Human adipose tissue levels of persistent organic pollutants and metabolic syndrome components: combining a cross-sectional with a 10-year longitudinal study using a multi-pollutant approach.

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Abstract

We aimed to assess the influence of long-term exposure to POPs on the risk of metabolic syndrome, combining a cross-sectional with a 10-year longitudinal follow-up design. Residues of eight POPs were quantifiedin adipose tissue samples from387 participants recruited between 2003 and 2004 in Granada province (Spain). The outcome ("metabolically compromised") was defined as having ≥1 diagnosis of type2 diabetes, hypertension, hypertriglyceridemia, and/or low HDLcholesterol.The cross-sectional analysis was conducted in the initial cohort, while the 10-year longitudinal analysis was conducted in 154 participants free of any of the so-mentioned metabolic diseases and classified as "metabolically healthy" at recruitment.Statistical analyses were performed using single and multi-pollutantapproaches through logistic and Cox regression analyses with elastic net penalty.After adjusting for confounders, β-hexachlorocyclohexane (β-HCH) and hexachlorobenzene (HCB) were independently associated with an increased risk of being metabollicaly compromised (unpenalized ORs=1.17, 95% CI=1.01-1.36 and 1.17, 95% CI=0.99-1.38, respectively). Very similar resultswere foundin the 10-year longitudinal analysis [HRs=1.28, 95% CI=1.01-1.61 (β-HCH); 1.26, 95% CI=1.00-1.59 (HCB)] and were in line with those obtained using elastic net regression. Finally, when the arithmetic sum of both compounds was used as independent variable, risk estimates increased to OR=1.25, 95% CI=1.03-1.52 and HR=1.32, 95% CI=1.02-1.70.Our results suggest that historical exposure to HCB and β-HCH is consistently associated with the risk of metabolic disorders, and that these POPs might be partly responsible for the morbidity risk traditionally attributed to age and obesity.

Keywords

Metabolic syndrome; Metabolic disruption; Persistent organic pollutants; Organochlorine pesticides; Polychlorinated biphenyls.

1. Introduction

The emergent obesity epidemic is at the centre of worldwide public health concerns, along with its implications for chronic diseases (Guh et al. 2009). Although unhealthy dietary patternsand sedentary lifestyles are recognized as the main triggers of this epidemic, mounting evidence is signaling other environmental stressors, such as exposure to endocrine disrupting chemicals (EDCs), as an additional risk factor for obesity and metabolic disorders (Dhurandhar and Keith 2014).Thus, increasing data suggests that long-term exposure to a group of EDCs designated persistent organic pollutants (POPs) may have a relevant impact on a cluster of metabolic conditions (obesity, dyslipidemia, high blood pressure and insulin resistance) known as the metabolic syndrome (MetS) (Parikh and Mohan 2012).

POPs are highly lipophilic compounds that resist metabolism and biodegradation and therefore tend to have a relatively long half-life in the environment and to bioaccumulate and biomagnify in the food chain (Mrema et al. 2013). The result is the virtually universal exposure of living organisms, including humans (Jakszyn et al. 2009). These chemicals include organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs), which have been used in a variety of commercial products, e.g. insecticides (dichlorodiphenyltrichloroethane [DDT], dicofol, lindane), fungicides (hexachlorobenzene [HCB]), and coolant and heating exchange fluids (polychlorinated biphenyls [PCBs]). Although legal restrictions in most countries have caused a worldwide decline in the production and handling of many POPs, human exposure remains relevant to public health due to their ubiquity and because current generations might suffer the effects of accumulated exposure throughout their lives, especially during critical windows of development (Tang-Péronard et al. 2015). Moreover, part of the POP body burden istransferred to subsequent generations during gestation and

breastfeeding (Shen et al. 2007), and most studies have considered diet, especially fatty food, to be the main current source of exposure in the general population (Gasull et al. 2011; Arrebola et al. 2012). Other sources, such as indoor inhalation or dermal exposure, might also be important for certain POPs and population groups (Bräuner et al. 2016; Luo et al. 2014).

