive history and lifestyle habits. The study was approved by the Ethics Committee of the corresponding hospital. All study data were coded for confidentiality. Table 1 shows the characteristics of the study population.

2.2. Sampling and chemical analyses

Single spot urine samples were used to assess exposure to the target compounds. First-morning urine samples were collected in polypropylene containers pretested to ensure that they did not contain or leach any of the compounds under study. All urine samples were frozen at −20 °C until they were shipped on dry ice (−70 °C) to San Cecilio University Hospital, in Granada (Spain), where they were analyzed.

The applied methodology for the analysis of BPA, parabens (MP, EP, PP and BP) and BP-type UV filters (BP-1, BP-2, BP-3, BP-6, BP-8 and 4-OH-BP) was performed by dispersive liquid–liquid microextraction (DLLME) and ultra-high performance liquid chromatography with tandem mass spectrometry detection (UHPLC-MS/MS), as described in a previous study (Vela-Soria et al., 2014) with slight modifications. In brief, urine samples were thawed completely at room temperature, centrifuged at 2600 *g* for 10 min to sediment particulate matter and 2.5 mL were taken to carry out the analysis. In order to determine total amounts (free plus conjugated) of target compounds in urine, each sample was spiked with 50 μL of enzyme solution (β-glucuronidase/sulfatase). The enzyme solution was prepared daily by dissolving 10 mg of β-glucuronidase/sulfatase ($3\ 10^6$ U g solid^{-1}) in 1.5 mL of 1 M ammonium acetate buffer (pH 5.0). After mixing, the sample was incubated at

Table 1
Characteristics of the study population.

	N(%)		N(%)
Age (years)		Parity	
<45 years	17 (50.0%)	*Nulliparous*	7 (20.6%)
>45 years	17 (50.0%)	*Multiparous*	27 (79.4%)
Body mass index (BMI)		Age at menarche	
Normal weight (BMI < 25 kg/m²)	10 (29.4%)	*<13 years*	17 (50.0%)
Over weight (25 <BMI < 30 kg/m²)	17 (50.0%)	*>13 years*	17 (50.0%)
Obese (BMI> 30 kg/m2	7 (20.6%)	Accumulated lactation time	
Marital status		*<14 months*	18 (52.9%)
Single	5 (14.7%)	*>14 months*	16 (47.1%)
Married/divorced	29 (85.3%)	Vegetables consumer	
Occupational class		*Yes*	34 (100.0%)
Homemakers	10 (29.4%)	*No*	0 (0.0%)
Outside workers	24 (70.6%)	Dairy products consumption	
Type of residence		*<1 per day*	17 (50.0%)
Rural	6 (17.6%)	*Everyday*	17 (50.0%)
Urban	28 (82.4%)	Meat consumption	
Location		*<6 days per week*	16 (47.1%)
North	31 (91.2%)	*>6 days per week*	18 (52.9%)
South	3 (8.8%)	Cereals consumption	
Menopausal status		*<4 days per week*	17 (50.0%)
Premenopausal	22 (64.7%)	*>4 days per week*	17 (50.0%)
Postmenopausal	12 (35.3%)		

SD: standard deviation; BMI: body mass index (kg/m^2)

37 °C for 24 h. The treated urine was placed in a 15 mL screw-cap glass tube and spiked with 20 μL of the surrogate standard solution (5 mg L^{-1} of EP-$^{13}C_6$, 2 mg L^{-1} of BPA-d_{16}, and 2 mg L^{-1} of BP-d_{10}). Urine was diluted to 10.0 mL with 7% NaCl aqueous solution (w/v) and the pH was adjusted to 2.0 with HCl 0.5 M. Next, 0.5 mL of acetone (disperser solvent) and 0.75 mL of trichloromethane (extraction solvent) were mixed and injected rapidly into the aqueous sample with a syringe. The mixture was gently shaken for 10 s, and centrifuged for 10 min at 2600 *g*. All sedimented phase volume was transferred to a clean glass vial using a 1.0 mL micropipette. The organic phase was evaporated under a nitrogen stream. The residue was dissolved with 100 μL of a mixture consisting of methanol (0.1% ammonia)/water (0.1% ammonia), 60:40 (v/v), vortexed for 30 s and placed in a 1.5 mL Eppendorf tube. It was then centrifuged for 15 min at 24,960 *g* and finally 5 μL was injected in the LC system.

2.3. UHPLC-MS/MS conditions

UHPLC-MS/MS analyses were performed using an ACQUITY UPLC™ H-Class (Waters, Manchester, UK), consisting of ACQUITY UPLC™ binary solvent manager and ACQUITY UPLC™ sample manager. A Xevo TQS tandem quadrupole mass spectrometer (Waters) equipped with an orthogonal Z-spray™ electrospray ionization (ESI) source was used for BPA, parabens and benzophenones detection. Chromatographic separation of compounds was performed using an ACQUITY UPLC BEH™ C18 (50 mm × 2.1 mm I.D., 1.7 μm particle size) from Waters. The gradient mobile phase consisted of 0.1% (v/v) ammonia aqueous solution (solvent A) and 0.1% (v/v) ammonia in methanol (solvent B). Gradient conditions were as follows: 0.0–3.5 min, 60% B; 3.5–4.0 min, 60–100% B; 4.0–6.5 min, 100%B and back to 60% in 0.1 min. Flow rate was 0.25 mL min^{-1}. The injection volume was 5 μL. The column temperature was maintained at 40 °C.

The mass spectrometer was operated in both, positive and negative ESI mode, using optimized MS/MS parameters as defined in a previous study (Vela-Soria et al., 2014).

2.4. Calibration and quality control

Matrix-matched calibration curves were constructed plotting the analyte/surrogate peak area ratio against the analyte concentration, in synthetic urine. The synthetic urine was prepared as a 1 kg stock solution according to Inn et al. (Inn et al., 2001). EP-$^{13}C_6$, BPA-d_{16}, and BP-d_{10} were used as surrogates. Limits of detections (LODs) obtained were 0.2 ng mL^{-1} and limits of quantification (LOQs) were 0.5 ng mL^{-1} for all the selected compounds.

Urine samples were analyzed in duplicate and extracted in batches of 6, with each batch containing 4 samples and 2 quality-control samples. These quality-control samples included one procedural blank of synthetic urine and one level home-made quality control (5 ng mL^{-1} for BPA and benzophenones and 15 ng mL^{-1} for parabens). Samples were frozen after extraction and injected in the LC-MS/MS in a single batch, in the same order as they were prepared. Recoveries for all the target compounds in the quality control spiked samples ranged between 89 and 108% and the coefficient of variation (CV) was < 15% for all of them.

2.5. Independent variables

Socio-demographic characteristics, reproductive history, tobacco and alcohol consumption were recorded using an ad-hoc questionnaire. A dietary section with information regarding habits and frequency of consumption of the main food groups (meat, dairy products, vegetables and cereals) was also included. None of the volunteers declared tobacco or alcohol consumption and no information regarding exposure to secondhand smoke is available. Occupational class was gathered and classified as homemakers and occupation outside the home. Participants were grouped according to their place of residence into women living in Northern Tunisia and women living in Southern Tunisia.

2.6. Statistical analysis

For the statistical analyses, EDC concentrations were log-transformed for minimizing the influence of outliers. Variables were described using geometric means (GM) and interquartile ranges. Urinary EDC concentrations below the LOD were assigned a value of LOD/2. Chemical concentrations were expressed in ng mL^{-1} and the statistical analyses were performed without adjustment for creatinine or gravity. Because ANOVA assumptions were not always fulfilled, the Mann–Whitney U-test was used to compare levels of selected EDCs between socio-demographic characteristics of the participants. The significance level was set at $p = 0.05$. The R statistical computing environment v3.0 (http://www.r-project.org/) was used for data analyses.

3. Results and discussion

3.1. Concentrations of studied EDCs

Frequencies of detection and concentrations of BPA, parabens and benzophenones are shown in Table 2. All analyzed samples were positive for at least one EDC residue.

3.1.1. Bisphenol A

BPA was found in 70.6% of the analyzed samples with levels ranging from <LOD to 8.1 ng mL^{-1}, with a median concentration of 0.35 ng mL^{-1}. To our knowledge, this median value is one of the lowest ever reported. BPA levels found in Tunisia were lower than those reported in Egypt by Nahar and coworkers, who described a median concentration of 0.70 ng mL^{-1} in girls from 10 to 13 years old (Nahar et al., 2012). Frequency and range were also slightly higher in this Egyptian population (79%, range: <LOD to 12.0 ng mL^{-1}). BPA was also higher detected (86.2%) in urine samples from general population from Saudi Arabia, with levels ranging from 0.30 to 177 ng mL^{-1}, and with a median concentration of 2.01 ng mL^{-1} (Asimakopoulos et al., in press).

It is also worth noting that BPA values obtained in the present study are much lower than those recently found in women from European countries such as Denmark (Frederiksen et al., 2013), Sweden (Larsson et al., 2014), France (Philippat et al., 2012), Germany (Kasper-Sonnenberg et al., 2012) and Spain (Casas et al., 2011). Frequency of detection for this European countries was 100%, or very close. Luxembourg is the only European country showing lower percentages of detection (44.6%) than our study (Covaci et al., 2015). This was probably due to the high LOQ of the analytical method employed (1.0 ng mL^{-1}). The low BPA exposure found in Tunisia may be due to specific characteristics and/or lifestyle habits of the study population or to the substitution of BPA in the last years for other compounds, like bisphenol S and bisphenol F.

Table 3 shows total EDC urine concentrations found in the present study together with those reported recently among women from Europe and some Arab countries.

Table 4 shows the Spearman correlation between urinary concentrations of the target compounds (BPA, parabens and benzophenones) in the analyzed samples. As described previously (Frederiksen et al., 2013; Larsson et al., 2014; Asimakopoulos et al., in press), we did not find correlation between BPA and the rest of compounds, suggesting that the sources of BPA exposure are different from those of parabens or benzophenones.

3.1.2. Parabens

Detection frequencies of parabens in urine of our study population indicates that exposure to parabens is widespread among Tunisian women. Levels of MP were found above the LOD in almost all samples (94.1%) at a median concentration of 34.9 ng mL^{-1}. EP and PP were detected in 67.6% and 70.6% of the samples, with a median concentration of 1.8 and 3.1 ng mL^{-1}, respectively. Among the selected parabens, BP showed the lowest detection rate (38.2%) with a median concentration <0.2 ng mL^{-1}. Urinary median concentrations of parabens in Tunisia were higher than in Saudi Arabia (Asimakopoulos et al., in press) (11.7, 0.23, 1.66 and 0.15 ng mL^{-1} for MP, EP, PP and BP, respectively), but with similar ranking of concentrations in both countries (MP>PP>EP>BP). In Saudi Arabia, frequencies of detection were higher for MP, EP and PP (100, 87.7 and 85.4%, respectively), and lower for BP (11.5%).

Studies conducted in several European countries including France (Philippat et al., 2012), Spain (Casas et al., 2011), Denmark (Frederiksen et al., 2013; de Renzy-Martin et al., 2014) and Belgium (Dewalque et al., 2014) reported that the highest concentrations corresponded to MP, followed by PP, EP and BP, with slight differences, for example, EP concentrations were higher than PP in Swedish women (Larsson et al., 2014). The median concentrations of MP and EP found in the present study were very similar to those reported for women in Belgium (32.4 and 1.9 ng mL^{-1}, respectively) and in Sweden (40 and 2.4 ng mL^{-1}, respectively) (Dewalque et al., 2014; Larsson et al., 2014). However, MP and PP levels were up to 5 times lower in Tunisian women than in Spanish pregnant women (191.0 and 8.8 ng mL^{-1}, respectively) (Casas et al., 2011). Regarding PP, the median concentration found in Tunisian women was similar to that found in Belgian women and in Danish pregnant women (3.3 ng mL^{-1} and 4.2 ng mL^{-1}, respectively) (Dewalque et al., 2014; de Renzy-Martin et al., 2014), but higher than the concentrations found in Danish and Swedish mothers (1.7 and 1.8 ng mL^{-1}, respectively) (Frederiksen et al., 2013; Larsson et al., 2014), and much lower than those found among Spanish and French pregnant women (12.5 ng mL^{-1} and 29.8 ng mL^{-1}, respectively) (Casas et al., 2011; Philippat et al., 2012), (Table 3).

Our findings are also in line with the low detection rates of BP found in Denmark (Frederiksen et al., 2013; de Renzy-Martin et al., 2014), Sweden (Larsson et al., 2014) and Saudi Arabia (Asimakopoulos et al., in press). In contrast, BP detection rates close to 90% were found in France (Philippat et al., 2012) and Spain (Casas et al., 2011).

In agreement with previous studies (Larsson et al., 2014; Asimakopoulos et al., in press), our work shows a correlation between urinary levels of MP and PP ($r = 0.54$, $p < 0.01$). This correlation is frequently found because MP and PP are usually used in combination in pharmaceuticals, personal care products and food processing (Cosmetic Ingredient Review Expert Panel, 2008). We also found correlation between urinary levels of MP and BP ($r = 0.65$, $p < 0.01$), EP and BP ($r = 0.67$, $p < 0.01$), PP and BP ($r = 0.56$, $p < 0.01$), and a weak but statistically significant correlation between EP and PP ($r = 0.37$, $p < 0.05$). No correlation was found in our study between MP and EP ($r = 0.29$, $p = 0.098$) (Table 4). In contrast with our results, Asimakopoulos et al.(in press) found no correlations between EP and BP, PP and BP, or EP and PP, but found a weak but statistically significant association between MP and EP ($r = 0.31$, $p < 0.05$). An explanation for this could be the differences between countries in terms of the paraben

Table 2
Levels of selected EDCs in urine samples (N = 34).

					Percentiles		
	%>LOD	Mean ± SD	Geometric mean	Range	P25	P50	P75
BP-1	31 (91.2%)	2.70 ± 3.06	1.33	11.55	0.53	1.82	3.78
BP-3	22 (64.7%)	4.76 ± 6.27	1.10	19.56	<LOD	1.73	7.96
BP-6	2 (5.9%)	<LOD	0.11	0.56	<LOD	<LOD	<LOD
BP-8	0 (0.0%)	<LOD	0.10	–	<LOD	<LOD	<LOD
BPA	24 (70.6%)	1.12 ± 1.77	0.44	7.96	<LOD	0.35	1.40
4-OH	0 (0.0%)	<LOD	0.10	–	<LOD	<LOD	<LOD
MP	32 (94.1%)	74.86 ± 101.27	30.10	404.15	15.76	34.94	80.91
EP	23 (67.6%)	5.86 ± 8.37	1.43	37.94	<LOD	1.77	9.90
PP	24 (70.6%)	12.72 ± 29.75	2.03	165.59	<LOD	3.06	12.54
BP	13 (38.2%)	4.62 ± 10.34	0.47	52.33	<LOD	<LOD	5.15

SD: standard deviation

Table 3
BPA, paraben and benzophenone concentrations – medians at ng mL^{-1} (%>LOD) – found in urine samples from women from Europe and some Arab countries.

Country	Year	N	Population	MP	EP	PP	BP	BPA	BP3	Ref
France	2002–2006	191	Pregnant women	97.8 (100%)	4.1 (67.7%)	12.5 (96.9%)	1.7 (87.6%)	2.7 (98.5%)	1.7 (80.5%)	Philippat et al. (2012)
The Netherlands	2004–2006	100	Pregnant women					1.2 (82%)		Ye et al. (2008)
Spain	2004–2008	120	Pregnant women	191.0 (100%)	8.8 (87.6%)	29.8 (97.3%)	2.4 (90.1%)	2.2 (91%)	3.4 (90.1%)	Casas et al. (2011)
Germany	2007–2009	104	Mothers					2.1 (100%)		Kasper-Sonnenberg et al. (2012)
Egypt	2009	57	Girls					0.70 (79%)		Nahar et al. (2012)
Denmark	2011	145	Mothers	14.0 (90%)	0.89 (66%)	1.7 (83%)	<LOD (39%)	2.1 (97%)	3.7 (98%)	Frederiksen et al. (2013)
Sweden	2011	76	Mothers	40 (100%)	2.4 (94.7%)	1.8 (88.2%)	<LOD (36.8%)	1.29 (100%)		Larsson et al. (2014)
Denmark	2011	200	Pregnant women	20.7 (95%)	1.01 (60%)	4.17 (83%)	<LOD (38%)	1.38 (89.5%)	3.20 (97%)	de Renzy-Martin et al. (2014).
Belgium	2011–2012	125	Pregnant women					2.30 (100%)		Covaci et al. (2015)
Denmark		143						2.10 (90.9%)		
Luxembourg		56						<LOQ (44.6%)		
Slovenia		106						1.97 (81.7%)		
Spain		113						2.26 (96.5%)		
Sweden		96						1.29 (100%)		
Tunisia	2012	34	Women	34.94 (94.1%)	1.77 (67.6%)	3.06 (70.6%)	<0.2 (38.2%)	0.35 (70.6%)	1.73 (64.7%)	Present study
Belgium	2013	138	Women [a]	32.4 (100%)	1.9 (97.1%)	3.3 (89.1%)	0.5 (58%)		1.7 (83.3%)	Dewalque et al. (2014)
Saudi Arabia	2014	130	General population	11.7 (100%)	0.23 (87.7)	1.66 (85.4)	0.15 (11.5)	2.01 (86.2%)	1.02 (85.4%)	Asimakopoulos et al., (in press)

[a] Children and adults

composition used in the manufacture of cosmetics, pharmaceuticals or in food and beverage products (Moos et al., 2014).

3.1.3. Benzophenones

Out of the five selected benzophenones, BP-1 and BP-3 were commonly detected in our study population (91.2 and 64.7%, respectively), whereas BP-6 was only found in 5.9% of the analyzed samples and neither BP-2 nor 4-OH-BP were detected. BP-1 concentrations ranged from <LOD to 11.7 ng mL^{-1}, at a median concentration of 1.8 ng mL^{-1}. BP-3 concentrations ranged from <LOD to 19.7 ng mL^{-1}, at a median concentration of 1.7 ng mL^{-1}. BP-3 and BP-1 have been habitually detected at high rates in urine (Asimakopoulos et al., in press; Casas et al., 2011; de Renzy-Martin et al., 2014; Wang and Kannan, 2013). Median concentrations of BP-3 and BP-1 found in our study were higher than those recently reported in Saudi Arabia (1.02 and 0.46 ng mL^{-1}, respectively). Differences in detection rates were also found [lower for BP-3 (85.4%) and higher for BP-1(100%) in Saudi Arabia]. In comparison with other studies in Europe (Table 3), BP-3 levels in this study were fairly similar to those observed in women from France (Philippat et al., 2012) and Belgium (Dewalque et al., 2014) but twofold lower than levels found in Spain (Casas et al., 2011) and Denmark (Frederiksen et al., 2013; de Renzy-Martin et al., 2014).

Additionally, the studies on the detection of BP-2, BP-8, BP-6 and 4-OH-BP are still scarce and provide differing results, particularly in detection rates (Asimakopoulos et al., in press; Kunisue et al., 2010; Vela-Soria et al., 2014). Our results differ from those reported in Saudi Arabia (Asimakopoulos et al., in press) where 4-OH-BP, BP-2 and BP-8 were frequently or moderately detected (100, 64.6 and 49.2%, respectively).

We found a statistically significant correlation between BP-3 and BP-1 ($r = 0.37$, $p < 0.05$), similar to that observed in Saudi Arabia ($r = 0.73$, $p = 0.0001$) (Asimakopoulos et al., in press). We also found a moderate but significant correlation between BP-3 and PP ($r = 0.41$, $p < 0.05$) and BP-1 and MP, EP, PP and BP ($r = 0.41$–0.61; $p = 0.016$–0.0001) (Table 4). These findings suggest that BP-3 is probably used in combination with parabens in some commonly used products. No associations were found between BP-3 or BP-1 and parabens in Saudi Arabia (Asimakopoulos et al., in press).

Table 4
Spearman correlation coefficient between urinary concentrations of selected EDCs

	BP-1	BP-3	BPA	MP	EP	PP	BP
BP-1	–						
BP-3	0.37**	–					
BPA	−0.04	0.08	–				
MP	0.43**	0.16	0.11	–			
EP	0.41**	0.1	0.20*	0.29	–		
PP	0.56***	0.41**	0.1	0.54***	0.37**	–	
BP	0.61***	0.23	−0.06	0.61***	0.67***	0.56***	–

*p-value <0.1; **p-value < 0.05; ***p-value <0.01

3.2. Predictors of BPA, parabens and benzophenones

The analysis that assesses the influence of demographical and dietary variables in the levels of exposure to the selected EDCs is shown in Table 5. Due to the low number of samples with concentrations above the LOD for BP-6, BP-8 and 4-OH-BP, analyses were not performed for these compounds. A negative association was found between age and urinary levels of PP, which was, however, only significant at a level of alpha-error <0.1 ($p = 0.09$). This was probably due to a more frequent use of personal care products in young adult women (<45 years) compared with older women.

To our knowledge, there is little research on the relation between exposure to BPA, parabens and bezophenones and reproductive characteristics. In our study we found that premenopausal women showed higher urinary levels of MP ($p = 0.07$), PP ($p < 0.01$), BP-1 ($p = 0.05$)

Table 5
Levels of selected EDCs by socio-demographic and dietary characteristics.

		BP-1		BP-3		BPA		MP		EP		PP		BP	
	N (%)	GM ± SD	P-value	GM ± SD	P-value	GM ± SD	P-value	GM ± SD	P-value	GM ± SD	P-value	GM ± SD	P-value	GM ± SD	P-value
Age															
<45 years	17 (50.0%)	1.76 ± 1.39	0.17	1.42 ± 1.97	0.56	0.44 ± 1.28	0.96	45.26 ± 1.02	0.38	0.85 ± 2.05	0.19	**4.43 ± 1.81**	**0.09** *	0.35 ± 2.17	0.60
>45 years	17 (50.0%)	1.02 ± 1.34		0.86 ± 2.22		0.44 ± 1.50		20.51 ± 2.28		2.32 ± 2.06		**1.02 ± 2.53**		0.61 ± 2.31	
BMI															
Normal weight	10 (29.4%)	1.17 ± 1.65	0.89	0.80 ± 1.98	0.50	0.71 ± 1.26	0.22	50.01 ± 1.31	0.52	1.32 ± 2.07	0.80	1.86 ± 2.61	0.89	0.31 ± 2.38	0.56
Overweight/obesity	24 (70.6%)	1.41 ± 1.27		1.26 ± 2.16		0.36 ± 1.40		24.14 ± 1.97		1.48 ± 2.14		2.11 ± 2.23		0.57 ± 2.18	
Marital status															
Single	5 (14.7%)	0.56 ± 1.76	0.23	**<LOD**	**0.03** **	0.71 ± 1.51	0.45	**92.27 ± 1.01**	**0.07** *	2.02 ± 2.14	0.90	1.82 ± 2.78	0.96	0.35 ± 2.80	0.76
Married/divorced	29 (85.3%)	1.55 ± 1.27		**1.53 ± 2.05**		0.41 ± 1.37		**24.65 ± 1.85**		1.34 ± 2.11		2.07 ± 2.28		0.49 ± 2.17	
Occupational class															
Homemakers	10 (29.4%)	0.82 ± 1.47	0.31	1.00 ± 2.03	0.80	0.32 ± 1.21	0.49	23.82 ± 2.29	0.95	1.12 ± 1.97	0.62	**0.60 ± 2.26**	**0.06** *	0.25 ± 1.82	0.39
Outside workers	24 (70.6%)	1.51 ± 1.32		1.01 ± 2.13		0.54 ± 1.43		29.57 ± 1.59		1.76 ± 2.13		**3.27 ± 2.20**		0.60 ± 2.36	
Residence															
Urban	28 (82.4%)	**1.09 ± 1.41**	**0.06** *	0.86 ± 2.06	0.21	0.45 ± 1.43	0.98	32.62 ± 1.61	0.60	**1.06 ± 2.16**	**0.09** *	2.07 ± 2.34	0.91	0.40 ± 2.20	0.52
Rural	6 (17.6%)	**3.19 ± 0.82**		3.21 ± 2.01		0.40 ± 1.22		20.99 ± 2.69		**5.57 ± 0.98**		1.86 ± 2.41		0.90 ± 2.43	
Location															
North	31 (91.2%)	1.26 ± 1.35	0.53	0.94 ± 2.08	0.19	0.41 ± 1.41	0.29	29.82 ± 1.87	0.98	1.23 ± 2.11	0.24	2.03 ± 2.33	0.95	0.41 ± 2.19	0.35
Center-south	3 (8.8%)	2.24 ± 1.76		5.18 ± 1.75		0.96 ± 0.67		33.11 ± 1.24		6.35 ± 1.31		2.07 ± 2.63		1.85 ± 2.59	
Menopausal status															
Premenopausal	22 (64.7%)	**1.75 ± 1.42**	**0.05** *	**1.82 ± 0.44**	**0.07** *	0.37 ± 1.24	0.43	**49.94 ± 0.22**	**0.07** *	1.60 ± 2.13	0.59	**5.77 ± 0.40**	**<0.01** ***	0.75 ± 2.31	0.20
Postmenopausal	12 (35.3%)	**0.82 ± 1.19**		**0.45 ± 0.58**		0.60 ± 1.60		**12.41 ± 0.72**		1.18 ± 2.08		**0.36 ± 0.60**		0.22 ± 1.93	
Parity															
Nulliparous	7 (20.6%)	0.79 ± 1.79	0.54	**0.28 ± 1.65**	**0.07** *	0.51 ± 1.57	0.80	76.22 ± 1.02	0.12	1.22 ± 2.28	0.73	2.49 ± 2.60	0.69	0.28 ± 2.56	0.59
Multiparous	27 (79.4%)	1.49 ± 1.28		**1.48 ± 2.08**		0.43 ± 1.36		24.49 ± 1.89		1.48 ± 2.09		1.94 ± 2.30		0.53 ± 2.19	
Age at menarche															
<13 years	17 (50.0%)	1.96 ± 1.03	0.17	**2.46 ± 2.01**	**0.02** **	**0.30 ± 1.49**	**0.09** *	31.85 ± 1.92	0.87	1.22 ± 2.34	0.99	**5.03 ± 2.11**	**0.04** **	0.76 ± 2.31	0.31
>13 years	17 (50.0%)	0.92 ± 1.57		**0.51 ± 1.91**		**0.64 ± 1.19**		28.55 ± 1.75		1.65 ± 1.88		**0.91 ± 2.24**		0.30 ± 2.13	
Lactation															
<14 months	18 (52.9%)	1.02 ± 1.36	0.22	1.22 ± 2.04	0.79	0.51 ± 1.35	0.46	32.43 ± 1.85	0.93	0.85 ± 2.15	0.13	**3.64 ± 2.33**	**0.08** *	0.24 ± 2.02	0.12
>14 months	16 (47.1%)	1.83 ± 1.37		0.97 ± 2.21		0.37 ± 1.44		27.53 ± 1.80		2.67 ± 1.89		**0.96 ± 2.14**		1.12 ± 2.25	
Dairy products consumption															
<1 per day	17 (50.0%)	1.26 ± 1.49	0.88	0.85 ± 1.97	0.66	0.44 ± 1.40	0.93	27.54 ± 1.88	0.75	1.39 ± 2.07	0.78	1.17 ± 2.43	0.26	0.45 ± 2.35	0.82
Everyday	17 (50.0%)	1.36 ± 1.33		1.18 ± 2.18		0.45 ± 1.45		34.00 ± 1.83		1.73 ± 2.12		3.03 ± 2.11		0.54 ± 2.21	
Meat consumption															
<6 days per week	16 (47.1%)	0.93 ± 1.57	0.17	1.25 ± 2.06	0.84	0.46 ± 1.13	0.73	35.83 ± 1.07	0.79	1.77 ± 2.01	0.66	2.35 ± 2.36	0.88	0.58 ± 2.25	0.68
>6 days per week	18 (52.9%)	1.80 ± 1.14		0.99 ± 2.16		0.43 ± 1.59		26.04 ± 2.27		1.19 ± 2.19		1.78 ± 2.33		0.39 ± 2.26	
Cereals consumption															
<4 days per week	17 (50.0%)	1.47 ± 1.11	0.93	1.63 ± 2.13	0.20	0.29 ± 1.19	0.12	**21.14 ± 1.76**	**0.07** *	0.86 ± 2.07	0.20	2.26 ± 2.21	0.77	0.35 ± 2.17	0.60
>4 days per week	17 (50.0%)	1.21 ± 1.61		0.75 ± 2.03		0.66 ± 1.45		**41.99 ± 1.83**		2.29 ± 2.06		1.84 ± 2.47		0.60 ± 2.31	

GM: geometric mean; SD: standard deviation; BM1: body mass index (kg/m): *p-value <0.1; **p-value <0.05; ***p-value <0.01

and BP-3 ($p = 0.07$) than postmenopausal women, suggesting an age-related association. A negative association was found between age of menarche and urinary levels of PP ($p = 0.04$) and BP-3 ($p = 0.02$). Furthermore, marital status was positively associated with urinary levels of BP-3 ($p = 0.03$).

Regarding urinary EDC levels and the place of residence, we found that women living in rural areas showed higher levels of EP and BP-1 ($p = 0.09$ and $p = 0.06$, respectively) than women living in urban areas. Regarding occupational class, we found that women working outside home showed higher urinary levels of PP than homeworkers ($p = 0.06$), suggesting a greater use of cosmetics and processed food in women working outside home.

Our data on dietary habits showed that cereal consumption was positively associated with urinary levels of MP ($p = 0.07$). This might be explained by the migration of MP from antibacterial plastic packaging (Lu et al., 2014).

