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Associations between persistent organic pollutants and endometriosis: A multiblock approach integrating metabolic and cytokine profiling

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ABSTRACT

Humans are exposed daily to complex mixtures of chemical pollutants through their environment and diet, some of which have the potential to disrupt the bodies' natural endocrine functions and contribute to reproductive diseases like endometriosis. Increasing epidemiological and experimental evidence supports the association between endometriosis and certain persistent organic pollutants (POPs) like dioxins; however, little is known about the underlying linking mechanisms. The main objective of this study is to proof the methodological applicability and discovery potential of integrating ultra-trace mass spectrometry (MS) profiling of POP biomarkers and endogenous biomarker profiling (MS metabolomics and cytokines) in a case-control study for the etiological research of endometriosis. The approach is applied in a pilot clinical-based study conducted in France where women with and without surgically confirmed endometriosis were recruited. Serum samples were analysed with high-resolution MS for about 30 polychlorinated biphenyls (PCBs), organochlorinated pesticides and perfluoroalkyl substances (PFAS). About 600 serum metabolites and lipids were identified with targeted metabolomics using tandem MS with the Biocrates MxP® Quant 500 Kit. A panel of 4 pro-inflammatory cytokines were analysed using ELISA-based 4-PLEX analyser. Statistical analysis included a battery of variable selection approaches, multivariate logistic regression for single-chemical associations, Bayesian kernel machine regressions (BKMR) to identify mixture effects of POPs and a multiblock approach to identify shared biomarker signatures among high risk clusters. The results showed the positive associations between some POPs and endometriosis risk, including the pesticide *trans*-nonachlor Odds Ratio (95% Confidence Interval) 3.38 (2.06–5.98), $p < 0.0001$ and PCB 114 OR (95% CI) 1.83 (1.17–2.93), $p = 0.009$. The BKMR approach showed a tendency of a positive cumulative effect of the mixture, however *trans*-nonachlor exhibited significant associations within the mixture and interacted with other PCBs, strengthening the effects at highest concentrations. Finally, the multiblock analysis, relating the various blocks of data, revealed a latent cluster of women with higher risk of endometrioma presenting higher concentrations of *trans*-nonachlor, PCB 114 and dioxin-like toxic equivalents from PCBs, together with an increased inflammatory profile (i.e. elevated interleukin-8 and monocyte chemoattractant protein-1). It was also highlighted a specific metabolic pattern characterized by dysregulation of bile acid homeostasis and lipase activity. Further research will be required with larger sample size to confirm these findings and gain insight on the underlying mechanisms between POPs and endometriosis.

1. Introduction

Humans today are daily exposed to a plethora of environmental contaminants, some of which have the potential to disrupt our bodies' natural hormonal regulations and pose a threat to human health (Diamanti-Kandarakis et al. 2009; Kahn et al. 2020). A group of endocrine

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disrupting chemicals (EDCs) known as Persistent Organic Pollutants (POPs) is of particular concern due to their bioaccumulative properties and impacts on human health (UNEP 2017). Women have been shown to be especially vulnerable to the effects of EDCs which have been related to a handful of female reproductive systems including hormone-sensitive cancers, infertility, disruption of menstrual cycles or endometriosis (Gore et al. 2015; Green et al. 2021; Kahn et al. 2020).

Epidemiologic and experimental evidence exists linking exposure to POPs with endometriosis (Cano-Sancho et al. 2019; Matta et al. 2021), an oestrogen regulated disease characterised by the presence of endometrial tissues outside of the uterus, and highly variable and non-specific symptoms like chronic pelvis pain, dysmenorrhea or dyschesia (Giudice 2010). Despite it is commonly considered a benign pathology, endometriosis is associated with higher risk of ovarian and breast cancers, asthma, and higher risk of several chronic diseases such as asthma and cardiovascular diseases (Kvaskoff et al. 2015; Kvaskoff et al. 2021). Endometriosis is estimated to impact 5–15% of women who menstruate (Shafrir et al. 2018).

The highly active 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is by far the most studied pollutant in association with endometriosis (Matta et al. 2021), with limited studies on the rest of POPs individually or as mixtures. Additionally, the mechanisms of action of POPs remains largely unknown partially due to the constraints of animal models to fully recapitulate the pathogenesis undergoing in women (Grümmer 2006). Over the past decades, new high throughput technologies have emerged, allowing us to study endometriosis with more molecular scrutiny, including metabolic profiling (e.g. metabolomics). The identification of predictive "omic" biomarkers of endometriosis has been an active field of research, motivated by the long and expensive diagnosis delays, lasting between 8 and 10 years. Some recent studies using metabolomic profiling have revealed an altered lipid profile associated to the development and progression of endometriosis (Cordeiro et al. 2015; Domínguez et al. 2017). In turn, metabolomics is also gaining popularity in the field of environmental health research as a cost-efficient means for linking external with internal exposures, biological responses and diseases (Vermeulen et al. 2020; Walker et al. 2019), proved as promising by few applied studies (Jin et al. 2020; Stratakis et al. 2020).

To our knowledge, no study has yet attempted to integrate biomarkers of exposure to POPs with metabolic and cytokine profiling to uncover signatures potentially informing about molecular pathways underlying the progression of endometriosis. Thus the main objective of this pilot study is to proof the methodological applicability and discovery potential of integrating ultra-trace profiling of POP biomarkers and endogenous biomarker profiling (metabolomics and cytokines) in the etiological research of endometriosis.

2. Methods and materials

2.1. Study design and recruitment

The ENDOXOMICS-β study is an ongoing clinical-based case-control study conducted at the Gynecology-Obstetrics Department and Biology and Reproductive Medicine Departments from the Univesity Hospital of Nantes, France. For the present study, 87 participants were recruited during 2018–2019 including only adult female patients (between 18 and 45 years) seeking surgical intervention on deep endometriosis without endometrioma (noOMA), with endometrioma (OMA) or other benign reproductive issues not related to endometriosis (controls). Endometriosis diagnoses for cases were performed by laparoscopy with histological confirmation Gynecology-Obstetrics Department as described elsewhere (Ploteau et al. 2017). All cases presented severe forms of endometriosis classified in stages III or IV according to the American Fertility Society reviewed-classification (AFSr) scale and often they were cases with rectosigmoid deep endometriosis (DE) exhibiting frozen pelvis with abundant adhesions. Controls comprised similar aged women consulting for non-endometriosis related benign gynaecological issues exhibiting no endometriosis-like symptoms, including tubal ligation or fertile women undergoing assisted reproductive technology for male infertility or oocyte donation. Fibroids or polycystic ovarian syndrome (PCOs) were not eligible. All patients gave informed consent to participate in the study; patients who did not provide written consent were excluded. The protocol was approved by the local biotethics committee Groupe Nantais d'éthique dans le Domaine de la Santé (GNEDS). Each participant provided a serum sample (at least 5 mL) that was stored at −80 °C until the analysis in aliquots to minimize the freeze–thaw cycling. Each participant was interviewed by the clinicians during consultation using a questionnaire was adapted from the World Endometriosis Research Foundation (WERF) Endometriosis Phenome and Biobanking Harmonization Project (EPHect) Questionnaire (Becker et al. 2014; Fassbender et al. 2014; Rahmioglu et al. 2014; Vitonis et al. 2014) about individual, socio-economic and demographic variables including age (years), body mass index (BMI, Kg/m^2), breast-feeding, parity, contraceptive use or type of infertility, among others.