Although adipose tissue was once considered a simple energy storage depot, it is now known to be a complex endocrine organ with autocrine, paracrine, and neuroendocrine actions that influence appetite, energy regulation, lipid oxidation, immune and vascular functions, and hormonal status (Galic et al. 2010). Adipose tissue also appears to have an important toxicological function by sequestering POPs and other lipophilic contaminants in order to protect other more sensitive lipophilic organs (e.g., the brain) from an overload (La Merrill et al. 2013). Therefore, adipose tissue constitutes a reservoir for long-term POP accumulation and can act as a source of chronic exposure to POPs through their slow release into the bloodstream, which might have relevant consequences in several chronic diseases (La Merrill et al. 2013).

Adipose tissue is itself a target of pollutants, and some authors have suggested that POPs are taken up by adipocytes and accumulate within lipid droplets, where they might exert a major local effect by interfering with lipid metabolism, insulin sensitivity, and endocrine function (Bourez et al. 2013; La Merrill et al. 2013). Given the relative frequency of clinical exceptions to the paradigm "more fat means more metabolic disease" (Muñoz-Garach et al. 2016), lipophilic contaminants are therefore increasingly seen as potentially explaining, at least in part, the link with adipose tissue inflammation and dysfunction, the underlying mechanisms thought to determine whether obese individuals remain metabolically healthy or not (Muñoz-Garach et al. 2016).

The action mechanisms proposed for POPs include interaction with nuclear receptors such asperoxisome proliferator-activated receptor gamma (PPAR-γ) and aryl hydrocarbon receptor (AhR) (La Merrill et al. 2013), endogenous endocrine-related enzymes, oxidative stress, inflammation pathways, and epigenetic modulation (Mrema et al. 2013). The diverse action mechanisms of different POP families and the potential interaction of complex mixtures, with additive, synergistic and/or antagonistic effects, complicate elucidation of the effects of POPs on metabolism (Rajapakse et al. 2002; Biemann et al. 2014). Furthermore, direct metabolic disrupting effects may coexist with long-term obesogenic effects that would lead to increased adiposity and therefore higher metabolic risk (Heindel et al. 2015; Lee et al. 2011).

Further complications are introduced by the simultaneous exposure of humans to complex low-level mixtures of EDCs that can potentially interfere with metabolism, including POPs (CDC 2015; Braun et al. 2014). The short and long-term health risks posed by these mixtures remain unclear and are causes of increasing concern. However, the vast majority of epidemiological studies have considered exposure to chemicals in a one-compound-at-a-time approach that may not address the true effect of chemical mixtures on human health (Braun et al. 2016).Consequently, one of the goals of current environmental epidemiology is the development of alternative and complementary multi-pollutant approaches to disentangle independent associations among several co-exposures and assess their combined effect (Lenters et al. 2016).

Few epidemiological studies have analyzed the association between POP exposure and MetS (Lee et al. 2007b; Lee et al. 2011; Lee YM et al. 2014; Park et al. 2010; Tomar et al. 2013). The present study was prompted by previous reports on associations between exposure to individual POPs and the risk of diabetes, hypertension, and elevated serum lipids in this same population (GraMo cohort) (Arrebola et al. 2013, 2014, 2015a). The

study objectives were to assess the relationship between long-term exposure to eight POPs and the risk of developing ≥ 1 MetS component/s, and secondly, to examine whether POP exposure is in part responsible for the metabolic risk traditionally attributed to body mass index (BMI) and age. The causality of these relationships was explored by combining a cross-sectional with a 10-year longitudinal design, using both a single-chemical and a multi-pollutant approach.

2. Methods

2.1. Study cohort

This research is part of a wider hospital-based study that aims to characterize the exposure to POPs of an adult cohort from Southern Spain and to assess its potential health effects (GraMo cohort). The study design, recruitment, and methods are extensively described elsewhere (Arrebola et al. 2009, 2010). In brief, study subjects were recruited in two public hospitals from Granada province: San Cecilio University Hospital in the city of Granada (240.000 inhabitants, urban area) and Santa Ana Hospital in the town of Motril (50.000 inhabitants, semi-rural area). Participants were recruited between July 2003 and June 2004 from patients undergoing non-cancer-related surgery. Following the standard surgery protocols of the hospitals, all the participants were under 12-h fasting conditions at the moment of sample collection. Adipose tissue was obtained from three main localizations: abdominal wall (35%; e.g. gallbladder surgery, umbilical hernia, eventration, epigastric hernia), pelvic waist (37%; e.g. inguinal hernia, pilonidal sinus, caesarean section), and limbs (14%, e.g. varicose veins, knee prosthesis). Inclusion criteria were: age over 16 years, absence of cancer, non-receipt of hormonal therapy, and residence in one of the study areas for at least 10 years. All subjects signed their informed consent to participate in the study, which was approved by the ethics committees of both hospitals.