At present, there is little scientific literature regarding the association between exposure to BPA, parabens or benzophenones and specific products, activities and demographic variables. Some studies have reported increased concentrations of BPA in relation to consumption of bottled water (Li et al., 2013; Engel et al., 2014). The only study available about exposure to BPA in an African population reports a significant correlation between urinary BPA concentrations and food storage in plastic containers (Nahar et al., 2012). As with the present study, Nahar et al. did not find significant correlations between urinary BPA concentrations and individual covariates such as age, body mass index (BMI) or residential status. In contrast, some studies have reported a positive association between BPA exposure and BMI (Carwile and Michels, 2011; Shankar et al., 2012). Others have showed inconsistent findings in relation to BPA. For example, while some authors found that canned fish and exposure to smoke or second-hand smoke are important sources of exposure to BPA in the general population (Braun et al., 2011a; Casas et al., 2013; Berman et al., 2014), others have reported no such association (Lakind and Naiman, 2011). In the present study, smoking habits could not be assessed because all the volunteers declared being nonsmoker. Regarding parabens and benzophenones, studies focused on predictors of exposure are much more limited. These studies found lower concentrations of MP and EP in obese people than in people with normal BMI (Meeker et al., 2011; Smith et al., 2012), positive association between BP and age (Meeker et al., 2011), positive association between BP-3 concentrations and age, BP and parity and BP-3 and income status (Meeker et al., 2013). In the present study, income status was not assessed because all the volunteers were of the same income level (200–500 €/month). There are also inconsistent findings regarding parabens. Thus, whereas Meeker et al. found no association between paraben concentrations and smoking status (Meeker et al., 2011), Engel et al. found that PP and MP concentrations were significantly lower among current smokers compared with former or never-smokers (Engel et al., 2014). Recent studies have examined the relation between the use of personal care product and urinary concentrations of parabens and benzophenones. Higher concentrations of parabens were found in women who reported a higher use of personal care products (Braun et al., 2014) and higher BP-3 concentrations in those who reported use of sunscreens (Meeker et al., 2013).

The limitations of our study include the small sample size, which is not representative of the general Tunisian female population, and the lack of in-depth information regarding the use of personal care products and behavioral and dietary habits (consumption of food and drinks in cans and plastic containers, the heating of these containers, etc.). The small sample size is insufficient for integration with epidemiological studies. However, this is an exploratory study and despite the fact that more research is needed, the results could set the basis for further epidemiological studies.

On the basis of the above, further studies like this one, on the levels of exposure to BPA, parabens and benzophenones and on the predictors of exposure in different populations worldwide are needed, with special attention to developing countries where exposure to these compounds remains poorly characterized.

4. Conclusions

This is the first study that simultaneously describes levels of BPA, parabens and benzophenones in urine from Tunisian women. All analyzed samples were positive for at least one EDC residue being MP and BP-1 highly detected. In view of our results, further population-based biomonitoring studies are warranted to elucidate exposure levels of the Tunisian general population and to identify routes of exposure and population groups at higher risk.

Competing interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

This work would not have been possible without the generous collaboration of the volunteers who took part in this study. The authors are also grateful to Esther Moreno for her editorial assistance. This study was supported by the Regional Government of Andalusia (Projects of Excellence P09-CTS-4470, P09-CTS-5488 and project PI-0513/2012) and by the European Union FEDER (European Regional Development Fund). The authors are grateful to *Instituto de Salud Carlos III* for the postdoctoral research contract (Sara Borrell Program, no CD012/00462) granted to I. Jiménez-Díaz and to the Ministry of Higher Education and Scientific Research of Tunisia for the scholarship granted to H. Belhassen. F. Artacho-Cordón has a fellowship from the Spanish Ministry of Education (FPU12/02524) and JP. Arrebola is currently receiving a postdoctoral fellowship from the *Consejería de Igualdad, Salud y Políticas Sociales*, from the Regional Government of Andalusia (RH-0092-2013).

References

Arrebola, J.P., Belhassen, H., Artacho-Cordón, F., Ghali, R., Ghorbel, H., Boussen, H., Perez-Carrascosa, F.M., Expósito, J., Hedhili, A., Olea, N., 2015. Risk of female breast cancer and serum concentrations of organochlorine pesticides and polychlorinated biphenyls: a case–control study in Tunisia. Sci. Total Environ. 520, 106–113.

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

Asimakopoulos, A.G., Xue, J., Pereira de Carvalho, B., Iyer, A., Abualnaja, K.O., Yaghmoor, S.S., Kumosani, T.A., Kannan, K., 2016. Urinary biomarkers of exposure to 57 xenobiotics and its association with oxidative stress in a population in Jeddah, Saudi Arabia. Environ. Res. http://dx.doi.org/10.1016/j.envres.2015.11.029 (in press).

Bailin, P.D., Byrne, M., Lewis, S., Liroff, R., 2008. Public Awareness Drives Market for Safer Alternatives: Bisphenol A Market Analysis Report. Investor Environmental Health Network, Falls Church, VA (Document available on line at: ⟨http://www.iehn.org/documents/BPA%20market%20report%20Final.pdf⟩).

Belhassen, H., Jiménez-Díaz, I., Arrebola, J.P., Ghali, R., Ghorbel, H., Olea, N., Hedili, A., 2015. Zearalenone and its metabolites in urine and breast cancer risk: a case–control study in Tunisia. Chemosphere 128, 1–6.

Berman, T., Goldsmith, R., Göen, T., Spungen, J., Novack, L., Levine, H., Amitai, Y., Shohat, T., Grotto, I., 2014. Demographic and dietary predictors of urinary bisphenol A concentrations in adults in Israel. Int. J. Hyg. Environ. Health 217, 638–644.

Braun, J.M., Kalkbrenner, A.E., Calafat, A.M., Bernert, J.T., Ye, X., Silva, M.J., Barr, D.B., Sathyanarayana, S., Lanphear, B.P., 2011a. Variability and predictors of urinary bisphenol A concentrations during pregnancy. Environ. Health Perspect. 119, 131–137.

Braun, J.M., Kalkbrenner, A.E., Calafat, A.M., Yolton, K., Ye, X., Dietrich, K.N., Lanphear, B.P., 2011b. Impact of early-life bisphenol A exposure on behavior and executive function in children. Pediatrics 128, 873–882.

Braun, J.M., Just, A.C., Williams, P.L., Smith, K.W., Calafat, A.M., Hauser, R., 2014. Personal care product use and urinary phthalate metabolite and paraben concentrations during pregnancy among women from a fertility clinic. J. Expo. Sci. Environ. Epidemiol. 24, 459–466.

Carwile, J.L., Michels, K.B., 2011. Urinary bisphenol A and obesity: NHANES 2003–2006. Environ. Res. 111, 825–830.

Casas, L., Fernández, M.F., Lop, S., Guxens, M., Ballester, F., Olea, N., Izurzun, M.B., Marina Rodríguez, L.S., Riaño, I., Tardón, A., Vrijheid, M., Calafat, A.M., Sunyer, J., 2011. Urinary

concentrations of phthalates and phenols in a population of Spanish pregnant women and children. Environ. Int. 37, 858–866.
Casas, M., Valvi, D., Luque, N., Ballesteros-Gómez, A., Carsin, A.E., Fernández, M.F., Koch, H.M., Mendez, M.A., Sunyer, J., Rubio, S., Vrijheid, M., 2013. Dietary and sociodemographic determinants of bisphenol A urine concentrations in pregnant women and children. Environ. Int. 56, 10–18.
Charles, A.K., Darbre, P.D., 2013. Combinations of parabens at concentrations measured in human breast tissue can increase proliferation of MCF-7 human breast cancer cells. J. Appl. Toxicol. 33, 390–398.
Chen, J., Ahn, K.C., Gee, N.A., Gee, S.J., Hammock, B.D., Lasley, B.L., 2007. Antiandrogenic properties of parabens and other phenolic containing small molecules in personal care products. Toxicol. Appl. Pharmacol. 221, 278–284.
Cosmetic Ingredient Review Expert Panel, 2008. Final amended report on the safety assessment of methylparaben, ethylparaben, propylparaben, isopropylparaben, butylparaben, isobutylparaben and benzylparaben as used in cosmetic products. Int. J. Toxicol. 27, 1–82.
Covaci, A., Hond, E.D., Geens, T., Govarts, E., Koppen, G., Frederiksen, H., Knudsen, L.E., Morck, T.A., Gutleb, A.C., Guignard, C., Cocco, E., Horvat, M., Heath, E., Kosjek, T., Mazej, D., Tratnik, J.S., Castaño, A., Esteban, M., Cutanda, F., Ramos, J.J., Berglund, M., Larsson, K., Jönsson, B.A.G., Biot, P., Casteleyn, L., Joas, R., Joas, A., Bloemen, L., Sepai, O., Exley, K., Schoeters, G., Angerer, J., Kolossa-Gehring, M., Fiddicke, U., Aerts, D., Koch, H.M., 2015. Urinary BPA measurements in children and mothers from six European member states: overall results and determinants of exposure. Environ. Res. 141, 77–85.
Darbre, P.D., 2015. Endocrine Disruption and Human Health. first ed. Elsevier.
Darbre, P.D., Harvey, P.W., 2008. Paraben esters: review of recent studies of endocrine toxicity, absorption, esterase and human exposure, and discussion of potential human health risks. J. Appl. Toxicol. 28, 561–578.
de Renzy-Martin, K.T., Frederiksen, H., Christensen, J.S., Kyhl, H.B., Andersson, A.M., Husby, S., Barington, T., Main, K.M., Jensen, T.K., 2014. Current exposure of 200 pregnant Danish women to phthalates, parabens and phenols. Reproduction 147, 443–453.
Dewalque, L., Pirard, C., Charlier, C., 2014. Measurement of urinary biomarkers of parabens, benzophenone-3 and phthalates in a Belgian population. Biomed. Res. Int. 1–13, Article ID 649314.
El Hussein, S., Muret, P., Berard, M., Makki, S., Humbert, P., 2007. Assessment of principal parabens used in cosmetics after their passage through human epidermis–dermis layers (ex-vivo study). Exp. Dermatol. 16, 830–836.
Engel, L.S., Buckley, J.P., Yang, G., Liao, L.M., Satagopan, J., Calafat, A.M., Matthews, C.E., Cai, Q., Ji, B.T., Cai, H., Engel, S.M., Wolff, M.S., Rothman, N., Zheng, W., Xiang, Y.B., Shu, X.O., Gao, Y.T., Cho, W.H., 2014. Predictors and variability of repeat measurements of urinary phenols and parabens in a cohort of Shanghai women and men. Environ. Health Perspect. 122, 733–740.
Fernández, M.F., Arrebola, J.P., Jiménez-Díaz, I., Saenz, J.M., Molina-Molina, J.M., Ballesteros, O., Kortenkamp, A., Olea, N., 2016. Bisphenol A and other phenols in human placenta from children with cryptorchidism or hypospadias. Reprod. Toxicol. 59, 89–95.
Frederiksen, H., Nielsen, J.K.S., Mork, T.A., Hansen, P.W., Jensen, J.F., Nielsen, O., Andersson, A.M., Knudsen, L.E., 2013. Urinary excretion of phthalate metabolites, phenols and parabens in rural and urban Danish mother–child pairs. Int. J. Hyg. Environ. Health 216, 772–783.
Geens, T., Aerts, D., Berthot, C., Bourguignon, J.P., Goeyens, L., Lecomte, P., Maghuin-Rogister, G., Pironnet, A.M., Pussemier, L., Scippo, M.L., Van Loco, J., Covaci, A., 2012. A review of dietary and non-dietary exposure to bisphenol A. Food Chem. Toxicol. 50, 3725–3740.
Hayden, C.G.J., Cross, S.E., Anderson, C., Saunders, N.A., Roberts, M.S., 2005. Sunscreen penetration of human skin and related keratinocyte toxicity after topical application. Pharmacol. Physiol. 18, 170–174.
Inn, K.G.W., McCurdy, D., Kuruvilla, L., Barss, N.M., Pietrzak, R., Kaplan, E., Inkret, W., Efurd, W., Rokop, D., Lewis, D., Gautier, P., Bell, R.T., 2001. Intercomparison study of inductively coupled plasma mass spectrometry, thermal ionization mass spectrometry and fission track analysis of mu Bq quantities of Pu-239 in synthetic urine. J. Radioanal. Nucl. Chem. 249, 121–131.
Kasper-Sonnenberg, M., Wittsiepe, J., Koch, H.M., Fromme, H., Wilhelm, M., 2012. Determination of Bisphenol A in urine from mother–child pairs—results from the Duisburg birth cohort study, Germany. J. Toxicol. Environ. Health 75, 429–437.
Krause, M., Klit, A., Jensen, M.B., Søeborg, T., Frederiksen, H., Schlumpf, M., Lichtensteiger, W., Skakkebaek, N.E., Drzewiecki, K.T., 2012. Sunscreens are they beneficial for health? An overview of endocrine disrupting properties of UV-filters. Int. J. Androl. 35, 424–436.
Kunisue, T., Wu, Q., Tanabe, S., Aldousa, K.M., Kannan, K., 2010. Analysis of five benzophenone-type UV filters in human urine by liquid chromatography-tandem mass spectrometry. Anal. Methods 2, 707–713.
Lakind, J.S., Naiman, D.Q., 2011. Daily intake of bisphenol A and potential sources of exposure: 2005–2006 National Health and Nutrition Examination Survey. J. Expo. Sci. Environ. Epidemiol. 21, 272–279.
Lang, I.A., Galloway, T.S., Scarlett, A., Henley, W.E., Depledge, M., Wallage, R.B., Melzer, D., 2008. Association of urinary bispenol A concentration with medical disorders and laboratory abnormalities in adults. J. Am. Med. Assoc. 300, 1303–1310.
Larsson, K., Björklund, K.L., Palm, B., Wennberg, M., Kaj, L., Lindh, C.H., Jönsson, Bo.A.G., Berglund, M., 2014. Exposure determinants of phthalates, parabens, bisphenol A and triclosan in Swedish mothers and their children. Environ. Int. 73, 323–333.
Li, X., Ying, G.G., Zhao, J.L., Chen, Z.F., Lai, H.J., Su, H.C., 2013. 4-Nonylphenol, bisphenol-A and triclosan levels in human urine of children and students in China, and the effects of drinking these bottled materials on the levels. Environ. Int. 52, 81–86.
Lu, L., Xiong, W., Li, X., Lv, S., Tang, X., Chen, M., Zou, Z., Lin, Z., Qiua, B., Chena, G., 2014. Determination of the migration of eight parabens from antibacterial plastic packaging by liquid chromatography-electrospray ionization-tandem mass spectrometry. Anal. Methods 6, 2096–2101.
Meeker, J.D., Yang, T., Ye, X., Calafat, A.M., Hauser, R., 2011. Urinary concentrations of parabens and serum hormone levels, semen quality parameters, and sperm DNA damage. Environ. Health Perspect. 119, 252–257.
Meeker, J.D., Cantonwine, D.E., Rivera-González, L.O., Ferguson, K.K., Mukherjee, B., Calafat, A.M., Ye, X., Anzalota Del Toro, L.V., Crespo, N., Jiménez-Vélez, B., Alshawabkeh, A.N., Cordero, J.F., 2013. Distribution, variability and predictors of urinary concentrations of phenols and parabens among pregnant women in Puerto Rico. Environ. Sci. Technol. 47, 3439–3447.
Molina-Molina, J.M., Amaya, E., Grimaldi, M., Sáenz, J.M., Real, M., Fernández, M.F., Balaguer, P., Olea, N., 2013. In vitro study on the agonistic and antagonistic activities of bisphenol-S and other bisphenol-A congeners and derivatives via nuclear receptors. Toxicol. Appl. Pharmacol. 272, 127–136.
Moos, R.K., Angerer, J., Wittsiepe, J., Wilhelm, M., Brüning, T., Koch, H.M., 2014. Rapid determination of nine parabens and seven other environmental phenols in urine samples of German children and adults. Int. J. Hyg. Environ. Health 217, 845–853.
Nahar, M.S., Soliman, A.S., Colacino, J.A., Calafat, A.M., Battige, K., Hablas, A., Seifeldin, I.A., Dolinoy, D.C., Rozek, L.S., 2012. Urinary Bisphenol A Concentrations in Girls from Rural and Urban Egypt: A Pilot Study.
Oishi, S., 2002. Effects of propyl paraben on the male reproductive system. Food Chem. Toxicol. 40, 1807–1813.
Pérez-Lobato, R., Mustieles, V., Calvente, I., Jiménez-Díaz, I., Ramos, R., Caballero-Casero, N., López-Jiménez, F.J., Rubio, S., Olea, N., Fernández, M.F., 2016. Exposure to Bisphenol A and behavior in school-age children. NeuroToxicology 53, 12–19.
Philippat, C., Mortamais, M., Chevrier, C., Petit, C., Calafat, A.M., Ye, X., Silva, M.J., Brambilla, C., Pin, I., Charles, M.A., Cordier, S., Slama, R., 2012. Exposure to phthalates and phenols during pregnancy and offspring size at birth. Environ. Health Perspect. 120, 464–470.
Schecter, A., Malik, N., Haffner, D., Smith, S., Harris, T.R., Paepkem, O., Birnbaum, L., 2010. Bisphenol A (BPA) in U.S. food. Environ. Sci. Technol. 44, 9425–9430.
Schlumpf, M., Kypke, K., Wittassek, M., Angerer, J., Mascher, H., Mascher, D., Vökt, C., Birchler, M., Lichtensteiger, W., 2010. Exposure patterns of UV filters, fragrances, parabens, phthalates, organochlorine pesticides, PBDEs, and PCBs in human milk: correlation of UV filters with use of cosmetics. Chemosphere 81, 1171–1183.
Shankar, A., Teppala, S., Sabanayagam, C., 2012. Urinary bisphenol A levels and measures of obesity: results from the national health and nutrition examination survey 2003–2008. ISRN Endocrinol., Article ID 965243
Sharpe, R.M., Drake, A.J., 2010. Bisphenol A and metabolic syndrome. Endocrinology 151, 2404–2407.
Smith, K.W., Braun, J.M., Williams, P.L., Ehrlich, S., Correia, K.F., Calafat, A.M., Ye, X., Ford, J., Keller, M., Meeker, J.D., Hauser, R., 2012. Predictors and variability of urinary paraben concentrations in men and women, including before and during pregnancy. Environ. Health Perspect. 120, 1538–1543.
Soni, M.G., Carabin, I.G., Burdock, G.A., 2005. Safety assessment of esters of p-hydroxybenzoic acid (parabens). Food Chem. Toxicol. 43, 985–1015.
Sonnenschein, C., Soto, A.M., 1998. An updated review of environmental estrogen and androgen mimics and antagonists. J. Steroid Biochem. Mol. Biol. 65, 143–150.
Susiarjo, M., Sasson, I., Mesaros, C., Bartolomei, M.S., 2013. Bisphenol A exposure disrupts genomic imprinting in the mouse. PLoS Genet. 9, e1003401.
Vandenberg, L.N., Hauser, R., Marcus, M., Olea, N., Welshons, W.V., 2007. Human exposure to Bisphenol A (BPA). Reprod. Toxicol. 24, 139–177.
Vandenberg, L.N., Chahoud, I., Heindel, J.J., Padmanabhan, V., Paumgartten, F.J., Schoenfelder, G., 2010. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to Bisphenol A. Environ. Health Perspect. 118, 1055–1070.
Vela-Soria, F., Ballesteros, O., Zafra-Gómez, A., Ballesteros, L., Navalón, A., 2014. UHPLC–MS/MS method for the determination of bisphenol A and its chlorinated derivatives, bisphenol S, parabens, and benzophenones in human urine samples. Anal. Bioanal. Chem. 406, 3773–3785.
Wang, L., Kannan, K., 2013. Characteristic profiles of Benzonphenone-3 and its derivatives in urine of children and adults from the United States and China. Environ. Sci. Technol. 47, 12532–12538.
Yang, J., Shi, S., Xua, F., Nie, J., 2013. Synthesis and photopolymerization kinetics of benzophenone sesamol one-component photoinitiator. Photochem. Photobiol. Sci. 12, 323–329.
Ye, X., Bishop, A.M., Reidy, J.A., Needham, L.L., Calafat, A.M., 2006. Parabens as urinary biomarkers of exposure in humans. Environ. Health Perspect. 114, 1843–1846.
Ye, X., Pierik, F.H., Hauser, R., Duty, S., Angerer, J., Park, M.M., Burdorf, A., Hofman, A., Jaddoe, V.W.V., Mackenbach, J.P., Steegers, E.A.P., Tiemeier, H., Longnecker, M.P., 2008. Urinary metabolite concentrations of organophosphorous pesticides, bisphenol A, and phthalates among pregnant women in Rotterdam, the Netherlands: the generation R study. Environ. Res. 108, 260–267.

7.3. Objective 3. Estimate the association between levels of single POPs and the risk of breast cancer in a Tunisian population.

RISK OF FEMALE BREAST CANCER AND SERUM CONCENTRATIONS OF ORGANOCHLORINE PESTICIDES AND POLYCHLORINATED BIPHENYLS: A CASE–CONTROL STUDY IN TUNISIA

*Arrebola JP, Belhassen H, **Artacho-Cordón F**, Ghali R, Ghorbel H, Boussen H, Perez-Carrascosa FM, Expósito J, Hedhili A, Olea N*

Science of the Total Environment; Impact factor 4.099; 1st Quartile; 1st Decile

ABSTRACT

The aim of this study was to investigate the association between serum concentrations of a group of organochlorine pesticides/polychlorinated biphenyls with xenoestrogenic potential and the risk of breast cancer in a female population from Tunisia.

The relationship between serum levels of the pollutants and the risk of cancer was assessed using logistic regression analyses. In the unadjusted models, β-hexachlorocyclohexane (β-HCH), hexachlorobenzene, heptachlor, polychlorinated biphenyl congeners 138, 153, and 180, and *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE) were positively associated with breast cancer risk. However, when the models were further adjusted for the selected covariates, only β-HCH and *p,p'*-DDE remained statistically significant, and heptachlor was borderline significant. In addition, analyses using POP concentration tertiles corroborated a positive dose–response relationship that was significant for *p,p'*-DDE (p-trend = 0.020) and borderline significant for heptachlor (p-trend= 0.078). A similar trend was also confirmed for β-HCH, in which concentrations ≥ limit of detection were positively associated with breast cancer risk (vs. concentrations < limit of detection, OR = 3.44, $p < 0.05$). Finally, the relative influence of each chemical in the presence of the others was assessed by entering the three chemicals in a single model with all covariates, and only β-HCH remained positively associated with the risk of cancer (OR:1.18, 95%CI: 1.05–1.34).

Our findings suggest a potential association between exposure to at least one organochlorine pesticide and breast cancer risk. However, our results should be interpreted with caution, and further research is warranted to confirm these findings.

Science of the Total Environment 520 (2015) 106–113

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Risk of female breast cancer and serum concentrations of organochlorine pesticides and polychlorinated biphenyls: A case–control study in Tunisia

Juan P. Arrebola [a,b,*], Hidaya Belhassen [c], Francisco Artacho-Cordón [b], Ridha Ghali [c], Hayet Ghorbel [c], Hamouda Boussen [d], Francisco M. Perez-Carrascosa [a], José Expósito [a], Abderrazek Hedhili [c], Nicolás Olea [b]

[a] Radiation Oncology Department, Virgen de las Nieves University Hospital, Granada, Spain
[b] San Cecilio University Hospital, Instituto de Investigación Biosanitaria ibs. Granada, University of Granada, CIBERESP, Spain
[c] Center of Urgent Medical Assistance of Tunis, Laboratory of Toxicology and Environment (LR12SP07), Montfleury, Tunis 1008, Tunisia
[d] Cancer Center of Ariana, Tunisia

HIGHLIGHTS

- We studied the association between persistent pollutants in serum and breast cancer.
- We performed a case–control study in a Tunisian population.
- Three organochlorine pesticides were individually associated with cancer risk.

ARTICLE INFO

Article history:
Received 8 January 2015
Received in revised form 10 March 2015
Accepted 10 March 2015
Available online xxxx

Editor: Adrian Covaci

Keywords:
Breast cancer
Organochlorine pesticides
Polychlorinated biphenyls
Persistent organic pollutants
Endocrine disruptors

ABSTRACT

The aim of this study was to investigate the association between serum concentrations of a group of organochlorine pesticides/polychlorinated biphenyls with xenoestrogenic potential and the risk of breast cancer in a female population from Tunisia.

The relationship between serum levels of the pollutants and the risk of cancer was assessed using logistic regression analyses. In the unadjusted models, β-hexachlorocyclohexane (β-HCH), hexachlorobenzene, heptachlor, polychlorinated biphenyl congeners 138, 153, and 180, and p,p′-dichlorodiphenyldichloroethylene (p,p′-DDE) were positively associated with breast cancer risk. However, when the models were further adjusted for the selected covariates, only β-HCH and p,p′-DDE remained statistically significant, and heptachlor was borderline significant. In addition, analyses using POP concentration tertiles corroborated a positive dose–response relationship that was significant for *p,p′*-DDE (p-trend = 0.020) and borderline significant for heptachlor (p-trend = 0.078). A similar trend was also confirmed for β-HCH, in which concentrations ≥ limit of detection were positively associated with breast cancer risk (vs. concentrations < limit of detection, OR = 3.44, $p < 0.05$). Finally, the relative influence of each chemical in the presence of the others was assessed by entering the three chemicals in a single model with all covariates, and only β-HCH remained positively associated with the risk of cancer (OR:1.18, 95%CI: 1.05–1.34).

Our findings suggest a potential association between exposure to at least one organochlorine pesticide and breast cancer risk. However, our results should be interpreted with caution, and further research is warranted to confirm these findings.

1. Introduction

Breast cancer represents 33% of female cancers in Tunisia, with approximately 1600 new cases per year. Although this disease remains less frequent in Tunisia than in European countries (Maalej et al., 2008), the crude incidence increased from 25.5 cases/100,000 women in 1995–1998 (RCNT, 1998) to 32.3 in 2004–2006 (RCNT, 2006). Breast cancer is the leading cause of cancer death among Tunisian women

Abbreviations: POPs, Persistent Organic Pollutants; PCBs, polychlorinated biphenyls; *p,p′*-DDE, *p,p′*-dichlorodiphenyldichloroethylene; HCB, hexachlorobenzene; β-HCH, hexachlorocyclohexane; LOD, limit of detection; OR, odds ratio; CI, confidence interval.

* Corresponding author at: Radiology and Physical Medicine Department, University ofGranada, Spain, 18071 Granada, Spain.
E-mail address: jparrebola@ugr.es (J.P. Arrebola).

http://dx.doi.org/10.1016/j.scitotenv.2015.03.045
0048-9697/

(Lazaar-Ben et al., 2011) and is estimated to be responsible for 22% of the total cancer mortality (WHO, 2014).

This increasing incidence of breast cancer, which has been documented in most countries (Parkin et al., 2005; World Cancer Research Fund International, 2014), can only be partially explained by improvements in screening programs (Charlier et al., 2003a), and genetic factors account for a small proportion of the incidence in women (Knower et al., 2014). Hence, environmental factors are estimated to play an important role in the pathogenesis of the disease.

The development of cancer is known to be a multifactorial process, with many reported risk factors that have a hormonal component, including age at menopause, menarche, and first pregnancy, parity, accumulated lactation time, time of reproductive life, use of hormonal contraception, hormone replacement therapy, or body weight (McPherson et al., 2000). In fact, previous research has shown that breast cancer is highly influenced by long-term elevated estrogen levels (Key et al., 2002), which can be metabolized to mutagenic elements that eventually stimulate tissue growth and cause the initiation, promotion, and progression of carcinogenesis (Yager and Davidson, 2006). This led researchers to hypothesize that long-term exposure to environmental pollutants with xenoestrogenic potential might make a significant contribution to the process of carcinogenesis (Knower et al., 2014). In fact, a recent epidemiological study concluded that women occupationally exposed to chemicals with hormonal activity (e.g., agriculture, industry) had an increased risk of developing breast cancer (Brophy et al., 2012).

POPs are a wide group of highly lipophilic environmental pollutants that tend to accumulate and biomagnify in food chains, resulting in the considerable exposure of living organisms (UNEP, 2003). POPs include organochlorine pesticides, which have long been widely used in agriculture and public health as highly effective pest control agents (UNEP, 2003), and also polychlorinated biphenyls (PCBs), used worldwide in numerous industrial and commercial applications (La Rocca and Mantovani, 2006). Between 1980 and 1984, the Tunisian Government banned the use and import of the organochlorine pesticides included in this study (APEK, 2005). PCBs were widely used in electrical transformers in Tunisia from the 1970s until their prohibition in 1986. A situation report estimated that there were 132 dump sites containing OCPs/PCBs in the country (APEK, 2005). A previous study revealed that approximately 89% of the stocks of obsolete pesticides were stored in unsatisfactory conditions (Dasgupta et al., 2010), with the consequent risk of contaminating the environment and humans.

POPs have been detected in virtually all human populations and environmental matrices, and diet (especially fatty food) has been reported to be the main route for human exposure (Arrebola et al., 2009; Brauner et al., 2012). Once absorbed, POPs are mainly stored in adipose tissue, where they can be released or persist for long periods of time (Yu et al., 2011).

There is evidence that exposure to some POPs can cause estrogen-related effects, including an increase in uterine weight (Adami et al., 1995) or the promotion of estrogen-related tumors (Scribner and Mottet, 1981). Among the suspected mechanisms of action, in vitro studies have shown that many POPs can interact with estrogen and/or androgen receptors and exert significant effects (Andersen et al., 2002; Bonefeld-Jorgensen et al., 2001; Grunfeld and Bonefeld-Jorgensen, 2004; Soto et al., 1994), which might be a consequence of their interaction with other chemicals as well as with endogenous hormones (Arrebola et al., 2012). Additionally, there is evidence that POPs might cause cancer by other mechanisms not related to estrogen receptors, such as oxidative stress (Karami-Mohajeri and Abdollahi, 2011).

The aim of this study was to investigate the association between serum concentrations of a group of organochlorine pesticides/PCBs with (anti-)estrogenic potential and the risk of breast cancer in a female population from Tunisia. These chemicals were chosen on the basis of their suspected hormonal effects, the reported existence of obsolete stocks in the region (APEK, 2005), and the frequency of their detection in other populations.

