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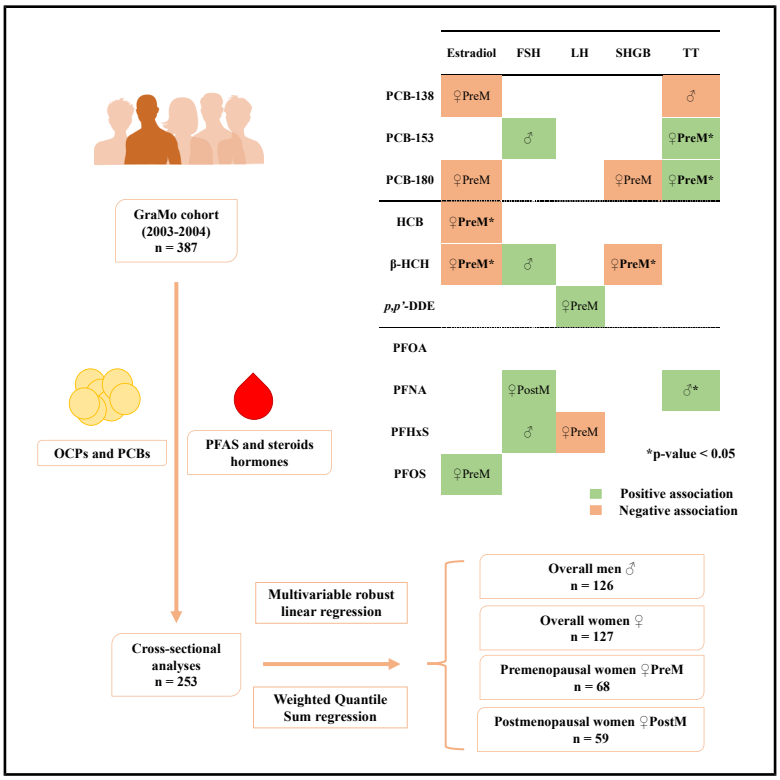
Associations of internal persistent organic pollutant levels with sex hormones: An analysis by sex and menopausal status in a Spanish cohort

Graphical abstract



Highlights

- Data analyses were performed for men, and overall, pre- and post-menopausal women
- POPs were measured in adipose tissue (PCBs and OCPs) or serum (PFAS)
- We evidenced relevant individual associations, mostly in pre-menopausal women
- POP mixtures were associated to TT in women and FSH in men

Authors

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In brief

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Associations of internal persistent organic pollutant levels with sex hormones: An analysis by sex and menopausal status in a Spanish cohort

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SUMMARY

The aim of this study was to investigate associations between human exposure to persistent organic pollutants and sex hormone levels. The study population (n = 253) was a subsample of GraMo adult cohort, recruited in 2003–2004 in two hospitals from Granada, Spain. Exposure was estimated by analyzing samples of adipose tissue (3 organochlorine pesticides and 3 polychlorinated biphenyls) and serum (4 per- and polyfluoroalkyl substances). Data analyses included robust linear regression and weighted quantile sum regression. In men, PFNA (positively) and PCB-138 (negatively) were associated with testosterone. In premenopausal women, OCPs and PCBs were negatively associated with estradiol and SHBG. PFHxS was inversely related to luteinizing hormone. Additionally, PCB-153 and -180 was positively associated with testosterone. The mixture of PCBs/OCPs was positively associated with testosterone in women and with FSH in men. Our results highlight the potential of chemical mixture exposure to alter sex hormone homeostasis, depending on sex and menopausal status.

INTRODUCTION

Persistent organic pollutants (POPs) are anthropogenic chemicals that remain for extremely long periods in the environment and accumulate in flora and fauna around the planet, entering the food chain and contaminating food and water supplies. Within this group of pollutants, we can find polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), and per- and polyfluoroalkyl substances (PFAS). PCBs include 209 congeners that were manufactured since the 1930s for several industrial applications and electronic devices. OCPs were extensively used in agriculture and public health campaigns to prevent vector-borne diseases until they were restricted or banned in the 1980s due to their high toxicity, persistence, and bioaccumulation. PFAS are used in industry for obtaining non-stick and waterproof coatings. The list of POPs in the United Nations Environment Program's Stockholm Convention, aimed at taking measures to eliminate or reduce the release of POPs into the environment by participating countries, includes several of these pollutants. OCPs and PCBs were added in 2001, while perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) were added in 2019.[1]

Despite regulation attempts, virtually all humans are still exposed to POPs due to its high persistence in the organism. In this regard, human biomonitoring is a key tool in the assessment of the body burden of pollutants, taking into account that internal levels of pollutants represent all exposure routes simultaneously, as well as their potential health implications.[2] Human adipose tissue, where many POPs accumulate due to their lipophilicity, gives meaningful information about the long-term accumulation of some of these chemicals and also plays a fundamental role in the production and regulation of certain hormones,[3] specifically sex steroids.[4] In addition, sex hormones regulate adipose tissue activity in a sex-specific way.[5] In fact, men and women differ in the amount and type of fat in their bodies, with women generally having a higher fat proportion and less visceral adipose tissue than men. However, this difference tends to vanish after women's menopause.[6]

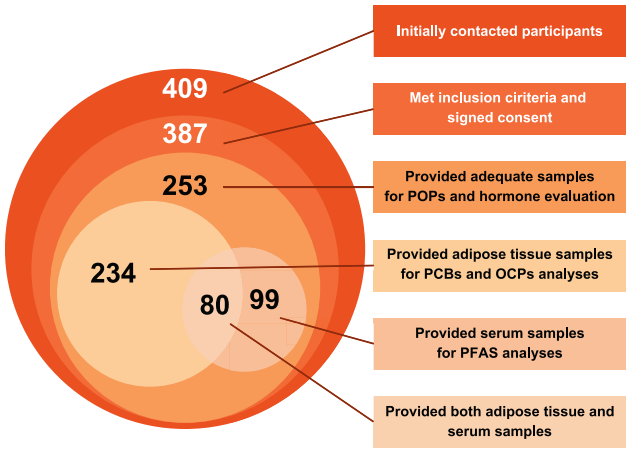

Figure 1. Diagram of the sample selection process and subgroups for individual pollutant and multipollutant analyses

POPs: persistent organic pollutants; PCBs: polychlorinated biphenyls; OCPs: organochlorine pesticides; PFAS: per- and polyfluoroalkyl substances.