Out of the 409 individuals who were contacted, 387 agreed to participate and were included in the initial cohort. All analyzed AT biopsies were collected at recruitment

(n=387) and were used for the cross-sectional analyses in the present work.From this initial cohort, all participants free of any metabolic disease at recruitment (n=169) were subsequently included in the 10-year follow-up. Out of these participants, 15 were excluded from the follow-up due to missing information or discrepancies in their clinical records, leaving a final subsample of 154 individuals. All participants were users of the public health system. No statistically significant differences in sex distribution or age were found between participants and non-participants (data not shown in tables). Main characteristics of the study population are summarized in Table 1.

2.2. Exposure assessment

Samples of 5-10g of adipose tissue were intra-operatively collected and immediately coded and stored at -80ºC until chemical analysis. Sample analysis and purification procedures were conducted as previously described (Rivas et al. 2001; Moreno Frías et al. 2004).In brief, chemical extraction with n-hexane was conducted on 200 mg of adipose tissue, and the solution was then purified through 2g of alumina in a glass column. All extracts were stored in glass tubes at -80ºC.

POPs were quantified by high-resolution gas chromatography coupled with a mass spectrometry detector in tandem mode, using a Saturn 2000 ion trap system (Varian, Walnut Creek, CA). We employed a 2m x 0.25mm silica capillary column (Bellefonte, PA) coupled with a 30m x 0.25mm analytical column (Factor FOUR vf-5MS, Varian Inc., Walnut Creek, CA). The limit of detection (LOD) was set at 0.01µg/L for all POPs under study. Chromatographic concentrations below the limit of detection were assigned a random value between 0 and the LOD. Residues of *p,p´*-dichlorodiphenyldichloroethylene (*p,p´*-DDE, the main metabolite of the pesticide

186 DDT), HCB, dicofol, α- and β-hexachlorocyclohexane (α- and β-HCH, respectively),
187 and PCB congeners -138, -153 and -180 were quantified. Recovery of the POPs from
188 adipose tissue was studied to assess the extraction efficiency of the method and ranged
189 from 90-98%.

190 Lipid content in adipose tissue samples was quantified gravimetrically as reported by
191 Rivas et al. (2001), including a homogenization step of 100mg adipose tissue with 5mL
192 chloroform:methanol:hydrochloric acid (20:10:0.1) and acidification with 0.1N
193 hydrochloric acid before collection and weighing of the organic phase. Lipid-basis POP
194 concentrations were calculated and expressed in nanograms per gram of lipid (ng/g
195 lipid).

196 *2.3 Outcome assessment*

197 The outcome ("metabolically compromised") was defined as having ≥1 diagnosis of the
198 following components: type 2 diabetes (fasting glucose ≥126 mg/dl and/or prescription
199 of anti-diabetic therapy); hypertension (systolic blood pressure >140 mmHg and/or
200 diastolic blood pressure >90 mmHg and/or prescription of antihypertensive therapy);
201 hypertriglyceridemia (serum triglycerides (TG) ≥150 mg/dl and/or prescription of lipid-
202 lowering treatment); or low high-density lipoprotein cholesterol (HDL-C), defined by
203 serum level <50 mg/dl in females or <40 mg/dl in males (Alberti et al. 2009).Thus, we
204 compared metabolically healthy individuals (free of any MetS component) with
205 thosemetabolically compromised (≥1MetS component).This was the final outcome
206 analyzed in both the cross-sectional and longitudinal analyses. The follow-up period
207 was from the date of recruitment to diagnosis of the first metabolic condition, death of
208 the participant, or December 31 2013 (the cohort remains under study).