2. Materials and methods

2.1. Study population

This case–control study is part of a wider research project designed to characterize the exposure to environmental pollutants in Tunisia and related health outcomes (Artacho-Cordon et al., in press; Belhassen et al., 2015). The study population was consecutively recruited between May and October 2012 from among patients attending the two main specialist cancer centers in the country, Salah Azaiz Hospital (Tunis state) and Ariana Hospital (Ariana state), which are both in the Grand Tunis metropolitan area (Northern Tunisia). Cases were women with breast cancer admitted to hospital for mastectomy, tumorectomy (Salah Azaiz Hospital), or chemotherapy (Cancer Center of Ariana). Out of the 96 eligible cases, 69 (72%) were finally included and provided signed consent and a blood sample. Patients were included if they were aged 18 years or over and able to give informed consent and complete a questionnaire and excluded if they had a previous history of cancer or evidenced distant metastasis at diagnosis. The control group was randomly selected from among healthy female hospital visitors, hospital staff, or blood donors who were aged 18 years and able to give informed consent and complete a questionnaire. Out of the 77 women invited to participate as controls, 56 (70%) were finally enrolled in the study. Biological samples were collected before surgery or chemotherapy. No significant differences were found between included and excluded participants in age, marital status, or occupational class (data not shown in tables).

All participants signed their informed consent to participate in the study, which was approved by the ethics committees of the hospitals.

2.2. Sample collection and extraction

Human serum samples were obtained under 12-h fasting conditions. Samples from cases were collected at the time of diagnosis and before any specific treatment. 2 mL serum was extracted using the methodology described by Turci et al. (2010) with slight modifications. Briefly, serum was spiked with *p*-chlorodibenzophenone as internal standard and further extracted with methanol and hexane/ethyl ether (1:1 v/v). The organic phase was reconstituted in 1 mL hexane and allowed to pass through a Bond Elut-PCB cartridge. The sample elution was performed with 3 ml hexane and 3 ml hexane/diethyl ether (1:1 v/v). Finally, the eluate was evaporated to dryness under a stream of nitrogen and stored at −80 °C until chemical analysis.

2.3. Chemical analyses and lipid quantification

A group of organochlorine pesticides (*p,p′*-dichlorodiphenyldichloroethylene [*p,p′*-DDE], hexachlorobenzene [HCB], β-hexachlorocyclohexane [β-HCH], α-endosulfan, endosulfan ether, heptachlor, and oxychlordane) and PCBs (congeners 138, 153, and 180) were quantified in the extracts by gas chromatography with micro-electron capture detection, using a VARIAN CP-3800 equipped with a ^{63}Ni electron capture detector (Walnut Creek, CA, US).

Procedural blanks were extracted with the same methodology and analyzed in the gas chromatograph, always yielding a negative result. Inter- and intra-day variabilities were calculated by analyzing fortified samples within the same day (repeatability) and on different days (intermediate precision), respectively, and were always <20%. Different concentrations of laboratory-fortified matrix samples were used for the quality control. The LOD was determined as the smallest amount of the analyte that gave a signal-to-noise ratio ≥ 3 and was set at 0.05 μg/L for each POP. The recovery of POPs from serum was also studied to assess the extraction efficiency of the methods, spiking 10 serum samples with target analytes at an intermediate point on the calibration curve and processing them as described above. Recoveries ranged from 90 to 98%.

POP concentrations were calculated by using matrix-matched calibration. Concentrations below the limit of detection (LOD) were assigned a random value between zero and the LOD, which was calculated by using the random numbers function of SPSS. In addition, we repeated the multivariable analyses considering concentrations < LOD as one half of the LOD; no differences were observed with the associations observed using the random value between zero and the LOD (data not shown in tables).

Total cholesterol and triglyceride levels were enzymatically quantified in 10 μL of serum from each participant using a Covas 400 machine (Roche, Switzerland). Total serum lipids were calculated by applying the short formula of Phillips et al. (1989): TL = 2.27 TC + TG + 0.623, where TL is total lipids, TC is total cholesterol, and TG is triglycerides, all expressed in units of g/L.

2.4. Covariates

Covariates were gathered in a questionnaire administered face-to-face by a trained interviewer. It was completed by each participant and collected information on their socio-demographic characteristics, reproductive history, and lifestyle. A participant was considered a smoker (past or present) at any level of daily tobacco consumption (≥1 cig/day). Age at menarche (years), accumulated breastfeeding time (months), alcohol consumption (glasses/week), and age at last breast feeding (years) were self-reported by the participants and recorded as continuous variables. Parity was recorded as both a continuous (number of children) and dichotomous variable (nulliparous/≥1 children). The BMI, calculated as weight/height squared (kg/m^2), served as a measure of obesity.

Participants were classified according to their occupation as manual workers, non-manual workers, or homemakers and according to their residence in an urban or rural area. Self-reported information was also collected on marital status, educational level, and menopausal status. Data were also gathered from the clinical records of cases on the size of the nodule, the presence of metastasis, and any family history of breast cancer.

2.5. Statistical analysis

Descriptions of the study variables in cases and controls were performed using means, standard deviations and percentiles (quantitative variables), and frequencies (categorical variables). Bivariate analyses for the comparison between cases and controls were performed using Mann–Whitney's *U*-test for continuous variables and Fisher's exact test for categorical variables. The linear correlation between pairs of POP concentrations was assessed with Spearman's correlation test. All tests in the bivariate analyses were two-tailed, and the significance level was set at $p \leq 0.05$.

The relationship between POP serum levels and breast cancer risk was assessed by using unconditional logistic regression analyses, entering POP concentrations as continuous variables. In addition, the associations found were further explored by entering POP concentrations in the models as tertiles, with the exception of β-HCH and α-endosulfan, which were entered as dichotomous variables (<LOD/ ≥LOD) because of the low frequency of their detection. Bivariate models with individual POP concentrations as independent variable were created and then adjusted for variables whose inclusion produced changes of >10% in beta coefficients and for those described as relevant factors in the literature. Finally, a single adjusted model was created that included all covariates and POP concentrations significantly associated with breast cancer risk in the previous adjusted models, i.e., β-HCH, heptachlor, and *p,p′*-DDE. Odds ratios (ORs) for the risk of breast cancer with their corresponding 95% confidence intervals (95% CIs) were calculated, and trends were evaluated with Mantel–Haenszel's chi-square test for linear trend. In order to facilitate interpretation of the coefficients in Table 3, wet-basis concentrations were all entered as ng/mL with the exception of *p,p′*-DDE (ng/dL), while lipid-basis concentrations were all entered as mg/g lipid with the exception of *p,p′*-DDE (ng/g lipid). We considered that POP concentrations were significantly associated with the risk of breast cancer when the 95% CIs of the OR in the adjusted models did not overlap the null value (1). Data were stored and processed using SPSS Statistics 22.0 (IBM, Chicago, IL) and R statistical computing environment v3.1 (http://www.r-project.org/).

3. Results

The main characteristics of cases and controls are summarized in Table 1. In comparison to controls, cases were older (median: 49 vs. 44 yrs, respectively) and had given birth to more children (median of 3 vs. 2 children, respectively). Among the cases, there was a larger proportion (vs. controls) of homemakers (71.0% vs. 27.8%, respectively), women with only primary schooling or less (76.8% vs. 35.2%, respectively), post-menopausal women (50.7% vs. 25.9%), and residents in rural areas (42.0% vs. 11.1%, respectively). No member of the control group reported a family history of breast cancer, while 17 (24.6%) cases declared at least one case of breast cancer in their family.

Table 2 exhibits the serum POP concentrations and detection frequencies in cases and controls. Concentrations of β-HCH, heptachlor, PCB 138, PCB 180, and *p,p′*-DDE were significantly or borderline significantly higher in cases than in controls. Due to the low number of positive samples, endosulfan-ether and oxychlordane were not further considered in the statistical models. Spearman correlation tests among individual POP concentrations are shown as Supplementary Material.

Table 3 exhibits the results of the logistic regression analyses for both wet- and lipid-basis POP concentrations, which showed very similar associations. In the unadjusted models, β-HCH, HCB, heptachlor, PCB 138, PCB 153, PCB 180, and *p,p′*-DDE were positively associated with the risk of breast cancer, i.e., the risk increased with higher exposure levels. However, when the models were further adjusted for the selected covariates, only β-HCH and *p,p′*-DDE remained statistically significant, and heptachlor was borderline significant. In addition, analyses using POP concentration tertiles corroborated a positive dose–response relationship that was significant for *p,p′*-DDE (p-trend = 0.020) and borderline significant for heptachlor (p-trend = 0.078). A similar trend was also confirmed for β-HCH, in which concentrations ≥ LOD were positively associated with breast cancer risk (vs. concentrations < LOD, OR = 3.44, $p < 0.05$). Finally, the relative influence of each chemical in the presence of the others was assessed by entering the three chemicals as continuous variables in a single model with all covariates, and only β-HCH remained positively associated with the risk of cancer (OR:1.18, 95%CI: 1.05–1.34, wet-basis concentrations, data not shown in tables). In an attempt to assess the potential effect modification of the associations found, we searched for interactions between POP concentrations in the final model by entering the following interaction terms: β-HCH × heptachlor, β-HCH × *p,p′*-DDE, and heptachlor × *p,p′*-DDE; however, no statistically significant interaction was found (data not shown in tables).

4. Discussion

In our study, high serum concentrations of β-HCH, *p,p′*-DDE, and heptachlor were associated with a greater risk of breast cancer in the adjusted models, and only β-HCH remained positively associated when the three chemicals were entered in a single model with all covariates. Many OCPs and PCBs have proven to interact with estrogen and/or androgen receptors (Bonefeld Jorgensen et al., 1997; Bonefeld-Jorgensen et al., 2001; Grunfeld and Bonefeld-Jorgensen, 2004; Sonnenschein and Soto, 1998; Soto et al., 1998), and epidemiological efforts have traditionally focused on hormone-dependent cancers, e.g., breast and prostate tumors (Xu et al., 2010). In this regard, the endocrine disrupting properties of these chemicals have been demonstrated in previous studies. β-HCH has been reported to promote

Table 1
Description of the study population.

	Cases (n = 69)					Controls (n = 54)					p-Value
	Mean	SD[a]	Percentiles			Mean	SD[a]	Percentiles			
			25th	50th	75th			25th	50th	75th	
Age (years)	49.9	11.0	42.0	49.0	57.5	43.9	8.7	38.0	43.5	50.0	0.001
Body mass index (kg/m^2)	27.1	5.5	22.9	26.2	30.5	26.7	4.4	23.8	26.1	28.6	0.967
Age at menarche (years)	13.0	1.6	12.0	13.0	14.0	12.9	1.5	12.0	13.0	13.3	0.881
Number of children	2.8	2.0	1.5	3.0	4.0	2.2	1.4	1.0	2.0	3.0	0.056
Accumulated breastfeeding time (months)	31.4	38.5	2.0	18.0	48.0	23.3	28.3	5.3	12.0	36.0	0.663
Age at last breastfeeding (years)	27.1	14.8	27.0	32.0	38.0	28.5	13.0	28.3	32.5	36.8	0.901
Size of first nodule (cm)	2.3	2.6	0.3	1.8	3.5	–	–	–	–	–	
		n	%				n	%			
Marital status											0.999
Single		10	14.5				8	14.8			
Married/divorced		59	85.5				46	85.2			
Occupational class											<0.001
Homemaker		49	71.0				15	27.8			
Manual worker		5	7.3				8	14.8			
Non-manual worker		15	21.7				31	57.4			
Education											<0.001
Up to primary		53	76.8				19	35.2			
Secondary		4	5.8				22	40.7			
University		12	17.4				13	24.1			
Residence											<0.001
Urban		40	58.0				48	88.9			
Rural		29	42.0				6	11.1			
Menopausal status											0.006
Pre menopausal		34	49.3				40	74.1			
Post menopausal		35	50.7				14	25.9			
Parity											0.133
Nulliparous		14	20.3				8	14.8			
≥ 1 children		55	79.7				46	85.2			
Family history of breast cancer		17	24.6				0	0.0			–
Smoker		2	2.9				2	3.7			0.999
Alcohol consumption		0	0.0				0	0.0			–

[a] Standard deviation.

tumors in mice (Wong and Matsumura, 2007), as well as the transformation and invasiveness of human MCF-7 breast cancer cells (Zou and Matsumura, 2003). In addition, heptachlor has been shown to induce estrogenic effects in various in vitro assays (Chow et al., 2013) or human breast preneoplastic and cancerous cell lines (Shekhar et al., 1997), and to modulate estrogenic function in rainbow trout hepatocytes (Okoumassoun et al., 2002). However, Kim et al. (2011) found no evidence of estrogenic activity induced by heptachlor in a stably

Table 2
POP concentrations in cases and controls.

		Cases (n = 69)						Controls (n = 54)						p-Value
		% ≥ LOD[a]	Mean	SD[b]	Percentiles			% ≥ LOD[a]	Mean	SD[b]	Percentiles			
					25th	50th	75th				25th	50th	75th	
β-HCH	ng/mL	55.1	0.14	0.32	<LOD	0.05	0.11	33.3	<LOD	–	<LOD	<LOD	0.05	0.005
	ng/g lipid		25.17	57.74	<LOD	9.29	18.06		<LOD	–	<LOD	<LOD	9.51	0.003
α-Endosulfan	ng/mL	26.1	<LOD	–	<LOD	<LOD	0.05	33.3	<LOD	–	<LOD	<LOD	0.05	0.508
	ng/g lipid		–	–	<LOD	<LOD	7.76		<LOD	–	<LOD	<LOD	7.92	0.531
Endosulfan-ether	ng/mL	0.0	<LOD	–	<LOD	<LOD	<LOD	0.0	<LOD	–	<LOD	<LOD	<LOD	–
	ng/g lipid		<LOD	–	<LOD	<LOD	<LOD		<LOD	–	<LOD	<LOD	<LOD	–
HCB	ng/mL	94.2	0.19	0.16	0.09	0.13	0.22	100	0.14	0.06	0.09	0.12	0.16	0.151
	ng/g lipid		33.48	29.23	17.09	21.60	40.24		23.91	10.02	18.25	19.98	28.22	0.201
Heptachlor	ng/mL	89.9	0.13	0.09	0.07	0.12	0.15	79.6	0.09	0.06	0.05	0.08	0.11	0.001
	ng/g lipid		22.49	15.12	13.05	19.90	26.57		14.88	14.86	6.36	12.39	16.33	0.001
Oxychlordane	ng/mL	7.2	<LOD	–	<LOD	<LOD	<LOD	3.7	<LOD	–	<LOD	<LOD	<LOD	0.740
	ng/g lipid		<LOD	–	<LOD	<LOD	<LOD		<LOD	–	<LOD	<LOD	<LOD	0.582
PCB 138	ng/mL	98.6	0.21	0.10	0.14	0.18	0.23	100	0.17	0.08	0.13	0.15	0.18	0.003
	ng/g lipid		37.74	19.37	25.68	31.45	42.60		28.59	16.64	22.26	26.08	31.33	0.002
PCB 153	ng/mL	100	0.72	0.27	0.53	0.68	0.83	100	0.68	0.21	0.54	0.62	0.76	0.463
	ng/g lipid		131.54	51.58	94.13	118.95	149.07		119.07	35.96	88.90	111.99	149.57	0.412
PCB 180	ng/mL	98.6	0.23	0.12	0.15	0.19	0.27	98.1	0.18	0.06	0.14	0.17	0.23	0.087
	ng/g lipid		41.35	24.57	26.05	34.78	49.01		31.74	11.00	22.76	29.84	40.44	0.095
p,p′-DDE	ng/mL	100	2.10	3.05	0.60	1.07	2.08	98.1	1.07	1.18	0.38	0.60	1.53	0.009
	ng/g lipid		381.97	562.49	106.18	196.49	362.86		215.05	214.72	66.02	127.59	276.93	0.008

[a] Limit of detection.
[b] Standard deviation.

Table 3
Serum POP concentrations and risk of breast cancer. Logistic regression analyses.

	Wet-basis model						Lipid-basis model					
	Unadjusted model			Adjusted model[a]			Unadjusted model			Adjusted model[a]		
	OR	95% CI[b]		OR	95% CI[b]		OR	95% CI[b]		OR	95% CI[b]	
		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper
β-HCH (ng/dL)	1.09**	1.02	1.19	1.16**	1.05	1.30	1.05**	1.01	1.11	1.10**	1.03	1.18
<LOD	1.00	–	–	1.00	–	–	1.00	–	–	1.00	–	–
>LOD	2.45**	1.18	5.21	3.44**	1.30	9.72	2.45**	1.18	5.21	3.44**	1.30	9.72
α-Endosulfan (ng/dL)	0.99	0.92	1.07	0.96	0.87	1.06	1.00	0.95	1.04	0.98	0.92	1.03
<LOD	1.00	–	–	1.00	–	–	1.00	–	–	1.00	–	–
>LOD	0.71	0.32	1.54	0.72	0.25	1.98	0.71	0.32	1.54	0.72	0.25	1.98
HCB (ng/dL)	1.04**	1.01	1.09	1.04	0.98	1.12	1.03**	1.00	1.06	1.02	0.99	1.07
T1	1.00	–	–	1.00	–	–	1.00	–	–	1.00	–	–
T2	0.91	0.38	2.16	1.95	0.58	6.99	0.55	0.23	1.32	1.21	0.36	4.15
T3	2.05	0.84	5.13	2.73	0.62	13.01	1.89	0.77	4.80	2.94	0.68	13.80
Heptachlor (ng/dL)	1.09**	1.03	1.17	1.06*	1.00	1.15	1.05**	1.01	1.09	1.03*	1.00	1.08
T1	1.00	–	–	1.00	–	–	1.00	–	–	1.00	–	–
T2	1.34	0.56	3.24	1.31	0.42	4.14	1.34	0.56	3.24	1.27	0.40	4.07
T3	5.02**	1.97	13.74	3.24*	0.92	12.18	5.02**	1.97	13.74	2.87*	0.93	10.63
PCB 138 (ng/dL)	1.06**	1.01	1.12	1.03	0.97	1.10	1.04**	1.01	1.07	1.02	0.99	1.06
T1	1.00	–	–	1.00	–	–	1.00	–	–	1.00	–	–
T2	1.48	0.62	3.58	2.06	0.58	7.77	1.22	0.51	2.92	2.31	0.67	8.54
T3	4.38**	1.74	11.67	4.40	0.90	18.88	3.96**	1.58	10.52	2.99	0.75	13.42
PCB 153 (ng/dL)	1.01*	0.99	1.02	1.01	0.98	1.03	1.01*	1.00	1.02	1.01	0.99	1.02
T1	1.00	–	–	1.00	–	–	1.00	–	–	1.00	–	–
T2	1.01	0.42	2.39	1.08	0.30	3.91	0.91	0.38	2.16	0.63	0.17	2.16
T3	1.35	0.56	3.28	1.23	0.29	5.31	1.50	0.62	3.66	1.25	0.31	5.11
PCB 180 (ng/dL)	1.05**	1.01	1.10	1.03	0.96	1.10	1.03**	1.01	1.05	1.01	0.98	1.06
T1	1.00	–	–	1.00	–	–	1.00	–	–	1.00	–	–
T2	1.34	0.56	3.23	1.28	0.36	4.67	1.48	0.62	3.58	1.33	0.40	4.45
T3	1.82	0.76	4.46	1.62	0.37	7.32	1.64	0.69	3.99	1.11	0.26	4.64
p,p′-DDE (ng/mL)	1.33**	1.06	1.83	1.72**	1.11	3.13	1.18**	1.04	1.42	1.37**	1.07	1.94
T1	1.00	–	–	1.00	–	–	1.00	–	–	1.00	–	–
T2	3.73**	1.52	9.57	7.79**	2.04	35.31	2.71**	1.12	6.75	6.26**	1.62	28.33
T3	3.00**	1.24	7.54	7.08**	1.15	38.47	3.01**	1.24	7.58	9.65**	1.81	63.33

In order to facilitate interpretation of the coefficients, wet-basis concentrations were all entered in units of ng/mL, with the exception of *p,p′-DDE* (ng/dL), while lipid-basis concentrations were all entered in units of mg/g lipid, with the exception of *p,p′-DDE* (ng/g lipid).
NS: Not statistically significant. T: Tertiles of POP concentrations.
[a] Adjusted for age, body mass index, occupational class, residence, education, accumulated lactation time, parity, menopausal status, family history of breast cancer, total serum lipids.
[b] Confidence interval.
* $p < 0.100$.
** $p < 0.050$.

transfected human estrogen receptor-α transcriptional activation assay. Furthermore, researchers using MCF-7 breast cancer cells observed that several constituents of the pesticide DDT (i.e., *o,p′*-DDT, *o,p′*-DDE, *p,p′*-DDT, and *p,p′*-DDE) induced cell proliferation (Andersen et al., 1999; Shekhar et al., 1997; Soto et al., 1995).

In addition, there is a growing evidence that these chemicals might also cause cancer via other action mechanisms, including: disruption of the epigenomic landscape in cancers (reviewed by: Knower et al., 2014), the induction of enzymes that produce genotoxic intermediates and DNA adduct (Yanez et al., 2004), and an increase in reactive oxygen and nitrogen species through the induction of cytochrome P450 or mitochondrial alterations (Karami-Mohajeri and Abdollahi, 2011). This issue becomes even more complex if we take into account evidence of potential gene–environment interactions affecting the putative relationship between POP exposure and breast cancer, which include a potential modifying effect of cytochrome P4501A1 (CYP1A1) and the p53 gene on the effect of PCBs on cancer (Hoyer et al., 2002; Laden et al., 2002; Moysich et al., 1999; Zhang et al., 2004).

At an epidemiological level, a number of studies have investigated the association between human exposure to POPs and the risk of breast cancer, but their conclusions have been controversial, with some authors reporting positive associations but many others finding no evidence to support a causal association (Cassidy et al., 2005; Charlier et al., 2003a,2003b; Demers et al., 2000, 2002; Gatto et al., 2007; Hoyer et al., 2001; Itoh et al., 2009; Laden et al., 2001; Lopez-Carrillo et al., 1997, 2002; Lopez-Cervantes et al., 2004; Olaya-Contreras et al., 1998; Recio-Vega et al., 2011; Snedeker, 2001; Ward et al., 2000; Wolff et al., 2000a,2000b; Zheng et al., 1999a,1999b). Hoyer et al. (2001) found no association between organochlorine pesticides and breast cancer but observed that they might contribute to a worse prognosis. These discrepancies may be attributable to various factors, including differences in study designs, in biological matrices used to estimate exposure, and in target populations, with highly varied historical and current exposure levels to POPs and distinct ethnicities, age groups and/or dietary patterns. Epidemiological studies reporting positive associations also differ in the chemicals responsible for the observed effect, including POPs such as DDT and HCB (Charlier et al., 2003a), *p,p′*-DDE (Olaya-Contreras et al., 1998), or PCBs (Recio-Vega et al., 2011).

We cannot rule out that the associations found with single chemicals are a surrogate of exposure to other unmeasured pollutants with similar physicochemical properties or even to mixtures of pollutants that exert a combined effect. In fact, heptachlor epoxide levels (an oxidation product and one of the most important metabolites of heptachlor) were previously found to be positively associated with the prevalence of breast cancer in biopsies and to contribute to the initiation, promotion, and progression of cancer (Cassidy et al., 2005). Likewise, the risk of breast cancer was found to increase with higher adipose tissue concentrations of the pesticide lindane (in which β-HCH is commonly present) in a case–control study of a female population recruited in Southern Spain (Ibarluzea et al., 2004). The concentrations of many POPs are often positively correlated, which poses methodological problems in the statistical modeling (Holford et al., 2000). In this regard, our population

showed positive correlations between β-HCH and HCB levels (Spearman coefficient = 0.27, $p < 0.05$, Supplementary Material) and between heptachlor and the concentrations of the three PCB congeners (Spearman coefficients = 0.2–0.3, $p < 0.05$, Supplementary Material). In our study, no statistically significant interaction was found between POP concentrations in the final model. However, there is a need to developing biomarkers of combined effects in order to improve our understanding of potential interactions among chemicals. Assessment of the effects of human exposure to environmental chemicals is a highly complicated issue, given that most individuals are exposed to complex mixtures of chemicals that can exert synergic and/or antagonistic effects (Aube et al., 2011) and can interact with endogenous hormones (Bonefeld-Jorgensen et al., 2014; Sonnenschein and Soto, 1998; Soto et al., 1998). In a study of a population from the Canary Islands (Spain), Boada et al. (2012) concluded that a combination of aldrin, *p,p′*-DDE and dichlorodiphenyldichloroethane may represent a risk factor for breast cancer.

The development and treatment of cancer usually implies modifications in metabolism and adipose tissue mobilization, which might alter serum POP concentrations (Boada et al., 2012). Hence, it is theoretically possible that the higher concentrations observed in cases are a consequence of the disease rather than the other way round. However, as argued by Charlier et al. (2003b), this seems unlikely given that samples were taken at the time of diagnosis in individuals with no history of cancer and before the start of chemotherapy or radiotherapy.

The use of serum POP concentrations has some limitations in comparison to adipose tissue. It has been reported that serum POP levels are good estimators of ongoing exposure but not always of long-term exposure, because they can be affected by lipid mobilization and current exposure levels (Archibeque-Engle et al., 1997; Crinnion, 2009). However, in the present study, all women were sampled under 12-h fasting conditions and adjustment for total serum lipids was performed in the multivariable models, which are known methods for minimizing this bias, at least in part (Charlier et al., 2003b; Schisterman et al., 2005).

No adjustment for food intake was performed in the present study. Diet, especially of animal origin, has been acknowledged as an important risk factor for cancer (Vieira et al., 2011) but is also responsible for most of the POP exposure in the general population (Agudo et al., 2009). Our hypothesis is that previously observed positive associations between the consumption of certain fatty foods and cancer risk (Jordan et al., 2013; Khodarahmi and Azadbakht, 2014) might be partially caused by the POP present in these food items. Therefore, we consider that adjustment for diet would imply the inclusion of covariates that are in the same causal pathway (diet ➔ POPs ➔ cancer).

In the present study, serum concentrations of organochlorine pesticides were comparable to those reported previously in some populations in Tunisia (Ben Hassine et al., 2014; Ennaceur and Driss, 2010) and other Mediterranean countries, such as Egypt (Ahmed et al., 2002), Italy (Bergonzi et al., 2009), Spain (Llop et al., 2010), or Greece (Vafeiadi et al., 2014). However, PCB concentrations were in general higher than those reported in other Tunisian populations and were comparable to those reported in more industrialized Mediterranean countries such as Italy (Bergonzi et al., 2009) and Spain (Llop et al., 2010), which may be attributable to the recent rapid technological development of Tunisia.

Although the sample size of our study was relatively limited, it yielded significant results that were consistent in both bivariate and multivariable models. The findings of the present study suggest a potential association between baseline serum concentrations of at least one organochlorine pesticide and breast cancer, underlining the need for measures to reduce current exposure levels. However, our results should be interpreted with caution and need to be verified in further studies.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.03.045.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors gratefully acknowledge scientific and technical assistance provided by Richard Davies. Dr J.P. Arrebola is currently receiving a postdoctoral fellowship from the Consejería de Igualdad, Salud y Políticas Sociales, Junta de Andalucía, Spain (RH-0092-2013). F Artacho-Cordón has a fellowship from the Spanish Ministry of Education (AP2012-2524). This study was supported in part by research grants from CIBER de Epidemiología and Junta de Andalucía (SAS 01/264, P09-CTS-5488, SAS PI-0675-2010, EF-0708-2013, and PI-0513/2012) and by the Ministry of Higher Education and Scientific Research of Tunisia, who granted H. Belhassen with a scholarship. The results would not have been achieved without the selfless collaboration of the patients who took part in the study.

References

Adami, H.O., Lipworth, L., Titus-Ernstoff, L., Hsieh, C.C., Hanberg, A., Ahlborg, U., Baron, J., Trichopoulos, D., 1995. Organochlorine compounds and estrogen-related cancers in women. Cancer Causes Control 6, 551–566.

Agudo, A., Goni, F., Etxeandia, A., Vives, A., Millan, E., Lopez, R., Amiano, P., Ardanaz, E., Barricarte, A., Chirlaque, M.D., Dorronsoro, M., Jakszyn, P., Larranaga, N., Martinez, C., Navarro, C., Rodriguez, L., Sanchez, M.J., Tormo, M.J., Gonzalez, C.A., 2009. Polychlorinated biphenyls in Spanish adults: determinants of serum concentrations. Environ. Res. 109, 620–628.

Ahmed, M.T., Loutfy, N., El, S.E., 2002. Residue levels of DDE and PCBs in the blood serum of women in the Port Said region of Egypt. J. Hazard Mater. 89, 41–48.

Andersen, H.R., Andersson, A.M., Arnold, S.F., Autrup, H., Barfoed, M., Beresford, N.A., Bjerregaard, P., Christiansen, L.B., Gissel, B., Hummel, R., Jorgensen, E.B., Korsgaard, B., Le, G.R., Leffers, H., McLachlan, J., Moller, A., Nielsen, J.B., Olea, N., Oles-Karasko, A., Pakdel, F., Pedersen, K.L., Perez, P., Skakkeboek, N.E., Sonnenschein, C., Soto, A.M., 1999. Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. Environ. Health Perspect. 107 (Suppl. 1), 89–108.

Andersen, H.R., Vinggaard, A.M., Rasmussen, T.H., Gjermandsen, I.M., Bonefeld-Jorgensen, E.C., 2002. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. Toxicol. Appl. Pharmacol. 179, 1–12.

APEK, 2005. Tunisia Country Situation Report. The International POPs Elimination Project, Tunisia.

Archibeque-Engle, S.L., Tessari, J.D., Winn, D.T., Keefe, T.J., Nett, T.M., Zheng, T., 1997. Comparison of organochlorine pesticide and polychlorinated biphenyl residues in human breast adipose tissue and serum. J. Toxicol. Environ. Health 52, 285–293.