Many POPs are considered endocrine disrupting chemicals (EDCs). EDCs are capable of interacting with hormone receptors and interfering with their signal transduction, causing epigenetic alterations and disrupting hormone synthesis, transport and breakdown among others.[7] As a result, human exposure to continuous low doses of EDCs is thought to be linked to several diseases including reproductive impairment, neurological disorders, metabolic diseases, and even cancer. However, there are still a number of uncertainties regarding the extent of these potential effects in general population.[8–13]

Alterations in sex hormones are related to several disorders in human health. For example, estradiol is not only crucial for the correct regulation of women menstrual cycle, but also for the cardiovascular, neurological, skeletal, and vascular system, among others.[14] In addition, estradiol levels in men have been linked to lower total testosterone (TT) secretion and obesity,[15] metabolic syndrome and cardiovascular diseases.[16] Moreover, low levels of sex hormone-binding globulin (SHBG)—which contributes to the transportation of androgens and estrogens—are associated with metabolic and cardiovascular diseases.[17] In addition, dysregulation in follicle-stimulating hormone (FSH) and luteinizing hormone (LH), could lead to infertility[18] and, specifically for FSH, increased inflammation, insulin resistance, and atherosclerosis.[19]

Based on the aforementioned knowledge gaps regarding the hormonal implications of long-term exposure to POPs, this study aimed to explore the associations of OCP, PCB, and PFAS exposure with sex hormone levels in adults from a Spanish cohort, considering sex and menopausal status.

MATERIALS AND METHODS

This manuscript was prepared in close adherence to the STROBE guidelines.[20]

Study design and population

This study is a cross-sectional research conducted within the GraMo adult cohort (Granada, Southern Spain), for which an extensive description of population characteristics and pollutant exposure levels has been published elsewhere.[21–26] The province of Granada encompasses rural, semi-rural, and urban areas, with a total population of over 915,000 inhabitants spread across an area of 12,531 km^2.

The characteristics of GraMo cohort and enrollment procedure have been thoroughly described elsewhere.[24] Briefly, recruitment was performed during 2003 and 2004 in two public hospitals: Clínico San Cecilio University Hospital in Granada city (inland urban area) and Santa Ana Hospital in the town of Motril (semi-rural area in the Mediterranean coast). The cohort included patients undergoing non-cancer-related surgery, namely hernias (41%), gallbladder diseases (21%), varicose veins (12%), and other conditions (26%).

As described in Figure 1, from a first set of 409 individuals contacted, 387 accepted the invitation to participate. The inclusion criteria were: aged older than 16 years, not having received hormone therapy and having resided in the study areas for at least 10 years before recruitment. Patients with cancer and those with a hormonal disease related to the hypothalamic axis were excluded. A final group of 253 participants was included in the study, as they provided samples for hormone assessment and some sample for POP measurement. 234 of them provided sufficient adipose tissue sample for OCP and PCB analyses (Subgroup 1), while 99 had enough sample volume for PFAS quantification in serum (Subgroup 2). A total of 80 individuals had both serum and adipose tissue analyses. Women who had menstruated at least once in the last 12 months were included in the premenopausal women group. Specific data about sociodemographic, lifestyle, and diet characteristics for the whole population participating in this study are provided in Table 1. The full description of subgroups 1 and 2 is shown in supplemental information (Tables S1 and S2, respectively).

All participants enrolled in this research signed an informed consent and the study protocol was approved by the Ethics Committee of Granada (Comité Ético de Investigación Provincial de Granada), dated December 7th, 2020.

Sampling and biomarker analyses

OCP and PCB analysis

During the surgery, 5–10 g of adipose tissue were intra-operatively collected mainly from pelvic waist (42%), front abdominal wall (39%), limbs (13%) and another (6%), and immediately coded and stored at −80°C. Levels of OCPs and PCBs in adipose tissue were quantified in 2005 at the Laboratorio Analítico Bioclínico (Almería, Spain) using the technique described by Rivas et al. (2002).[27] PCBs and OCPs were quantified by gas chromatography with a mass spectrometry detector in tandem mode (GC-MS/MS), using a Saturn 2000 ion trap system (Varian, WalnutCreek, CA, USA). A 2 m × 0.25 mm silica capillary column (Bellefonte, PA) coupled to a 30 m × 0.25 mm analytical column (Factor FOUR VF-5MS, Varian Inc., Walnut Creek, CA) was used.[28] The pollutants selected, because they were the most frequent in the population, were: (1) PCBs congeners −138, −153, and −180; (2) OCPs: hexachlorobenzene (HCB), *alpha*-hexachlorocyclohexane (α-HCH), *beta*-hexachlorocyclohexane (β-HCH), *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE), and dicofol. OCPs and PCBs adipose tissue concentrations were expressed in nanograms per gram of lipid (ng/g lipid).

The individual concentrations of OCPs and PCBs in adipose tissue of the GraMo cohort have already been described in previous publications.[22–24]

PFAS analysis

Blood samples were collected in S-Monovette neutral tubes (Sarstedt, Nümbrecht, Germany), centrifuged and keep at 4°C, as described by Esteban et al.,[29] and stored until analysis at −20°C. PFAS analysis was carried out at the Centro de Sanidad Ambiental of the Instituto de Salud Carlos III (Madrid, Spain) in 2021 by high resolution liquid chromatography with a mass spectrometry detector in tandem mode (HPLC-MS/MS), TSQ Vantage (Thermo Fisher Scientific, Waltham, MA, USA) in the serum samples. Quantification was carried out by isotope dilution method and labeled standards were used for internal quality purposes, as well.[30]

Twelve PFAS compounds were analyzed: perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUdA), perfluorododecanoic acid (PFDoA), perfluorobutane sulfonic acid (PFBS), perfluorohexane sulfonic acid (PFHxS), perfluoroheptane sulfonic acid (PFHpS), and perfluorooctane

Table 1. Full study population characteristics (*n* = 253)

Characteristics [n (%)]	Men 126 (49.8)	Women 127 (50.2)	Premenopausal women 68 (26.9)	Postmenopausal women 59 (23.3)
Sociodemographic characteristics				
Age [median (IQR)]	51.0 (35.0–63.0)	47.0 (34.0–59.5)	36.0 (27.5–42.5)	60.0 (54.5–68.5)
BMI [median (IQR)]	27.2 (25.0–29.4)	26.1 (23.4–29.5)	23.9 (22.4–27.7)	27.5 (25.1–30.9)
Education level [n (%)]				
No formal education	40 (31.7)	36 (28.3)	7 (10.3)	29 (49.2)
Primary education	50 (39.7)	59 (46.5)	33 (48.5)	26 (44.1)
≥ Secondary education	36 (28.6)	32 (25.2)	28 (41.2)	4 (6.8)
Occupation [n (%)]				
Manual worker	27 (21.4)	25 (19.7)	20 (29.4)	5 (8.5)
Non-manual worker	88 (69.8)	102 (80.3)	48 (70.6)	54 (91.5)
Retired	11 (8.7)	0 (0.0)	0 (0.0)	0 (0.0)
Lifestyle				
Smoking [n (%)]				
Non-smoker	28 (22.2)	74 (58.3)	26 (38.2)	48 (81.4)
Current smoker	57 (45.2)	34 (26.8)	26 (38.2)	8 (13.6)
Former smoker	41 (32.5)	19 (15.0)	16 (23.5)	3 (5.1)
Alcohol consumer [n (%)]	98 (77.8)	41 (32.3)	31 (45.6)	10 (16.9)
Women reproductive health				
Oral contraceptives [n (%)]		51 (40.2)	39 (57.4)	12 (20.3)
Children [n (%)]				
No children		23 (18.1)	19 (27.9)	4 (6.8)
1-2		52 (40.9)	37 (54.4)	15 (25.4)
3-9		52 (40.9)	12 (17.6)	40 (67.8)
Ever breastfed [n (%)]		87 (68.5)	39 (57.4)	48 (81.4)

BMI, Body mass index. Age is expressed in years and BMI in kg/m^2.

sulfonic acid (PFOS). PFAS serum concentrations were expressed in micrograms per liter (μg/L).