2.2. Laboratory analysis

2.2.1. Characterization of exposure biomarkers of POPs

Concentrations of 20 polychlorinated biphenyls (PCBs), and 30 organochlorine pesticides (OCPs) were analysed in thawed serum samples using gas chromatography coupled to high resolution mass spectrometry (GC-HRMS) as detailed elsewhere (Koual et al. 2019; Ploteau et al. 2017). Serum concentrations of 14 perfluoroalkyl substances (PFAS) were quantified using liquid chromatography with tandem-mass spectrometry (LC-MS/MS) as previously detailed (Mancini et al. 2020). Briefly, serum samples underwent a preliminary alkaline digestion followed by a two-stage Solid Phase Extraction purification using polymeric Oasis® HLB and graphitised carbon (ENVI-Carb®) cartridges, and were subsequently analysed by LC-ESI(-)-MS/MS for PFAS. For dioxin-like (DL) and non-dioxin-like (NDL) PCBs, extracts were analysed by gas chromatography (7890A; Agilent Technologies, USA) coupled to a high-resolution mass spectrometer, double sector (JMS-700D and 800D; Jeol, Japan) operating at a resolution of 10,000 (10% valley) (Rivière et al. 2014). Pesticides were separated by capillary gas chromatography (Trace GC Ultra Thermo Scientific) equipped with a programmed temperature vaporiser (PTV) injector and coupled to a Quantum XLS Triple Quadrupole (GC–MS/MS). The mass spectrometer was operated in electron ionization (EI) mode. All analyses were performed with blinding under ISO17025:2005 standard conditions.

Concentration of organochlorinated POPs (PCBs and OCPs) were normalised based on total serum lipids, due to the bioaccumulative properties. Serum lipid content was determined with enzymatic kits (Biolabo, Maizy, France) as the sum of phospholipids, triglycerides, total cholesterol and free cholesterol (Akins et al. 1989). Concentrations below the limit of detection (LOD) were assigned a value of LOD/2 (medium bound approach).

2.2.2. Characterization of endogenous biomarkers

2.2.2.1. Targeted metabolomics profiling. Targeted metabolomics profiling was performed using the MxP® Quant 500 kit (Biocrates Life Sciences AG, Innsbruck, Austria) in thawed serum aliquots. The kit allows the identification and quantification up to 630 endogenous metabolites and small molecules from 26 biochemical classes, combining the high throughput capacities of non-targeted methods with the absolute quantification capacities of classical targeted methods. Among the metabolites includes 14 classes of small molecules: alkaloids (1), amine oxides (1), amino acids (20), amino acid related (30), bile acids (14), biogenic amines (9), carbohydrates and related (1), carboxylic acids (7), cresols (1), fatty acids (12), hormones and related (4), indoles and derivatives (4), nucleobases and related (2), and vitamins and cofactors

(1); and 12 classes of lipids: acylcarnitines (40), lysophosphatidylcholines (14), phosphatidylcholines (76), sphingomyelins (15), ceramides (28), dihydroceramides (8), hexosylceramides (19), dihexosylceramides (9), trihexosylceramides (6), cholesteryl esters (22), diglycerides (44), and triglycerides (242). Small molecules were measured by LC-MS/MS and lipids were measured by flow injection analysis (FIA)-MS/MS using a 6500 + QTRAP® instrument (AB Sciex, Darmstadt, Germany) with an electrospray ionization source, using the 96-well plate kit. The targeted metabolomics analysis methods using the Biocrates Kit followed the extensively documented protocol in the user manual and is described below. For the purposes of the present study only FIA metabolites were used and related methodology is described.

FIA Solvent constituted the Biocrates FIA Mobile Phase Additive and 290 mL methanol (MeOH). The column was prewashed with 95% Solution B for 20 min with a flow rate of 0.5 mL/min. For the system suitability test (SST) and instrument calibration, the column was equilibrated with starting condition: 100% Solvent A with a flow rate of 800 μL/min at 50° C. SST for FIA consisted of injection of: 3 blanks, 3 test mixes, followed by 2 blanks, with 20 μL injection volume. Quality controls (QCs) were dissolved in 100 μL HPLC grade water. Internal standard (ISTD) mix was dissolved in 1200 μL HPLC water. All vials (Cal, QCs, and ISTD) were shaken for 15 min at 1200 rpm and vortexed. QCs and serum samples were centrifuged for 5 min at 2750 × *g* at 4° C. 10 μL of the ISTD mix was added to each well on the kit (except in the first double blank well) using an Eppendorf Multipette® E3 at maximum dispensing speed. 10 μL of the phosphate buffered saline solution, Cal, and QC were then pipetted according to the well plate layout. All wells were dried under nitrogen for 30 min using a nitrogen evaporator.

The derivatisation solution was prepared using 1900 μL of each: ethanol, water, and pyridine, and vortexed rigorously with 300 μL phenylisothiocyanate (PITC) until clear. 50 μL of this was added to each well using the Multipette® at maximum dispensing speed. The plate was then covered and left to incubate at room temperature for 20 min, and then dried under nitrogen for one hour. Then, 300 μL of the extraction solvent was added to each well using an 8-channel pipette. The plate was then shaken for 30 min at 450 rpm (or "low speed") to ensure no spillover, and then centrifuged for 2 min at 500*g* so the fluid would pass through the upper filter plate into the capture plate.

After removing the filter plate, 10 μL of the extracts were transferred into the other 96 deep well plate for FIA and diluted with 490 μL FIA Solvent. The two deep well plates were then covered with airtight silicon mats and shaken for 10 min at 600 rpm. The LC-MS system was equilibrated with starting condition: 100% FIA Solvent, with a flow rate of 30 μL/min at 50° C with 20 μL injection volume. Retention time windows were manually adjusted, and technical validity was assessed in MetIDQ™ software and using Sciex Analyst®.

Biocrates' add-on software tool Metabo*INDICATOR*™ also provides information on up to an additional 230 biologically relevant metabolite sums and ratios associated with biochemical pathways. The analysis of sums and ratios of quantified metabolites may reveal patterns and disruptions of relative proportions that were not apparent by the individual metabolite concentrations.

2.2.2.2. Cytokine profiling. Considering the central role of inflammation in the POPs-endometriosis link, we extended the molecular profiling with a panel of inflammatory cytokines (Interleukin-6, −8, MCP-1 and TNF-α) analysed for a subset of samples using 4-PLEX technology in the Center for Immuno Monitoring Nantes Atlantic (CINMA) platform under ISO9001 and ISO15189 standards.

2.3. Data analysis

Demographic characteristics were summarised using mean and standard deviation (SD), as well as median and interquartile range (IQ) for continuous variables and frequency and percentage for categorical variables. Statistical comparison between groups (i.e. controls, cases without endometrioma (noOMA), and cases with endometrioma (OMA)) was performed using Kruskal-Wallis and Mann-Whitney Tests for continuous variables and Fisher's exact test for categorical variables.