The classification criteria wasbased on the harmonized definition of the International Diabetes Federation (IDF) (Alberti et al. 2009), and the same criteria were used in the cross-sectional and the longitudinal analyses. However, we did not include obesity in the MetS components criteria for two main reasons: i) Our secondary aim was to disentangle the metabolic effect of POPs from the metabolic risk traditionally attributed to obesity, as it has been suggested (Lee et al. 2007a; Gauthier et al. 2014); ii)BMI is a crucial and widely recognized confounder of the association between POPs and metabolic diseases because it strongly and positively correlates with both the exposure and the outcome (Vaclavik et al. 2006; Arrebola et al. 2010).

The prevalence of MetS components were gathered using both self-reported information and by using DIRAYA clinical record database (no discrepancies were found between the two sources), while the incidence was gathered by using DIRAYA database. DIRAYA, which was implemented in 2003, integrates all clinical information for each public health system user, including primary and specialized care, storing data on diagnostic tests performed and pharmaceutical treatments received. The aim of this system is to facilitate clinical procedures and epidemiological research.

2.4. Covariates

Data on socio-demographic characteristics, lifestyle, and health status were collected in face-to-face interviews conducted by trained personnel at the time of recruitment during the hospital stay. Questionnaire and research procedures were standardized and validated in a pilot study with 50 subjects. The questionnaire was designed and validated in a previous research, (REFs).

Comentado [u1]: añadir referencias de las respuestas

Participant´s weight and height were measured and BMI was calculated as weight/height squared (kg/m^2). A subject was considered a smoker or alcohol consumer

with any level of daily tobacco (≥ 1 cig/day) or weekly alcohol (≥ 1 drink/week) consumption. Residence in the city of Granada at the time of the surgery was considered "urban" and residence in the area of Motril was considered "semi-rural".

The dietary section was composed of a semi-quantitative questionnaire on food consumption habits which included the following food groups: meat, cold meats, fats, fish, eggs, milk, dairy products, cheese, vegetables, pulses, fruit, bread, and pasta. The frequency of food consumption was gathered in four categories: <1 portion/week, 1 portion/week, 2–6 portions/week, or >7 portions/week. We also recorded the type of milk predominantly consumed (skimmed/ semi-skimmed/ whole) and the types of fish (lean/fatty) and meat (white/ red) consumed.

2.5. Statistical methods

Descriptive analysis included the calculation of medians and 25/75th percentiles for the interval variables and percentages for the categorical variables. ∑(HCB+β-HCH) was calculated as the arithmetic sum of the two chemical concentrations.

For the overall cohort (n=387), the magnitude of associations between POPs and the outcome was analyzed by binomial unconditional logistic regression, calculating multivariable-adjusted odds ratios (ORs) with their corresponding 95% confidence intervals (CIs). Then, those participants that were free of any MetS component at recruitment were followed (n=154), and the magnitude of associations between POPs and the 10-year incidence of MetS components was evaluated using Cox-regression models with time-to-events as the time variable, calculating hazard ratios (HRs) with their corresponding 95% CIs. Estimations of time-to-events were based on the dates of recruitment, diagnosis, and end of follow-up. Data on participants who died before the

observation of a study outcome were censored; therefore, only their disease-free time was considered in the analyses.

The shape of the relationships between individual POP concentrations and the outcome was evaluated through locally weighted scatterplot smoothing (LOWESS) and Generalized Additive Models (GAM). The influence of extreme values was minimized by log-transforming the concentrations of all POPs and treating them as continuous variables (for POPs whose LOD ranged between 86-100%), with the exception of dicofol (%>LOD=20%) and α-HCH (%>LOD=22%), which were treated as dichotomous (≥LOD/<LOD) due to their low detection level. In addition, the comparability was enhanced by adjusting both the logistic- and Cox-regression models for the same group of covariates, which included variables whose inclusion in any model produced changes >10% in beta coefficients and/or those reported as relevant confounders in previous studies, i.e., BMI (kg/m^2), age (years), sex (male/female), residence (urban/semi-rural), education (primary schooling not completed/primary or higher), alcohol consumption (consumer/non-consumer), and smoking habit (smoker/non-smoker). The hypothesized causal pathway between exposure and outcome, as well as the role of the potential confounders are graphically depicted in Figure S1 of the supplementary material. The potential modifying effect of sex, age, BMI, residence, education, smoking habit, and alcohol consumption on the associations found was studied by entering the interaction terms (POP levels * each potential modifier) in the models. No significant interactions were found. Multivariable models displayed in the manuscript were performed using lipid-basis POP concentrations (ng/g lipid), although they were repeated using wet-basis concentrations (ng/g adipose tissue), with no relevant changes in the associations found (data not shown in tables).