More information on the methodology and quality control of the POPs analyses is shown in the STAR Methods (method details).

Hormone analysis

Serum sex hormone concentrations (estradiol, pg/mL; FSH and LH, U/mL; SHBG, nmol/L; and TT, ng/mL) were evaluated in 2018 at Instituto de Investigación Biosanitaria de Granada (ibs.GRANADA) (Granada, Spain), by means of immunoassays performed in an electrochemiluminescence bioanalyzer cobas e−411 (F. Hoffmann-La Roche Ltd, Basel, Switzerland).

Covariates

Data on socio-demographic characteristics, lifestyle, and health status were collected through face-to-face interviews conducted by trained staff during recruitment of the study population, while participants were at the hospital. Previously validated questionnaires were used.[31,32] Age was expressed in years and body mass index (BMI) as weight/height squared (kg/m^2). Current smoker was defined as any level of daily tobacco consumption at the time of recruitment (≥1 cig/day), and alcohol consumers at any level of weekly alcohol intake (≥1 glass/week). A woman was considered to be an oral contraceptive user if she had used oral contraceptives at any time in her life. Missing lifestyle data (<0.4%) from participants were imputed using the median for quantitative variables and the most frequent level for categorical variables.

Statistical analyses

Data analyses were performed using R v 4.3.1[33] with RStudio v 2023.09.0,[34] and SPSS Statistics v 20.0.[35] Plots were created using ggplot2[36] library, while the correlation matrix plot was generated using the corrplot R-package version 0.92.[37] Generalized additive models (GAMs) were fitted using the mgcv[38] package. Linear regression models were performed using the MASS package in R.[39] Additionally, directed acyclic graphs (DAGs) were constructed using the DAGitty software V.3.1[40] to identify potential causal relationships among variables and guide covariate selection for statistical modeling.

The shape of the associations between POP concentrations and sex hormone levels was explored using GAMs that showed an approximately linear pattern in the associations observed, with non-linearity appearing only at the extremes. Potentially influential observations were assessed using Cook's distance and leverage diagnostics. However, none were excluded, as they were not consistent across all models and their removal could have compromised comparability between groups. In order to minimize the influence of outliers and obtain more reliable estimates, the associations between individual POPs (independent variables) and sex hormones (dependent variables) were assessed using multivariable robust linear regression models based on MM estimators. These estimators are less likely influenced by the outliers and do not require normally distributed residuals, which allowed us to avoid variable transformation and to retain their original scale for interpretation.[41]

Only POPs detected in more than 75% of samples (i.e., PCB-138, PCB-153, PCB-180, HCB, β-HCH, *p,p'*-DDE, PFOA, PFNA, PFHxS, and PFOS) were included in the statistical analyses, where concentrations <LOD were imputed with a random value between 0 and the LOD (Table S3). Analyses were stratified by sex, and for women, further stratified by menopausal status.

Models for men were adjusted for age (years), BMI (kg/m^2), smoking habit (smoker or former smoker vs. non-smoker), alcohol consumption (consumer vs. non-consumer) and education level (primary or secondary/higher levels vs. unfinished primary level). Models for women were additionally adjusted by number of children, breastfeeding (yes vs. no) and use of contraceptives (Figure S1). Adjustment for smoking habit and alcohol consumption was performed across all models, as both factors have been associated with

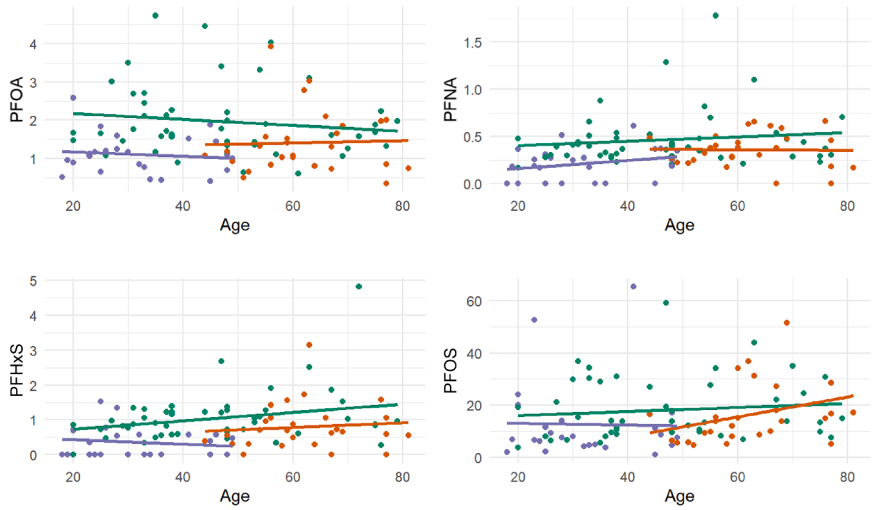

Figure 2. Relationship between serum per- and polyfluoroalkyl substances (PFAS) concentrations (μg/L) and age

Scatterplots represent unadjusted linear regression models non-adjusted for confounders variables. PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid; PFHxS: perfluorohexane sulfonic acid; PFOS: perfluorooctane sulfonic acid.

alterations in sex hormone levels and may also influence internal POPs levels.[42–47] PFAS models were not adjusted for BMI to avoid potential overadjustment, since PFAS are considerably less lipophilic and, consequently, less prone to accumulate in fat tissues compared to OCPs and PCBs. In addition, BMI might be present in the causal pathway for PFAS → hormones, since these pollutants have been acknowledged as possible obesogens,[48,49] and elevated BMI has been related to altered hormone levels.[50]

To explore potential mixture effects, weighted quantile sum (WQS) regressions with repeated holdout validation were fitted for each sex hormone using the R-package gWQS,[51] including log-transformed concentrations of PCBs and OCPs (n = 234) in the combined index. No transformation was applied to any covariate. PFAS levels, as well as the subgroups of premenopausal and postmenopausal women, were not included in the WQS analyses due to the limited number of participants with available chemical data, which would have compromised validity and robustness of the analyses. Considering that WQS regressions need to set *a priori* the expected direction of the association, we calculated two models for mixture effects for each outcome, i.e., one assuming a positive influence of the mixture on each hormone (positive model) and the other assuming a negative effect (negative model). The models were set by including chemical concentrations ranked in quartiles, 25% of dataset for training and 75% for validation, 100 bootstrap samples and 100 sets of repeated holdout validation.

The R script used for the analyses is shown in supplemental information (Data S1).