Data analysis encompassed three principle sections (Fig. 1). First, each data block (i.e. exposome [1.a], metabolome [1.b]) was analysed separately with respect to endometriosis status as the outcome. Then, the associations linking the blocks (interblock correlation) were explored irrespective of endometriosis status. Lastly, the data blocks were integrated all together to analyse their combined associations against the outcome.

2.3.1. Single-block models

2.3.1.1. POPs-endometriosis models (Path 1a)

2.3.1.1.1. Data exploration and pre-processing. Pre-processing and exploration of POPs data was conducted with a similar approach described for metabolome, integrating specific models for the assessment of joint effects of mixtures. Bivariate correlation analysis was measured using the Spearman's rank correlation. Principal Components Analysis (PCA) and Latent Variable Clustering (LVC) analysis were carried out to visualise the structure of the data and identify potential outliers (R packages *FactoMineR* and *ClustVarLV*, respectively) (Vigneau et al. 2015). Distribution analysis included inspection of distribution histograms and conducting Shapiro–Wilk tests. Exposure data was log transformed, scaled to the standard-deviation and mean centred. Selection of confounding variables, age and BMI, was conducted *a priori* based on the evidence supporting their association with POP internal levels and endometriosis risk (Cano-Sancho et al. 2019). Breastfeeding and parity were not considered confounding variables because their causal role in the pathway between POPs and endometriosis.

2.3.1.1.2. Variable selection models. Considering the high correlation of POP data and "a priori" unknown link function with endometriosis risk, we first conducted a variable selection step with three complementary methods (Lazarevic et al. 2020; Lenters et al. 2018). ENET was performed with the '*glmnet*' package, with 100x repeated 5-fold cross validation. Covariates age and BMI were set as fixed variables by setting *penalty.factor* to 0. Gradient Boosting Machine (GBM) (R package '*dismo*') was conducted using with 100 bootstrap replications for robustness considering the parameters: number of iterations = 10000, number of trees = 5, tree complexity = 2, step size = 1, learning rate = 0.01 and bag fraction = 0.8. Bayesian Kernel Machine Regression (BKMR) was conducted with R package '*bkmr*' using the probit (binomial) link function in compound-wise variable selection mode considering default *prior* parameters and 10,000 iterations to ensure convergence (Bobb et al. 2015). To guarantee robustness, we replicated 100 times BKMR models with different seeds and then, mean (SD) posterior inclusion probabilities (PIPs), between 0 (never included) and 1 (always included), were used as measure of variable importance for each variable across the replicates. Variables retained by at least 2 models were finally retained for subsequent analysis, including single pollutant models with multivariate logistic regression (MLR), refined BKMR models and integrative multiblock models.

2.3.1.1.3. Joint effects of chemical mixtures. For the subset of chemicals consistently identified through the variable selection process, BKMR was further used to examine the joint effects of the chemical ensemble, and any interactions between the chemicals, and their associations with endometriosis status. The exposure–response function (h) was modelled using a kernel machine regression that captures complex, non-linear and non-additive, exposure–response relationships, adjusting for covariates age and BMI (Bobb et al. 2015). Default *prior* parameters and 10,000 iterations were considered to ensure convergence.

2.3.1.1.4. Pooled analysis with EndoTox study. In order to increase statistical power, we combined the data from EndoxOmics- β study with data from our previous analysis of EndoTox conducted in the same

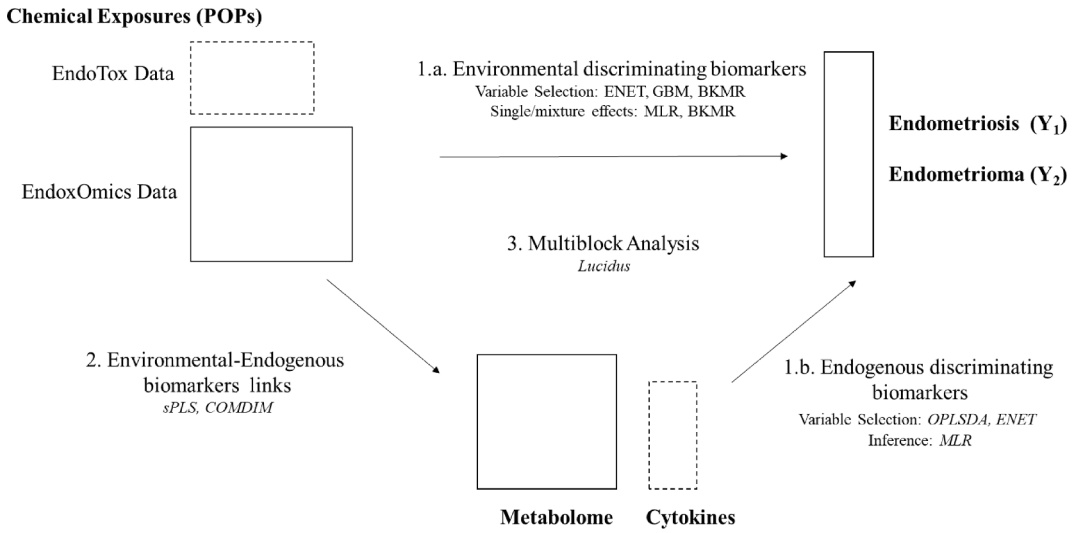

Fig. 1. Statistical workflow diagram. Path 1a and 1b (Single Block) focuses on the associations between each individual block and the outcome (Y). Path 2 (Interblock) focuses on the correlation between the exposome (E) and the metabolome (M). Lastly, Path 3 (Multiblock) integrates both E and M blocks in relation to Y. Boxes with doted borders depict datasets with incomplete observations (e.g. Cytokines) or biomarkers (e.g. EndoTox Data) for the pooled analysis. Methods used in each path are identified with abbreviations in Italics: BKMR, Bayesian Kernel Machine Regression; ENET, elastic-net regression; COMDIM, common dimension analysis; GBM, gradient boosting-machine; LUCIDus, latent unknown clustering with integrated data; MLR, multivariate logistic regression; OPLS, orthogonal partial least squares; sPLS, sparse partial least squares.

clinical setting and the same analytical platform previously reported and published elsewhere (Cano-Sancho et al. 2018; Ploteau et al. 2016; Ploteau et al. 2017). Briefly, the additional data comes from a case-control study (EndoTox) involving 99 women (55 cases, 44 controls), recruited from the same setting (Gynecology-Obstetrics Department of CHU-Nantes) during 2013–2015 resulting a final pool of 186 women. Endometriosis status was diagnosed via surgical confirmation by the same surgeon using the same clinical protocol. Internal exposure levels of target POPs were quantified in serum and adipose tissue with same methods described above (Ploteau et al. 2016; Ploteau et al. 2017). In order to account any variability between collection periods we included the study cohort as covariate in the models. Demographic distributions of this combined dataset are available in Table S1.

2.3.1.2. Metabolome-endometriosis models (Path 1.b)

2.3.1.2.1. Data pre-processing. Metabolites retained for statistical analysis were first selected on the basis of a stringent quality assessment based on internal validity (coefficient of variation $< 20\%$) and detection rate $> 75\%$. Selected metabolites as well as numerical covariates age and body mass index (BMI) were log transformed to approximate normal distributions, then centred and scaled. Normality was assessed for each data block using the Shapiro-Wilk test. Variables with non-logarithmic distributions were transformed using the package '*bestNormalize*' in R, which estimates the optimal normalisation technique (including Yeo-Johnson or Box Cox, among others). Batch-effect was corrected beforehand using *NOREVA* package (Li et al. 2017).