Arrebola, J.P., Martin-Olmedo, P., Fernandez, M.F., Sanchez-Cantalejo, E., Jimenez-Rios, J.A., Torne, P., Porta, M., Olea, N., 2009. Predictors of concentrations of hexachlorobenzene in human adipose tissue: a multivariate analysis by gender in Southern Spain. Environ. Int. 35, 27–32.

Arrebola, J.P., Fernandez, M.F., Molina-Molina, J.M., Martin-Olmedo, P., Exposito, J., Olea, N., 2012. Predictors of the total effective xenoestrogen burden (TEXB) in human adipose tissue. A pilot study. Reprod. Toxicol. 33, 45–52.

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

Aube, M., Larochelle, C., Ayotte, P., 2011. Differential effects of a complex organochlorine mixture on the proliferation of breast cancer cell lines. Environ. Res. 111, 337–347.

Belhassen, H., Jiménez-Díaz, I., Arrebola, JP, Ghali, R, Ghorbel, H., Olea, N., Hedili, A., 2015. Zearalenone and its metabolites in urine and breast cancer risk: A case-control study in Tunisia. Chemosphere 128C, 1–6. http://dx.doi.org/10.1016/j.chemosphere.2014.12.055.

Ben Hassine, S., Hammami, B., Ben, A.W., El, M.Y., Barhoumi, B., El, A.R., Driss, M.R., 2014. Concentrations of organochlorine pesticides and polychlorinated biphenyls in human serum and their relation with age, gender, and BMI for the general population of Bizerte, Tunisia. Environ. Sci. Pollut. Res. Int. 21, 6303–6313.

Bergonzi, R., Specchia, C., Dinolfo, M., Tomasi, C., De, P.G., Frusca, T., Apostoli, P., 2009. Distribution of persistent organochlorine pollutants in maternal and foetal tissues: data from an Italian polluted urban area. Chemosphere 76, 747–754.

Boada, L.D., Zumbado, M., Henriquez-Hernandez, L.A., Almeida-Gonzalez, M., Alvarez-Leon, E.E., Serra-Majem, L., Luzardo, O.P., 2012. Complex organochlorine pesticide mixtures as determinant factor for breast cancer risk: a population-based case-control study in the Canary Islands (Spain). Environ. Health 11, 28.

Bonefeld Jorgensen, E.C., Autrup, H., Hansen, J.C., 1997. Effect of toxaphene on estrogen receptor functions in human breast cancer cells. Carcinogenesis 18, 1651–1654.

Bonefeld-Jorgensen, E.C., Andersen, H.R., Rasmussen, T.H., Vinggaard, A.M., 2001. Effect of highly bioaccumulated polychlorinated biphenyl congeners on estrogen and androgen receptor activity. Toxicology 158, 141–153.

Bonefeld-Jorgensen, E.C., Ghisari, M., Wielsoe, M., Bjerregaard-Olesen, C., Kjeldsen, L.S., Long, M., 2014. Biomonitoring and hormone-disrupting effect biomarkers of

persistent organic pollutants in vitro and ex vivo. Basic Clin. Pharmacol. Toxicol. 115, 118–128.
Brauner, E.V., Raaschou-Nielsen, O., Gaudreau, E., Leblanc, A., Tjonneland, A., Overvad, K., Sorensen, M., 2012. Predictors of adipose tissue concentrations of organochlorine pesticides in a general Danish population. J. Exposure Anal. Environ. Epidemiol. 22, 52–59.
Brophy, J.T., Keith, M.M., Watterson, A., Park, R., Gilbertson, M., Maticka-Tyndale, E., Beck, M., Abu-Zahra, H., Schneider, K., Reinhartz, A., Dematteo, R., Luginaah, I., 2012. Breast cancer risk in relation to occupations with exposure to carcinogens and endocrine disruptors: a Canadian case-control study. Environ. Health 11, 87.
Cassidy, R.A., Natarajan, S., Vaughan, G.M., 2005. The link between the insecticide heptachlor epoxide, estradiol, and breast cancer. Breast Cancer Res. Treat. 90, 55–64.
Charlier, C., Albert, A., Herman, P., Hamoir, E., Gaspard, U., Meurisse, M., Plomteux, G., 2003a. Breast cancer and serum organochlorine residues. Occup. Environ. Med. 60, 348–351.
Charlier, C., Pitance, F., Plomteux, G., 2003b. PCB residues in a breast cancer patient population. Bull. Environ. Contam. Toxicol. 71, 887–891.
Chow, W.S., Chan, W.K., Chan, K.M., 2013. Toxicity assessment and vitellogenin expression in zebrafish (Danio rerio) embryos and larvae acutely exposed to bisphenol A, endosulfan, heptachlor, methoxychlor and tetrabromobisphenol A. J. Appl. Toxicol. 33, 670–678.
Crinnion, W.J., 2009. Chlorinated pesticides: threats to health and importance of detection. Altern. Med. Rev. 14, 347–359.
Dasgupta, S., Meisner, C., Wheeler, D., 2010. Stockpiles of obsolete pesticides and cleanup priorities: a methodology and application for Tunisia. J. Environ. Manag. 91, 824–830.
Demers, A., Ayotte, P., Brisson, J., Dodin, S., Robert, J., Dewailly, E., 2000. Risk and aggressiveness of breast cancer in relation to plasma organochlorine concentrations. Cancer Epidemiol. Biomarkers Prev. 9, 161–166.
Demers, A., Ayotte, P., Brisson, J., Dodin, S., Robert, J., Dewailly, E., 2002. Plasma concentrations of polychlorinated biphenyls and the risk of breast cancer: a congener-specific analysis. Am. J. Epidemiol. 155, 629–635.
Ennaceur, S., Driss, M.R., 2010. Serum organochlorine pesticide and polychlorinated biphenyl levels measured in delivering women from different locations in Tunisia. Int. J. Environ. Anal. Chem. 90, 821–828.
Gatto, N.M., Longnecker, M.P., Press, M.F., Sullivan-Halley, J., McKean-Cowdin, R., Bernstein, L., 2007. Serum organochlorines and breast cancer: a case-control study among African-American women. Cancer Causes Control 18, 29–39.
Grunfeld, H.T., Bonefeld-Jorgensen, E.C., 2004. Effect of in vitro estrogenic pesticides on human oestrogen receptor alpha and beta mRNA levels. Toxicol. Lett. 151, 467–480.
Holford, T.R., Zheng, T., Mayne, S.T., Zahm, S.H., Tessari, J.D., Boyle, P., 2000. Joint effects of nine polychlorinated biphenyl (PCB) congeners on breast cancer risk. Int. J. Epidemiol. 29, 975–982.
Hoyer, A.P., Jorgensen, T., Rank, F., Grandjean, P., 2001. Organochlorine exposures influence on breast cancer risk and survival according to estrogen receptor status: a Danish cohort-nested case-control study. BMC Cancer 1, 8.
Hoyer, A.P., Gerdes, A.M., Jorgensen, T., Rank, F., Hartvig, H.B., 2002. Organochlorines, p53 mutations in relation to breast cancer risk and survival. A Danish cohort-nested case-controls study. Breast Cancer Res. Treat. 71, 59–65.
Ibarluzea, J.J., Fernandez, M.F., Santa-Marina, L., Olea-Serrano, M.F., Rivas, A.M., Aurrekoetxea, J.J., Exposito, J., Lorenzo, M., Torne, P., Villalobos, M., Pedraza, V., Sasco, A.J., Olea, N., 2004. Breast cancer risk and the combined effect of environmental estrogens. Cancer Causes Control 15, 591–600.
Itoh, H., Iwasaki, M., Hanaoka, T., Kasuga, Y., Yokoyama, S., Onuma, H., Nishimura, H., Kusama, R., Tsugane, S., 2009. Serum organochlorines and breast cancer risk in Japanese women: a case-control study. Cancer Causes Control 20, 567–580.
Jordan, I., Hebestreit, A., Swai, B., Krawinkel, M.B., 2013. Dietary patterns and breast cancer risk among women in northern Tanzania: a case-control study. Eur. J. Nutr. 52, 905–915.
Karami-Mohajeri, S., Abdollahi, M., 2011. Toxic influence of organophosphate, carbamate, and organochlorine pesticides on cellular metabolism of lipids, proteins, and carbohydrates: a systematic review. Hum. Exp. Toxicol. 30, 1119–1140.
Key, T., Appleby, P., Barnes, I., Reeves, G., 2002. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J. Natl. Cancer Inst. 94, 606–616.
Khodarahmi, M., Azadbakht, L., 2014. The association between different kinds of fat intake and breast cancer risk in women. Int. J. Prev. Med. 5, 6–15.
Kim, T.S., Kim, C.Y., Lee, H.K., Kang, I.H., Kim, M.G., Jung, K.K., Kwon, Y.K., Nam, H.S., Hong, S.K., Kim, H.S., Yoon, H.J., Rhee, G.S., 2011. Estrogenic activity of persistent organic pollutants and parabens based on the stably transfected human estrogen receptor-alpha transcriptional activation assay (OECD TG 455). Toxicol. Res. 27, 181–184.
Knower, K.C., To, S.Q., Leung, Y.K., Ho, S.M., Clyne, C.D., 2014. Endocrine disruption of the epigenome: a breast cancer link. Endocr. Relat. Cancer 21, T33–T55.
La Rocca, C., Mantovani, A., 2006. From environment to food: the case of PCB. Ann. Ist. Super. Sanita 42, 410–416.
Laden, F., Collman, G., Iwamoto, K., Alberg, A.J., Berkowitz, G.S., Freudenheim, J.L., Hankinson, S.E., Helzlsouer, K.J., Holford, T.R., Huang, H.Y., Moysich, K.B., Tessari, J.D., Wolff, M.S., Zheng, T., Hunter, D.J., 2001. 1,1-Dichloro-2,2-bis(p-chlorophenyl)ethylene and polychlorinated biphenyls and breast cancer: combined analysis of five U.S. studies. J. Natl. Cancer Inst. 93, 768–776.
Laden, F., Ishibe, N., Hankinson, S.E., Wolff, M.S., Gertig, D.M., Hunter, D.J., Kelsey, K.T., 2002. Polychlorinated biphenyls, cytochrome P450 1A1, and breast cancer risk in the Nurses' Health Study. Cancer Epidemiol. Biomarkers Prev. 11, 1560–1565.
Lazaar-Ben, G.H., Hajjem, S., Aounallah-Skhiri, H., Achour, N., Hsairi, M., 2011. Mortality from cancer in Tunisia: calculating years of life lost. Sante Publique 23, 31–40.
Llop, S., Ballester, F., Vizcaino, E., Murcia, M., Lopez-Espinosa, M.J., Rebagliato, M., Vioque, J., Marco, A., Grimalt, J.O., 2010. Concentrations and determinants of organochlorine levels among pregnant women in Eastern Spain. Sci. Total Environ. 408, 5758–5767.
Lopez-Carrillo, L., Blair, A., Lopez-Cervantes, M., Cebrian, M., Rueda, C., Reyes, R., Mohar, A., Bravo, J., 1997. Dichlorodiphenyltrichloroethane serum levels and breast cancer risk: a case-control study from Mexico. Cancer Res. 57, 3728–3732.
Lopez-Carrillo, L., Lopez-Cervantes, M., Torres-Sanchez, L., Blair, A., Cebrian, M.E., Garcia, R.M., 2002. Serum levels of beta-hexachlorocyclohexane, hexachlorobenzene and polychlorinated biphenyls and breast cancer in Mexican women. Eur. J. Cancer Prev. 11, 129–135.
Lopez-Cervantes, M., Torres-Sanchez, L., Tobias, A., Lopez-Carrillo, L., 2004. Dichlorodiphenyldichloroethane burden and breast cancer risk: a meta-analysis of the epidemiologic evidence. Environ. Health Perspect. 112, 207–214.
Maalej, M., Hentati, D., Messai, T., Kochbati, L., El, M.A., Mrad, K., Romdhane, K.B., Ben, A.M., Zouari, B., 2008. Breast cancer in Tunisia in 2004: a comparative clinical and epidemiological study. Bull. Cancer 95, E5–E9.
McPherson, K., Steel, C.M., Dixon, J.M., 2000. ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. BMJ 321, 624–628.
Moysich, K.B., Shields, P.G., Freudenheim, J.L., Schisterman, E.F., Vena, J.E., Kostyniak, P., Greizerstein, H., Marshall, J.R., Graham, S., Ambrosone, C.B., 1999. Polychlorinated biphenyls, cytochrome P4501A1 polymorphism, and postmenopausal breast cancer risk. Cancer Epidemiol. Biomarkers Prev. 8, 41–44.
Okoumassoun, L.E., Averill-Bates, D., Gagne, F., Marion, M., Denizeau, F., 2002. Assessing the estrogenic potential of organochlorine pesticides in primary cultures of male rainbow trout (*Oncorhynchus mykiss*) hepatocytes using vitellogenin as a biomarker. Toxicology 178, 193–207.
Olaya-Contreras, P., Rodriguez-Villamil, J., Posso-Valencia, H.J., Cortez, J.E., 1998. Organochlorine exposure and breast cancer risk in Colombian women. Cad. Saude Publica 14 (Suppl. 3), 125–132.
Parkin, D.M., Bray, F., Ferlay, J., Pisani, P., 2005. Global cancer statistics, 2002. CA Cancer J. Clin. 55, 74–108.
Phillips, D.L., Pirkle, J.L., Burse, V.W., Bernert Jr., J.T., Henderson, L.O., Needham, L.L., 1989. Chlorinated hydrocarbon levels in human serum: effects of fasting and feeding. Arch. Environ. Contam. Toxicol. 18, 495–500.
RCNT, 1998. Registre des cancers Nord Tunisie (RCNT), données (1995–1998).
RCNT, 2006. Registre des cancers Nord Tunisie (RCNT), données (2004–2006). Available at. http://www.insp.rns.tn/doc/cancer/cancer17.pdf.
Recio-Vega, R., Velazco-Rodriguez, V., Ocampo-Gomez, G., Hernandez-Gonzalez, S., Ruiz-Flores, P., Lopez-Marquez, F., 2011. Serum levels of polychlorinated biphenyls in Mexican women and breast cancer risk. J. Appl. Toxicol. 31, 270–278.
Schisterman, E.F., Whitcomb, B.W., Louis, G.M., Louis, T.A., 2005. Lipid adjustment in the analysis of environmental contaminants and human health risks. Environ. Health Perspect. 113, 853–857.
Scribner, J.D., Mottet, N.K., 1981. DDT acceleration of mammary gland tumors induced in the male Sprague–Dawley rat by 2-acetamidophenanthrene. Carcinogenesis 2, 1235–1239.
Shekhar, P.V., Werdell, J., Basrur, V.S., 1997. Environmental estrogen stimulation of growth and estrogen receptor function in preneoplastic and cancerous human breast cell lines. J. Natl. Cancer Inst. 89, 1774–1782.
Snedeker, S.M., 2001. Pesticides and breast cancer risk: a review of DDT, DDE, and dieldrin. Environ. Health Perspect. 109 (Suppl. 1), 35–47.
Sonnenschein, C., Soto, A.M., 1998. An updated review of environmental estrogen and androgen mimics and antagonists. J. Steroid Biochem. Mol. Biol. 65, 143–150.
Soto, A.M., Chung, K.L., Sonnenschein, C., 1994. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environ. Health Perspect. 102, 380–383.
Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N., Serrano, F.O., 1995. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ. Health Perspect. 103 (Suppl. 7), 113–122.
Soto, A.M., Michaelson, C.L., Prechtl, N.V., Weill, B.C., Sonnenschein, C., Olea-Serrano, F., Olea, N., 1998. Assays to measure estrogen and androgen agonists and antagonists. Adv. Exp. Med. Biol. 444, 9–23.
Turci, R., Balducci, C., Brambilla, G., Colosio, C., Imbriani, M., Mantovani, A., Vellere, F., Minoia, C., 2010. A simple and fast method for the determination of selected organohalogenated compounds in serum samples from the general population. Toxicol. Lett. 192, 66–71.
UNEP, 2003. Regionally based assessment of persistent toxic substances. Mediterranean Regional Report. United Nations Environment Programme on Chemicals (www.chem.unep.ch/pts/regreports/mediterranean.pdf (last accesed June 25th 2014)).
Vafeiadi, M., Vrijheid, M., Fthenou, E., Chalkiadaki, G., Rantakokko, P., Kiviranta, H., Kyrtopoulos, S.A., Chatzi, L., Kogevinas, M., 2014. Persistent organic pollutants exposure during pregnancy, maternal gestational weight gain, and birth outcomes in the mother-child cohort in Crete, Greece (RHEA study). Environ. Int. 64, 116–123.
Vieira, F.G., Di Pietro, P.F., Boaventura, B.C., Ambrosi, C., Rockenbach, G., Fausto, M.A., Crippa, C.G., Da Silva, E.L., 2011. Factors associated with oxidative stress in women with breast cancer. Nutr. Hosp. 26, 528–536.
Ward, E.M., Schulte, P., Grajewski, B., Andersen, A., Patterson Jr., D.G., Turner, W., Jellum, E., Deddens, J.A., Friedland, J., Roeleveld, N., Waters, M., Butler, M.A., DiPietro, E., Needham, L.L., 2000. Serum organochlorine levels and breast cancer: a nested case-control study of Norwegian women. Cancer Epidemiol. Biomarkers Prev. 9, 1357–1367.
WHO, 2014. World Health Organization. Cancer Country Profiles, 2014.
Wolff, M.S., Berkowitz, G.S., Brower, S., Senie, R., Bleiweiss, I.J., Tartter, P., Pace, B., Roy, N., Wallenstein, S., Weston, A., 2000a. Organochlorine exposures and breast cancer risk in New York City women. Environ. Res. 84, 151–161.
Wolff, M.S., Zeleniuch-Jacquotte, A., Dubin, N., Toniolo, P., 2000b. Risk of breast cancer and organochlorine exposure. Cancer Epidemiol. Biomarkers Prev. 9, 271–277.

Wong, P.S., Matsumura, F., 2007. Promotion of breast cancer by beta-hexachlorocyclohexane in MCF10AT1 cells and MMTV-neu mice. BMC Cancer 7, 130.
World Cancer Research Fund International, 2014. http://www.wcrf.org/index.php (last accesed June 25th 2014).
Xu, X., Dailey, A.B., Talbott, E.O., Ilacqua, V.A., Kearney, G., Asal, N.R., 2010. Associations of serum concentrations of organochlorine pesticides with breast cancer and prostate cancer in U.S. adults. Environ. Health Perspect. 118, 60–66.
Yager, J.D., Davidson, N.E., 2006. Estrogen carcinogenesis in breast cancer. N. Engl. J. Med. 354, 270–282.
Yanez, L., Borja-Aburto, V.H., Rojas, E., de la Fuente, H., Gonzalez-Amaro, R., Gomez, H., Jongitud, A.A., Diaz-Barriga, F., 2004. DDT induces DNA damage in blood cells. Studies in vitro and in women chronically exposed to this insecticide. Environ. Res. 94, 18–24.
Yu, G.W., Laseter, J., Mylander, C., 2011. Persistent organic pollutants in serum and several different fat compartments in humans. J. Environ. Public Health 2011, 417980.
Zhang, Y., Wise, J.P., Holford, T.R., Xie, H., Boyle, P., Zahm, S.H., Rusiecki, J., Zou, K., Zhang, B., Zhu, Y., Owens, P.H., Zheng, T., 2004. Serum polychlorinated biphenyls, cytochrome P-450 1A1 polymorphisms, and risk of breast cancer in Connecticut women. Am. J. Epidemiol. 160, 1177–1183.
Zheng, T., Holford, T.R., Mayne, S.T., Tessari, J., Owens, P.H., Zahm, S.H., Zhang, B., Dubrow, R., Ward, B., Carter, D., Boyle, P., 1999a. Environmental exposure to hexachlorobenzene (HCB) and risk of female breast cancer in Connecticut. Cancer Epidemiol. Biomarkers Prev. 8, 407–411.
Zheng, T., Holford, T.R., Mayne, S.T., Ward, B., Carter, D., Owens, P.H., Dubrow, R., Zahm, S.H., Boyle, P., Archibeque, S., Tessari, J., 1999b. DDE and DDT in breast adipose tissue and risk of female breast cancer. Am. J. Epidemiol. 150, 453–458.
Zou, E., Matsumura, F., 2003. Long-term exposure to beta-hexachlorocyclohexane (beta-HCH) promotes transformation and invasiveness of MCF-7 human breast cancer cells. Biochem. Pharmacol. 66, 831–840.

7.4. Objective 4. Investigate whether the combined effect of xenoestrogens circulating in the blood is associated with the risk of breast cancer in a population-based multicase-control study in Spain

TOTAL EFFECTIVE XENOESTROGEN BURDEN IN SERUM SAMPLES AND RISK FOR BREAST CANCER IN A POPULATION-BASED MULTICASE-CONTROL STUDY IN SPAIN

*Pastor-Barriuso R, Fernández MF, Castaño-Vinyals G, Whelan D, Pérez-Gómez B, Llorca J, Villanueva CM, Guevara M, Molina-Molina JM, **Artacho-Cordón F**, Barriuso-Lapresa L,Tusquets I, Dierssen-Sotos T, Aragonés N, Olea N, Kogevinas M, Pollán M*

Environmental Health Perspectives; Impact factor 7.980; 1st Quartile; 1st Decile

ABSTRACT

BACKGROUND: Most studies on endocrine disrupting chemicals and breast cancer have focused on single compounds with inconclusive findings.

OBJECTIVES: We assessed the combined estrogenic effect of mixtures of xenoestrogens in serum and its relation to breast cancer risk.

METHODS: A total of 186 incident pretreatment breast cancer cases and 196 frequency matched controls were randomly sampled from a large population-based multicase-control study in Spain. The total effective xenoestrogen burden attributable to organohalogenated xenoestrogens (TEXB-α) and endogenous hormones and more polar xenoestrogens (TEXB-β) were determined in serum samples by using high-performance liquid chromatography separation and E-Screen bioassay. Odds ratios for breast cancer comparing tertiles of serum TEXB-α and TEXB-β were estimated using logistic models, and smooth risk trends using spline models.

RESULTS: Cases had higher geometric mean TEXB-α and TEXB-β levels (8.32 and 9.94 Eeq pM/mL) than controls (2.99 and 5.96 Eeq pM/mL, respectively). The fully-adjusted odds ratios for breast cancer (95% confidence intervals) comparing the second and third tertiles of TEXB-α with the first tertile were 1.77 (0.76, 4.10) and 3.45 (1.50, 7.97), and those for TEXB-β were 2.35 (1.10, 5.03) and 4.01 (1.88, 8.56). A steady increase in risk was evident across all detected TEXB-α levels and a sigmoidal trend for TEXB-β. Individual xenoestrogens showed weak and opposed associations with breast cancer risk.

CONCLUSIONS: This is the first study to show a strong positive association between serum total xenoestrogen burden and breast cancer risk, thus highlighting the importance of evaluating xenoestrogen mixtures, rather than single compounds, when studying hormone-related cancers.

Accepted: 05th of May, 2016. Environmental Health Perspectives

Total Effective Xenoestrogen Burden in Serum Samples and Risk for Breast Cancer in a Population-Based Multicase-Control Study in Spain

Roberto Pastor-Barriuso,[1,2*] Mariana F. Fernández,[2,3*] Gemma Castaño-Vinyals,[2,4,5,6] Denis Whelan,[1,7,8] Beatriz Perez-Gomez,[1,2] Javier Llorca,[2,9] Cristina M. Villanueva,[2,4,5,6] Marcela Guevara,[2,10] Jose-Manuel Molina Molina,[3] Francisco Artacho-Cordon,[3] Laura Barriuso-Lapresa,[2,10] Ignasi Tusquets,[5,11,12] Trinidad Dierssen-Sotos,[2,9] Nuria Aragonés,[1,2] Nicolás Olea,[2,3] Manolis Kogevinas,[2,4,5,6] and Marina Pollan[1,2]

[1]National Center for Epidemiology, Carlos III Institute of Health, Madrid, Spain; [2]Consortium for Biomedical Research in Epidemiology and Public Health (CIBERESP), Madrid, Spain; [3]Biosanitary Institute of Granada (ibs.GRANADA), University of Granada, Granada, Spain; [4]Center for Research in Environmental Epidemiology (CREAL), Barcelona, Spain; [5]Hospital del Mar Medical Research Institute (IMIM), Barcelona, Spain; [6]Pompeu Fabra University (UPF), Barcelona, Spain; [7]USA-Spain Fulbright Commission for Cultural, Educational, and Scientific Exchange, Madrid, Spain; [8]Department of Biostatistics and Bioinformatics, Rollins School of Public Health, Emory University, Atlanta, Georgia, USA; [9]Division of Epidemiology and Computational Biology, School of Medicine, University of Cantabria, Santander, Spain; [10]Public Health Institute of Navarra, Pamplona, Spain; [11]Medicine Department, Autonomous University of Barcelona, Barcelona, Spain; [12]Medical Oncology Department, Hospital del Mar, Barcelona, Spain

*These authors contributed equally to this work.

Corresponding author: M. Pollán, National Center for Epidemiology, Carlos III Institute of Health, Monforte de Lemos 5, 28029 Madrid, Spain. Telephone: +34 91 822 26 35. E-mail: mpollan@isciii.es

Running title: Serum total xenoestrogen burden and breast cancer

Acknowledgments

This study was partially supported by Acción Transversal del Cancer, approved by the Spanish Ministry Council on October 11, 2007; Carlos III Institute of Health (grants PI08/1770, PI09/00773-Cantabria, PI11/00610, PI12/00265, PI12/00488, PI12/00715, and PI12/01270); and Fundación Marqués de Valdecilla (grant API 10/09). Biological samples were stored at biobanks supported by the Carlos III Institute of Health and the European Regional Development Fund (MAR Biobank RD09/0076/00036 and ISCIII Biobank RD09/0076/00108).

Competing financial interests

The authors declare they have no actual or potential competing financial interests.

41 **Abstract**

42 BACKGROUND: Most studies on endocrine disrupting chemicals and breast cancer have focused
43 on single compounds with inconclusive findings.

44 OBJECTIVES: We assessed the combined estrogenic effect of mixtures of xenoestrogens in serum
45 and its relation to breast cancer risk.

46 METHODS: A total of 186 incident pretreatment breast cancer cases and 196 frequency-matched
47 controls were randomly sampled from a large population-based multicase-control study in Spain.
48 The total effective xenoestrogen burden attributable to organohalogenated xenoestrogens
49 (TEXB-α) and endogenous hormones and more polar xenoestrogens (TEXB-β) were determined
50 in serum samples by using high-performance liquid chromatography separation and E-Screen
51 bioassay. Odds ratios for breast cancer comparing tertiles of serum TEXB-α and TEXB-β were
52 estimated using logistic models, and smooth risk trends using spline models.

53 RESULTS: Cases had higher geometric mean TEXB-α and TEXB-β levels (8.32 and 9.94 Eeq
54 pM/mL) than controls (2.99 and 5.96 Eeq pM/mL, respectively). The fully-adjusted odds ratios
55 for breast cancer (95% confidence intervals) comparing the second and third tertiles of TEXB-α
56 with the first tertile were 1.77 (0.76, 4.10) and 3.45 (1.50, 7.97), and those for TEXB-β were
57 2.35 (1.10, 5.03) and 4.01 (1.88, 8.56). A steady increase in risk was evident across all detected
58 TEXB-α levels and a sigmoidal trend for TEXB-β. Individual xenoestrogens showed weak and
59 opposed associations with breast cancer risk.

60 CONCLUSIONS: This is the first study to show a strong positive association between serum total
61 xenoestrogen burden and breast cancer risk, thus highlighting the importance of evaluating
62 xenoestrogen mixtures, rather than single compounds, when studying hormone-related cancers.

63

64 **Introduction**

65 Malignant breast tumors are the leading cause of cancer in women worldwide in terms of
66 incidence and mortality (Ferlay et al. 2013). In spite of efforts to elucidate breast cancer etiology,
67 genetic determinants and well-established risk factors explain a limited amount of the global
68 burden of this disease (Barnes et al. 2011; Howell et al. 2014; Sprague et al. 2008). It is
69 noteworthy that most recognized determinants of breast cancer, such as reproductive history,
70 alcohol intake, obesity, and use of hormone therapy, exert their effect, at least in part, by
71 modifying the time and intensity of the exposure of the mammary gland to steroidal hormones
72 (Brown and Hankinson 2015; Hilakivi-Clarke et al. 2013; MacMahon 2006; Renehan et al. 2015;
73 Seitz et al. 2012).

74 Laboratory studies, specifically rodent models, support the implication of environmental
75 pollutants in breast cancer development (Dhimolea et al. 2014; Rudel et al. 2007). Endocrine
76 disrupting chemicals (EDCs) are among the 17 chemical groups prioritized for evaluation in
77 epidemiological studies on breast cancer (Rudel et al. 2014) due to their potential to act as
78 xenoestrogens or modulate the estrogenic activity via different pathways (Gibson and Saunders
79 2014; WHO/UNEP 2013). Hundreds of EDCs are present in human breast tissue, but
80 epidemiological evidence linking these substances with breast cancer is inconclusive
81 (WHO/UNEP 2013). Most previous studies have focused on individual EDCs with weak
82 estrogenic effects, thus failing to consider multiple exposures and interactions involving different
83 EDCs and physiological hormones (Fernandez et al. 2014). Functional tests measuring the
84 combined estrogenic activity of mixtures of EDCs offer a promising approach for an aggregated
85 exposure assessment. A case-control study reported a positive association between the combined
86 effect of environmental estrogens in human adipose tissue and breast cancer risk (Ibarluzea et al.