RESULTS AND DISCUSSION

Concentrations of POPs and PFAS in the study population

Adipose tissue concentrations of POPs in men and overall/pre-/postmenopausal women are summarized in Tables S4 and S5. The distribution of OCPs and PCBs in the GraMo cohort, as well as their comparison with other studies, has been widely discussed elsewhere.[22–24,26,52] Briefly, PCB-153 showed the highest detection rates and median concentrations in the study population, in comparison with previous studies.[53] Postmenopausal women showed the highest median levels of PCBs and OCPs, while men showed the lowest, in accordance with previously demonstrated positive correlations of human bioaccumulation and biomagnification of most OCPs and PCBs with age, both in the GraMo cohort[22–24] as well as in other studies.[54] The results also point to a potential sex-specific accumulation of OCPs and PCBs in adipose tissue, with higher levels in women than in men, that has been previously discussed elsewhere.[22–24]

PFOS and PFOA were detected in 100% of the participants, followed by PFNA and PFHxS in 80.91% and 82.83% of the participants, respectively (Table S3). PFAS serum concentrations were most frequently higher in men than in women, and in postmenopausal than premenopausal women (Table S5). These results corroborate findings of previous studies,[30,55,56] and could be explained by the elimination of PFAS through breastfeeding and menstruation.[57,58] In fact, the elimination of PFAS in premenopausal women through these two pathways would explain the observed negative regressions with age in the study population, with the exception of PFNA (Figure 2). In the case of postmenopausal women and men, positive associations between PFAS and age were evidenced, in agreement with findings obtained by Bartolomé et al.[30] in a representative sample of Spanish labor force. Additional exceptions were found for PFNA, which showed no significant associations in the subgroup of postmenopausal women, and for PFOA in men, which was negatively correlated with age as younger men showed higher concentrations of this pollutant. The negative association with PFOA levels with age could be partially explained by the increased consumption of fast food (e.g., pizza, microwave popcorn) among youth.[59]

Correlations between pairs of persistent pollutants are shown in Figure 3. As previously evidenced in the GraMo cohort,[26] strong correlations ($\rho \geq 0.6$) were found within the PCBs and OCPs analyzed, as well as among PFAS compounds and some PCBs congeners and OCPs. For example, despite the different biological matrices, a negative association was found between PFOA and HCB concentrations, and a weaker positive association between PFNA levels and the three PCBs congeners.

Sex hormone levels in the study population

Sex hormone concentrations are summarized in Table 2. As expected, estradiol levels were markedly higher in premenopausal women, followed by men and postmenopausal women. In addition, women showed increased SHBG and markedly decreased TT levels compared to men, in agreement with previous research.[60–62] Furthermore, LH and FSH concentrations were substantially higher in postmenopausal women compared with men and premenopausal women. These findings were in line with

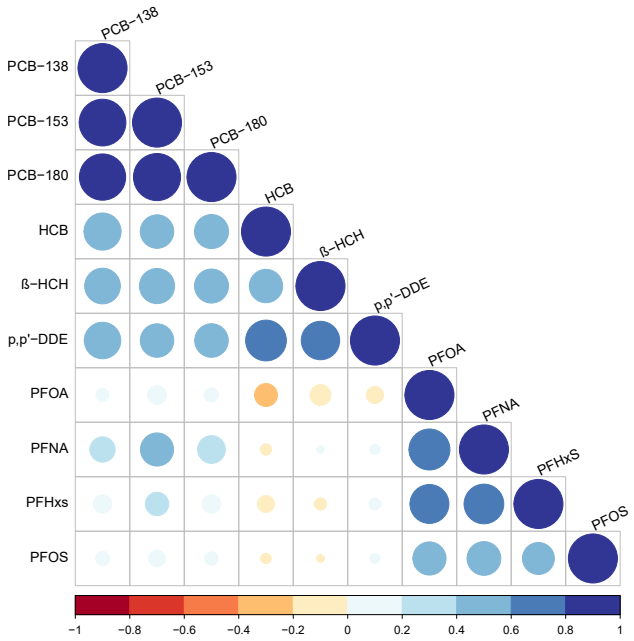

Figure 3. Correlation matrix between POPs in the study population
Cells display the Spearman's correlation test rho value, whose value ranges between 1 (blue) and - 1 (red). PCB: polychlorinated biphenyl; HCB: hexachlorobenzene; β-HCH: beta-hexachlorocyclohexane; *p,p'*-DDE: *p,p'*-dichlorodiphenyldichloroethylene; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid; PFHxS: perfluorohexane sulfonic acid; PFOS: perfluorooctane sulfonic acid.

Kawakita et al., [63] that evidenced an increase in LH and FSH levels with the progress of the menopause.

Associations of POPs with sex hormone levels

The results of the multivariable linear regression analyses between individual POPs and sex hormones for each population group are detailed in Tables 3 and 4, as well as in Tables S6–S9 .

Associations of PCBs and OCPs with sex hormone levels

Figure 4 summarizes the significant results of the WQS multi-pollutant regression models of PCBs and OCPs. Specific data of the coefficients for each model is shown in Tables S10 and S11. No relevant results were observed in pre- and postmenopausal women.

For men, only marginally significant positive associations of β-HCH and PCB-153 with FSH levels were observed (Table S6), as well as a significant mixture effect of PCBs and OCPs (Figure 4). These associations could have implications on men fertility, since higher levels of FSH and LH have been reported to be associated with decreased fertility in previous studies.[64] However, our results should be taken with caution since, to the best of our knowledge, they have not been observed in previous studies. [65–67] Our findings might either be either false positives or explained by the aforementioned differential biological meaning of POP concentrations measured in adipose tissue.

Furthermore, in men, we only observed a marginally significant negative association between PCB-138 and TT levels (Table S6). Goncharov et al. reported positive associations between PCB-153 and the total sum of PCBs in 257 adult Mohawk men, but not for PCB-138,[68] which aligns with findings from other epidemiological studies.[66,69] Lower TT levels could be caused by an alteration of the primary function of Leyding cells, which would compromise TT production and secretion. This could be produced by a reduction in steroidogenic enzyme activity within these cells or by a decrease in pituitary LH secretion secondary to alteration of the hypothalamic-pituitary-gonadal axis.[69] Another mechanism that could explain the lower TT concentrations is that PCB exposure would reduce pituitary responses to low androgen levels, as seen in experimental studies.[70] Thus, TT levels are reduced by attenuating negative feedback inhibition within the hypothalamic-pituitary-gonadal axis.[71] This warrants further research since, unlike our research, previous biomonitoring studies in blood/serum/plasma samples have found positive associations between both *p,p'*-DDE and α-HCH and SHBG, estradiol and TT levels.[72–75]

In the overall group of women, a significant positive mixture effect with TT in the WQS models (Figure 4). In the individual analyses, no significant associations with TT levels were found (Table S7), but significantly positive associations were evidenced in the group of premenopausal women with PCB-180, as well as with PCB-153 (Table 3; Table S8). To date, evidence regarding the effect of mixtures of PCBs and OCPs in women is very limited, highlighting the need for further investigation on this issue. Nevertheless, an *in vitro* study conducted on ovarian follicular cells found that when *p,p'*-DDE, the second largest contributor in our positive WQS model, was added to the investigated mixture of POPs, a strong inhibitory effect on TT secretion was observed. A possible mechanism of action would involve the activation of CYP17 (responsible for the biosynthesis