Variability of metabolome due to confounders like age and BMI was assessed using PCA. In order to account for related variability, two approaches were considered in all models involving the metabolome. The first approach considered the confounders in the models as covariates, and the second, a two-stage residual inclusion (2SRI) estimation beforehand, wherein models were run on the residuals after accounting for the variability explained by the covariates (Terza et al. 2008). A linear regression model was fitted to each chemical independently, adjusting for continuous variables age and BMI, and the residuals for each chemical were retained for subsequent analysis.

2.3.1.2.2. Variable selection and regression models. A supervised variable selection step was introduced to reduce data dimensionality of metabolites data, removing redundant variables and identifying the most informative metabolites using two methods ENET logistic regression and orthogonal partial least squares discriminating analysis (OPLS-DA). ENET was conducted as described above with R '*glmnet*' package, using 10-fold cross-validation for tuning the parameters in 'binomial' mode. OPLS-DA was performed using the R Bioconductor '*ropls*" package considering a cross-validation with 7 segments and 20 random permutations. Overlapping selected metabolites from ENET (with non-zero coefficients) and metabolites retained by OPLS/DA with variable importance in projection (VIP) above 1.5 were retained for multivariate logistic regression models (MLR) and integrative multiblock models. Herein, MLR was used to assess the associations between each individual chemical exposure and endometriosis, accounting for age and BMI.

2.3.2. Inter-block analysis to identify links between POPs and metabolome (Path 2)

Associations between exposure and metabolomic profiles were explored by maximizing their variance–covariance, using sparse partial least-squares regression (sPLS) within R '*mixOmics*' package. This approach allows modelling the high dimensional structures to identify the main contributors within a matrix (e.g. POPs) on the prediction of another (e.g. Metabolome) based on latent projection methods (Jain et al. 2018). The method was complemented with another multiblock method called Common Dimension (ComDim) which aims at exhibiting simultaneously common components among the various blocks and specific weights highlighting the importance of the dimension for each block (Cariou et al. 2018).

2.3.3. Multi block integrative analysis (Path 3)

Latent Unknown Clustering with Integrated Data (LUCID) approach (R '*LUCIDus*' Package) (Peng et al. 2019) was used to gain insight about the joint signature of biomarkers (POPs, metabolome and cytokine) underlying the discrimination of endometriosis subgroups. Briefly, the method integrates two main blocks of variables, (e.g. exposure variables and endogenous biomarkers) under a predefined causal structure, accounting for confounding variables to estimate a joint distribution through latent clustering. The parameters of the model and the posterior probability of being assigned to the latent clusters are estimated via the expectation–maximization algorithm, accommodating high-dimensional and collinear data via regularization. The number of

clusters K were identified using the Bayesian Information Criteria, without penalty parameters since the subset of variables underwent a comprehensive variable selection process.

2.3.4. Software

All statistical analyses were performed using R version 4.0.3. Pathway enrichment analysis was further performed with MetaboAnalyst v 5.0 in order to identify metabolic pathways which may potentially be associated with endometriosis and/or POPs.

3. Results

3.1. Population characteristics

Participant demographic data are displayed in Table 1. Neither age nor BMI significantly differed across groups of endometriosis status (Kruskal-Wallis test, $p = 0.29$ and 0.47, respectively). Approximately half (44%) of the participants were never smokers; smoking status did not significantly differ across groups (Fisher's test, $p = 0.75$). Of the 43 participants who reported use of hormonal contraception (e.g. combined oral contraceptives, progesterone only pills, vaginal ring, hormonal intrauterine devices), most (n = 35) used for 8 or more years, which was significant across the groups (Fisher's test, $p < 0.05$). Data on non-hormonal contraception use (e.g. condoms, copper intrauterine devices) was collected but not considered to be a potential confounder. Fewer than half of the women had become pregnant (45%), given birth (35%), or breastfed for any length of time (17%). In turn the control group had lower levels of IL-6 and IL-8.

3.2. Associations between POPs exposure with endometriosis status (Path 1.a)

Concentrations of seven PFAs, eight DL PCBs, six NDL PCBs, and six OCPs were quantified in>75 % of participants and retained for statistical analysis. Distribution of POPs and cytokine levels in serum are summarized in Supplemental Table S2 showing that endometriosis groups had significantly larger levels of PCB 157, sum of TEQ PCBs and pesticide *trans*-nonachlor ($p < 0.001$), compared to the control group. Bivariate analysis using Spearman's rank correlation revealed generally high correlations within chemical families specially for PCBs (Fig. 2). Correlations between the concentrations of the chemicals were higher amongst controls than cases, with little difference between cases OMA and noOMA (Fig. S1). Latent clustering analysis revealed 2 main clusters within the exposure data, mostly corresponding with major exposure families with structural similarity, PFAS and organochlorinated chemicals (Fig. S3.A and S3.B). Only 58.7% of the variability could be explained by the first two components (Dim1 46%, Dim2 12.7%) from PCA (Fig. S3.C) with high overlapping of individual observations projected onto the first 2 components (Fig. S3.D).

3.2.1. Variable selection and single pollutant models

Ranking of variables computed from the three variable selection models are presented in Fig. 3 in descending order of specific model metrics including variable importance in case of ENET (Fig. 3A), contribution to the model fit for GBM (Fig. 3B) and PIPs in case of BKMR (Fig. 3B). For practical interpretation, ranking of variables from BKMR based on PIPs provide an ordering of exposure based on variable importance similar to GBM contribution or ENET, yet no shrinkage of coefficients to zero is conducted and a threshold must be defined

Table 1
Participant demographic characteristics of 87 participants enrolled within EndoxOmics-β study. Distributions between groups were compared statistically using Kruskal-Wallis test for continuous variables, and Fisher's exact test for categorical variables. Abbreviations: IL, interleukin; IQR, interquartile range; MCP, Monocyte Chemoattractant Protein; noOMA, deep endometriosis without endometrioma; OMA, deep endometriosis with endometrioma; TNF, tumor necrosis factor.