87 2004). Adipose tissue, however, is difficult to obtain in population-based studies, and it would be
88 of great practical value to assess the estrogenic potential of EDC mixtures present in blood
89 samples (Rudel et al. 2014).
90 In the present study, we measured the combined estrogenic activity of mixtures of
91 xenoestrogens in serum samples and evaluated its relation to breast cancer risk in a subsample of
92 cases and controls from a large population-based multicase-control study in Spain (MCC-Spain).
93 **Methods**
94 ***Study population***. MCC-Spain (http://www.mccspain.org) is a population-based multicase-
95 control study conducted between 2008–2013 in 12 Spanish provinces to identify environmental,
96 personal, and genetic factors related to five common cancers, including breast, prostate,
97 colorectal, stomach, and chronic lymphocytic leukemia. The study design has been previously
98 reported (Castano-Vinyals et al. 2015). Briefly, the study recruited 6,082 histologically
99 confirmed incident cancer cases aged 20–85 years, including 1,750 breast cancers, 1,115 prostate
100 cancers, 2,171 colorectal cancers, 492 gastro-oesophageal cancers, and 554 cases of leukemia, as
101 well as a single set of 4,101 population controls. The response rates were 69% among breast
102 cancer cases and 54% among female controls. All participants completed computer-assisted
103 personal interviews on sociodemographic factors, self-reported anthropometric data, lifestyle,
104 reproductive history, hormonal factors, medications, and personal and family medical history.
105 Blood samples were collected from 76% of participants. The study was approved by the ethics
106 committees of the participating institutions. Written informed consent was obtained from each
107 participant.
108 For this analysis, we randomly selected 204 breast cancer cases among those who agreed to
109 donate blood samples in the provinces of Madrid, Barcelona, Navarra, and Cantabria and 204

110 female controls frequency-matched to cases by province, 5-year age interval, and 2-unit category
111 of body mass index.
112 ***Biochemical analyses.*** We measured the total effective xenoestrogen burden (TEXB) in serum
113 samples by using a standardized bioassay for the combined estrogenic effect of mixtures of
114 xenoestrogens (Fernandez et al. 2004), which has been applied to extracts of adipose tissue,
115 blood, and placenta (Fernandez et al. 2007; Ibarluzea et al. 2004; Lopez-Espinosa et al. 2009;
116 Sonnenschein et al. 1995). Three mL of serum were added to the same volume of methanol and
117 the solution was extracted with 5 mL of hexane/ethyl ether (1:1 v/v). The organic phase was then
118 passed through a Bond Elut PCB cartridge (Varian), previously conditioned with 1.5 mL hexane.
119 The eluate obtained was dried at reduced pressure under a stream of nitrogen. Serum dried
120 extracts were reconstituted in 200 µL hexane, halved, and eluted in duplicate by high-
121 performance liquid chromatography (HPLC). This semipreparative HPLC method was
122 developed to efficiently separate organohalogenated lipophilic xenoestrogens [organochlorine
123 pesticides and metabolites, polychlorinated biphenyls (PCBs), and halogenated bisphenols,
124 among others] eluting in the alpha fraction from endogenous hormones and more polar
125 xenoestrogens (non-halogenated bisphenols, polyphenols, phytoestrogens, and mycoestrogens)
126 eluting in the beta fraction, using a normal-phase column and a gradient with two mobile phases
127 [n-hexane (phase A) and n-hexane:methanol:2-isopropanol (40:45:15 v/v) (phase B)], with the
128 most lipophilic compounds eluting in the shortest time. After HPLC fractionation, duplicated dry
129 extracts of each fraction were joined, resuspended in experimental steroid-free medium (phenol
130 red-free medium, supplemented with 2.5 mL of charcoal-dextran fetal bovine serum), and tested
131 for estrogenic activity in the E-Screen bioassay (Soto et al. 1995). The combined estrogenic
132 activity of all compounds included in each fraction was analyzed from its proliferative effect on

MCF-7 human breast cancer cells. Each fraction extract was assayed at three different dilutions (1:1, 1:5, and 1:10), together with a negative control (experimental steroid-free medium) and a positive control (treated with 100 pM of estradiol) in each culture plate. The proliferative effects of alpha and beta fractions were calculated as the difference in MCF-7 cell proliferation between the fraction extract and the steroid-free control, divided by the highest difference in proliferation between the estradiol-treated and steroid-free control cells. These relative proliferative effects were transformed into estradiol equivalent units by reading from a sigmoidal dose-response curve prepared with estradiol at concentrations of 0.1–1000 pM, and they were expressed as the estradiol equivalent concentration in picomolar per milliliter of serum (Eeq pM/mL) that would produce the same cell proliferation in the bioassay (Fernandez et al. 2007). Thus, TEXB of the alpha fraction (TEXB-α) can be regarded as a biomarker of the combined estrogenic effect of mixtures of organohalogenated lipophilic xenoestrogens, whereas TEXB of the beta fraction (TEXB-β) represents the combined estrogenic activity of endogenous hormones and more polar xenoestrogens.

The limit of detection for TEXB-α and TEXB-β was 0.1 Eeq pM/mL, which corresponded to the minimum concentration needed to produce a significantly different proliferative effect from that observed in steroid-free control cells. For 6.3% and 3.7% of participants with TEXB-α and TEXB-β determinations below the limit of detection, a level equal to the limit of detection divided by the square root of 2 was imputed. TEXB-α and TEXB-β levels could not be determined in 44.2% and 35.1% of serum samples, respectively, because MCF-7 cells treated with their extracts grew less than steroid-free control cells, which hampered reading the proliferative effect in the estradiol dose-response curve. For quality control, 10 serum samples were analyzed in triplicate through independent extraction, HPLC fractionation, and E-Screen

156 bioassay. The interassay coefficients of variation for TEXB-α and TEXB-β were 18.5% and
157 11.1%, respectively.
158 Specific organohalogenated compounds present in the alpha HPLC fraction, such as PCB-
159 138, PCB-153, PCB-180, hexachlorobenzene (HCB), and *p,p'*-dichlorodiphenyldichloroethylene
160 (*p,p'*-DDE), were quantified by high-resolution gas chromatography with micro-electron capture
161 detection, using *p*-chlorodibenzophenone as internal standard. The limit of detection for all these
162 chemicals was set at 0.05 ng/mL, representing the smallest analyte amount that gave a signal-to-
163 noise ratio greater than 3. For 5.2%, 1.8%, 3.4%, 8.4%, and 2.1% of participants with serum
164 concentrations of PCB-138, PCB-153, PCB-180, HCB, and *p,p'*-DDE below the limit of
165 detection, respectively, a level equal to the limit of detection divided by the square root of 2 was
166 imputed. Total cholesterol and triglycerides were enzymatically quantified in 10 μL of serum
167 using a Cobas 400 analyzer (Roche), and total lipids were derived from the short formula based
168 on these measured lipid species (Phillips et al. 1989).
169 ***Statistical analysis***. Participants were grouped into tertiles of serum TEXB-α and TEXB-β levels
170 based on their distributions among controls. Odds ratios for breast cancer and 95% confidence
171 intervals (CIs) comparing the second and third tertiles with the first tertile of serum TEXB-α and
172 TEXB-β were estimated using logistic regression models. We also estimated the odds ratio for
173 women with undetermined estrogenicity in the bioassay compared with all other women with
174 determined estrogenicity. Tests for linear risk trend across serum TEXB-α and TEXB-β tertiles
175 were performed by including an ordinal variable with the median level of each tertile among
176 controls in logistic regression models. To further explore the shape of the dose-response relations
177 of serum TEXB-α and TEXB-β levels with breast cancer risk, we used restricted quadratic
178 splines for log-transformed TEXB-α and TEXB-β levels with knots at the 10th, 50th, and 95th

179 percentiles of their control distributions (the first knot was set at the 10th percentile to exceed
180 levels below the limit of detection) (Greenland 1995). We also estimated odds ratios for breast
181 cancer comparing tertiles of specific organohalogenated compounds (PCB-138, PCB-153, PCB-
182 180, HCB, and *p,p'*-DDE) based on their control distributions.
183 Logistic regression models were fitted with increasing degrees of adjustment. The first model
184 adjusted for province (Madrid, Barcelona, Navarra, or Cantabria), age (continuous), body mass
185 index (continuous), education level (primary or less, high school, or college), and serum total
186 lipid levels (continuous). The second model further adjusted for breast cancer risk factors,
187 including smoking status (never, former, or current), number of births (nulliparous, 1–2, or ≥ 3),
188 age at first birth (continuous), menopausal status (premenopausal or postmenopausal), use of
189 hormone replacement therapy (never or ever), previous breast biopsy (no or yes), and family
190 history of breast cancer (no, second-degree relative, or first-degree relative). Finally, the third
191 model mutually adjusted serum TEXB-α and TEXB-β levels for each other. Effect modifications
192 were contrasted by including interaction terms of serum TEXB-α and TEXB-β tertiles with each
193 of the above covariates in logistic regression models. Analyses were performed using Stata,
194 version 13.1 (StataCorp) and R, version 2.15 (R Foundation for Statistical Computing).
195 **Results**
196 From the 408 randomly selected women (204 breast cancer cases and 204 controls), we excluded
197 5 prevalent or recurrent cases of breast cancer at baseline interview, 5 cases who initiated chemo
198 or hormone therapy before blood extraction, one case who withdrew initial consent, and 15
199 additional women (7 cases and 8 controls) with insufficient serum samples. Thus, the final
200 sample included 186 incident pretreatment cases of breast cancer (166 invasive and 20 ductal
201 carcinoma in situ) and 196 population-based controls with available serum samples for

202 estrogenicity analyses. The mean age and body mass index among cases and frequency-matched
203 controls were 59.8 years and 26.3 kg/m^2, respectively. Compared with controls, cases were more
204 likely to be nulliparous, ever smokers, ever users of hormone therapy, have lower education
205 level, and have higher prevalences of breast biopsies and affected first-degree relatives, though
206 only the difference in the prevalence of breast biopsies was statistically significant (Table 1). The
207 geometric mean serum levels of TEXB-α and TEXB-β were significantly higher in breast cancer
208 cases (8.32 and 9.94 Eeq pM/mL) than in controls (2.99 and 5.96 Eeq pM/mL, respectively).
209 Samples with undetermined estrogenicity in the bioassay were equally distributed among cases
210 and controls. Regarding specific organohalogenated compounds, cases had marginally lower
211 HCB concentrations and similar levels of PCB-138, PCB-153, PCB-180, and *p,p'*-DDE than
212 controls (Table 1).
213 Serum levels of TEXB-α and TEXB-β were moderately correlated among controls (Pearson
214 correlation coefficient for log-transformed variables of 0.34; 95% CI: 0.14, 0.51). Serum
215 concentrations of PCB-138, PCB-153, PCB-180, HCB, and *p,p'*-DDE were weakly correlated
216 with TEXB-α levels among controls (Pearson correlations for log-transformed variables of -0.21,
217 -0.01, -0.18, -0.18, and -0.17, respectively) and virtually uncorrelated with TEXB-β levels (-0.05,
218 -0.03, -0.07, -0.02, and 0.03). Apart from differences by geographic region, no other significant
219 trend in breast cancer risk factors or serum chemical concentrations was observed across tertiles
220 of serum TEXB-α and TEXB-β levels among controls, partially due to the limited number of
221 control women within each tertile (Table 2). Compared with controls with determined
222 estrogenicity in serum samples, controls with undetermined TEXB-α and TEXB-β had similar
223 risk factor distributions, but significantly higher serum concentrations of PCB-138, PCB-180,
224 HCB, and *p,p'*-DDE (Table 2).

225 In models adjusted for sociodemographic and traditional breast cancer risk factors (Table 3),
226 the risk for breast cancer increased with increasing serum levels of both TEXB-α and TEXB-β (*p*
227 for linear trend = 0.003 and 0.001, respectively). Compared with the first tertile, the odds ratios
228 for the second and third tertiles of serum TEXB-α were 1.77 (95% CI: 0.76, 4.10) and 3.45 (95%
229 CI: 1.50, 7.97), and those for the second and third tertiles of serum TEXB-β were 2.35 (95% CI:
230 1.10, 5.03) and 4.01 (95% CI: 1.88, 8.56). The increase in breast cancer risk was marked and
231 sustained over all serum TEXB-α levels above 0.5 Eeq pM/mL (Figure 1A). However, a
232 sigmoidal risk trend was observed across serum TEXB-β levels, with a sharp increase in risk
233 between 2–40 Eeq pM/mL and a downturn at higher levels (Figure 1B). When serum TEXB-α
234 and TEXB-β levels were mutually adjusted for each other, the association of TEXB-α with breast
235 cancer risk was substantially attenuated, while that for TEXB-β remained virtually unchanged
236 (Table 3 and Figure 1). The risk for breast cancer did not differ among women with
237 undetermined TEXB-α and TEXB-β compared with women with determined estrogenicity
238 (fully-adjusted odds ratios of 0.73 and 0.97, respectively) (Table 3).
239 Individual organohalogenated xenoestrogens contained in the alpha fraction showed weak and
240 opposed associations with breast cancer risk (Table 4). In models adjusted for sociodemographic
241 and traditional risk factors, the odds ratios for breast cancer comparing the third with the first
242 tertile were 1.73 (95% CI: 0.96, 3.14) for PCB-138, 1.36 (95% CI: 0.75, 2.45) for PCB-153, 1.01
243 (95% CI: 0.55, 1.87) for PCB-180, 0.84 (95% CI: 0.45, 1.58) for *p,p'*-DDE, and 0.60 (95% CI:
244 0.32, 1.15) for HCB.
245 In subgroup analyses, the increased risk for breast cancer in the third versus the first tertile of
246 serum TEXB-α tended to be higher in women with normal weight and those with family history
247 of breast cancer (subgroup-specific odds ratios of 6.37 and 5.78, respectively), although none of

these effect modifications was statistically significant (Figure 2). The positive association of serum TEXB-β with breast cancer risk was quite homogeneous across all subgroups.

Discussion

This is the first study showing a graded positive association between serum total xenoestrogen burden, as determined by the alpha fraction of the TEXB bioassay, and breast cancer risk. Women in the third tertile of serum TEXB-α had a 3.45-fold increase in breast cancer risk compared with those in the first tertile. The beta fraction of the TEXB bioassay was also positively associated with the risk for breast cancer, a result somewhat expected taking into account that circulating endogenous estrogens are included in this fraction. Finally, none of the individual organohalogenated xenoestrogens analyzed in this study was significantly associated with breast cancer risk.

Most studies in this field have focused on serum or adipose concentrations of a single chemical or a reduced number of chemicals, ignoring the cumulative effects of mixtures. Recognizing this limitation, the World Health Organization Report has concluded that it is critical to move beyond the analysis of one chemical at a time to explore the effects of EDC mixtures (WHO/UNEP 2013). The TEXB bioassay is an alternative technique that directly measures the combined estrogenic effect of all compounds included in either alpha or beta HPLC fractions. Since additive, synergistic, or antagonistic mechanisms may be present in these complex mixtures (Evans et al. 2012; Scholze et al. 2014), this approach constitutes a more efficient way to explore the cumulative impact of these compounds. In fact, cell culture studies have shown that EDC mixtures can produce a significant proliferative effect even at concentrations of individual chemicals that alone do not produce detectable effects (Rajapakse et

al. 2002). Thus, the estrogenic potential of EDCs, when tested individually, is likely to be underestimated (Kortenkamp 2007).

Most women in this study (83.5%) had detectable serum concentrations of all the measured organochlorine chemicals, reflecting the ubiquity of their exposure in our population. We found no differences in PCB, HCB, or *p,p'*-DDE concentrations between cases and controls, and none of these single compounds was positively correlated with serum TEXB-α levels among controls, reflecting their modest contribution to the total xenoestrogen burden. Similarly, a previous study has reported no correlation between individual organohalogenated xenoestrogens and their combined estrogenic activity in adipose tissue (Ibarluzea et al. 2004).

The extensive HPLC fractionation previous to the TEXB bioassay was designed to separate organohalogenated lipophilic xenoestrogens in the alpha fraction from endogenous hormones and more polar xenoestrogens in the beta fraction (Fernandez et al. 2004). In our study, serum TEXB-α and TEXB-β levels were positively correlated among controls, which resulted in an attenuation of the association between TEXB-α and breast cancer risk after adjusting for TEXB-β. The causal diagram of Figure 3 displays the assumed causal relations among TEXB-α, TEXB-β, breast cancer, and other relevant exposures, which provides a valuable tool for identifying potential sources of bias and their control. Serum TEXB-α and TEXB-β levels are assumed to be affected by an unspecified common exposure to both lipophilic and polar xenoestrogens (Fernandez et al. 2004), as suggested by the observed association between TEXB-α and TEXB-β. Serum TEXB-β levels are also affected by unmeasured endogenous hormones, which are independent of xenoestrogen exposure and directly influence breast cancer risk. According to this diagram, the causal effect of TEXB-α on breast cancer is confounded by correlated xenoestrogens present in the beta fraction, whose upward bias can be controlled by adjusting for

293 TEXB-β. However, this adjustment induces a negative conditional association between
294 xenoestrogens and endogenous hormones, which results in a downward selection bias that can be
295 as severe as the controlled confounding if endogenous hormone effects are strong (Greenland
296 2003). Thus, without further information on xenoestrogen exposure or endogenous hormones, we
297 can just conclude that the underlying effect of TEXB-α on breast cancer lies between the
298 estimated associations with and without adjustment for TEXB-β.
299 While the risk for breast cancer increased progressively across all detected TEXB-α levels
300 above 0.5 Eeq pM/mL, the association for TEXB-β followed a sigmoidal trend, with a sharp
301 increase in risk between 2–40 Eeq pM/mL and a downturn at higher levels. Nonmonotonic
302 responses are remarkably common in studies of natural hormones and EDCs (Engstrom et al.
303 2015; Vandenberg et al. 2012). The upward-then-downward risk trend for TEXB-β could be
304 explained by receptor competition between endogenous hormones and polar xenoestrogens
305 included in the beta fraction (Vandenberg et al. 2012). At low-to-intermediate TEXB-β levels,
306 natural hormone concentrations do not saturate receptors and xenoestrogens bind to unoccupied
307 receptors to increase the overall cellular response; but at high TEXB-β levels, xenoestrogens can
308 outcompete natural ligands and, due to their weaker estrogenic activity, result in an attenuation
309 of the overall biological response.
310 Though the study had limited power to detect effect modifications, we observed somewhat
311 higher effects of TEXB-α on breast cancer risk in women with normal weight and those with
312 family history of breast cancer. A stronger effect in leaner women was also evident in a previous
313 case-control study using the TEXB bioassay in adipose tissue (Ibarluzea et al. 2004), which was
314 attributed to a greater relative impact of EDCs in women with lower levels of endogenous
315 hormones accumulated in their fat. Regarding the stronger association between TEXB-α and

316 breast cancer risk in women with family history of breast cancer, this group was too small to
317 draw further conclusions and larger studies are needed to confirm this potential effect-measure
318 modification.

319 Contrary to previous findings in adipose tissue extracts (Fernandez et al. 2004; Ibarluzea et al.
320 2004), the combined estrogenic activity in serum samples was not associated with age, body
321 mass index, or any other women characteristic in our study, with the exception of the differences
322 observed by geographical region. Control women from the province of Navarra had higher
323 TEXB-α and TEXB-β levels, while those from Cantabria presented lower estrogenic activity in
324 both fractions. We have no clear explanation for these geographical differences. Previous studies
325 have reported higher serum concentrations of PCBs in healthy adults from the northern Spanish
326 regions (Agudo et al. 2009; Huetos et al. 2014) and elevated HCB levels in the province of
327 Navarra (Jakszyn et al. 2009). However, these single chemicals contributed little to the total
328 xenoestrogen burden in women of this study and can hardly explain the observed regional
329 variations. Navarra is also one of the Spanish regions with higher prevalence of postmenopausal
330 hormone therapy use (Isidoro et al. 2015), but our study found no differences in TEXB-α or
331 TEXB-β levels between never and ever users of hormone therapy. Thus, larger population-based
332 studies are required to identify determinants of serum TEXB levels that contribute to explain
333 their geographical distribution.

334 The strengths of this study include the population-based case-control design and the use of a
335 reliable biomarker for the combined estrogenic effect of EDC mixtures. However, several
336 limitations must be mentioned. First, the response rate among population controls was moderate,
337 with higher participation rates among women with higher education level. To control for this
338 potential selection bias, all analyses were adjusted for education level. Second, owing to the

339 case-control design, serum samples were collected after diagnosis in breast cancer cases, which
340 might have led to a reverse causation bias if serum concentrations of hormones or xenoestrogens
341 had changed after disease onset. To minimize the potential for reverse causation, we restricted
342 the analysis to incident cases of breast cancer who did not receive neoadjuvant chemo or
343 hormone therapy before blood extraction. However, as most growing breast tumors are estrogen-
344 demanding, serum estrogen levels might have decreased after disease onset, leading to a
345 potential dilution in the associations, particularly for TEXB-β since endogenous hormones have
346 higher binding affinity to estrogen receptors and shorter biological half-lives than xenoestrogens.
347 Third, adipose tissue extracts were not collected in our study and TEXB in serum samples was
348 taken as a surrogate of the overall estrogenic activity at the mammary gland. While many EDCs
349 are lipophilic and accumulate in the breast fatty tissue, their concentrations in serum are
350 relatively low and depend on serum lipid content. For this reason, all analyses relating serum
351 TEXB levels with breast cancer risk were adjusted for serum total lipids. Fourth, estrogenicity
352 could not be determined in over one third of serum samples because breast cancer cells treated
353 with their extracts grew less than steroid-free control cells in the TEXB bioassay. Although there
354 is no clear explanation for this lack of growth, samples with undetermined estrogenicity had
355 significantly higher levels of all measured organohalogenated xenoestrogens, so that they might
356 also have elevated concentrations of other unmeasured common-source xenobiotics that
357 prevented or hampered cellular growth. Nevertheless, since undetermined estrogenicity was
358 unrelated to case-control status, our analyses based on determined samples will provide an
359 unbiased estimate of the association between TEXB and breast cancer risk. Finally, the inherent
360 time-consuming and serum-demanding characteristics of the TEXB bioassay, together with the
361 substantial proportion of undetermined samples, heavily limited the effective sample size and

power of the present study, which precluded more extensive analyses according to tumor subtypes.

Conclusions

The combined estrogenic activity of mixtures of organohalogenated xenoestrogens in serum samples was positively associated with breast cancer risk, even though no single compound showed a significant effect when analyzed separately. The increase in risk was strong and progressive across all detected estrogenic levels. Our findings show the importance of evaluating mixtures of EDCs, rather than single chemicals, in epidemiological studies on hormone-related cancers. This study provides new evidence linking breast cancer with combined exposures to EDCs to be considered by policy agencies in charge of controlling their production and distribution.

374 **References**

375 Agudo A, Goni F, Etxeandia A, Vives A, Millan E, Lopez R, et al. 2009. Polychlorinated
376 biphenyls in Spanish adults: determinants of serum concentrations. Environ Res 109:620-628.
377 Barnes BB, Steindorf K, Hein R, Flesch-Janys D, Chang-Claude J. 2011. Population attributable
378 risk of invasive postmenopausal breast cancer and breast cancer subtypes for modifiable and
379 non-modifiable risk factors. Cancer Epidemiol 35:345-352.
380 Brown SB, Hankinson SE. 2015. Endogenous estrogens and the risk of breast, endometrial, and
381 ovarian cancers. Steroids 99:8-10.
382 Castano-Vinyals G, Aragones N, Perez-Gomez B, Martin V, Llorca J, Moreno V, et al. 2015.
383 Population-based multicase-control study in common tumors in Spain (MCC-Spain): rationale
384 and study design. Gac Sanit 29:308-315.
385 Dhimolea E, Wadia PR, Murray TJ, Settles ML, Treitman JD, Sonnenschein C, et al. 2014.
386 Prenatal exposure to BPA alters the epigenome of the rat mammary gland and increases the
387 propensity to neoplastic development. PLoS One 9:e99800;
388 doi:10.1371/journal.pone.0099800.
389 Engstrom W, Darbre P, Eriksson S, Gulliver L, Hultman T, Karamouzis MV, et al. 2015. The
390 potential for chemical mixtures from the environment to enable the cancer hallmark of
391 sustained proliferative signalling. Carcinogenesis 36(suppl 1):S38-S60.
392 Evans RM, Scholze M, Kortenkamp A. 2012. Additive mixture effects of estrogenic chemicals
393 in human cell-based assays can be influenced by inclusion of chemicals with differing effect
394 profiles. PLoS One 7:e43606; doi:10.1371/journal.pone.0043606.

395 Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, et al. 2013. GLOBOCAN
396 2012, Cancer Incidence and Mortality Worldwide. IARC CancerBase No. 11, version 1.0.
397 Available: http://globocan.iarc.fr [accessed 7 January 2016].
398 Fernandez MF, Rivas A, Olea-Serrano F, Cerrillo I, Molina-Molina JM, Araque P, et al. 2004.
399 Assessment of total effective xenoestrogen burden in adipose tissue and identification of
400 chemicals responsible for the combined estrogenic effect. Anal Bioanal Chem 379:163-170.
401 Fernandez MF, Roman M, Arrebola JP, Olea N. 2014. Endocrine disruptors: time to act. Curr
402 Environ Health Rep 1:325-332.
403 Fernandez MF, Santa-Marina L, Ibarluzea JM, Exposito J, Aurrekoetxea JJ, Torne P, et al. 2007.
404 Analysis of population characteristics related to the total effective xenoestrogen burden: a
405 biomarker of xenoestrogen exposure in breast cancer. Eur J Cancer 43:1290-1299.
406 Gibson DA, Saunders PT. 2014. Endocrine disruption of oestrogen action and female
407 reproductive tract cancers. Endocr Relat Cancer 21:T13-T31.
408 Greenland S. 1995. Dose-response and trend analysis in epidemiology: alternatives to categorical
409 analysis. Epidemiology 6:356-365.
410 Greenland S. 2003. Quantifying biases in causal models: classical confounding vs collider-
411 stratification bias. Epidemiology 14:300-306.
412 Hilakivi-Clarke L, de Assis S, Warri A. 2013. Exposures to synthetic estrogens at different times
413 during the life, and their effect on breast cancer risk. J Mammary Gland Biol Neoplasia 18:25-
414 42.
415 Howell A, Anderson AS, Clarke RB, Duffy SW, Evans DG, Garcia-Closas M, et al. 2014. Risk
416 determination and prevention of breast cancer. Breast Cancer Res 16:446.

417 Huetos O, Bartolome M, Aragones N, Cervantes-Amat M, Esteban M, Ruiz-Moraga M, et al.
418 2014. Serum PCB levels in a representative sample of the Spanish adult population: the
419 BIOAMBIENT.ES project. Sci Total Environ 493:834-844.
420 Ibarluzea JJ, Fernandez MF, Santa-Marina L, Olea-Serrano MF, Rivas AM, Aurrekoetxea JJ, et
421 al. 2004. Breast cancer risk and the combined effect of environmental estrogens. Cancer
422 Causes Control 15:591-600.
423 Isidoro B, Lope V, Whelan D, Pedraz C, Sanchez-Contador C, Santamarina C, et al. 2015. Use
424 of hormone therapy and isoflavones and mammographic density in Spain. Menopause;
425 doi:10.1097/gme.0000000000000569 [Online 28 December 2015].
426 Jakszyn P, Goni F, Etxeandia A, Vives A, Millan E, Lopez R, et al. 2009. Serum levels of
427 organochlorine pesticides in healthy adults from five regions of Spain. Chemosphere 76:1518-
428 1524.
429 Kortenkamp A. 2007. Ten years of mixing cocktails: a review of combination effects of
430 endocrine-disrupting chemicals. Environ Health Perspect 115(suppl 1):98-105.
431 Lopez-Espinosa MJ, Silva E, Granada A, Molina-Molina JM, Fernandez MF, Aguilar-Garduno
432 C, et al. 2009. Assessment of the total effective xenoestrogen burden in extracts of human
433 placentas. Biomarkers 14:271-277.
434 MacMahon B. 2006. Epidemiology and the causes of breast cancer. Int J Cancer 118:2373-2378.
435 Phillips DL, Pirkle JL, Burse VW, Bernert JT, Henderson LO, Needham LL. 1989. Chlorinated
436 hydrocarbon levels in human serum: effects of fasting and feeding. Arch Environ Contam
437 Toxicol 18:495-500.

438 Rajapakse N, Silva E, Kortenkamp A. 2002. Combining xenoestrogens at levels below individual
439 no-observed-effect concentrations dramatically enhances steroid hormone action. Environ
440 Health Perspect 110:917-921.
441 Renehan AG, Zwahlen M, Egger M. 2015. Adiposity and cancer risk: new mechanistic insights
442 from epidemiology. Nat Rev Cancer 15:484-498.
443 Rudel RA, Ackerman JM, Attfield KR, Brody JG. 2014. New exposure biomarkers as tools for
444 breast cancer epidemiology, biomonitoring, and prevention: a systematic approach based on
445 animal evidence. Environ Health Perspect 122:881-895.
446 Rudel RA, Attfield KR, Schifano JN, Brody JG. 2007. Chemicals causing mammary gland
447 tumors in animals signal new directions for epidemiology, chemicals testing, and risk
448 assessment for breast cancer prevention. Cancer 109:2635-2666.
449 Scholze M, Silva E, Kortenkamp A. 2014. Extending the applicability of the dose addition model
450 to the assessment of chemical mixtures of partial agonists by using a novel toxic unit
451 extrapolation method. PLoS One 9:e88808; doi:10.1371/journal.pone.0088808.
452 Seitz HK, Pelucchi C, Bagnardi V, La Vecchia C. 2012. Epidemiology and pathophysiology of
453 alcohol and breast cancer: update 2012. Alcohol Alcohol 47:204-212.
454 Sonnenschein C, Soto AM, Fernandez MF, Olea N, Olea-Serrano MF, Ruiz-Lopez MD. 1995.
455 Development of a marker of estrogenic exposure in human serum. Clin Chem 41:1888-1895.
456 Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. 1995. The E-
457 SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental
458 pollutants. Environ Health Perspect 103(suppl 7):113-122.