Most POP concentrations were highly correlated, showing variance inflation factors of 3.2-5.4 when entered simultaneously in a model. Therefore, in addition to single-pollutant analyses, elastic-net regression analyses were conducted to select the most relevant predictors, accounting for simultaneous co-exposures (Friedman et al. 2010; Simon et al. 2011; Tibshirani et al. 2012). The degree of penalization was estimated using cross-validation (Friedman et al. 2008), and the process was repeated 100 times to calculate the combination of α and γ that produced a cross-validation error which is one standard error of the minimum (error.1se) (Zou et al. 2005; Hastie et al. 2009).

Data were stored and processed using the R statistical computing environment v3.2.3 (http://www.r-project.org/), and elastic-net models were built with the *glmnet* package. The significance level was set at $p \leq 0.05$, and all tests were two-tailed.

3. Results

3.1. Characteristics of study population

Table 1 summarizes the characteristics of the study population and the results of the chemical analyses. In comparison to the initially recruited population, the individuals included in the follow-up were considerably younger (median age: 52 *vs*42 years, respectively), had a similar BMI (median BMI: 26.6 *vs*26.0 Kg/m^2, respectively), and there was a higher proportion of males (61.7% vs 50.9%, respectively). There was also a higher proportion of alcohol consumers in the follow-up study population (66.2% vs 51.7%, respectively).Out of the 387 initially recruited participants, 218 (56.3%) were classified as "metabolically compromised" (≥1 MetS component). Out of the 154 metabolically healthy individuals at recruitment, 53 (34.4%) developed at least one MetS component during the 10-year follow-up and were consequently classified as “at

risk of MetS" in the longitudinal analysis. The median follow-up time, including censored and non-censored data, was 117.5 months. Levels of accumulated POPs in the adipose tissue samples from our cohort were comparable to other contemporary European and Asian populations (Malarvannan et al. 2013; Pauwels et al. 2000: Shen et al. 2009) and have been extensively described elsewhere (Arrebola et al. 2009, 2010, 2013).On the other hand, POP concentrations in the subsample included in the follow-up were 12-24% lower for PCBs and 35-45% lower for OCPs in comparison to levels in the initial cohort.

3.2. Prevalence at recruitment and incidence of MetS components

The prevalence and incidence of MetS components are reported as supplementary material (Table S1). At recruitment, 136 (35.1%) participants presented one metabolic component, 63 (16.3%) two components, 17 (4.4%) three components, and only 2 (0.5%) presented with the four metabolic components considered in our classification criteria. During the 10-year follow-up, one of the four metabolic components was developed by43 participants (27.9%), two by 9 (5.9%), and three by 1 (0.6%). Among the four components, hypertension and low HDL-C were more prevalent at recruitment, whereas incident hypertension predominated over the other three MetS components at the follow-up.

3.3. Associations between POP exposure and risk of MetS

Figures 1a and 1b depict POP levels according to the number of MetS components at recruitment in the study population. We found that median β-HCH and HCB concentrations increased in parallel with the number of MetS components (Figure 1a), but this trend was not so obvious for the rest of POPs (Figure 1b).

Table 2 displays the results of the logistic regression analyses of the association between POPs and MetS components. Among the eight POPs measured, individual models using single chemical concentrations as independent variables showed that β-HCH was positively and significantly associated with the outcome (OR=1.17, 95% CI=1.01-1.36). Exposure to HCB showed a similar positive association, although the association was only marginally significant (OR=1.17, 95% CI=0.99-1.38). When all POPs were included in elastic net regression analyses, coefficients for β-HCH [exp(beta)=1.09] and HCB [exp(beta)=1.03] differed from 0, suggesting that both chemicals were associated with the outcome. Thus, when ∑(HCB+β-HCH) was analyzed as an independent variable, we observed a stronger OR than that found for each chemical alone (OR=1.25, 95% CI=1.03-1.52).