	Control* (N = 12)	NoOMA (N = 26)	OMA (N = 49)	p-value
Age (years)				0.288
Mean (SD)	32.5 (2.92)	34.6 (6.15)	34.2 (4.79)	
Median [Min, Max]	32.4 [27.6, 35.9]	35.0 [24.0, 48.0]	34.0 [26.0, 47.0]	
BMI (kg/m^2)				0.466
Mean (SD)	22.7 (2.53)	22.9 (3.94)	24.1 (3.75)	
Median [Min, Max]	22.1 [18.3, 27.0]	21.9 [16.9, 31.2]	23.4 [16.7, 32.1]	
Smoking Status				0.754
Never	7 (58.3%)	12 (46.2%)	19 (38.8%)	
Ever	4 (33.3%)	10 (38.5%)	19 (38.8%)	
NA	1 (8.3%)	4 (15.4%)	11 (22.4%)	
Hormonal Contraceptive Use				0.044
0	0 (0%)	3 (11.5%)	4 (8.2%)	
0–7 yr	2 (16.7%)	4 (15.4%)	2 (4.1%)	
8–10 yr	5 (41.7%)	6 (23.1%)	13 (26.5%)	
> 10 yr	4 (33.3%)	2 (7.7%)	5 (10.2%)	
NA	1 (8.3%)	11 (42.3%)	25 (51.0%)	
Parity				0.715
0	7 (58.3%)	18 (69.2%)	26 (53.1%)	
1	2 (16.7%)	4 (15.4%)	10 (20.4%)	
2+	2 (16.7%)	4 (15.4%)	8 (16.3%)	
NA	1 (8.3%)	0 (0%)	5 (10.2%)	
Breastfeeding				0.842
0	9 (75.0%)	18 (69.2%)	31 (63.3%)	
< 3 mo	2 (16.7%)	3 (11.5%)	4 (8.2%)	
> 3 mo	0 (0%)	1 (3.8%)	5 (10.2%)	
NA	1 (8.3%)	4 (15.4%)	9 (18.4%)	
Serum lipids (g/dL)				
Median (IQR)	0.52 (0.46–0.62)	0.50 (0.42–0.57)	0.51 (0.48–0.57)	0.56
Cytokine profiles (pg/mL)	Missing (N = 0)	Missing (N = 2)	Missing (N = 22)	
IL-6	0.00 (0.00–0.00)	0.90 (0.00–6.10)	1.79 (0.00–7.96)	0.010
IL-8	4.10 (2.26–6.91)	16.59 (6.39–278.25)	9.04 (5.45–75.74)	0.017
MCP-1	258.26 (233.46–351.83)	350.29 (272.67–561.88)	343.91 (288.75–675.37)	0.091
TNF-alpha	2.47 (0.00–5.88)	4.13 (2.25–6.67)	3.84 (0.02–7.73)	0.47

* Control group were women attending surgery for tubal ligation or fertile women undergoing assisted reproductive technology for male infertility or oocyte donation. Fibroids or polycystic ovarian syndrome were not eligible.

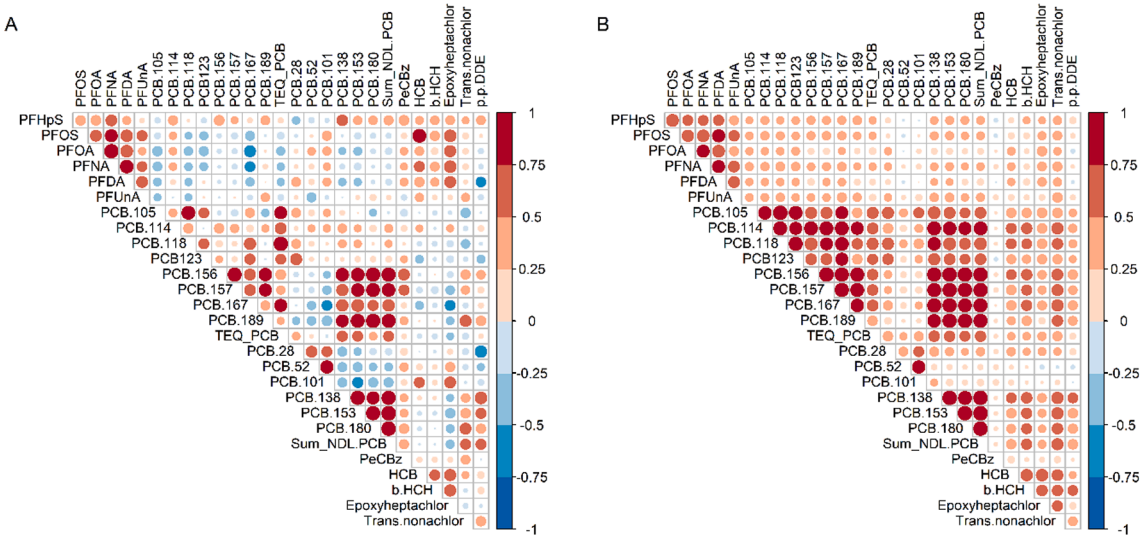

Fig. 2. Spearman's rank correlation heatmaps among serum concentrations of 29 biomarkers of exposure to POPs among controls (A) and cases (B).