459 Sprague BL, Trentham-Dietz A, Egan KM, Titus-Ernstoff L, Hampton JM, Newcomb PA. 2008.
460 Proportion of invasive breast cancer attributable to risk factors modifiable after menopause.
461 Am J Epidemiol 168:404-411.
462 Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Lee DH, et al. 2012. Hormones
463 and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses.
464 Endocr Rev 33:378-455.
465 WHO/UNEP (World Health Organization/United Nations Environment Programme). 2013. State
466 of the Science of Endocrine Disrupting Chemicals - 2012. Available:
467 http://www.who.int/ceh/publications/endocrine/en/index.html [accessed 7 January 2016].
468

469 **Table 1.** Main characteristics and serum levels of total effective xenoestrogen burden (TEXB)
470 and specific organohalogenated compounds in breast cancer cases and controls ($n = 382$).

Characteristic	Controls	Breast cancer cases	p-Value[a]
No. of women	196	186	
Province			
Madrid	84 (42.9)	71 (38.2)	
Barcelona	34 (17.3)	33 (17.7)	
Navarra	26 (13.3)	27 (14.5)	
Cantabria	52 (26.5)	55 (29.6)	
Age (years)	59.8 ± 10.7	59.7 ± 11.1	
Body mass index[b] (kg/m^2)	26.2 ± 4.5	26.4 ± 4.5	
Education level			0.40
Primary or less	94 (48.0)	102 (54.8)	
High school	72 (36.7)	59 (31.7)	
College	30 (15.3)	25 (13.5)	
Smoking status[b]			0.28
Never	124 (63.3)	103 (55.6)	
Former	33 (16.8)	41 (22.2)	
Current	39 (19.9)	41 (22.2)	
No. of births			0.38
Nulliparous	33 (16.9)	39 (21.1)	
1–2	105 (53.9)	102 (55.1)	
≥ 3	57 (29.2)	44 (23.8)	
Age at first birth[c] (years)	26.5 ± 4.4	26.7 ± 5.4	0.81
Menopausal status			0.43
Premenopausal	28 (14.3)	32 (17.2)	
Postmenopausal	168 (85.7)	154 (82.8)	
Use of hormone replacement therapy			0.46
Never	177 (94.7)	167 (92.8)	
Ever	10 (5.3)	13 (7.2)	
Previous breast biopsy			0.02
No	189 (96.4)	168 (90.8)	
Yes	7 (3.6)	17 (9.2)	
Family history of breast cancer			0.27
No	166 (84.6)	147 (79.0)	
Second-degree relative	15 (7.7)	16 (8.6)	
First-degree relative	15 (7.7)	23 (12.4)	
Serum total lipids (mg/mL)	7.67 ± 1.87	7.42 ± 1.60	0.17
Serum TEXB-α[d] (Eeq pM/mL)	2.99 (7.86)	8.32 (5.72)	< 0.001
Undetermined estrogenicity	90 (45.9)	79 (42.5)	0.50
Serum TEXB-β[d] (Eeq pM/mL)	5.96 (5.65)	9.94 (4.57)	0.01
Undetermined estrogenicity	70 (35.7)	64 (34.4)	0.79

23

Serum PCB-138[e] (ng/mL)	0.89 (3.25)	1.04 (3.13)	0.21
Serum PCB-153[e] (ng/mL)	1.37 (3.28)	1.62 (2.92)	0.15
Serum PCB-180[e] (ng/mL)	0.72 (3.07)	0.71 (2.71)	0.97
Serum HCB[e] (ng/mL)	0.68 (3.40)	0.53 (4.11)	0.06
Serum *p,p*'-DDE[e] (ng/mL)	2.69 (5.04)	2.45 (4.42)	0.56

Values are means ± SDs or numbers (percentages).
[a]*p*-Value for homogeneity of means or proportions between breast cancer cases and controls. [b]Body mass index and smoking status one year before baseline interview. [c]Age at first birth among parous women. [d]Geometric mean (geometric SD) serum levels of the total effective xenoestrogen burden of alpha (TEXB-α) and beta (TEXB-β) fractions, together with numbers (percentages) of samples with undetermined estrogenicity in the bioassay.
[e]Geometric mean (geometric SD) serum concentrations of polychlorinated biphenyl congeners 138 (PCB-138), 153 (PCB-153), 180 (PCB-180), hexachlorobenzene (HCB), and *p,p*'-dichlorodiphenyldichloroethylene (*p,p*'-DDE).

Table 2. Main characteristics and serum concentrations of specific organohalogenated compounds by tertile of total effective xenoestrogen burden of alpha (TEXB-α) and beta (TEXB-β) fractions among controls (n = 196).

Characteristic	Serum TEXB-α[a] (Eeq pM/mL)						Serum TEXB-β[a] (Eeq pM/mL)					
	Tertile 1 (≤2.62)	Tertile 2 (2.63–8.75)	Tertile 3 (≥8.76)	p for trend[b]	Undetermined estrogenicity	p-Value[c]	Tertile 1 (≤4.56)	Tertile 2 (4.57–11.27)	Tertile 3 (≥11.28)	p for trend[b]	Undetermined estrogenicity	p-Value[c]
No. of control women	35	36	35		90		42	42	42		70	
Median serum level (Eeq pM/mL)	0.07	4.78	15.18				2.29	7.41	19.89			
Province				0.002		< 0.001				0.08		< 0.001
Madrid	31.4	61.1	48.6		37.8		47.6	50.0	61.9		24.3	
Barcelona	17.2	2.8	14.3		24.4		11.9	14.3	2.4		31.4	
Navarra	11.4	27.8	25.7		3.3		19.1	11.9	28.6		1.4	
Cantabria	40.0	8.3	11.4		34.5		21.4	23.8	7.1		42.9	
Age (years)	57.0	61.8	58.9	0.73	60.5	0.40	57.7	60.6	59.9	0.46	60.7	0.40
Body mass index (kg/m^2)	27.5	25.3	26.5	0.65	25.9	0.39	26.0	25.5	26.5	0.50	26.4	0.54
High school education or more	62.9	44.4	54.3	0.68	50.0	0.60	57.1	52.4	59.5	0.72	44.3	0.11
Ever smoker	45.7	36.1	37.1	0.55	33.3	0.36	35.7	40.5	38.1	0.90	34.3	0.60
Nulliparous	22.9	11.1	14.3	0.45	18.0	0.72	14.3	19.0	7.1	0.21	23.2	0.09
Age at first birth (years)	26.1	26.9	26.8	0.70	26.4	0.74	26.8	27.0	27.0	0.88	25.7	0.08
Postmenopausal	71.4	94.4	88.6	0.11	86.7	0.73	81.0	85.7	92.9	0.10	84.3	0.67
Ever use of hormone therapy	3.1	3.0	2.9	0.95	8.0	0.12	7.1	2.4	7.1	0.80	4.8	0.83
Previous breast biopsy	2.9	5.6	0.0	0.30	4.4	0.54	7.1	4.8	0.0	0.05	2.9	0.68
Family history of breast cancer	25.7	16.7	17.1	0.45	10.0	0.05	16.7	19.0	21.4	0.59	8.6	0.04
Serum total lipids (mg/mL)	7.73	7.36	7.93	0.51	7.66	0.97	7.35	8.04	7.82	0.47	7.54	0.50
Serum PCB-138[d] (ng/mL)	0.98	0.77	0.58	0.11	1.09	0.03	0.73	0.95	0.66	0.55	1.17	0.02
Serum PCB-153[d] (ng/mL)	1.16	1.51	1.27	0.91	1.45	0.56	1.18	1.88	1.11	0.53	1.40	0.83
Serum PCB-180[d] (ng/mL)	0.61	0.42	0.60	0.82	1.01	< 0.001	0.56	0.69	0.53	0.67	1.03	0.001
Serum HCB[d] (ng/mL)	0.62	0.56	0.45	0.30	0.90	0.003	0.53	0.83	0.42	0.19	0.93	0.007
Serum p,p'-DDE[d] (ng/mL)	2.69	1.37	1.54	0.26	4.37	< 0.001	1.45	3.08	1.89	0.82	4.42	0.001

Values are means or percentages.
[a]Tertiles of the total effective xenoestrogen burden of alpha (TEXB-α) and beta (TEXB-β) fractions, together with serum samples with undetermined estrogenicity in the bioassay. [b]p-Value for linear trend in means or proportions across tertiles of the total effective xenoestrogen burden of alpha and beta fractions; except for province, which corresponds to the p-value for homogeneity of province distributions among tertiles. [c]p-Value for homogeneity of means or

485 proportions comparing serum samples with determined estrogenicity in the bioassay (all tertiles combined) with those undetermined. [d]Geometric mean serum
486 concentrations of polychlorinated biphenyl congeners 138 (PCB-138), 153 (PCB-153), 180 (PCB-180), hexachlorobenzene (HCB), and *p,p'*-
487 dichlorodiphenyldichloroethylene (*p,p'*-DDE).
488

Table 3. Odds ratios for breast cancer (95% confidence intervals) by tertile of total effective xenoestrogen burden of alpha (TEXB-α) and beta (TEXB-β) fractions ($n = 382$).

TEXB	Tertile 1	Tertile 2	Tertile 3	p for trend[a]	Undetermined estrogenicity[b]
Serum TEXB-α[c] (Eeq pM/mL)	≤ 2.62	2.63–8.75	≥ 8.76		
No. of controls/breast cancer cases	35/18	36/32	35/57		90/79
Model 1[d]	1.00 (reference)	1.64 (0.74, 3.62)	3.04 (1.38, 6.70)	0.005	0.83 (0.52, 1.32)
Model 2[e]	1.00 (reference)	1.77 (0.76, 4.10)	3.45 (1.50, 7.97)	0.003	0.73 (0.45, 1.20)
Model 3[f]	1.00 (reference)	1.50 (0.55, 4.08)	1.80 (0.63, 5.09)	0.32	
Serum TEXB-β[c] (Eeq pM/mL)	≤ 4.56	4.57–11.27	≥ 11.28		
No. of controls/breast cancer cases	42/21	42/43	42/58		70/64
Model 1[d]	1.00 (reference)	2.14 (1.06, 4.35)	3.27 (1.62, 6.61)	0.002	0.86 (0.52, 1.41)
Model 2[e]	1.00 (reference)	2.35 (1.10, 5.03)	4.01 (1.88, 8.56)	0.001	0.97 (0.58, 1.65)
Model 3[f]	1.00 (reference)	1.75 (0.65, 4.71)	3.53 (1.24, 10.0)	0.02	

[a]p-Value for linear risk trend across tertiles based on an ordinal variable with the median level of each tertile. [b]Odds ratio for breast cancer comparing women with undetermined estrogenicity in the bioassay with all other women with determined estrogenicity. [c]Serum levels of the total effective xenoestrogen burden of alpha (TEXB-α) and beta (TEXB-β) fractions. [d]Adjusted for province (Madrid, Barcelona, Navarra, or Cantabria), age (continuous), body mass index (continuous), education level (primary or less, high school, or college), and serum total lipid levels (continuous). [e]Further adjusted for smoking status (never, former, or current), number of births (nulliparous, 1–2, or ≥ 3), age at first birth (continuous), menopausal status (premenopausal or postmenopausal), use of hormone replacement therapy (never or ever), previous breast biopsy (no or yes), and family history of breast cancer (no, second-degree relative, or first-degree relative). [f]Further adjusted for the other fraction of total effective xenoestrogen burden (tertiles).

499 **Table 4.** Odds ratios for breast cancer (95% confidence intervals) by tertile of specific organohalogenated compounds ($n = 382$).

Organohalogenated compound	Tertile 1	Tertile 2	Tertile 3	p for trend[a]
Serum PCB-138[b] (ng/mL)	≤0.80	0.81–1.59	≥1.60	
No. of controls/breast cancer cases	65/52	65/60	66/74	
Model 1[c]	1.00 (reference)	1.27 (0.75, 2.14)	1.63 (0.93, 2.85)	0.09
Model 2[d]	1.00 (reference)	1.30 (0.74, 2.27)	1.73 (0.96, 3.14)	0.07
Model 3[e]	1.00 (reference)	1.34 (0.64, 2.81)	1.64 (0.78, 3.46)	0.20
Serum PCB-153[b] (ng/mL)	≤0.90	0.91–2.07	≥2.08	
No. of controls/breast cancer cases	63/50	68/78	65/58	
Model 1[c]	1.00 (reference)	1.54 (0.92, 2.58)	1.21 (0.69, 2.12)	0.85
Model 2[d]	1.00 (reference)	1.42 (0.83, 2.42)	1.36 (0.75, 2.45)	0.46
Model 3[e]	1.00 (reference)	0.90 (0.45, 1.82)	1.33 (0.64, 2.75)	0.36
Serum PCB-180[b] (ng/mL)	≤0.52	0.53–1.17	≥1.18	
No. of controls/breast cancer cases	65/63	66/56	65/67	
Model 1[c]	1.00 (reference)	0.82 (0.48, 1.41)	1.04 (0.59, 1.85)	0.73
Model 2[d]	1.00 (reference)	0.82 (0.46, 1.43)	1.01 (0.55, 1.87)	0.81
Model 3[e]	1.00 (reference)	0.96 (0.47, 1.98)	1.09 (0.49, 2.43)	0.81
Serum HCB[b] (ng/mL)	≤0.43	0.44–1.25	≥1.26	
No. of controls/breast cancer cases	65/75	66/58	65/53	
Model 1[c]	1.00 (reference)	0.69 (0.41, 1.15)	0.56 (0.30, 1.02)	0.09
Model 2[d]	1.00 (reference)	0.69 (0.41, 1.18)	0.60 (0.32, 1.15)	0.18
Model 3[e]	1.00 (reference)	0.63 (0.32, 1.24)	0.64 (0.27, 1.50)	0.38
Serum p,p'-DDE[b] (ng/mL)	≤1.37	1.38–6.76	≥6.77	
No. of controls/breast cancer cases	65/56	66/86	65/44	
Model 1[c]	1.00 (reference)	1.50 (0.90, 2.49)	0.72 (0.40, 1.31)	0.06
Model 2[d]	1.00 (reference)	1.59 (0.94, 2.70)	0.84 (0.45, 1.58)	0.20

Model 3[e]	1.00 (reference)	1.61 (0.81, 3.21)	0.63 (0.27, 1.46)	0.10

[a]*p*-Value for linear risk trend across tertiles based on an ordinal variable with the median level of each tertile. [b]Serum concentrations of polychlorinated biphenyl congeners 138 (PCB-138), 153 (PCB-153), 180 (PCB-180), hexachlorobenzene (HCB), and *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE). [c]Adjusted for province (Madrid, Barcelona, Navarra, or Cantabria), age (continuous), body mass index (continuous), education level (primary or less, high school, or college), and serum total lipid levels (continuous). [d]Further adjusted for smoking status (never, former, or current), number of births (nulliparous, 1–2, or ≥ 3), age at first birth (continuous), menopausal status (premenopausal or postmenopausal), use of hormone replacement therapy (never or ever), previous breast biopsy (no or yes), and family history of breast cancer (no, second-degree relative, or first-degree relative). [e]Further adjusted for the total effective xenoestrogen burden of beta fraction (tertiles).

508 **Figure legends**

509 **Figure 1.** Odds ratios for breast cancer by serum levels of total effective xenoestrogen burden of
510 alpha (*A*) and beta fractions (*B*).

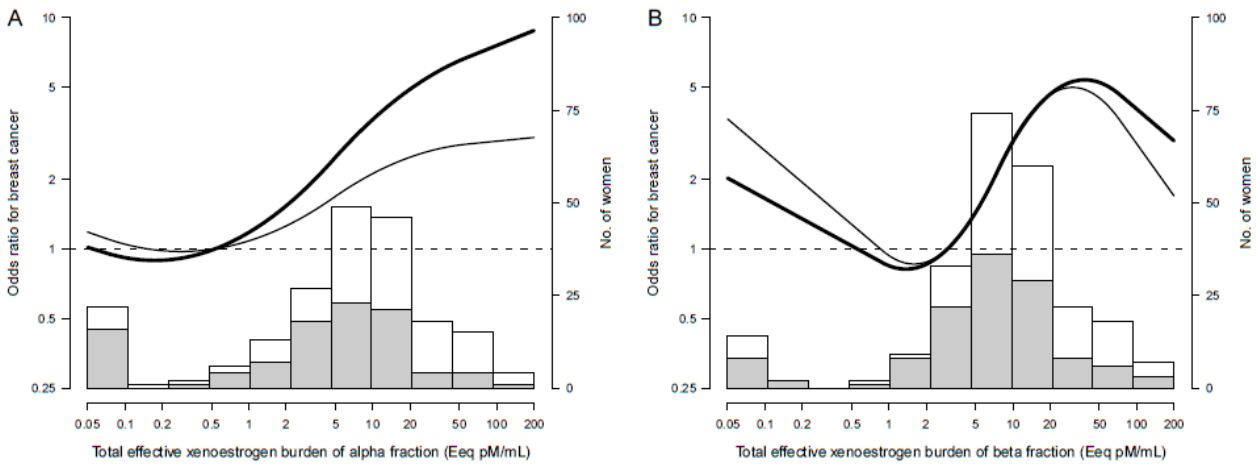
511 Curves represent adjusted odds ratios based on restricted quadratic splines for log-transformed levels of total
512 effective xenoestrogen burden of alpha and beta fractions with knots at the 10th, 50th, and 95th percentiles. The
513 reference value (odds ratio = 1) was set at the 20th percentile of each fraction distribution among controls (0.54 and
514 2.97 Eeq pM/mL for alpha and beta fractions, respectively). Odd ratios were adjusted for province, age, body mass
515 index, education level, serum total lipid levels, smoking status, number of births, age at first birth, menopausal
516 status, use of hormone replacement therapy, previous breast biopsy, and family history of breast cancer (bold
517 curves), and further adjusted for the other fraction of total effective xenoestrogen burden (thin curves). Histograms
518 represent each fraction distribution among controls (shaded bars) and breast cancer cases (white bars).

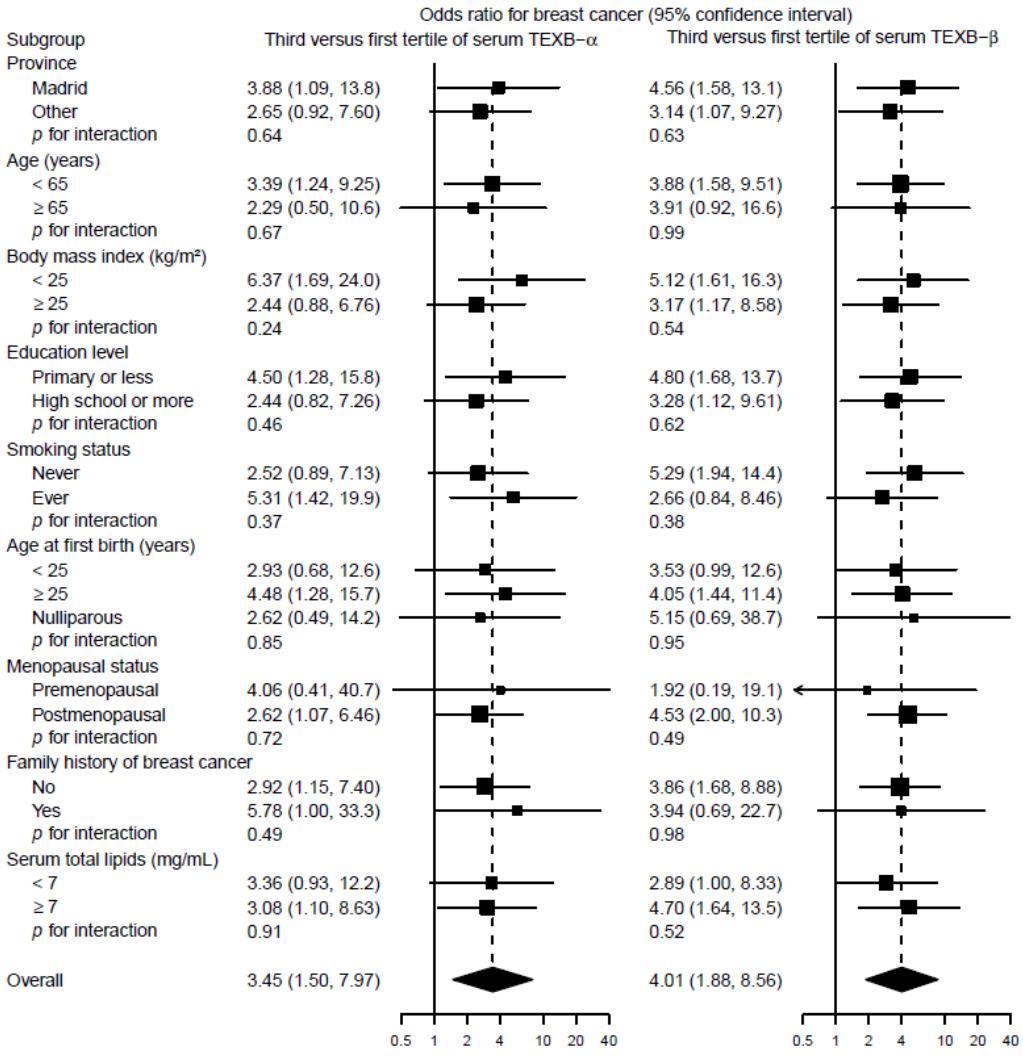
519 **Figure 2.** Odds ratios for breast cancer comparing the third with the first tertile of total effective
520 xenoestrogen burden of alpha (TEXB-α) and beta (TEXB-β) fractions by subgroup.

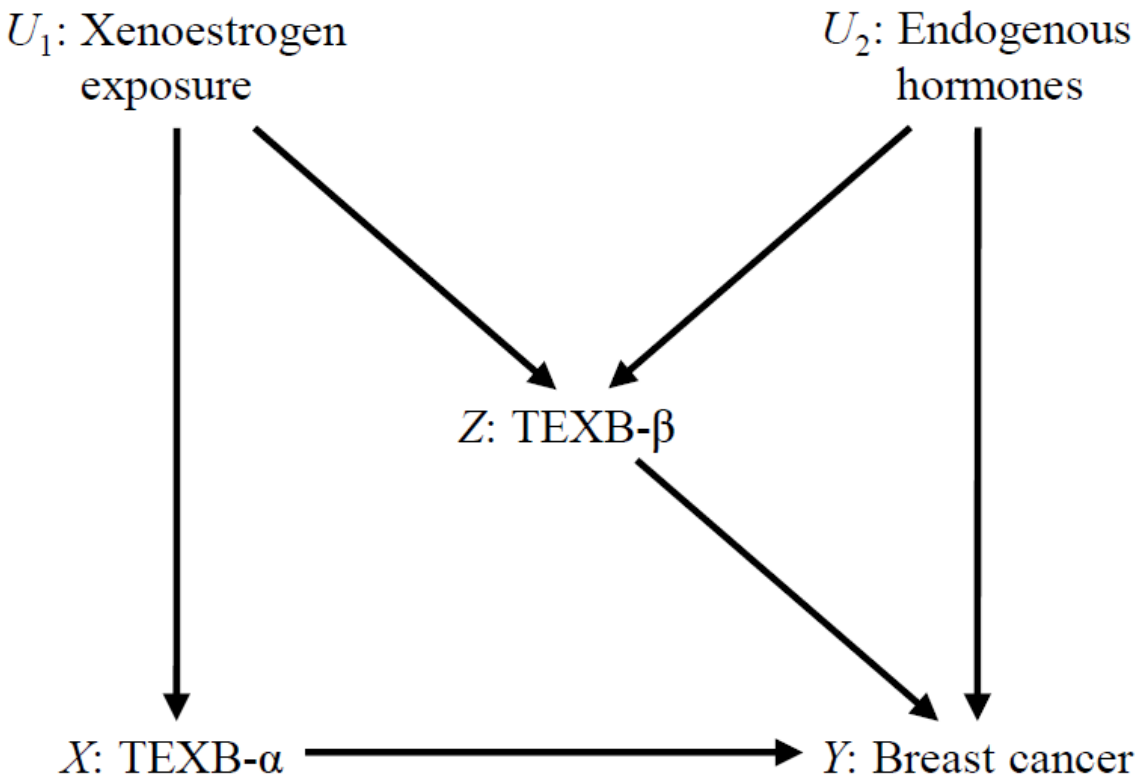
521 Subgroup-specific odds ratios (squares with area inversely proportional to the variance) and their 95% confidence
522 intervals (horizontal lines) were obtained from logistic regression models with interaction terms of serum TEXB-α
523 and TEXB-β tertiles with the corresponding subgroup indicators and adjusted for province, age, body mass index,
524 education level, serum total lipid levels, smoking status, number of births, age at first birth, menopausal status, use
525 of hormone replacement therapy, previous breast biopsy, and family history of breast cancer.

526 **Figure 3.** Diagram with causal relations among total effective xenoestrogen burden of alpha
527 (TEXB-α) and beta (TEXB-β) fractions, breast cancer, and unmeasured exposures to
528 xenoestrogens and endogenous hormones.

529 The causal path from TEXB-α to breast cancer $X \rightarrow Y$ is confounded by the undirected path $X \leftarrow U_1 \rightarrow Z \rightarrow Y$, which can
530 be blocked by adjusting for TEXB-β. However, conditioning on TEXB-β unblocks the other undirected path
531 $X \leftarrow U_1 \rightarrow Z \leftarrow U_2 \rightarrow Y$, thus resulting in selection bias.


A
Odds ratio for breast cancer
No. of women
Total effective xenoestrogen burden of alpha fraction (Eeq pM/mL)
B
Odds ratio for breast cancer
No. of women
Total effective xenoestrogen burden of beta fraction (Eeq pM/mL)


Odds ratio for breast cancer (95% confidence interval)
Subgroup
Third versus first tertile of serum TEXB−α
Third versus first tertile of serum TEXB−β
Province
Madrid 3.88 (1.09, 13.8) 4.56 (1.58, 13.1)
Other 2.65 (0.92, 7.60) 3.14 (1.07, 9.27)
p for interaction 0.64 0.63
Age (years)
< 65 3.39 (1.24, 9.25) 3.88 (1.58, 9.51)
≥ 65 2.29 (0.50, 10.6) 3.91 (0.92, 16.6)
p for interaction 0.67 0.99
Body mass index (kg/m²)
< 25 6.37 (1.69, 24.0) 5.12 (1.61, 16.3)
≥ 25 2.44 (0.88, 6.76) 3.17 (1.17, 8.58)
p for interaction 0.24 0.54
Education level
Primary or less 4.50 (1.28, 15.8) 4.80 (1.68, 13.7)
High school or more 2.44 (0.82, 7.26) 3.28 (1.12, 9.61)
p for interaction 0.46 0.62
Smoking status
Never 2.52 (0.89, 7.13) 5.29 (1.94, 14.4)
Ever 5.31 (1.42, 19.9) 2.66 (0.84, 8.46)
p for interaction 0.37 0.38
Age at first birth (years)
< 25 2.93 (0.68, 12.6) 3.53 (0.99, 12.6)
≥ 25 4.48 (1.28, 15.7) 4.05 (1.44, 11.4)
Nulliparous 2.62 (0.49, 14.2) 5.15 (0.69, 38.7)
p for interaction 0.85 0.95
Menopausal status
Premenopausal 4.06 (0.41, 40.7) 1.92 (0.19, 19.1)
Postmenopausal 2.62 (1.07, 6.46) 4.53 (2.00, 10.3)
p for interaction 0.72 0.49
Family history of breast cancer
No 2.92 (1.15, 7.40) 3.86 (1.68, 8.88)
Yes 5.78 (1.00, 33.3) 3.94 (0.69, 22.7)
p for interaction 0.49 0.98
Serum total lipids (mg/mL)
< 7 3.36 (0.93, 12.2) 2.89 (1.00, 8.33)
≥ 7 3.08 (1.10, 8.63) 4.70 (1.64, 13.5)
p for interaction 0.91 0.52
Overall 3.45 (1.50, 7.97) 4.01 (1.88, 8.56)
0.5 1 2 4 10 20 40
0.5 1 2 4 10 20 40


U_1: Xenoestrogen exposure
U_2: Endogenous hormones
Z: TEXB-β
X: TEXB-α
Y: Breast cancer

8. GENERAL DISCUSSION

The results of this PhD thesis suggest that (1) although both matrices (serum and AT) seem useful for the assessment of internal POP burden in BC patients, the determination of the POP burden in each of the matrices may provide relevant information to evaluate the association of POP burden with BC risk, and therefore, consideration of the individual features of each of the matrices and of the covariates that may differentially act as confounding factors in the association with the disease should be taken into account; (2) the levels of exposure to EDCs (mainly the significant levels of PCB-153) found in the Tunisian female population, as well as the patterns of exposure found such as the South–North gradient, which may correlate to the particular distribution of obsolete stockpiles of pesticides, would warrant the design of biomonitoring programs; and (3) serum xenoestrogen burden, measured as single chemicals or as total estrogenic activity, was associated with an increased BC risk.

Because of the amount of evidence that supports the relationship with NHL, lindane has been classified as "human carcinogen" (Group 1) by the International Agency for Research on Cancer (IARC) (Loomis et al. 2015). In the same report, the IARC also classifies DDT as "probably carcinogenic to humans" (Group 2A), based on the evidence that DDT causes cancer in experimental animals and on the limited evidence of its carcinogenic effect in humans. Epidemiological studies found positive associations between exposure to DDT and to NHL and testicular and liver cancer. However, these studies have also reported that there is not enough evidence to support any association between BC and DDT levels measured in blood or fat samples. Moreover, IARC has also recently classified PCBs as "carcinogenic to humans" (Group 1) due to the positive associations found with malignant melanoma, NHL and BC (Lauby-Secretan et al. 2016). Our data shed light on the evidence of the potential carcinogenic, or at the very least co-carcinogenic, effect of some EDCs.