Table 2 also exhibits the results of Cox regression analyses on the influence of historical exposure to POPs and the 10-year incidence of MetS components in the individuals classified as "metabolically healthy" at recruitment. Multivariable models with single POPs showed that β-HCH and HCB were again positively and significantly associated with the risk of MetS components (HRs=1.28, 95% CI=1.01-1.61 and 1.26, 95% CI=1.00-1.59, respectively). In addition, elastic net regression analyses again showed positive beta coefficients for both β-HCH [exp(beta)=1.02] and HCB [exp(beta)=1.01]. Furthermore, and similar to the cross-sectional analyses, ∑(HCB+β-HCH) was more strongly associated with the outcome in comparison to each individual compound (HR=1.32, 95% CI=1.02-1.70).

In order to test the potential influence of the adipose tissue source in the associations found, we adjusted all the multivariable models for this variable (abdominal wall/pelvic waist/limbs), finding no relevant modification in model coefficients (data not shown in tables).

In addition, we examined whether POP exposure might account for part of the contribution of age and obesity to the risk of MetS components. Thus, Table 3 and Figure 2 represents risk estimates when BMI or Age is used as the independent variable in relation to the outcome (dependent variable), in both cross-sectional and longitudinal analyses, at different levels of POP adjustment. In summary, β-HCH and HCB concentrations might account for 14-25% of the effect of the association between BMI or age with the risk of presenting or developing the outcome.

Given that diet, especially animal fatty food, is considered a major contributor to both metabolic health and POP exposure in the general population, we conducted sensitivity analyses of the final models (both logistic and Cox regression), which were sequentially adjusted for selected dietary components potentially associated with both the exposure and the outcome (i.e., milk, blue fish, cheese, meat, fruit, and vegetable consumption). These adjustments for dietary variables did not substantially affect the initial associations shown in Table 2, with only *p-p'*-DDE changing to a borderline significant association (Supplementary material, Table S2).

4. Discussion

According to the results of this study, accumulated levels of β-HCH in combination with HCB were consistently associated with the risk of MetS. Moreover, these pollutants might account for part of the risk of morbidity traditionally attributed to obesity and age. To our best knowledge, this investigation is one of the first prospective studies to analyze associations between POP exposure and MetS components as a whole and the first to combine cross-sectional with longitudinal analyses.

375 Our findings are in line with those of Lee et al. (2007b), who studied five POP
376 subclasses and observed that serum OCP levels, especially β-HCH levels, showed the
377 strongest and most consistent association with MetS. Park et al. (2010) found that β-
378 HCH and heptachlor were associated with MetS in a case-control study, while Lee YM
379 et al. (2014) reported that levels of β-HCH and HCB (as well as oxychlordane and
380 heptachlor) predicted the risk of MetS in a nested case-control study. Moreover, Tomar
381 et al. (2013) found higher serum levels of nine OCPs in MetS patients compared to
382 controls, although only β-HCH and aldrin were significantly associated with the risk of
383 MetS.

384 There is also increasing evidence on the relationship between POP exposure and
385 individual MetS components. Thus, β-HCH and HCB have been associated with the
386 prevalence of type 2 diabetes (Al-Othman et al. 2014; Dirinck et al. 2011; Evangelou et
387 al. 2016), and both β-HCH and HCB (as well as PCB-138 and PCB-153) were
388 associated with incident hypertension in the GraMo cohort (Arrebola et al. 2015a).
389 Similar findings have been reported in vulnerable groups, with findings of positive
390 associations between serum POP concentrations and insulin resistance in pregnant
391 women (Arrebola et al., 2015b) and between HCB exposure during pregnancy and
392 higher plasma insulin levels in female offspring at 5 years of age (Tang-Péronard et al.
393 2015). Furthermore, higher plasma concentrations of HCB (among other POPs) were
394 found to increase the likelihood of insulin resistance in pre-pubertal boys (Burns et al.
395 2014). In contrast, Lee et al. (2016) reported positive associations between PCB
396 exposure and blood pressure and/or triglyceride levels among children but found no
397 association with OCPs. There has even been a report of an inverse association between
398 β-HCH levels and hypertension in adults (Valera et al. 2013).