Table 2. Summary of sex hormones serum levels in the study population (*n* = 253)

		Population group [n (%)]			
	Sex Hormones	Men 126 (49.8)	Women 127 (50.2)	Premenopausal women 68 (26.9)	Postmenopausal women 59 (23.3)
Median (IQR)	Estradiol (pg/mL)	21.5 (9.7–34.5)	21.3 (5.0–75.6)	59.6 (6.5–106.0)	5.3 (5.0–21.8)
	FSH (U/mL)	6.8 (5.0–12.3)	19.3 (6.2–59.7)	6.7 (4.4–11.0)	59.7 (47.6–75.0)
	LH (U/mL)	7.8 (5.6–13.2)	11.9 (5.4–26.5)	6.8 (4.1–15.2)	21.9 (9.0–33.1)
	SHBG (nmol/L)	53.9 (35.9–66.3)	63.7 (45.0–86.8)	66.0 (43.8–92.9)	61.5 (47.5–79.4)
	TT (ng/mL)	4.3 (2.9–5.6)	0.3 (0.2–0.5)	0.3 (0.3–0.6)	0.2 (0.2–0.4)

IQR, interquartile range; FSH, follicle-stimulating hormone; LH, luteinizing hormone; SHBG, sex hormone binding globulin; TT, total testosterone.

Table 3. Associations of organochlorine pesticides and polychlorinated biphenyls with sex hormones levels in premenopausal women (n = 68)

	Estradiol		FSH		LH		SHGB		TT	
	β (SE)	p value	β (SE)	p value	β (SE)	p value	β (SE)	p value	β (SE)	p value
PCB-138	−0.214 (0.117)	0.072	−0.0084 (0.013)	0.518	−0.0035 (0.014)	0.808	−0.1402 (0.086)	0.110	0.0006 (0.000)	0.175
PCB-153	−0.0648 (0.048)	0.186	−0.006 (0.005)	0.256	−0.0034 (0.006)	0.568	−0.0488 (0.036)	0.175	0.0004 (0.000)	0.037
PCB-180	−0.1047 (0.059)	0.081	−0.0074 (0.007)	0.263	−0.0113 (0.007)	0.111	−0.0725 (0.043)	0.099	0.0007 (0.000)	0.001
HCB	−1.8512 (0.279)	<0.001	−0.0215 (0.037)	0.562	−0.019 (0.040)	0.640	−0.2784 (0.240)	0.251	0.0007 (0.001)	0.586
β-HCH	−1.7495 (0.724)	0.019	0.003 (0.088)	0.973	−0.0325 (0.095)	0.733	−1.2459 (0.561)	0.030	0.0005 (0.003)	0.869
p,p'-DDE	0.0068 (0.077)	0.930	0.0029 (0.008)	0.729	0.0156 (0.008)	0.067	−0.069 (0.055)	0.217	0.0003 (0.000)	0.356

SE, standard error; PCB, polychlorinated biphenyl; HCB, hexachlorobenzene; β-HCH, beta-hexachlorocyclohexane; p,p'-DDE, p,p'-dichlorodiphenyldichloroethylene; FSH, follicle-stimulating hormone; LH, luteinizing hormone; SHBG, sex hormone binding globulin; TT, total testosterone.
Models were adjusted for age, BMI, smoking habit, alcohol consumption, education level, number of children, breastfeeding and use of contraceptives.
Continuous variables were entered in the models without any transformation.

of androstenedione, a precursor for testosterone production) and the activation or inhibition of CYP19 (aromatase), a key enzyme in the conversion of androgens to estrogens, which can be induced by certain PCBs.[76]

Moreover, in premenopausal women (Table 3; Table S8), PCB-138 and PCB-180 showed a negative (yet marginally significant) association with estradiol. *In vivo/in vitro* studies have shown different results in relation to the estrogenic potential of PCBs depending on the congener studied.[77–79] In general, it has been observed that PCBs can increase the bioavailability of estradiol in target tissues that express estrogen receptors, thus exerting an indirect estrogenic effect not necessarily associated with a significant reduction in circulating levels of estradiol.[77] It has also been observed that in the uterine tract of mice, the PCB mixture (Aroclor 1254) could negatively regulate the MMTV integration site family of wingless type, member 7A, which also occurred with synthetic estrogens at low levels.[78] In another study carried out a human-breast-cancer estrogen-sensitive MCF7-BUS cell line, antiestrogenic activity by individual PCBs was made by endogenous estradiol depletion, as well as through an estrogen receptor-dependent pathway. However, when a mixture of PCB congeners was administered, this effect disappeared. It was hypothesized that exposure to mixtures of PCBs could have synergistic effects on their antiestrogenicity through an estrogen receptor-independent pathway.[79] For these reasons, although PCBs are typically associated with an antiestrogenic effect, as suggested by the found marginally significant associations, no significant associations could be detected in any of the groups studied.

For OCPs, in the premenopausal women group (Table 3; Table S8), negative significant associations of HCB and β-HCH with estradiol were found. This association was also observed previously for HCB levels (but not HCH) in a cross-sectional study carried out in pregnant women.[80] A possible mechanism of action for the lower estradiol levels associated with HCB could be an inhibitory action of HCB on the CYP19 enzyme involved in oestradiol synthesis.[81]

Moreover, a positive association between p,p'-DDE and LH levels was also found. In contrast with our results, Freire et al.[82] observed that not only serum p,p'-DDE but also HCB levels were associated with lower LH levels in a sample of 300 Brazilian women of all ages. Finally, in our study, β-HCH was negatively associated with SHBG in premenopausal women group, an association that was not previously reported.[83]

Associations of individual PFAS with sex hormone levels

In the men group (Table 4; Table S6), we observed a significant positive association of PFHxS with FSH levels. Previous studies have not only evidenced this association, but have also described positive relationships between PFOA and PFDA levels with FSH.[84,85]

A positive significant association between PFNA and TT levels was also found in men. There are only few epidemiological studies investigating the relationship between serum PFAS levels and different sex hormones in different population groups. Thus, a previous cross-sectional study found a positive association between PFOS and TT levels in men aged 20–49 years, and over 50 years. A particularly positive association was also observed with PFNA levels, but only in the second quartile.[86] In contrast,

Table 4. Associations of per- and polyfluoroalkyl substances (PFAS) with sex hormones levels in men (*n* = 48)