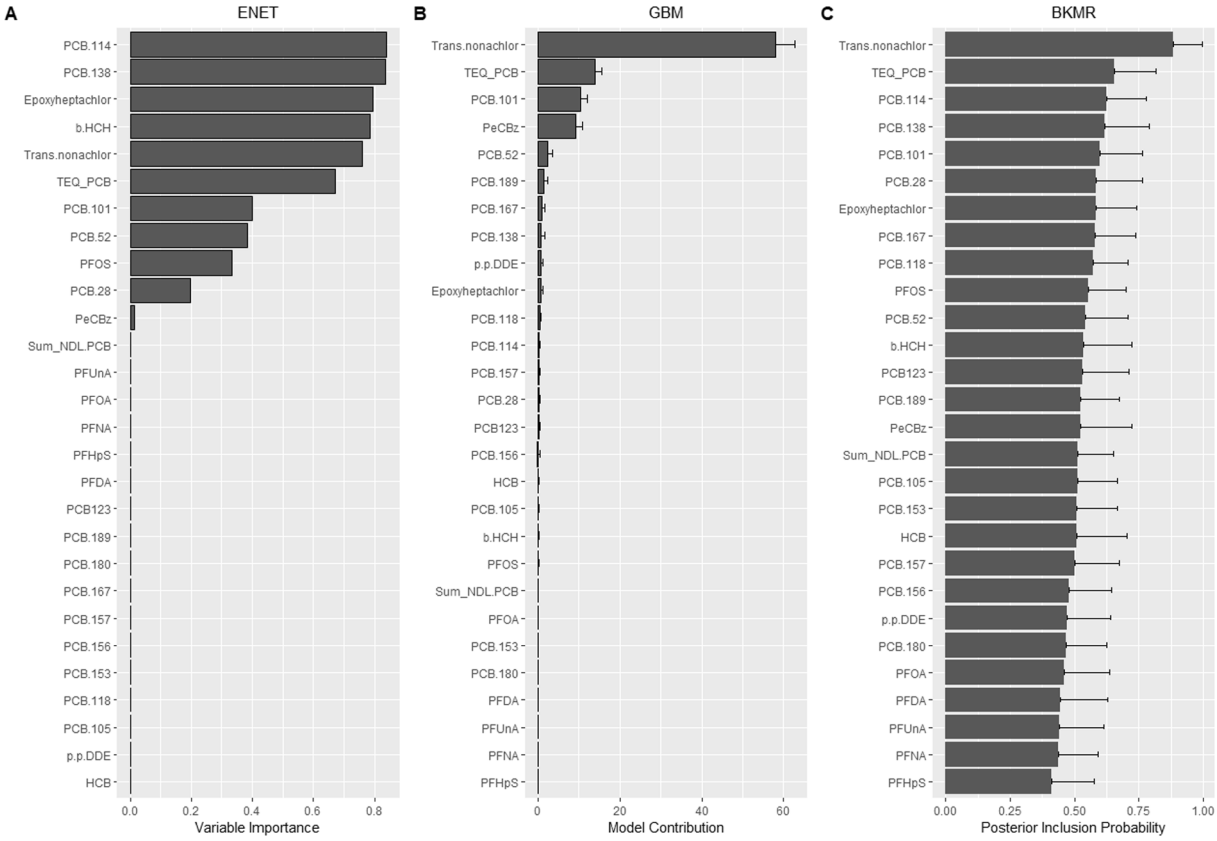

Fig. 3. Variable importance rankings measuring the association between individual exposure marker levels measured in serum and endometriosis status using (A) Elastic-net (ENET) - Data is presented as weighted non-zero coefficients of selected variables following 100x repeated 5-fold cross validation, (B) Gradient Boosting Machine (GBM) - Data are presented as the distribution (mean ± SD) of the scaled percentage contribution to the model following 100-fold replications, and (C) Bayesian Kernel Machine Regression (BKMR) - Data are presented as the distribution (mean ± SD) of the Posterior Inclusion Probabilities (PIPs) following of 10,000 iterations and replicated 100 times. All models considered age and BMI as covariates.

(Lazarevic et al. 2020). Top ranked chemicals by GBM and BKMR were consistent *trans*-nonachlor and Toxic Equivalents (TEQ) for dioxin-like PCB (TEQ-PCBs), with slight differences with ENET. Nonetheless, the retained biomarkers were quite consistent across models irrespectively of minor differences of ordering, with major presence of non-dioxin-like PCBs and pesticides (Table S3). Conversely, small associations were found between most PFAS and endometriosis, with the exception of PFOS that ranked high with ENET and BKMR. Results from GBM, expressed as variable importance (Fig. 3B), showed that *trans*-nonachlor (mean 43.6 ± 3.9) singly outranked all other chemicals, with a mean over fourfold the next most contributory variables. The profile of PIPs from BKMR showed larger probabilities for *trans*-nonachlor with

smoother differences between the rest of chemicals, challenging the variable selection procedure. We observed some instability of PIPs for most chemicals with the exception of *trans*-nonachlor, motivating us the replication of models 100 times to gain robustness. In order to support the refinement of subsequent models we considered a threshold of PIP of 0.55 allowing a balance between model complexity and variance. The three models replicated on the residuals of the 2SRI analysis did not significantly alter the results of the variable selection (Results not shown).

Retained POPs in the variable selection step were modelled with MLR in order compute inferential statistics with minor impact of false discovery rates. Results from MLR are summarized in Fig. 4A for the estimates from EndoxOmics (n = 87) and Fig. 4B from the pooled analysis with EndoTox (n = 186). Overall the results from both analyses were quite consistent, being *trans*-nonachlor the most relevant exposure biomarker positively associated with overall endometriosis risk followed by TEQ PCB in case of EndoxOmics and PCB 114 in the pooled analysis. Globally, the pooled analysis provided robustness and precision of estimates with narrower confidence intervals and larger statistical significance. The statistically significant biomarkers were *trans*-nonachlor, OR (95% CI) 3.38 (2.06–5.98), $p < 0.0001$; PCB 114 OR (95% CI) 1.83 (1.17–2.93), $p = 0.009$; PCB 118 OR (95% CI) 1.6 (1.08–2.42), $p = 0.02$ and PCB 167 OR (95% CI) 1.64 (1.02–2.66), $p = 0.04$. In turn PCB 28 was statistically negatively associated with endometriosis risk OR (95% CI) 0.69 (0.48–0.98), $p = 0.04$.

3.2.2. *Joint effects of chemical mixtures*

The refined model mixture model was built with the top 11 chemicals with larger PIPs in the BKMR variable selection step (Fig. 3C) and the results are summarized in Fig. 5. The trends of the exposure–response functions h(z) for each of the 11 included chemicals are shown in Fig. S4A, which can be interpreted as the relationship between chemicals and a latent continuous marker of endometriosis status when all other chemicals are held at their median levels. Trans-nonachlor exhibited a non-monotonic inverted-U trend in its associations with endometriosis. PCB 114 and TEQ-PCBs showed the steeper positive associations whereas PCB 28, 101 and 138 the negative ones.

Fig. S4B depicts the overall risk summary between the combined exposures and endometriosis status as a continuous link function. The overall risk summary followed a flattened S-curve, suggesting a slightly non-monotonic increase in overall risk with increasing percentiles of exposure to the mixture of this chemical subset, but not significantly (credible intervals overlapping the null). Individual variable contributions to the overall risk are depicted in Fig. S4C when the rest of the mixture was fixed at the percentiles 25, 50 and 75. Though no individual variable's risk contributions were statistically significant, there were some observable trends. Most notably, *trans*-nonachlor had an increasing and overall positive contribution to the risk associated with the endometriosis outcome with increasing quartiles that would suggest a potential interaction. Conversely, PCB 28 showed a decreasing and overall negative risk contribution with increasing quartiles of the mixture. We further explored the exposure–response function of each chemical while holding a second predictor at fixed quantiles to explore potential two-way interactions of exposures (Fig. S5). Analyses indicated the presence of a non-linear relationship between exposure to pesticides *trans*-nonachlor and endometriosis risk. Two-way synergic interactions were suggested between *trans*-nonachlor and PCB 101, 138 and 167, which exhibited more striking effects in the higher quantiles of *trans*-nonachlor.

The results from the pooled analysis with EndoTox data are depicted in Fig. 5. Slight differences were noticed in the variable selection step with pooled data, for instance the pesticide p,p'-DDE was retained instead of Epoxy-heptachlor, whereas TEQ-PCBs did not exhibit larger PIPs supporting the inclusion in the refined model. The individual trends of PCB 114, 167 and Trans-nonachlor were similarly positive, whereas PCB 28 was also negatively related with endometriosis risk (Fig. 5.A). Interestingly, the cumulative effect was steeper in the pooled analysis showing credible intervals almost above the null in the higher quantiles (Fig. 5.B). Individual variable contributions to the overall risk did not reveal interactive trends (Fig. 5.C), however did show that *trans*-nonachlor has a contribution to the overall risk since the estimates fall above the null in any of fixed quantiles.