399 In addition, the above epidemiological data on POP exposure and the metabolic
400 syndrome are supported by experimental findings (La Merrill et al. 2013; Lee DH et al.
401 2014). When Ruzzin et al. (2010) exposed rats to an environmentally relevant mixture
402 of POPs from fish oil (farmed Atlantic salmon), animals developed insulin resistance,
403 abdominal obesity, and hepatosteatosis. Moreover, these results were confirmed with
404 differentiated adipocytes. A similar study in exposed mice also found an increased
405 insulin resistance linked to visceral adiposity and inflammatory markers (Ibrahim et al.,
406 2011). More recently, Zhang et al. (2015) reported a POP-induced modification of gut
407 microbiota-host metabolic homeostasis in mice *via* activation of the AhR. These effects
408 are in agreement with mechanistic data on POPs, suggesting interplay among
409 endocrine-related mechanisms, chronic low-grade inflammation, and mitochondrial
410 dysfunction (Kim et al. 2012; La Merrill et al. 2013; Lim et al. 2010). Although still
411 remains the need for experimental data regarding the effect of isolated POPs on
412 metabolic outcomes (including β-HCH and HCB),a recent systems biology approach
413 has revealed converging molecular mechanisms that link three different POP families
414 with common metabolic diseases (Ruiz et al. 2016).

415 Interestingly, the present data indicate that β-HCH and HCB levels might partially
416 account for the effects of age and BMI as MetS risk factors (Table 3, Figure 2).
417 Although this finding needs to be further investigated and confirmed in future studies, it
418 is consistent with the relatively new concept of the metabolically healthy obese
419 phenotype. In this line, Gauthier et al. (2014) found significantly higher levels of 12 out
420 of 18 POPs in metabolically unhealthy *versus* healthy obese women, despite having a
421 similar age, BMI, and fat mass. Moreover, Lee et al. (2007a) found that, among obese
422 individuals, only those with high levels of OCPs were at increased risk of insulin

423 resistance, and there was no apparent association with the lowest quartile of OCP
424 concentrations.

425 Although the ability of specific POPs to interfere with lipid and glucose metabolism is
426 well documented (Evangelou et al. 2016; Lee DH et al. 2014; Lee YM et al. 2014), the
427 isolated interpretation of specific compounds may provide apparently
428 inconsistentresults (Lee DH et al. 2014). This can be due to the existence of different
429 patterns of POP mixtures between and within populations and/or the possibility that the
430 observed effect of a specific chemical might be a surrogate of a POP combined effect.
431 The influence of isolated individual compounds therefore has limited meaning, because
432 humans are exposed to complex mixtures of POPs and the biological concentrations of
433 many PCBs and OCPs are highly correlated, as observed in the present population
434 (Spearman coefficients ranging from 0.6 to 0.9, Supplementary Material, Figure S2). In
435 this line, our elastic-net regression models demonstrated that both β-HCH and HCB
436 were independently associated with the outcome. Additionally, the fact that risk
437 estimates for ∑(HCB+β-HCH) were stronger than for the individual chemicals might
438 point to an additive effect. This possibility is supported by the similar chemical
439 structures of these two compounds. Thus, the equatorial position of the chlorine atoms
440 in the molecule of β-HCH causes a more planar conformation that is highly similar to
441 that of HCB (Figure 3) (Mrema et al. 2013). This structural and conformational analogy
442 might explain their similar half-lives and bioaccumulation patterns, as well as their
443 comparable pharmacokinetics and mechanisms of action (Mrema et al. 2013).