Our results highlight the relevance of the selection of an adequate biomarker of exposure, together with a number of other confounding factors (as described in the Introduction chapter). Although AT was described as the primary site of long-term storage of POPs, which makes it the most adequate biological matrix for measuring chronic exposure to POPs, most of the studies have used the serum because (1) blood collection is easier than the invasive procedures necessary to isolate AT and (2) serum has been described as a proxy value of adipose burden under physiological equilibrium. However, serum burden is considered by some authors as a less robust measurement because it can be affected by enhanced lipolysis during substantial metabolic changes and by the current exposure. Our results suggest that serum and AT may provide relevant information, being differentially affected by external factors. Hence, we have detected that, unlike adipose POP burden, levels in serum are differentially affected by weight loss and neoadjuvant QT administered before sample collection. Therefore, these variables

should be included as covariates in further studies aimed at investigating a possible association between POP exposure and BC.

Despite the fact that most experts in the field agree that the overall xenoestrogenic effect is not the result of a simple compound, but rather a result of the combined, additive, synergistic and antagonist effect of the internal burden of POPs, most of the studies have usually addressed the exposure at a single compound level, or at the very best, the sum of some compounds. According to this single-chemical approach, we have found a positive and significant association between at least one organochlorine pesticide (β-HCH) and higher risk of BC, considering the exposure either as a quantitative variable and categorized by tertiles. These results are in line with those reported previously, corroborating its carcinogenic potential in breast (Mussalo-Rauhamaa et al. 1990; Ibarluzea et al. 2004), prostate cancer (Mills and Yang 2003) and NHL (Rothman et al. 1997).

However, some evidence has emerged in recent years that emphasize the importance of the mixtures (Kortenkamp 2006). In this regard, we reported in a previous work that the total xenoestrogenic activity exerted by the internal POP burden (TEXB) stored in breast tissue was positively associated with the risk of BC in a case-control study carried out in Southern Spain (Ibarluzea et al. 2004). Nevertheless, difficulties associated to the invasive procedures required to isolate AT for epidemiological studies precluded us to compare our results to those obtained in most studies which used serum as a biomarker of exposure. After the optimization of the TEXB biomarker for serum samples, we carried out a new population-based multicase-control study on 204 BC cases and 204 female controls recruited in four Spanish provinces. In addition to the positive association found previously between xenoestrogenic burden in AT and BC risk and despite the fact that circulating levels of POPs seem to be differentially affected by those in the AT, these new results reflect that serum TEXB burden is also related to BC. Moreover, these results clearly demonstrate that the combined effect, and not the single-chemical effect, is responsible for the carcinogenic effect of EDCs. In this regard, whereas we found no association between serum PCB, HCB, or *p,p'*-DDE concentrations and serum TEXB-α levels (reflecting their modest contribution to the total xenoestrogen burden) or BC risk, we found that the risk for BC increased with increasing serum levels of TEXB-α (p for linear trend = 0.003), in models adjusted for sociodemographic and traditional BC risk factors. Compared with the first tertile, the odds ratio for the third tertile of serum TEXB-α was 3.45 (95% CI: 1.50, 7.97).

Hanahan and Weinberg (2000) described that the transition from normal to neoplastic cell is a process in which cells acquire a succession of capabilities during the multistep development of tumors. As a crucial orchestrator in the initiation of breast carcinogenesis, prolonged exposure to high levels of estrogens in women has been shown to stimulate cellular proliferation through

the estrogen receptor mediated hormonal activity, increase mutation rates through a cytochrome P450-mediated metabolic activation (exerting direct genotoxic effects), and induce aneuploidy (Russo and Russo 2006), which leads to the neoplastic transformation of human breast epithelial cells. Although estrogen-mimicking EDCs were described as low-potency inductors of estrogen-related pathways, it is known that homeostasis of the hormone system is very sensitive to subtle changes in hormone levels. Therefore much effort has been made to elucidate the role of xenoestrogens in human BC [reviewed in: (Snedeker 2001)], though the discrepancies in the results precluded the scientific community to reach an agreement about their participation in BC initiation (Kortenkamp 2006).

In addition to that role attributed to POPs in BC initiation, some *in vitro* studies have addressed new pathways of action of these compounds in tumor cell biology. In this respect, it has been demonstrated that POPs are able to induce angiogenesis (Bratton et al. 2012; Pontillo et al. 2015) and metastasis (Pestana et al. 2015) by activating enzymes involved in collagen turnover such as matrix metalloproteinases (MMPs) (Zou and Matsumura 2003; Pontillo et al. 2013). Moreover, associations have been found between POP exposure and alterations in microRNA expression profiles (Tilghman et al. 2012), interactions in the MAPK (mitogen-activated protein kinase) (Bratton et al. 2012), or modifications in the growth factor beta (TGF-beta) pathway (Valerón et al. 2009). Epidemiological evidence is still scarce, with some studies showing that most exposed subjects had higher levels of MMPs (Kim et al. 2011), or decreased global methylation in leukocyte DNA (Itoh et al. 2014). There is also some evidence on the effect of POPs in expression of ER (Muñoz-de-Toro et al. 2006) or p53 (Al-Anati et al. 2014). In this regard, although these are preliminary results that have not been published yet, we have recently found that POP levels were related to more aggressive breast tumors, with increased expression of ER, PR and HER2/neu oncogene, and lower expression of p-53 and E-cadherin. It has also been reported that the probability of lymph-node invasion and tumor size in BC cases increases with serum *p,p'*-DDE levels (Demers et al. 2000). Moreover, it has been found that serum DDT was associated with BC-related mortality at 5 [HR 2.72 (95% CI 1.04-7.13)] and 15 years [HR 1.42 (95% CI 0.99-2.06)] after diagnosis (Parada et al. 2016).

While the information about levels and sources of exposure to EDCs as well as their role in BC is abundant especially in European and North American countries, there is still limited information regarding exposure to EDCs in developing countries, with expected great differences in the magnitude of the exposure, type of pesticides, and routes of exposure between developing and developed countries (Shakeel et al. 2010). Unlike to developed countries, developing countries use more HCH, DDT and its derivatives (Shakeel et al. 2010). In this regard, India is unarguably a hotspot of DDT and HCH contamination where pesticidal POPs were in widespread use in agriculture until recent years and where DDTs is still used for malaria

control (Sharma et al. 2014). India is not the only country where illegal use of DDTs and other POPs in agriculture and industrial sector has been reported (UNEP/POPS/COP.7/36 2015). In this regard, previous studies have reported increased environmental levels of POPs (Gioia et al. 2014) in different African countries, which have resulted in increased levels in humans (Luzardo et al. 2014). Although Tunisia banned the use and import of POPs three decades ago, the government has recently confirmed that banned compounds are still being illegally imported from neighboring countries (APEK 2005). In line with these declarations, we have detected a potential source of human exposure to *p,p'*-DDE through cereal consumption, that may reflect illegal use of DDT in agriculture. We have also found increased levels of PCB-153, which may be a consequence of the rapid transformation in the IT-field and of the considerable amount of PCB-containing equipment that is still being used in Tunisia (World Bank 2012). Moreover, we have also detected substantial exposure rates to parabens and some benzophenones in Tunisian women, with urine concentrations of these npEDCs in a similar range to those measured in European populations (Casas et al. 2013; Frederiksen et al. 2013; Dewalque et al. 2014). Therefore, our results would warrant the design of biomonitoring programs in Tunisia that would help to identify population groups at risk of exposure, to characterize the different sources of exposure and to design prevention programs. Environmental health policies and strategies in Northern and Southern Mediterranean countries should be, at least, complementary. However, given the financial limitations of some African countries, Northern Mediterranean countries should collaborate with the developing countries in the development of studies aimed at the general population.

Regarding the influence of EDCs in the incidence of BC in developing countries, information is even scarcer. In this sense, it is clear that the wide differences between developed and developing countries regarding EDCs do not allow us to generalize the results. In this sense, while most studies carried out in developed countries failed to find positive association, 4 out of 8 studies carried out in developing countries such as Mexico or India found significant association between POPs and BC (Shakeel et al. 2010). In our study, high serum concentrations of β-HCH, *p,p'*-DDE, and heptachlor were associated with a greater risk of BC in Tunisian women, although only β-HCH remained positively associated when the three chemicals were entered in a single model with all covariates. However, due to the limited sample size of our study, these results should be interpreted with caution, and further research is warranted to confirm this association in Tunisia and other developing countries, where the ban on these chemicals is more recent.

The increasing evidence that links human disease to exposure to EDC has recently led to estimate the burden and disease costs of exposure to EDC in European countries (Trasande et al. 2015). This study only included those diseases with the highest probability of causation such as

intellectual disability, autism, attention-deficit hyperactivity disorder, childhood and adult obesity, adult diabetes, male infertility and mortality associated with reduced testosterone and estimated a median cost of €157 billion (or $209 billion, corresponding to 1.23% of EU gross domestic product) annually. However, it is known that cost-of-illness approaches fail to capture the complete scope of economic costs associated with illness (especially psychological and other indirect or intangible costs that are difficult to assess). Moreover, in the near future, other highly prevalent and chronic diseases such as hypertension and breast or prostate cancer (which have been already studied) may be included in this estimation which will result in a considerable increase of the estimated costs for the Health Systems. These costs will accrue until harmful exposures cease. Thus, regulatory action to limit exposure to the most prevalent and potentially hazardous EDCs is likely to result in substantial economic benefits. These economic benefits should inform decision-making on measures to protect public health (Trasande et al. 2015). Moreover, these results also suggest the need to expand the estimation of the costs attributable to EDC exposure to developing countries where the policies on chemical use and practices to protect human health and the environment are insufficient as well as to implement measures to reduce current exposure levels (Dasgupta et al. 2010).

9. CONCLUSIONS

The primary objective of this PhD thesis was to assess exposure to a selected group of EDCs (including HCB, β-HCH, DDT and *p,p'*-DDE, and PCBs) and to determine whether exposure to these chemicals is a contributing factor in female BC in developed and developing countries.

The main conclusions are:

1. Serum and AT burden of POPs provide relevant, but different information in the assessment of exposure in BC studies. We consider that it is relevant to take into account the individual features of blood and fat tissue, because we have identified some factors that differentially influence POP levels in both matrices. In this regard, 'recent weight loss' and neoadjuvant chemotherapy was negatively associated with serum POP levels but no (or positively) associated with AT concentrations of POPs. Therefore, factors such as recent weight loss, QT and neoadjuvant therapy should be taken into account in the adjustment of statistical models in future studies when addressing the association between exposure to POPs and risk of BC because they may differentially act as confounding factors.

2. Exposure to POPs in Tunisian women is frequent and the magnitude of this exposure is different to other countries. Hence, while serum concentrations were comparable to those of developed countries for most of studied POPs, the target population of this study showed PCB-153 concentrations in serum higher than other countries, which is consistent with the inadequate electronic waste management recently declared by Tunisian authorities. This is the first study that assesses potential sources of exposure to POPs in a Tunisian population. In this regard, we have identified a geographical gradient in the levels of exposure to PCBs, with higher levels of exposure in people living in the North. Additionally, we also have detected a positive association between cereal consumption and levels of *p,p'*-DDE, which may be related to illegal use in some regions, as illegal import of DDT from neighbouring countries has been reported.

3. Detectable levels of non-persistent EDCs were found in most of Tunisian women, including bisphenol-A, benzophenone-3 and methylparaben. Although the amount of personal care products and processed food consumption (the most relevant determinants of the exposure to these chemicals) are expected to be lower than in developed countries, levels of exposure to non-persistent EDCs identified in Tunisia were in a similar range than those found in women living in European countries with the exception of bisphenol-A which was found in lower

concentration. Our results confirm the high exposure of Tunisian women to npEDCs, and therefore, they would warrant biomonitoring studies to assess the levels of exposure to these compounds and to identify sources of exposure and population groups at higher risk.

4. Risk of BC is associated with serum levels of at least one POP with demonstrated estrogenic activity in Tunisian women. Although only β-HCH remained positively associated in the adjusted multi-residue model, higher serum concentrations of *p,p'*-DDE and heptachlor were also associated with a higher risk of BC. These results confirm the hypothesis that exposure to synthetic chemicals with xenoestrogenic activity increases the risk of developing BC. In line with the few other studies that have demonstrated a significant association between human exposure to POPs and risk of BC in developing countries, our study also urges the international community to collaborate with the developing countries to establish biomonitoring studies in these regions and to take actions to reduce inadvertent exposure.

5. In a population based multicase-control study carried out in Spain, BC patients had increased serum xenoestrogenic burden (TEXB) in comparison with healthy women. We have found that women in the third tertile of serum TEXB-α (the chromatographic fraction where most POPs elute) had higher risk for BC compared with those in the first tertile. We found no statistically significant association between serum levels of each single EDC and both the levels of TEXB-α and the risk of BC, which reflects their modest contribution to TEXB burden. Our results suggest the relevance of considering effects of mixtures of combination of EDCs rather than the exposure to single EDCs in the evaluation of their role in hormone-related cancers. Therefore, this holistic and singular approach should be adopted in order to improve the knowledge of the role of EDCs in hormone-dependent disease.

10. REFERENCES

Agudo A, Goni F, Etxeandia A, Vives A, Millán E, López R, et al. 2009. Polychlorinated biphenyls in Spanish adults: determinants of serum concentrations. Environmental Research 109(5): 620-628.

Ahmedin Jemal D, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, et al. 2008. Cancer statistics, 2004.

Airaksinen R, Rantakokko P, Eriksson JG, Blomstedt P, Kajantie E, Kiviranta H. 2011. Association between type 2 diabetes and exposure to persistent organic pollutants. Diabetes Care 34(9): 1972-1979.

Al-Anati L, Kadekar S, Högberg J, Stenius U. 2014. PCB153, TCDD and estradiol compromise the benzo [a] pyrene-induced p53-response via FoxO3a. Chemico-biological interactions 219: 159-167.

Aneck-Hahn NH, Schulenburg GW, Bornman MS, Farias P, Jager C. 2007. Impaired semen quality associated with environmental DDT exposure in young men living in a malaria area in the Limpopo Province, South Africa. Journal of Andrology 28(3): 423-434.

Anway MD, Cupp AS, Uzumcu M, Skinner MK. 2005. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308(5727): 1466-1469.

APEK. 2005. Tunisia Country Situation Report. The International POPs Elimination Project. Tunisia.

Aronson KJ, Miller AB, Woolcott CG, Sterns EE, McCready DR, Lickley LA, et al. 2000. Breast adipose tissue concentrations of polychlorinated biphenyls and other organochlorines and breast cancer risk. Cancer Epidemiology Biomarkers & Prevention 9(1): 55-63.

Arrebola J, Cuellar M, Claure E, Quevedo M, Antelo S, Mutch E, et al. 2012a. Concentrations of organochlorine pesticides and polychlorinated biphenyls in human serum and adipose tissue from Bolivia. Environmental Research 112: 40-47.

Arrebola J, Mutch E, Cuellar M, Quevedo M, Claure E, Mejía L, et al. 2012b. Factors influencing combined exposure to three indicator polychlorinated biphenyls in an adult cohort from Bolivia. Environmental Research 116: 17-25.

Arrebola JP, Fernández MF, Martin-Olmedo P, Bonde JP, Martín-Rodriguez JL, Expósito J, et al. 2015. Historical exposure to persistent organic pollutants and risk of incident hypertension. Environmental Research 138: 217-223.

Arrebola JP, Fernández MF, Olea N, Ramos R, Martin-Olmedo P. 2013a. Human exposure to p, p′-dichlorodiphenyldichloroethylene (p, p′-DDE) in urban and semi-rural areas in southeast Spain: A gender perspective. Science of the total environment 458: 209-216.

Arrebola JP, Ocaña-Riola R, Arrebola-Moreno AL, Fernández-Rodríguez M, Martin-Olmedo P, Fernández MF, et al. 2014. Associations of accumulated exposure to persistent organic pollutants with serum lipids and obesity in an adult cohort from Southern Spain. Environmental Pollution 195: 9-15.

Arrebola JP, Pumarega J, Gasull M, Fernandez MF, Martin-Olmedo P, Molina-Molina JM, et al. 2013b. Adipose tissue concentrations of persistent organic pollutants and prevalence of type 2 diabetes in adults from Southern Spain. Environmental Research 122: 31-37.

ATSDR. 2002. Toxicological profile for DDT, DDE and DDT.Agency for Toxic Substances and Disease Registry.

ATSDR. 2015. Toxicological Profile for Hexachlorobenzene.Agency for Toxic Substances and Disease Registry.

Aubé M, Larochelle C, Ayotte P. 2011. Differential effects of a complex organochlorine mixture on the proliferation of breast cancer cell lines. Environmental Research 111(3): 337-347.

Ayotte P, Giroux S, Dewailly É, Avila MH, Farias P, Danis R, et al. 2001. DDT spraying for malaria control and reproductive function in Mexican men. Epidemiology 12(3): 366-367.

Baron JA, Newcomb PA, Longnecker MP, Mittendorf R, Storer BE, Clapp RW, et al. 1996. Cigarette smoking and breast cancer. Cancer Epidemiology Biomarkers & Prevention 5(5): 399-403.

Bay K, Asklund C, Skakkebaek NE, Andersson A-M. 2006. Testicular dysgenesis syndrome: possible role of endocrine disrupters. Best Practice & Research Clinical Endocrinology & Metabolism 20(1): 77-90.

Bernstein L. 2002. Epidemiology of endocrine-related risk factors for breast cancer. Journal of mammary gland biology and neoplasia 7(1): 3-15.

Bernstein L. 2008. Identifying population-based approaches to lower breast cancer risk. Oncogene 27: S3-S8.

Berntssen MH, Julshamn K, Lundebye A-K. 2010. Chemical contaminants in aquafeeds and Atlantic salmon (Salmo salar) following the use of traditional-versus alternative feed ingredients. Chemosphere 78(6): 637-646.

Boada L, Henríquez-Hernández L, Zumbado M, Almeida-González M, Álvarez-León E, Navarro P, et al. 2016. Organochlorine pesticides exposure and bladder cancer: evaluation from a gene-environment perspective in a hospital-based case-control study in the Canary Islands (Spain). Journal of agromedicine 21(1): 34-42.

Boada LD, Zumbado M, Henríquez-Hernández LA, Almeida-González M, Álvarez-León EE, Serra-Majem L, et al. 2012. Complex organochlorine pesticide mixtures as determinant factor for breast cancer risk: a population-based case-control study in the Canary Islands (Spain). Environ Health 11(28): 6-7.

Bodenstein G. 1972. Disposal of wastes from Lindane manufacture. Lindane monograph of an insecticide Schillinger, Freiburg im Breisgau, Germany: 23-77.

Bonefeld-Jørgensen EC, Andersen HR, Rasmussen TH, Vinggaard AM. 2001. Effect of highly bioaccumulated polychlorinated biphenyl congeners on estrogen and androgen receptor activity. Toxicology 158(3): 141-153.

Bonefeld-Jorgensen EC, Long M, Hofmeister MV, Vinggaard AM. 2007. Endocrine-disrupting potential of bisphenol A, bisphenol A dimethacrylate, 4-n-nonylphenol, and 4-n-octylphenol in vitro: new data and a brief review. Environmental Health Perspectives 115: 69.

Botella B, Crespo J, Rivas A, Cerrillo I, Olea-Serrano MF, Olea N. 2004. Exposure of women to organochlorine pesticides in Southern Spain. Environmental Research 96(1): 34-40.

Bratton MR, Frigo DE, Segar HC, Nephew KP, McLachlan JA, Wiese TE, et al. 2012. The organochlorine o, p'-DDT plays a role in coactivator-mediated MAPK crosstalk in MCF-7 breast cancer cells. Environmental Health Perspectives 120(9): 1291.

Campoy C, Jimenez M, Olea-Serrano M, Frias MM, Canabate F, Olea N, et al. 2001. Analysis of organochlorine pesticides in human milk: preliminary results. Early Human Development 65: S183-S190.

Carreño J, Rivas A, Granada A, Lopez-Espinosa MJ, Mariscal M, Olea N, et al. 2007. Exposure of young men to organochlorine pesticides in Southern Spain. Environmental Research 103(1): 55-61.

Carroll KK. 1975. Experimental evidence of dietary factors and hormone-dependent cancers. Cancer Research 35(11 Part 2): 3374-3383.

Casas M, Valvi D, Luque N, Ballesteros-Gomez A, Carsin A-E, Fernandez MF, et al. 2013. Dietary and sociodemographic determinants of bisphenol A urine concentrations in pregnant women and children. Environment International 56: 10-18.

Caspersen I, Haugen M, Schjølberg S, Vejrup K, Knutsen H, Brantsæter A, et al. 2016. Maternal dietary exposure to dioxins and polychlorinated biphenyls (PCBs) is associated with language delay in 3year old Norwegian children. Environment International 91: 180-187.

CDC. 2009. Fourth National Report on Human Exposure to Environmental Chemicals Atlanta, USA:Department of Health and Human Services, Centers for Disease Control and Prevention

Černá M, Krsková A, Čejchanová M, Spěváčková V. 2012. Human biomonitoring in the Czech Republic: an overview. International journal of hygiene and environmental health 215(2): 109-119.

Cerrillo I, Olea-Serrano M, Ibarluzea J, Exposito J, Torne P, Laguna J, et al. 2006. Environmental and lifestyle factors for organochlorine exposure among women living in Southern Spain. Chemosphere 62(11): 1917-1924.

Cohn BA, La Merrill M, Krigbaum NY, Yeh G, Park J-S, Zimmermann L, et al. 2015. DDT exposure in utero and breast cancer. The Journal of Clinical Endocrinology & Metabolism 100(8): 2865-2872.

Collaborative Group on Hormonal Factors in Breast Cancer. 1997. Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52 705 women with breast cancer and 108 411 women without breast cancer. The Lancet 350(9084): 1047-1059.

Collaborative Group on Hormonal Factors in Breast Cancer. 2002. Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50 302 women with breast cancer and 96 973 women without the disease. The Lancet 360(9328): 187-195.

Conzen SG, TA; Olopade, OI 2008. Cancer of the Breast: The molecular biology of breast cancer In book: Cancer: Principles and Practice of Oncology, Edition: 8th edition, Chapter: 43, Editors: edDeVita et al, pp.74 chapters, 3039 pp. In: Cancer: Principles and Practice of

Oncology, Vol. Volume 2, (Jr. JWP, ed). Philadelphia, USA:Wolters Kluwer Health, 1595 - 1606.

Crinnion WJ. 2009. Chlorinated pesticides: threats to health and importance of detection. Alternative medicine review 14(4): 347-360.

Charlier C, Albert A, Herman P, Hamoir E, Gaspard U, Meurisse M, et al. 2003. Breast cancer and serum organochlorine residues. Occupational and Environmental Medicine 60(5): 348-351.

Charlier C, Foidart J-M, Pitance F, Herman P, Gaspard U, Meurisse M, et al. 2004. Environmental dichlorodiphenyltrichlorethane or hexachlorobenzene exposure and breast cancer: is there a risk? Clinical chemistry and laboratory medicine 42(2): 222-227.

Charlier CJ, Foidart J-M. 2005. Comparative study of dichlorodiphenyldichloroethylene in blood and semen of two young male populations: lack of relationship to infertility, but evidence of high exposure of the mothers. Reproductive Toxicology 20(2): 215-220.

Dasgupta S, Meisner C, Wheeler D. 2010. Stockpiles of obsolete pesticides and cleanup priorities: a methodology and application for Tunisia. Journal of environmental management 91(4): 824-830.

Davis DL, Bradlow HL, Wolff M, Woodruff T, Hoel DG, Anton-Culver H. 1993. Medical hypothesis: xenoestrogens as preventable causes of breast cancer. Environmental Health Perspectives 101(5): 372.

Demers A, Ayotte P, Brisson J, Dodin S, Robert J, Dewailly E. 2000. Risk and aggressiveness of breast cancer in relation to plasma organochlorine concentrations. Cancer Epidemiology Biomarkers & Prevention 9(2): 161-166.

DeSantis C, Ma J, Bryan L, Jemal A. 2014. Breast cancer statistics, 2013. CA: a cancer journal for clinicians 64(1): 52-62.

Dewalque L, Pirard C, Charlier C. 2014. Measurement of urinary biomarkers of parabens, benzophenone-3, and phthalates in a Belgian population. BioMed research international 2014.

Diamanti-Kandarakis E, Bourguignon J-P, Giudice LC, Hauser R, Prins GS, Soto AM, et al. 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocrine reviews 30(4): 293-342.

Dirinck E, Jorens PG, Covaci A, Geens T, Roosens L, Neels H, et al. 2011. Obesity and persistent organic pollutants: possible obesogenic effect of organochlorine pesticides and polychlorinated biphenyls. Obesity 19(4): 709-714.

Djordjevic MV, Hoffmann D, Fan J, Prokopczyk B, Citron ML, Stellman SD. 1994. Assessment of chlorinated pesticides and polychiorinated biphenyls in adipose breast tissue using a supercritical fluid extraction method. Carcinogenesis 15(11): 2581-2585.

Duggan C, Irwin ML, Xiao L, Henderson KD, Smith AW, Baumgartner RN, et al. 2010. Associations of insulin resistance and adiponectin with mortality in women with breast cancer. Journal of Clinical Oncology: JCO. 2009.2026. 4473.

Dvorská A, Šír M, Honzajková Z, Komprda J, Čupr P, Petrlík J, et al. 2012. Obsolete pesticide storage sites and their POP release into the environment—an Armenian case study. Environmental Science and Pollution Research 19(6): 1944-1952.

Eyster JT, Humphrey HE, Kimbrough RD. 1983. Partitioning of polybrominated biphenyls (PBBs) in serum, adipose tissue, breast milk, placenta, cord blood, biliary fluid, and feces. Archives of Environmental Health: An International Journal 38(1): 47-53.

Falck Jr F, Ricci Jr A, Wolff MS, Godbold J, Deckers P. 1991. Pesticides and polychlorinated biphenyl residues in human breast lipids and their relation to breast cancer. Archives of Environmental Health 47(2): 143-146.

Fenton SE. 2006. Endocrine-disrupting compounds and mammary gland development: early exposure and later life consequences. Endocrinology 147(6): s18-s24.

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. International journal of cancer 127(12): 2893-2917.

Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh J, Comber H, et al. 2013. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. European journal of cancer 49(6): 1374-1403.

Fernández J, Arjol M, Cacho C. 2013. POP-contaminated sites from HCH production in Sabiñánigo, Spain. Environmental Science and Pollution Research 20(4): 1937-1950.

Fernandez M, Kiviranta H, Molina-Molina J, Laine O, Lopez-Espinosa M, Vartiainen T, et al. 2008. Polychlorinated biphenyls (PCBs) and hydroxy-PCBs in adipose tissue of women in Southeast Spain. Chemosphere 71(6): 1196-1205.

Fernandez MF, Olmos B, Granada A, López-Espinosa MJ, Molina-Molina J-M, Fernandez JM, et al. 2007a. Human exposure to endocrine-disrupting chemicals and prenatal risk factors for cryptorchidism and hypospadias: a nested case-control study. Environmental Health Perspectives.

Fernández MF, Rivas A, Olea-Serrano F, Cerrillo I, Molina-Molina JM, Araque P, et al. 2004. Assessment of total effective xenoestrogen burden in adipose tissue and identification of chemicals responsible for the combined estrogenic effect. Analytical and bioanalytical chemistry 379(1): 163-170.

Fernandez MF, Santa-Marina L, Ibarluzea JM, Exposito J, Aurrekoetxea JJ, Torne P, et al. 2007b. Analysis of population characteristics related to the total effective xenoestrogen burden: a biomarker of xenoestrogen exposure in breast cancer. European journal of cancer 43(8): 1290-1299.

Forns J, Torrent M, Garcia-Esteban R, Grellier J, Gascon M, Julvez J, et al. 2012. Prenatal exposure to polychlorinated biphenyls and child neuropsychological development in 4-year-olds: An analysis per congener and specific cognitive domain. Science of the total environment 432: 338-343.

Frederiksen H, Nielsen JKS, Mørck TA, Hansen PW, Jensen JF, Nielsen O, et al. 2013. Urinary excretion of phthalate metabolites, phenols and parabens in rural and urban Danish mother–child pairs. International journal of hygiene and environmental health 216(6): 772-783.

Frederiksen H, Skakkebaek NE, Andersson AM. 2007. Metabolism of phthalates in humans. Molecular nutrition & food research 51(7): 899-911.

Freire C, Lopez-Espinosa M-J, Fernández M, Molina-Molina J-M, Prada R, Olea N. 2011. Prenatal exposure to organochlorine pesticides and TSH status in newborns from Southern Spain. Science of the total environment 409(18): 3281-3287.

Frenich AG, Vidal J, Frías MM, Olea-Serrano F, Olea N. 2000. Quantitative determination of endocrine-disrupting polychlorinated biphenyls and organochlorinated pesticides in human serum using gas chromatography with electron-capture detection and tandem mass spectrometry. Journal of mass spectrometry 35(8): 967-975.

Freyberger A, Witters H, Weimer M, Lofink W, Berckmans P, Ahr H-J. 2010. Screening for (anti) androgenic properties using a standard operation protocol based on the human stably transfected androgen sensitive PALM cell line. First steps towards validation. Reproductive Toxicology 30(1): 9-17.

García MA, Peña D, Álvarez L, Cocca C, Pontillo C, Bergoc R, et al. 2010. Hexachlorobenzene induces cell proliferation and IGF-I signaling pathway in an estrogen receptor α-dependent manner in MCF-7 breast cancer cell line. Toxicology letters 192(2): 195-205.

Gasull M, Porta M, Pumarega J, Vioque J, de Basea MB, Puigdomènech E, et al. 2010. The relative influence of diet and serum concentrations of organochlorine compounds on K-ras mutations in exocrine pancreatic cancer. Chemosphere 79(7): 686-697.

Gioia R, Akindele AJ, Adebusoye SA, Asante KA, Tanabe S, Buekens A, et al. 2014. Polychlorinated biphenyls (PCBs) in Africa: a review of environmental levels. Environmental Science and Pollution Research 21(10): 6278-6289.

Govarts E, Nieuwenhuijsen M, Schoeters G, Ballester F, Bloemen K, De Boer M, et al. 2012. Birth weight and prenatal exposure to polychlorinated biphenyls (PCBs) and dichlorodiphenyldichloroethylene (DDE): a meta-analysis within 12 European Birth Cohorts. Environmental Health Perspectives 120(2): 162.