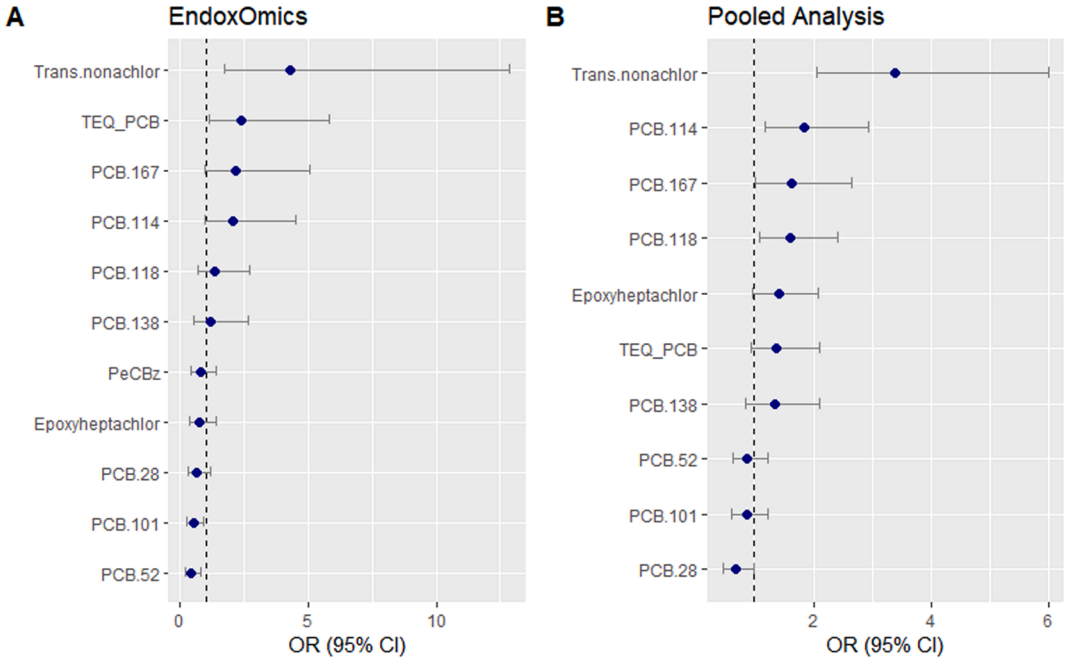

Fig. 4. Forest plot depicting the association (Odds Ratios [OR] and 95% confidence intervals) between individual exposure marker levels measured in serum and overall endometriosis risk in the EndoxOmics study (A) and pooled analysis with Endotox study (B) measured by multivariate logistic regression, adjusted by age and body mass index and study (EndoxOmics/EndoTox). The subset of POPs was identified by at least 2 models in the variable selection step (Table S3).

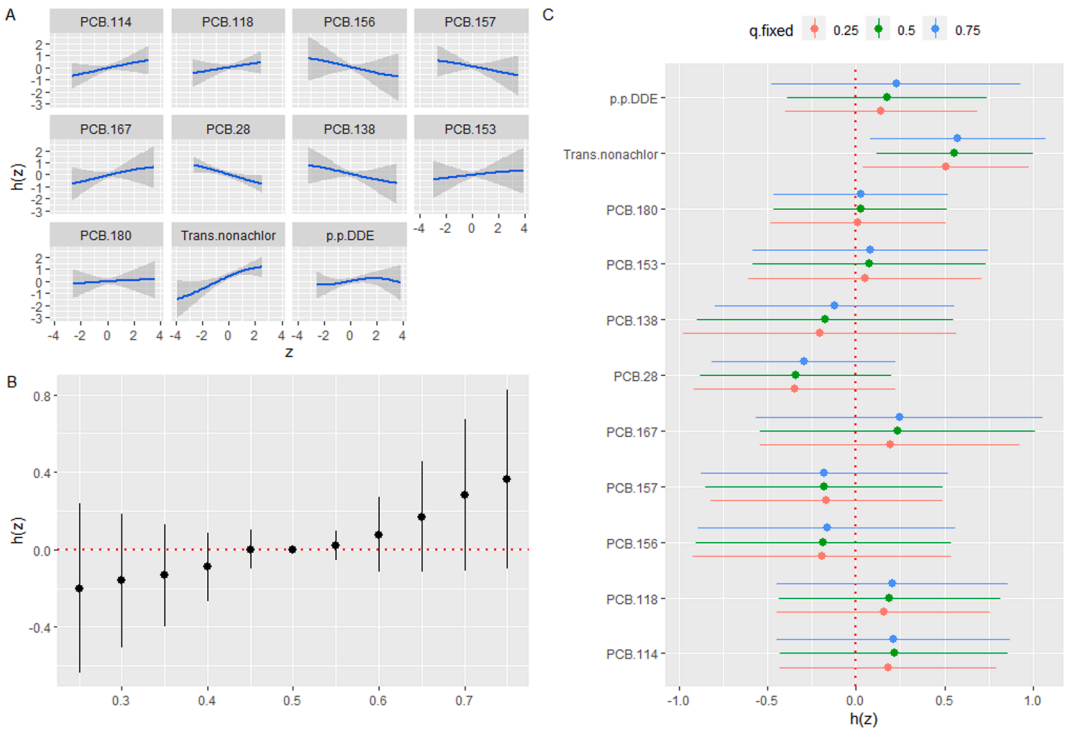

Fig. 5. Pollutant mixture analysis of endometriosis risk with the pooled EndoxOmics and EndoTox studies using BKMR depicted through (A) univariate exposure–response for each chemical (blue line) with 95% CI (grey silhouette) between the selection of variables and endometriosis. h(Z) can be interpreted as the relationship between chemicals and a latent continuous marker of endometriosis status, (B) joint effect overall risk summary of exposures on endometriosis by BKMR model when all chemicals at certain percentiles are compared to all other chemicals at the 50th percentile, and (C) single variable risk summary of each chemical by quartiles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Associations between POPs exposure with endometriosis status (Path 1.a)

3.3.1. Exploratory analysis

After stringent quality assessment, 352 lipids quantified across ten families were retained: acylcarnitines (n = 33), ceramides (n = 13), cholesterol esters (n = 16), diacylglycerols (n = 12), dihydroceramides (n = 1), glycerophospholipids (n = 87), glycosylceramides (n = 10), sphingolipids (n = 14), sugars (n = 1), and triacylglycerols (n = 165). Lipids were found to be highly correlated, especially within families (Fig. 6). 37 functional metabolite ratios were also quantified. A complete list of metabolites quantified can be found in Table S4.

3.3.2. Variable selection and regression.

Variable selection of metabolomic data conducted with ENET and OPLS-DA showed an overlapping of 26 top metabolites retained for MLR and multiblock analysis (Fig. 7A and B). Despite the order of metabolites was modified between methods, we found consistent identification of most relevant features. In any case, the discrimination capacity of OPLS-DA model remained low, suggesting little dysregulation of metabolome due to endometriosis status (Fig. 7C). Selected metabolites were further analyzed with MLR adjusting for age and BMI (Fig. 7D), showing that 8 metabolites out of 26 were statistically significant ($p < 0.05$) and other 8 metabolites with marginal significance ($p < 0.1$). These included the functional ratio of 12-alpha-hydroxylated bile acids to non-12-alpha-hydroxylated bile acids (ratio.12aOH.BAs.to.Non12aOH.Bas), ratio of diglycerides to triglycerides (Ratio.DGs.to.TGs) or ratio sphingomyelins to phosphatidylcholines (Ratio.SMs.to.PCs), with respective OR (95 %, p-value) of 3.12 (1.46–7.93, p = 0.007), 3.10 (1.42–7.99; p = 0.009) and 2.54 (1.29–5.68, p = 0.012) (see Fig. 7D).