	Estradiol		FSH		LH		SHGB		TT	
	β (SE)	*p* value	β (SE)	*p* value	β (SE)	*p* value	β (SE)	*p* value	β (SE)	*p* value
PFOA	0.4512 (2.922)	0.878	0.1938 (0.793)	0.808	−1.2788 (1.255)	0. 314	4.8417 (3.912)	0.223	0.2955 (0.315)	0.353
PFNA	11.1179 (8.820)	0.215	3.7531 (2.318)	0.113	−4.0380 (3.791)	0.293	18.9983 (11.699)	0.112	2.2856 (0.880)	0.013
PFHxS	2.0623 (3.659)	0.576	2.1042 (0.915)	0.027	−0.8764 (1.587)	0.584	−0.3681 (4.940)	0.941	0.4112 (0.4026)	0.313
PFOS	−0.0787 (0.214)	0.715	0.0687 (0.057)	0.233	−0.1355 (0.090)	0.140	0.3486 (0.279)	0.219	0.0311 (0.022)	0.174

SE, standard error; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid; PFOS, perfluorooctane sulfonic acid; FSH, follicle-stimulating hormone; LH, luteinizing hormone; SHBG, sex hormone binding globulin; TT, total testosterone.
Models were adjusted for age, smoking habit, alcohol consumption, and education level.
Continuous variables were entered in the models without any transformation.

other epidemiological studies have found no association of PFAS and TT levels in men.[87,88] Although we did not find a significant relationship, Petersen et al. found a positive association of PFOS measured in serum with LH levels in population from Faroe Islands.[89] These differences may be explained by the different matrices in which the exposure was measured (adipose tissue vs. serum), as well as the considerably younger mean age and higher exposure levels in the Faroese study.

For premenopausal women we only observed an inversely significant association between PFHxS and LH levels (Table S8). Despite previous studies have not found associations between PFAS and LH,[88] *in vivo* investigations observed increases in estradiol and LH levels in female adult and juvenile rodents after exposure to these chemicals, probably through an induction of the hypothalamic kisspeptin system (a potential regulator of GnRH activity induced by estradiol).[90,91] In fact, in our study, PFOS was marginally positive related to estradiol levels.[91] However, Harlow et al., evidenced negative associations of PFOA and PFNA with estradiol levels after 15 years of follow-up in women enrolled during their menopausal transition.[92] For this reason, the relevance of these findings in a human population level needs further elucidation.

We did not find any association of PFAS with FSH. This is in line with the cross-sectional study of Zhan et al.[93] that evidenced positive associations between plasma PFAS and serum FSH levels in Chinese women with premature ovarian insufficiency, but not in the healthy women group as in our study.

Finally, in the group of postmenopausal women, only a marginally significant association was found between PFOA and FSH levels (Table S9). The *in vitro* study by Houck et al.[94] suggested that the ability of PFAS to alter different sex hormones may be due to an endocrine disruption of the hypothalamic-pituitary-gonadal axis, mediated through the inhibition or activation of peroxisome proliferator-activated receptors α and γ, pregnane X receptor, constitutive androstane receptor, and estrogen receptor α, among others. Further research is needed to completely understand the mechanisms underlying the alteration of sex hormones due to PFAS exposure, as findings from *in vitro* studies cannot be directly extrapolated to *in vivo* conditions due to the different metabolism of PFAS in living organisms.

Limitations of the study

To the best of our knowledge, this study has explored for the first time the associations between mixtures of adipose tissue PCB/OCP concentrations and serum PFAS concentrations with sex hormones. The multi-pollutant approach, incorporating two different POP families (OCPs and PCBs), complements findings for individual pollutants and allows for the investigation of the combined effects of multiple simultaneous exposures. However, certain particularities of the WQS models used should be kept in mind. For example, only joint effects in the same direction can be simultaneously explored, and the model assumes additive effects.

The cross-sectional design limits causal inference, although the most probable direction of the associations is POPs → hormones, we cannot rule out the presence of reverse causality. Indeed, increased FSH levels have been reported to induce changes in the amount, composition and metabolism of adipose

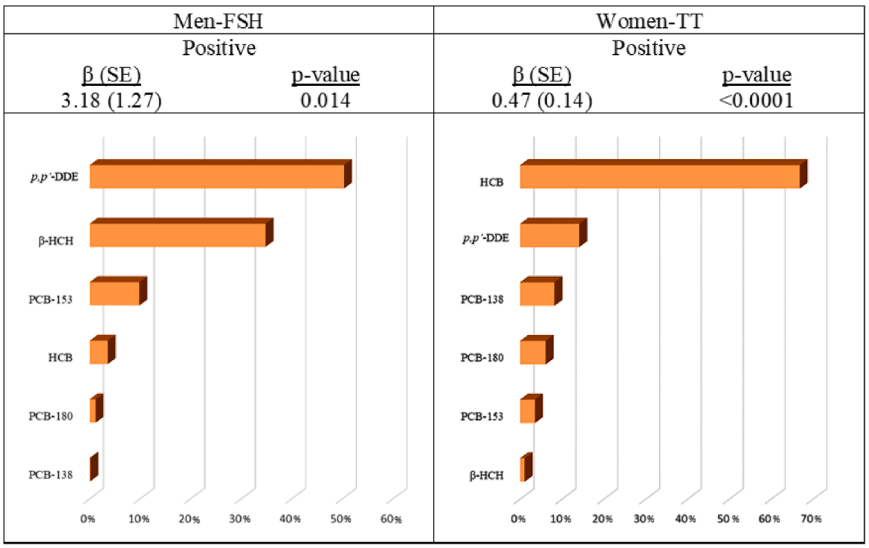

Figure 4. Percentage contribution of the mixture of PCBs and OCPs in the WQS significant models

Models for men were adjusted for age, BMI, smoking habit, alcohol consumption and education level. For women they were also adjusted for number of children, breastfeeding and use of contraceptives. The concentrations of PCBs/OCPs were logarithmically transformed. The covariates were not transformed. The bar length represents the weight of each contaminant in percentage. SE: standard error; WQS: weighted quantile sum; PCBs: polychlorinated biphenyls; OCPs: organochlorine pesticides; FSH: follicle-stimulating hormone; SHBG: sex hormone binding globulin; TT: total testosterone; HCB: hexachlorobenzene; β-HCH: beta-hexachlorocyclohexane; *p,p'*-DDE: *p,p'*-dichlorodiphenyldichloroethylene.

tissue.[95,96] This might in turn affect the POP concentrations bioaccumulated in the adipose tissue. Furthermore, when comparing our results with previous studies, it is important to consider potential differences in the biological meanings of adipose tissue POP concentrations (our study) vs. serum (the most frequently used matrix). Adipose tissue OCP and PCB concentrations are considered the most accurate estimators of long-term exposure to these pollutants, while serum concentrations, even after lipid standardization, might partially account for more recent exposures.[97–99] Lastly, adipose tissue is acknowledged to have a differential contribution to global steroid hormone balance according to factors such as age, obesity, sex or menopausal status.[100,101] Thus, we cannot completely rule out the differential meaning of adipose tissue POP concentrations according to the study subsample.