444 The main strength of this study is its combined design, which showed consistent results
445 between the cross-sectional and longitudinal analyses (despite differences in population
446 characteristics), thereby reinforcing causal inference and minimizing potential reverse
447 causality. In this context, some authors have noted that certain inflammatory processes

448 associated with metabolic diseases may lead to an alteration of POP metabolism, the so-
449 called disease progression bias (Porta 2014). However, the fact that similar findings
450 were observed in the follow-up group (even with lower POP levels, younger age and
451 healthier status) minimizes the aforementioned bias. A further strength is the utilization
452 of a complementary statistical multi-pollutant model that assesses several co-exposures
453 simultaneously, which represents a more realistic exposure scenario. Furthermore,
454 historical exposure to POPs was estimated by using samples of adipose tissue, which is
455 considered the most suitable matrix for the assessment of long-term accumulated
456 concentrations of these compounds, accounting for all routes and sources of exposure
457 (Kohlmeier and Kohlmeier, 1995; Quintana et al. 2004). In this regard, adipose tissue
458 POP concentrations have been reported to show less variability in comparison to serum
459 concentrations, which can be influenced by both point exposures and the mobilization
460 of POPs stored in fatty tissues (Archibeque-Engle et al. 1997; Gaskins and Schisterman,
461 2009). In the present study, the validity of adipose tissue POP concentrations as a
462 biomarker of long-term exposure is supported by the similar findings in the cross-
463 sectional and longitudinal analyses, which can be of relevance for future
464 epidemiological studies.

465 In addition, sensitivity analyses (Table S2) showed that adjustment for dietary items
466 didnot substantially affect the associations withβ-HCH and HCB. These diet-adjusted
467 results are timely,given a recent claim that the BMI might not be sufficient when
468 estimating the confounding effect of diet as a source of POPs and as a predictor of
469 metabolic disease (Tuomisto et al. 2016).

470 Among the limitations of our study is the relatively modest sample size, particularly in
471 the longitudinal analyses. However, the statistical power was sufficient to yield robust
472 associations and to reaffirm the results found in the cross-sectional analyses. Another

limitation is that the final models were not adjusted for physical activity or for diet as a global pattern. As previously mentioned, diet might be a potential confounder of the associations found. However, POP concentrations can be considered as intermediate variables on the causal path from fatty food consumption to MetS, and the inclusion of dietary variables and POP exposure in the same model might have led to an over-adjustment. Interestingly, sensitivity analyses adjusting for dietary items showed no relevant changes in the previously observed associations. Additionally, all models were adjusted for BMI, which may represent, at least partially, both an excessive caloric intake and a sedentary lifestyle. Therefore, diet does not seem to have been a relevant modifier of the associations found in our study. A further limitation is that we cannot exclude the potential influence of other groups of unmeasured environmental pollutants that may have partially contributed to the observed associations. In this regard, further analyses of different groups of persistent and non-persistent contaminants are currently being conducted in the GraMo cohort.

Conclusions

Our results show that historical exposure to β-HCH and/or HCB is consistently related to the risk of MetS components, and that these pollutants might account for part of the risk of morbidity traditionally attributed to obesity and age. Given the ubiquity of the exposure and the frequency of metabolic diseases, we consider these results to be of major public health importance.

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Conflicts of interest

The authors declare no actual or potential competing financial conflict of interests.

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713

714 **Figure Captions**

715 **Figures 1a and 1b.** POP levels according to the number of MetS components at
716 recruitment in the study population.

717 Median β-HCH and HCB concentrations increased in parallel with the number of MetS
718 components (Figure 1a), but this trend was not so obvious for the rest of POPs (Figure
719 1b).

720

721 **Figure 2.** Percentages of change in risk estimates of BMI and age for MetS components
722 at different levels of POP adjustment.

723 Each column depicts the risk estimates (Odd Ratios [ORs] or Hazard Ratios [HRs]) of
724 age and BMI in the models before and after adjustment for β-HCH and/or HCB
725 concentrations. Percentages on columns represent the relative decrease of the model
726 coefficients after adjustment for POPs. Model 1: adjusted for [age (years) or BMI
727 (Kg/m2)] + sex (male/ female) + smoking (yes/no) + alcohol (yes/no) + place of

residence (urban vs semi-rural) + education (primary or higher vs less than primary education). Model 2: model 1 + additional adjustment for β-HCH. Model 3: model 1 + additional adjustment for HCB. Model 4: model 1 + additional adjustment for Σ(HCB+β-HCH).

Figure 3. A: Hexachlorobenzene (HCB); **B:** β-hexachlorocyclohexane (β-HCH).

Interestingly, the equatorial position of the chlorine atoms in the molecule of β-HCH causes a more planar conformation, very similar to that of HCB.