Greenland S. 1995. Dose-response and trend analysis in epidemiology: alternatives to categorical analysis. Epidemiology: 356-365.

Guo YL, Emmett EA, Pellizzari ED, Rohde CA. 1987. Influence of serum cholesterol and albumin on partitioning of PCB congeners between human serum and adipose tissue. Toxicology and applied pharmacology 87(1): 48-56.

Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. cell 100(1): 57-70.

Hauser R, Chen Z, Pothier L, Ryan L, Altshul L. 2003. The relationship between human semen parameters and environmental exposure to polychlorinated biphenyls and p, p'-DDE. Environmental Health Perspectives 111(12): 1505.

Helzlsouer KJ, Alberg AJ, Huang H-Y, Hoffman SC, Strickland PT, Brock JW, et al. 1999. Serum concentrations of organochlorine compounds and the subsequent development of breast cancer. Cancer Epidemiology Biomarkers & Prevention 8(6): 525-532.

Herington AC, Chopin LK, Jeffery P, de Amorim L, Veveris-Lowe T, Bui L, et al. 2010. Hormone-dependent cancers: new approaches to identification of potential diagnostic and/or therapeutic biomarkers. Asia-Pacific Journal of Molecular Biology and Biotechnology 18(1): 63-66.

Høyer AP, Grandjean P, Jørgensen T, Brock JW, Hartvig HB. 1998. Organochlorine exposure and risk of breast cancer. The Lancet 352(9143): 1816-1820.

Ibarluzea JM, Fernández MF, Santa-Marina L, Olea-Serrano MF, Rivas AM, Aurrekoetxea JJ, et al. 2004. Breast cancer risk and the combined effect of environmental estrogens. Cancer Causes & Control 15(6): 591-600.

Itoh H, Iwasaki M, Kasuga Y, Yokoyama S, Onuma H, Nishimura H, et al. 2014. Association between serum organochlorines and global methylation level of leukocyte DNA among Japanese women: a cross-sectional study. Science of the total environment 490: 603-609.

Jansen MS, Nagel SC, Miranda PJ, Lobenhofer EK, Afshari CA, McDonnell DP. 2004. Short-chain fatty acids enhance nuclear receptor activity through mitogen-activated protein kinase activation and histone deacetylase inhibition. Proceedings of the National Academy of Sciences of the United States of America 101(18): 7199-7204.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. 2011. Global cancer statistics. CA: a cancer journal for clinicians 61(2): 69-90.

Kahlenborn C, Modugno F, Potter DM, Severs WB. Oral contraceptive use as a risk factor for premenopausal breast cancer: a meta-analysis. In: Proceedings of the Mayo Clinic Proceedings, 2006, Vol. 81. Elsevier, 1290-1302.

Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray LE, Kaattari S, et al. 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the US EPA-sponsored workshop. Environmental Health Perspectives 104(Suppl 4): 715.

Kelsey JL, Gammon MD, John EM. 1993. Reproductive factors and breast cancer. Epidemiologic reviews 15(1): 36.

Key T, Appleby P, Barnes I, Reeves G. 2002. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. Journal of the National Cancer Institute 94(8): 606-616.

Khodarahmi M, Azadbakht L. 2014. The association between different kinds of fat intake and breast cancer risk in women. International journal of preventive medicine 5(1): 6-15.

Kim M-J, Marchand P, Henegar C, Antignac J-P, Alili R, Poitou C, et al. 2011. Fate and complex pathogenic effects of dioxins and polychlorinated biphenyls in obese subjects before and after drastic weight loss. Environmental Health Perspectives 119(3): 377.

Knower KC, To SQ, Leung Y-K, Ho S-M, Clyne CD. 2014. Endocrine disruption of the epigenome: a breast cancer link. Endocrine-related cancer 21(2): T33-T55.

Kohlmeier L, Kohlmeier M. 1995. Adipose tissue as a medium for epidemiologic exposure assessment. Environmental Health Perspectives 103(Suppl 3): 99.

Kortenkamp A. 2006. Breast cancer, oestrogens and environmental pollutants: a re-evaluation from a mixture perspective. International journal of andrology 29(1): 193-198.

Kuruto-Niwa R, Nozawa R, Miyakoshi T, Shiozawa T, Terao Y. 2005. Estrogenic activity of alkylphenols, bisphenol S, and their chlorinated derivatives using a GFP expression system. Environmental toxicology and pharmacology 19(1): 121-130.

Lacey JV, Kreimer AR, Buys SS, Marcus PM, Chang S-C, Leitzmann MF, et al. 2009. Breast cancer epidemiology according to recognized breast cancer risk factors in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial Cohort. BMC cancer 9(1): 84.

Lahmann PH, Hoffmann K, Allen N, Van Gils CH, Khaw KT, Tehard B, et al. 2004. Body size and breast cancer risk: findings from the European Prospective Investigation into Cancer And Nutrition (EPIC). International journal of cancer 111(5): 762-771.

Lassen TH, Frederiksen H, Jensen TK, Petersen JH, Joensen UN, Main KM, et al. 2014. Urinary bisphenol A levels in young men: association with reproductive hormones and semen quality. Environmental Health Perspectives 122(5): 478.

Lauby-Secretan B, Loomis D, Baan R, El Ghissassi F, Bouvard V, Benbrahim-Tallaa L, et al. 2016. Use of mechanistic data in the IARC evaluations of the carcinogenicity of polychlorinated biphenyls and related compounds. Environmental Science and Pollution Research 23(3): 2220-2229.

Lemaire G, Mnif W, Mauvais P, Balaguer P, Rahmani R. 2006. Activation of α-and β-estrogen receptors by persistent pesticides in reporter cell lines. Life sciences 79(12): 1160-1169.

Loomis D, Guyton K, Grosse Y, El Ghissasi F, Bouvard V, Benbrahim-Tallaa L, et al. 2015. Carcinogenicity of lindane, DDT, and 2, 4-dichlorophenoxyacetic acid. The Lancet Oncology 16(8): 891.

López-Abente G, Mispireta S, Pollán M. 2014. Breast and prostate cancer: an analysis of common epidemiological features in mortality trends in Spain. BMC cancer 14(1): 874.

López-Carrillo L, Torres-Sánchez L, Blair A, Cebrián ME, Uribe M. 1999. The adipose tissue to serum dichlorodiphenyldichloroethane (DDE) ratio: some methodological considerations. Environmental Research 81(2): 142-145.

Lopez-Espinosa M-J, Granada A, Carreno J, Salvatierra M, Olea-Serrano F, Olea N. 2007. Organochlorine pesticides in placentas from Southern Spain and some related factors. Placenta 28(7): 631-638.

Lopez-Espinosa M-J, Silva E, Granada A, Molina-Molina J-M, Fernandez MF, Aguilar-Garduño C, et al. 2009. Assessment of the total effective xenoestrogen burden in extracts of human placentas. Biomarkers 14(5): 271-277.

Lopez-Espinosa M, Kiviranta H, Araque P, Ruokojärvi P, Molina-Molina J, Fernandez M, et al. 2008. Dioxins in adipose tissue of women in Southern Spain. Chemosphere 73(6): 967-971.

Luzardo OP, Boada LD, Carranza C, Ruiz-Suárez N, Henríquez-Hernández LA, Valerón PF, et al. 2014. Socioeconomic development as a determinant of the levels of organochlorine pesticides and PCBs in the inhabitants of Western and Central African countries. Science of the total environment 497: 97-105.

Ma J, Jemal A. 2013. Breast cancer statistics. In: Breast Cancer Metastasis and Drug Resistance:Springer, 1-18.

Maalej M, Hentati D, Messai T, Kochbati L, El May A, Mrad K, et al. 2008. Breast cancer in Tunisia in 2004: a comparative clinical and epidemiological study. Bulletin du cancer 95(2): 10005-10009.

Madigan MP, Ziegler RG, Benichou J, Byrne C, Hoover RN. 1995. Proportion of breast cancer cases in the United States explained by well-established risk factors. Journal of the National Cancer Institute 87(22): 1681-1685.

Mahoney MC, Bevers T, Linos E, Willett WC. 2008. Opportunities and strategies for breast cancer prevention through risk reduction. CA: a cancer journal for clinicians 58(6): 347-371.

Martínez-Vidal J, Moreno-Frías M, Frenich GA, Olea-Serrano F, Olea N. 2002. Determination of endocrine-disrupting pesticides and polychlorinated biphenyls in human serum by GC–ECD and GC–MS–MS and evaluation of contributions to the uncertainty of the results. Analytical and bioanalytical chemistry 372(7-8): 766-775.

McGraw JE, Waller DP. 2009. Fish ingestion and congener specific polychlorinated biphenyl and p, p'-dichlorodiphenyldichloroethylene serum concentrations in a great lakes cohort of pregnant African American women. Environment International 35(3): 557-565.

Mercado LA, Freille SM, Vaca-Pereira JS, Cuellar M, Flores L, Mutch E, et al. 2013. Serum concentrations of p, p′-dichlorodiphenyltrichloroethane (p, p′-DDE) in a sample of agricultural workers from Bolivia. Chemosphere 91(10): 1381-1385.

Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, et al. 1994. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 266(5182): 66-71.

Million Women Study Collaborators. 2003. Breast cancer and hormone-replacement therapy in the Million Women Study. The Lancet 362(9382): 419-427.

Mills PK, Yang R. 2003. Prostate cancer risk in California farm workers. Journal of Occupational and Environmental medicine 45(3): 249-258.

Moreno-Frías M, Jiménez-Torres M, Garrido-Frenich A, Martínez Vidal JL, Olea-Serrano F, Olea N. 2004. Determination of organochlorine compounds in human biological samples by GC-MS/MS. Biomedical Chromatography 18(2): 102-111.

Moysich KB, Shields PG, Freudenheim JL, Schisterman EF, Vena JE, Kostyniak P, et al. 1999. Polychlorinated biphenyls, cytochrome P4501A1 polymorphism, and postmenopausal breast cancer risk. Cancer Epidemiology Biomarkers & Prevention 8(1): 41-44.

Muñoz-de-Toro M, Durando M, Beldoménico PM, Beldoménico HR, Kass L, García SR, et al. 2006. Estrogenic microenvironment generated by organochlorine residues in adipose mammary tissue modulates biomarker expression in ERalpha-positive breast carcinomas. Breast Cancer Res 8(4): R47.

Mussalo-Rauhamaa H. 1991. Partitioning and levels of neutral organochlorine compounds in human serum, blood cells, and adipose and liver tissue. Science of the total environment 103(2): 159-175.

Mussalo-Rauhamaa H, Hasanen E, Pyysalo H, Antervo K, Kauppila R, Pantzar P. 1990. Occurrence of beta-hexachlorocyclohexane in breast cancer patients. Cancer 66(10): 2124-2128.

Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, et al. 2007. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science 317(5834): 121-124.

Neal J. 2016. How the endocrine system works, 2nd edition: Wiley Blackwell.

Okubo T, Yokoyama Y, Kano K, Kano I. 2001. ER-dependent estrogenic activity of parabens assessed by proliferation of human breast cancer MCF-7 cells and expression of ERα and PR. Food and Chemical Toxicology 39(12): 1225-1232.

Olea N, Fernández M, Araque P, Olea-Serrano F. 2002. Perspectivas en disrupción endocrina. Gaceta sanitaria 16(3): 650-656.

Olea N, Fernandez M, Martin-Olmedo P. 2001a. Endocrine Disrupters. The case of oestrogenic xenobiotics. Rev Salud Ambient 1: 6-11.

Olea N, Fernandez M, Martin-Olmedo P. 2001b. Endocrine Disrupters. The case of oestrogenic xenobiotics II: synthetic oestrogens. Rev Salud Ambient 1: 64-72.

Parada H, Wolff MS, Engel LS, White AJ, Eng SM, Cleveland RJ, et al. 2016. Organochlorine insecticides DDT and chlordane in relation to survival following breast cancer. International journal of cancer 138(3): 565-575.

Pauwels A, Covaci A, Weyler J, Delbeke L, Dhont M, De Sutter P, et al. 2000. Comparison of persistent organic pollutant residues in serum and adipose tissue in a female population in Belgium, 1996–1998. Archives of environmental contamination and toxicology 39(2): 265-270.

Pestana D, Teixeira D, Faria A, Domingues V, Monteiro R, Calhau C. 2015. Effects of environmental organochlorine pesticides on human breast cancer: Putative involvement on invasive cell ability. Environmental toxicology 30(2): 168-176.

Phillips DL, Pirkle JL, Burse VW, Bernert Jr JT, Henderson LO, Needham LL. 1989. Chlorinated hydrocarbon levels in human serum: effects of fasting and feeding. Archives of environmental contamination and toxicology 18(4): 495-500.

Pi N, Chia SE, Ong CN, Kelly BC. 2016. Associations of serum organohalogen levels and prostate cancer risk: Results from a case–control study in Singapore. Chemosphere 144: 1505-1512.

Pinzone JJ, Stevenson H, Strobl JS, Berg PE. 2004. Molecular and cellular determinants of estrogen receptor α expression. Molecular and cellular biology 24(11): 4605-4612.

Pontillo C, Español A, Chiappini F, Miret N, Cocca C, Alvarez L, et al. 2015. Hexachlorobenzene promotes angiogenesis in vivo, in a breast cancer model and neovasculogenesis in vitro, in the human microvascular endothelial cell line HMEC-1. Toxicology letters 239(1): 53-64.

Pontillo CA, Rojas P, Chiappini F, Sequeira G, Cocca C, Crocci M, et al. 2013. Action of hexachlorobenzene on tumor growth and metastasis in different experimental models. Toxicology and applied pharmacology 268(3): 331-342.

Porta M, Puigdomènech E, Ballester F, Selva J, Ribas-Fitó N, Domínguez-Boada L, et al. 2008a. Studies conducted in Spain on concentrations in humans of persistent toxic compounds. Gaceta sanitaria 22(3): 248-266.

Porta M, Puigdomènech E, Ballester F, Selva J, Ribas-Fitó N, Llop S, et al. 2008b. Monitoring concentrations of persistent organic pollutants in the general population: the international experience. Environment International 34(4): 546-561.

Puertas R, Lopez-Espinosa M-J, Cruz F, Ramos R, Freire C, Pérez-García M, et al. 2010. Prenatal exposure to mirex impairs neurodevelopment at age of 4 years. Neurotoxicology 31(1): 154-160.

Rafnsson V. 2006. Risk of non-Hodgkin's lymphoma and exposure to hexachlorocyclohexane, a nested case-control study. European journal of cancer 42(16): 2781-2785.

Rasmussen TH, Nielsen F, Andersen HR, Nielsen JB, Weihe P, Grandjean P. 2003. Assessment of xenoestrogenic exposure by a biomarker approach: application of the E-Screen bioassay to determine estrogenic response of serum extracts. Environmental Health 2(1): 12.

Ray A, Jain D, Yadav R, Naik S, Sharma S, Bahadur A, et al. 2001. Effect of cancer treatment modalities on serum lipids and lipoproteins among women with carcinoma of the breast. Indian journal of physiology and pharmacology 45(3): 337-344.

RCG RdCdG. 2014. Tendencias Temporales de la Incidencia de Cáncer en la provincia de Granada, 1985-2011.Escuela Andaluza de Salud Pública.

RCNT. 1998.Registre des cancers Nord Tunisie (RCNT), données (1995-1998).

RCNT. 2006 Registre des cancers Nord Tunisie (RCNT), données (2004-2006).

Reed L, Büchner V, Tchounwou PB. 2007. Environmental toxicology and health effects associated with hexachlorobenzene exposure. Reviews on environmental health 22(3): 213-244.

Rivas A, Fernandez MF, Cerrillo I, Ibarluzea J, Olea-Serrano MF, Pedraza V, et al. 2001. Human exposure to endocrine disrupters: standardisation of a marker of estrogenic exposure in adipose tissue. Apmis 109(S103): S189-S202.

Rivero J, Luzardo OP, Henríquez-Hernández LA, Machín RP, Pestano J, Zumbado M, et al. 2015. In vitro evaluation of oestrogenic/androgenic activity of the serum organochlorine pesticide mixtures previously described in a breast cancer case–control study. Science of the total environment 537: 197-202.

Romieu I, Hernandez-Avila M, Lazcano-Ponce E, Weber JP, Dewailly E. 2000. Breast cancer, lactation history, and serum organochlorines. American Journal of Epidemiology 152(4): 363-370.

Rothman N, Cantor KP, Blair A, Bush D, Brock JW, Helzlsouer K, et al. 1997. A nested case-control study of non-Hodgkin lymphoma and serum organochlorine residues. The Lancet 350(9073): 240-244.

Rudel RA, Ackerman JM, Attfield KR, Brody JG. 2014. New exposure biomarkers as tools for breast cancer epidemiology, biomonitoring, and prevention: a systematic approach based on animal evidence. Environmental Health Perspectives (Online) 122(9): 881.

Rüegg J, Penttinen-Damdimopoulou P, Mäkelä S, Pongratz I, Gustafsson J-Å. 2009. Receptors mediating toxicity and their involvement in endocrine disruption. In: Molecular, clinical and environmental toxicology:Springer, 289-323.

Rusiecki JA, Matthews A, Sturgeon S, Sinha R, Pellizzari E, Zheng T, et al. 2005. A correlation study of organochlorine levels in serum, breast adipose tissue, and gluteal adipose tissue among breast cancer cases in India. Cancer Epidemiology Biomarkers & Prevention 14(5): 1113-1124.

Russo J, Russo IH. 2006. The role of estrogen in the initiation of breast cancer. The Journal of steroid biochemistry and molecular biology 102(1): 89-96.

Rylander L, Strömberg U, Dyremark E, Östman C, Nllsson-Ehle P, Hagmar L. 1998. Polychlorinated biphenyls in blood plasma among Swedish female fish consumers in relation to low birth weight. American Journal of Epidemiology 147(5): 493-502.

Safe SH. 1994. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. Critical reviews in toxicology 24(2): 87-149.

Santini F, Vitti P, Ceccarini G, Mammoli C, Rosellini V, Pelosini C, et al. 2003. In vitro assay of thyroid disruptors affecting TSH-stimulated adenylate cyclase activity. Journal of endocrinological investigation 26(10): 950-955.

Schettgen T, Alt A, Esser A, Kraus T. 2015. Current data on the background burden to the persistent organochlorine pollutants HCB, p, p′-DDE as well as PCB 138, PCB 153 and PCB 180 in plasma of the general population in Germany. International journal of hygiene and environmental health 218(4): 380-385.

Schug T, Abagyan R, Blumberg B, Collins T, Crews D, DeFur P, et al. 2013. Designing endocrine disruption out of the next generation of chemicals. Green Chemistry 15(1): 181-198.

Shah FD, Shukla SN, Shah PM, Patel HR, Patel PS. 2008. Significance of alterations in plasma lipid profile levels in breast cancer. Integrative cancer therapies 7(1): 33-41.

Shakeel MK, George PS, Jose J, Jose J, Mathew A. 2010. Pesticides and breast cancer risk: a comparison between developed and developing countries. Asian Pac J Cancer Prev 11(1): 173-180.

Sharma BM, Bharat GK, Tayal S, Nizzetto L, Čupr P, Larssen T. 2014. Environment and human exposure to persistent organic pollutants (POPs) in India: A systematic review of recent and historical data. Environment International 66: 48-64.

Shelton JF, Geraghty EM, Tancredi DJ, Delwiche LD, Schmidt RJ, Ritz B, et al. 2014. Neurodevelopmental disorders and prenatal residential proximity to agricultural pesticides: the CHARGE study. Environ Health Perspect 122(10): 1103-1109.

Sieri S, Krogh V, Ferrari P, Berrino F, Pala V, Thiébaut AC, et al. 2008. Dietary fat and breast cancer risk in the European Prospective Investigation into Cancer and Nutrition. The American journal of clinical nutrition 88(5): 1304-1312.

Silva E, Rajapakse N, Kortenkamp A. 2002. Something from "nothing"-eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environmental science & technology 36(8): 1751-1756.

Singletary SE, Walsh G, Vauthey J-N, Curley S, Sawaya R, Weber KL, et al. 2003. A role for curative surgery in the treatment of selected patients with metastatic breast cancer. The Oncologist 8(3): 241-251.

Snedeker SM. 2001. Pesticides and breast cancer risk: a review of DDT, DDE, and dieldrin. Environmental Health Perspectives 109(Suppl 1): 35.

Søeborg T, Frederiksen H, Andersson A-M. 2014. Considerations for estimating daily intake values of nonpersistent environmental endocrine disruptors based on urinary biomonitoring data. Reproduction 147(4): 455-463.

Soto AM, Chung KL, Sonnenschein C. 1994. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environmental Health Perspectives 102(4): 380.

Soto AM, Sonnenschein C. 2010. Environmental causes of cancer: endocrine disruptors as carcinogens. Nature Reviews Endocrinology 6(7): 363-370.

Soto AM, Sonnenschein C. 2015. Endocrine disruptors: DDT, endocrine disruption and breast cancer. Nat Rev Endocrinol 11(9): 507-508.

Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. 1995. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environmental Health Perspectives 103(Suppl 7): 113.

Stabile LP, Davis ALG, Gubish CT, Hopkins TM, Luketich JD, Christie N, et al. 2002. Human non-small cell lung tumors and cells derived from normal lung express both estrogen receptor α and β and show biological responses to estrogen. Cancer Research 62(7): 2141-2150.

Steinmetz R, Young PC, Caperell-Grant A, Gize EA, Madhukar BV, Ben-Jonathan N, et al. 1996. Novel estrogenic action of the pesticide residue β-hexachlorocyclohexane in human breast cancer cells. Cancer Research 56(23): 5403-5409.

Stellman SD, Djordjevic MV, Muscat JE, Gong L, Bernstein D, Citron ML, et al. 1998. Relative abundance of organochlorine pesticides and polychlorinated biphenyls in adipose tissue and serum of women in Long Island, New York. Cancer Epidemiology Biomarkers & Prevention 7(6): 489-496.

Takatori S, Kitagawa Y, Oda H, Miwa G, Nishikawa J-i, Nishihara T, et al. 2003. Estrogenicity of Metabolites of Benzophenone Derivatives Examined by a Yeast Two-Hybrid Assay. Journal of health science 49(2): 91-98.

Tao S, Xu F, Wang X, Liu W, Gong Z, Fang J, et al. 2005. Organochlorine pesticides in agricultural soil and vegetables from Tianjin, China. Environmental science & technology 39(8): 2494-2499.

Térouanne B, Tahiri B, Georget V, Belon C, Poujol N, Avances C, et al. 2000. A stable prostatic bioluminescent cell line to investigate androgen and antiandrogen effects. Molecular and cellular endocrinology 160(1): 39-49.

The European Commission. 2011. amending Directive 2002/72/EC as regards the restriction of use of Bisphenol A in plastic infant feeding bottles. Fed Reg 2011/08/EU.

Tilghman SL, Bratton MR, Segar HC, Martin EC, Rhodes LV, Li M, et al. 2012. Endocrine disruptor regulation of microRNA expression in breast carcinoma cells. PloS one 7(3): e32754.

Tinwell H, Ashby J. 2004. Sensitivity of the immature rat uterotrophic assay to mixtures of estrogens. Environmental Health Perspectives 112(5): 575.

Tjønneland A, Christensen J, Olsen A, Stripp C, Thomsen BL, Overvad K, et al. 2007. Alcohol intake and breast cancer risk: the European Prospective Investigation into Cancer and Nutrition (EPIC). Cancer Causes & Control 18(4): 361-373.

Trasande L, Zoeller RT, Hass U, Kortenkamp A, Grandjean P, Myers JP, et al. 2015. Estimating burden and disease costs of exposure to endocrine-disrupting chemicals in the European Union. The Journal of Clinical Endocrinology & Metabolism 100(4): 1245-1255.

Travis LB, Hill DA, Dores GM, Gospodarowicz M, van Leeuwen FE, Holowaty E, et al. 2003. Breast cancer following radiotherapy and chemotherapy among young women with Hodgkin disease. Jama 290(4): 465-475.

Trejo-Acevedo A, Díaz-Barriga F, Carrizales L, Domínguez G, Costilla R, Ize-Lema I, et al. 2009. Exposure assessment of persistent organic pollutants and metals in Mexican children. Chemosphere 74(7): 974-980.

Trejo-Acevedo A, Rivero-Pérez Norma E, Flores-Ramírez R, Díaz-Barriga F, Ochoa-Angeles C, Nelinho P-MI. 2013. Assessment of persistent organic pollutants levels in blood samples

from Quintana Roo, Mexico. International journal of hygiene and environmental health 216(3): 284-289.

Turci R, Balducci C, Brambilla G, Colosio C, Imbriani M, Mantovani A, et al. 2010. A simple and fast method for the determination of selected organohalogenated compounds in serum samples from the general population. Toxicology letters 192(1): 66-71.

UNEP/POPS/COP.7/36. 2015. Report of the Conference of the Parties to the Stockholm Convention on Persistent Organic Pollutants on the work of its seventh meeting. Geneva (Switzerland):United Nations (UNEP).

UNEP/WHO. 2012. State of the Science of Endocrine Disrupting Chemicals 2012. Geneva, Switzerland: United Nations Environment Programme and the World Health Organization.

Valerón PF, Pestano JJ, Luzardo OP, Zumbado ML, Almeida M, Boada LD. 2009. Differential effects exerted on human mammary epithelial cells by environmentally relevant organochlorine pesticides either individually or in combination. Chemico-biological interactions 180(3): 485-491.

Vela-Soria F, Ballesteros O, Zafra-Gómez A, Ballesteros L, Navalón A. 2014. UHPLC–MS/MS method for the determination of bisphenol A and its chlorinated derivatives, bisphenol S, parabens, and benzophenones in human urine samples. Analytical and bioanalytical chemistry 406(15): 3773-3785.

Vilahur N, Bustamante M, Byun H-M, Fernandez MF, Santa Marina L, Basterrechea M, et al. 2014a. Prenatal exposure to mixtures of xenoestrogens and repetitive element DNA methylation changes in human placenta. Environment International 71: 81-87.

Vilahur N, Fernández MF, Bustamante M, Ramos R, Forns J, Ballester F, et al. 2014b. In utero exposure to mixtures of xenoestrogens and child neuropsychological development. Environmental Research 134: 98-104.

Vilahur N, Molina-Molina JM, Bustamante M, Murcia M, Arrebola JP, Ballester F, et al. 2013. Male specific association between xenoestrogen levels in placenta and birthweight. Environment International 51: 174-181.

Villalobos M, Olea N, Brotons JA, Olea-Serrano MF, De Almodovar JR, Pedraza V. 1995. The E-screen assay: a comparison of different MCF7 cell stocks. Environmental Health Perspectives 103(9): 844.

Vogel VG. 2008. Epidemiology, genetics, and risk evaluation of postmenopausal women at risk of breast cancer. Menopause 15(4): 782-789.

Waliszewski S, Carvajal O, Infanzon R, Trujillo P, Hart M. 2004. Copartition ratios of persistent organochlorine pesticides between human adipose tissue and blood serum lipids. Bulletin of environmental contamination and toxicology 73(4): 732-738.

Whitcomb BW, Schisterman EF, Buck GM, Weiner JM, Greizerstein H, Kostyniak PJ. 2005. Relative concentrations of organochlorines in adipose tissue and serum among reproductive age women. Environmental toxicology and pharmacology 19(2): 203-213.

Whitworth KW, Bornman RM, Archer JI, Kudumu MO, Travlos GS, Wilson RE, et al. 2014. Predictors of plasma DDT and DDE concentrations among women exposed to indoor residual spraying for malaria control in the South African Study of Women and Babies (SOWB). Environmental Health Perspectives 122(6): 545.

Wolff MS, Toniolo PG, Lee EW, Rivera M, Dubin N. 1993. Blood levels of organochlorine residues and risk of breast cancer. Journal of the National Cancer Institute 85(8): 648-652.

Wolff MS, Zeleniuch-Jacquotte A, Dubin N, Toniolo P. 2000. Risk of breast cancer and organochlorine exposure. Cancer Epidemiology Biomarkers & Prevention 9(3): 271-277.

Wong PS, Matsumura F. 2007. Promotion of breast cancer by β-Hexachlorocyclohexane in MCF10AT1 cells and MMTV-neu mice. BMC cancer 7(1): 130.

Woodruff TJ, Zota AR, Schwartz JM. 2011. Environmental chemicals in pregnant women in the United States: NHANES 2003-2004. Environmental Health Perspectives 119(6): 878.

World Bank. 2012. Demonstrating and promoting best techniques and practices for managing healthcare waste and polychlorinated biphenyls (PCBs) project.Sustainable Development Department Middle East and North Africa Region

Yáñez L, Borja-Aburto VcH, Rojas E, de la Fuente H, González-Amaro R, Gómez H, et al. 2004. DDT induces DNA damage in blood cells. Studies in vitro and in women chronically exposed to this insecticide. Environmental Research 94(1): 18-24.

Zacharewski T. 1998. Identification and assessment of endocrine disruptors: limitations of in vivo and in vitro assays. Environmental Health Perspectives 106(Suppl 2): 577.

Zhang J, Huang Y, Wang X, Lin K, Wu K. 2015. Environmental Polychlorinated Biphenyl Exposure and Breast Cancer Risk: A Meta-Analysis of Observational Studies. PloS one 10(11): e0142513.

Zheng T, Holford TR, Mayne ST, Tessari J, Owens PH, Zahm SH, et al. 1999. Environmental exposure to hexachlorobenzene (HCB) and risk of female breast cancer in Connecticut. Cancer Epidemiology Biomarkers & Prevention 8(5): 407-411.

Zheng T, Holford TR, Mayne ST, Ward B, Carter D, Ownes PH, et al. 1999. DDE and DDT in breast adipose tissue and risk of female breast cancer. American Journal of Epidemiology 150(5): 453-458.

Zou E, Matsumura F. 2003. Long-term exposure to β-hexachlorocyclohexane (β-HCH) promotes transformation and invasiveness of MCF-7 human breast cancer cells. Biochemical pharmacology 66(5): 831-840.