Although GraMo is a well-characterized cohort, the main limitations of this exploratory study include its hospital-based population and relatively small sample size, particularly in the analysis of women by menopausal status, which likely impact statistical power and external validity. However, our results suggest novel and potentially relevant associations from a public health perspective that may be applicable to the general population. For these reasons, marginally significant associations were marked as potential indicators of meaningful findings, and results were not adjusted for multiple comparisons to enhance sensitivity in detecting potential associations, despite the increased risk of false positives.[102,103] Based on the aforementioned considerations, these findings strongly warrant further confirmation in larger and more diverse populations.

In addition, the specific day of the menstrual cycle at the time of sample collection is a critical variable in the evaluation of sex hormone levels in premenopausal women; however, this information was not recorded. This may result in non-differential bias, potentially attenuating the associations observed in premenopausal women. Lastly, despite the extensive characterization of the cohort, the influence of residual confounding cannot be ruled out due to unmeasured or poorly characterized variables, such as physical activity or an adequate characterization of the diet.

Conclusions

We observed individual and mixture associations between POP exposure and sex hormone levels, with effects differing by sex and menopausal status. The associations were more pronounced in premenopausal women. If confirmed, these findings could have significant public health implications, as human exposure to these pollutants remains widespread, and disturbances in sex hormone levels are highly prevalent in contemporary society.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact Juan Pedro Arrebola (jparrebola@ugr.es).

Materials availability

The authors confirm that the materials are available from the lead contact upon reasonable request.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request.

The R script generated is shown in supplemental information (Data S1).

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

C.P.-D.: methodology, formal analysis, data curation, writing – original draft; R. E.: conceptualization, methodology, formal analysis, data curation, writing – original draft; F.M.P.C.: data curation; I.S.-B.: writing – review and editing; P. R.: writing – review and editing; R.B.-R.: writing – review and editing; J.J.R.: investigation, validation, writing – review and editing; N.O.: writing – review and editing; M.F.F.: writing – review and editing; P.M.-O.: writing – review and editing; J.P.A.: conceptualization, funding acquisition, project administration, writing – review and editing; investigation; methodology, supervision.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
R version 4.3.1	R Core Team[33]	https://www.r-project.org/
RStudio version 2023.09.0	RStudio Team[34]	https://posit.co/download/rstudio-desktop/
SPSS Statistics v20.0	IBM[35]	https://www.ibm.com/products/spss-statistics
ggplot2	Wickham [36]	https://ggplot2.tidyverse.org/
corrplot v0.92	Wei et al.[37]	https://cran.r-project.org/package=corrplot
MASS package	Ripley et al.[39]	https://cran.r-project.org/package=MASS
gWQS v3.0.4	Renzetti et al.[51]	https://cran.r-project.org/package=gWQS
Original R script	Data S1	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

This study is a cross-sectional research conducted within the GraMo adult cohort (Granada, Southern Spain), for which an extensive description of population characteristics and pollutant exposure levels has been published elsewhere.[21–26] The province of Granada encompasses rural, semi-rural, and urban areas, with a total population of over 915,000 inhabitants spread across an area of 12,531 km^2.

The characteristics of GraMo cohort and enrollment procedure have been thoroughly described elsewhere.[24] Briefly, recruitment was performed during 2003 and 2004 in two public hospitals: Clínico San Cecilio University Hospital in Granada city (inland urban area) and Santa Ana Hospital in the town of Motril (semi-rural area in the Mediterranean coast). The inclusion criteria were: aged older than 16 years, not having received hormone therapy and having resided in the study areas for at least 10 years before recruitment. Patients with cancer and those with a hormonal disease related to the hypothalamic axis were excluded. The cohort included patients undergoing non-cancer-related surgery, namely hernias (41%), gallbladder diseases (21%), varicose veins (12%), and other conditions (26%).

From a first set of 409 individuals contacted, 387 accepted the invitation to participate. A final group of 253 participants was included in the study, as they provided samples for hormone assessment and some sample for persistent organic pollutants (POP) measurement. 234 of them provided sufficient adipose tissue sample for organochlorine pesticides (OCPs) and polychlorobiphenyls (PCBs) analyses, while 99 had enough sample volume for per- and polyfluoroalkyl substances (PFAS) quantification in serum.

In our analysis, we stratified by sex, defined as a biological variable. This variable was obtained from participants' medical records. As for gender (complex sociocultural construct) given the marked influence of traditional gender roles in southern Spain during participant recruitment, we considered a concordance between sex and gender. Women who had menstruated at least once in the last 12 months were included in the premenopausal women group.

No data were collected on the ethnicity or race of the population, which may limit the generalisability of the results to the whole population and may obscure structural differences relevant to understanding how pollutants affect hormone health in different racial or ethnic groups. This study was conducted in the general hospital population in Spain during the years 2003–2004. In that context, the systematic collection of information on ethnicity or race was not part of standard practice in health information systems and was not considered an epidemiologically or clinically relevant criterion. Therefore, these data were not included in the study design or collection. However, it is recognized that their absence may limit the analysis of possible inequalities associated with racial-ethnic factors and restricts the ability to extrapolate results to particular population groups.

All participants enrolled in this research signed an informed consent and the study protocol was approved by the Ethics Committee of Granada (Comité Ético de Investigación Provincial de Granada), dated December 7th, 2020.

METHOD DETAILS

OCPs and PCBs analysis

During the surgery, 5–10 g of adipose tissue were intra-operatively collected mainly from pelvic waist (42%), front abdominal wall (39%), limbs (13%) and another (6%), and immediately coded and stored at −80°C.

The pollutants selected, because they were the most frequent in the population, were: 1) PCBs congeners −138, −153 and −180; 2) OCPs: hexachlorobenzene (HCB), alpha-hexachlorocyclohexane (α-HCH), beta-hexachlorocyclohexane (β-HCH), *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE) and dicofol. OCPs and PCBs adipose tissue concentrations were expressed in nanograms per gram of lipid (ng/g lipid).

Levels of OCPs and PCBs in adipose tissue were quantified in 2005 at the Laboratorio Analítico Bioclínico (Almería, Spain) using the technique described by Rivas et al. (2002).[27]

200 mg of adipose tissue was extracted using n-hexane, and the solution was then purified through 200 mg alumina in a glass column and kept in test tubes at −80°C. From each serum sample, 4 mL was extracted with acidified diethyl ether and n-hexane, and the cleaned-up extract was eluted through a solid phase silica extraction column (Sep-Pak, Waters).

PCBs and OCPs were quantified by high-resolution gas chromatography with a mass spectrometry detector in tandem mode (GC-MS/MS), using a Saturn 2000 ion trap system (Varian Inc., Walnut Creek, CA) and 2 m × 0.25 mm silica capillary column (Bellefonte, PA) coupled to a Factor Four VF-5MS 30 m × 0.25-mm i.d. analytical column (Varian Inc., Walnut Creek, CA).[104] For the quality control, laboratory fortified matrix samples at different concentrations were used. 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 0.01 μg/L for all PCBs/OCPs under study.

PCBs and OCPs recovery from adipose tissue samples was studied to assess the extraction efficiency of the method and ranged from 90 to 98%. Lipid content in adipose tissue samples was quantified gravimetrically: 100 mg of adipose tissue were homogenized in 2.5 mL of chloroform:methanol:hydrochloric acid (20:l0:0.L). After repeating the process, 5 mL of HC1 0.1N were added and centrifuged at 3000 rpm for 10 min. The organic phase was collected; the non-organic phase was extracted again and added to the first extraction product. After drying under a nitrogen stream, the tubes were weighed and the total lipid expressed in g of lipid per g of adipose tissue.

Normalized PCBs and OCPs concentrations were expressed in nanograms per gram of lipid (ng/g lipid).

A double-blinded procedure was followed so that neither the chemical analysts nor statistical staff knew the identity or characteristics of any study subject.

PFAS analysis

Blood samples were collected in S-Monovette neutral tubes (Sarstedt, Nümbrecht, Germany), centrifuged and transported at 4°C, as described by Esteban et al.,[29] and stored until analysis at −20°C.

Chemical analyses were performed in 2021 at Centro de Sanidad Ambiental of the Instituto de Salud Carlos III (Madrid, Spain). 100 μL of serum, 100 μL of ACN for protein precipitationand 25 μL of labeled standard solution were mixed. The final volume of 270 μL was achieved using MeOH. The mixed sample was vortexed for 15–20 s and centrifuged for 10 min at 13,500 rpm and 4°C. The supernatant organic layer was injected into the mass spectrometry detector in tandem mode (HPLC-MS/MS), TSQ Vantage (Thermo Fisher Scientific, Waltham, MA, USA). Quantification was carried out by isotope dilution method, and labeled standards were used for internal quality purposes, as well.[30]

The LOD was established using labeled standards. All PFAS were quantified between 0.16 and 0.34 μg/L. Internal quality control was performed with PFAS Standar and PFAS labeled Standard. Quality in the analytical procedure was assured by participating in G-EQUAS 51/2013 (German External Quality Assessment Scheme, Erlangen, Germany). All values were within the established range of tolerance, *Z* score b.

Twelve PFAS compounds were analyzed: perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUdA), perfluorododecanoic acid (PFDoA), perfluorobutane sulfonic acid (PFBS), perfluorohexane sulfonic acid (PFHxS), perfluoroheptane sulfonic acid (PFHpS) and perfluorooctane sulfonic acid (PFOS). PFAS serum concentrations were expressed in micrograms per liter (μg/L).

Hormone analysis

Serum sex hormone concentrations (estradiol, pg/mL; FSH and LH, U/mL; SHBG, nmol/L; and TT, ng/mL) were evaluated in 2018 at Instituto de Investigación Biosanitaria de Granada (ibs.GRANADA) (Granada, Spain), by means of immunoassays performed in an electrochemiluminescence bioanalyzer cobas e−411 (F. Hoffmann-La Roche Ltd, Basel, Switzerland).

Covariates

Data on socio-demographic characteristics, lifestyle, and health status were collected through face-to-face interviews conducted by trained staff during recruitment of the study population, while participants were at the hospital. Previously validated questionnaires were used.[31,32] Age was expressed in years and body mass index (BMI) as weight/height squared (kg/m^2). Current smoker was defined as any level of daily tobacco consumption at the time of recruitment (≥1 cig/day), and alcohol consumers at any level of weekly alcohol intake (≥1 glass/week). A woman was considered to be an oral contraceptive user if she had used oral contraceptives at any time in her life. Missing lifestyle data (<0.4%) from participants were imputed using the median for quantitative variables and the most frequent level for categorical variables.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analyses were performed using R v 4.3.1[33] with RStudio v 2023.09.0,[34] and SPSS Statistics v 20.0.[35] Plots were created using ggplot2[36] library, while the correlation matrix plot was generated using the corrplot R-package version 0.92.[37] Linear regression models were performed using the MASS package in R.[39]

The sample size was limited to individuals for whom a biological sample was available. Continuous variables were described by median and interquartile range (IQR), while categorical variables were presented as absolute frequencies and percentages (%).

The shape of the associations between POP concentrations and sex hormone levels was analyzed using generalized additive models, which showed an approximately linear pattern throughout most of the range, with non-linearity appearing only at the extremes. Potentially influential observations were assessed using Cook's distance and leverage diagnostics. However, none were excluded, as they were not consistent across all models and their removal could have compromised comparability between groups. Therefore, in order to minimize the influence of outliers and obtain more reliable estimates, the associations between individual POPs (independent variables) and sex hormones (dependent variables) were assessed using multivariable robust linear regression models based on MM estimators. These estimators are less likely influenced by the outliers and do not require normally distributed residuals, which allowed us to avoid variable transformation and to retain their original scale for interpretation.[41]

Only POPs detected in more than 75% of samples (i.e., PCB-138, PCB-153, PCB-180, HCB, β-HCH, *p,p'*-DDE, PFOA, PFNA, PFHxS and PFOS) were included in the statistical analyses, where concentrations <LOD were imputed with a random value between 0 and the LOD (Table S3).

Analyses were stratified by sex, and for women, further stratified by menopausal status (associations of POPs with sex hormone levels section, Tables 3 and 4). Models for men (n = 126) were adjusted for age (years), BMI (Kg/m^2), smoking habit (smoker or former smoker vs. non-smoker), alcohol consumption (consumer vs. non-consumer) and education level (primary or secondary/higher levels vs. unfinished primary level). Models for women (overall n = 127; premenopausal n = 68; postmenopausal n = 59) were additionally adjusted by number of children, breastfeeding (yes vs. no) and use of contraceptives (Figure S1). Adjustment for smoking habit and alcohol consumption was performed across all models, as both factors have been associated with alterations in sex hormone levels and may also influence internal POPs levels.[42–47] PFAS models were not adjusted for BMI to avoid potential overadjustment, since PFAS are considerably less lipophilic and, consequently, less prone to accumulate in fat tissues compared to OCPs and PCBs. In addition, BMI might be present in the causal pathway for PFAS → hormones, since these pollutants have been acknowledged as possible obesogens,[48,49] and elevated BMI has been related to altered hormone levels.[50]

To explore potential mixture effects, weighted quantile sum (WQS) regressions with repeated holdout validation were fitted for each sex hormone using the R-package gWQS,[51] including log-transformed concentrations of PCBs and OCPs (n = 234) in the combined index (associations of POPs with sex hormone levels section, Table 2). No transformation was applied to any covariate. PFAS levels, as well as the subgroups of premenopausal and postmenopausal women, were not included in the WQS analyses due to the limited number of participants with available chemical data, which would have compromised validity and robustness of the analyses. Considering that WQS regressions need to set *a priori* the expected direction of the association, we calculated two models for mixture effects for each outcome, i.e., one assuming a positive influence of the mixture on each hormone (positive model) and the other assuming a negative effect (negative model). The models were set by including chemical concentrations ranked in quartiles, 25% of dataset for training and 75% for validation, 100 bootstrap samples and 100 sets of repeated holdout validation.

Statistical significance was set at $p < 0.05$. Results with p-values between 0.05 and 0.10 were considered to indicate a trend or borderline significance and were interpreted with caution.