3.4. Associations between POPs exposure and metabolome profiles (Path 2a)

The associations between POPs and metabolic profiles were globally small and consistent across two approaches (ComDim and sPLS). Despite differences in number of components (3 in ComDim and 2 in sPLS), we found substantial overlapping of most relevant features retained from each data block. For instance, in both cases, a similar metabolic congener profile was related with one component associated with PCBs (related to triglycerides) and another with PFAS, highlighted by the presence of tetradecenoylcarnitine (C14:1) and hexosylceramide (HexCer) d18:2/24:0, among others (Fig. S7 for ComDim results and Fig. S8 for sPLS results). Specific findings were also highlighted by each approach, for instance ComDim revealed the links between PFOA, PeCBz and HCB in component 2 with a list of functional ratios, including the ratio of diacyl-hosphatidylcholines and cyl-alkyl-phosphatidylcholines to choline (Fig. S6D). In turn sPLS approach showed the central position of the ratio of hydroxylated sphingomyelins to non-hydroxylated sphingomyelins (Ratio.SMOHs.to.SMNonOHs) and HexCer 18:1/24:0 in the associations with PCBs and PFAs.

3.5. Associations between POPs exposures and cytokine profiles (Path 2b)

Summary results from analysis of associations between POP exposures and inflammatory status can be found in Supplemental Fig. S8. In a first step we attempted to discriminate the inflammation profiles (High vs Low) dichotomizing the levels of IL-8 based on the POP signature using PLSDA, showing moderate/low discrimination being *p,p*'DDE, PFHpS, TEQ-PCBs and b-HCH the congeners that scored with higher VIPs (Fig. S8.A and S8.B). The chemicals with higher VIPs (VIP > 1.25) were further modelled against IL-8 levels using multivariate linear regression adjusted by age, BMI and endometriosis status. The regression analysis confirmed the positive and statistically significant

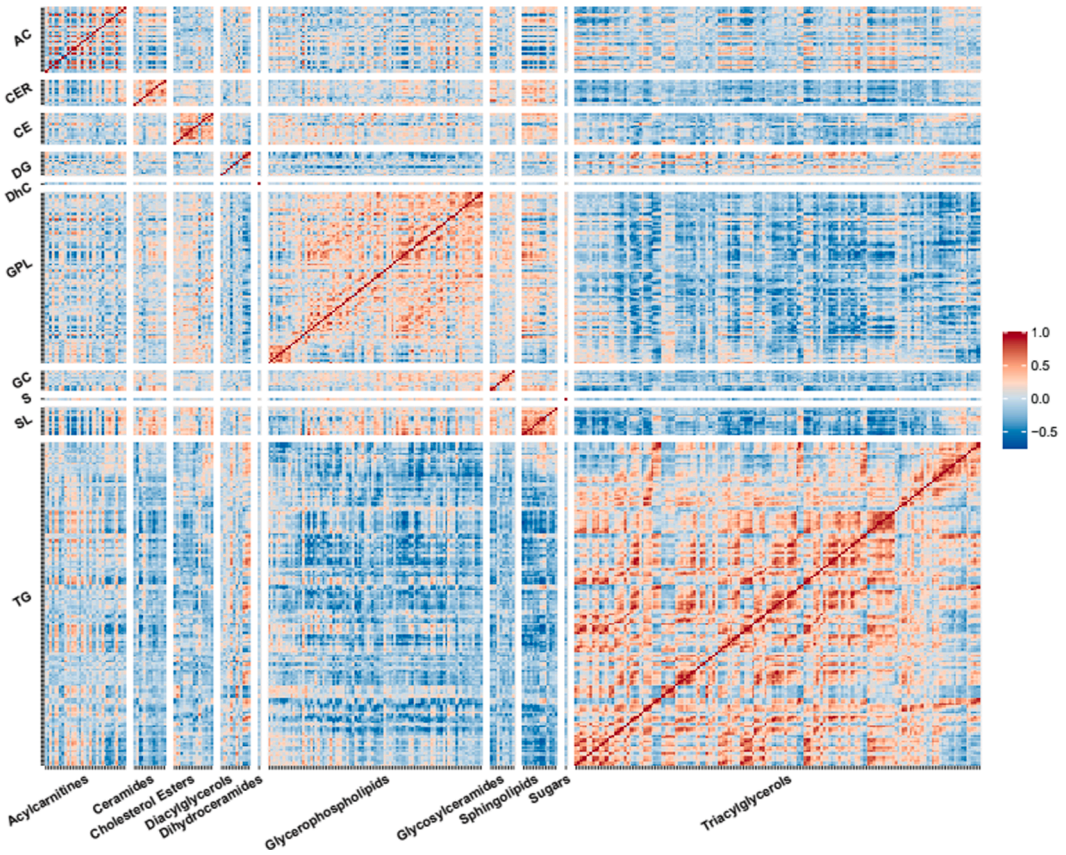

Fig. 6. Bivariate Spearman Correlations among serum concentrations of 354 lipid biomarkers, presented as $-1 < \rho < 1$. Complete list of lipids in Table S4. Abbreviations: Acylcarnitines (AC); Ceramides (CER); Cholesterol Esters (CE); Diacylglycerols (DG); Dihydroceramides (DhC); Glycerophospholipids (GPL); Glycosylceramides (GC); Sphingolipids (SL); Sugars (S); Triacylglycerols (TG).

associations between PFHpS and levels of IL-8, β (95 % CI) 0.26 (0.05–0.48), $p = 0.019$. The rest of POPs showed in general null results. In the sensitivity analysis, adjustment by endometriosis status did not exhibit a large impact on the estimates for PFHpS, regardless of considering two (cases vs controls) or three levels (OMA, noOMA vs controls). Nonetheless, for TEQ-PCBs and *p,p*'DDE the adjustment for endometriosis status notably reduced the magnitude coefficients (over 10%).

3.6. Integrated multiblock analysis (Path 3)

This analysis was conducted with the subset of 11 POPs and 26 metabolites selected in Path 1a and 1b respectively, identifying two main latent clusters (*K*) on the joint discrimination model (Fig. S9). Latent Cluster 2 (LC2) was associated with a higher risk (OR = 1.51) of endometriosis compared with the referent Cluster 1. POPs showed a major probability of being assigned to the high risk cluster LC2, being *trans*-nonachlor (OR = 1.88), PCB 167 (OR = 4.08) and PCBs 52 (OR = 2.08) the chemicals with larger positive ORs, whereas PCB 28 (OR = 0.83) and 138 (OR = 0.13) the ones with larger negative probabilities. High risk LC2 was also characterised by overall lower levels of most triglycerides (TG), lysophosphatidylcholine (lysoPC) a C20:4, ceramide (d18:1/18:0), ratio of phosphatidylcolines PCs to choline and the ratio of TG to fatty acids. In turn, LC2 had larger levels of (PCs), ratio SM to PCs, ratio DG to TG and ratio of 12-alpha-hydroxylated bile acids to non-12-alpha-hydroxylated bile acids (ratio.12aOH.BAs.to.Non12aOH.BAs).

In a secondary analysis, for a subset of samples (n = 67) with available quantitative cytokine profiles, we extended the integrative analysis and considered different endometriosis outcome groupings. This analysis was refined in order to include the most relevant variables identified in the variable step procedure, resulting in a subset of 6 POPs (Table S3), 8 metabolites and ratios and 2 cytokines. The results for overall endometriosis risk showed poor performance with a latent cluster (LC2) more associated with POPs but little association the outcome. More performant results were found for OMA, in terms of model convergence (Fig. 8). In this case, LC2 was also more positively associated with POPs including PCB 114, TEQ-PCBs and *trans*-nonachlor and negatively associated with PCB 101, PCB 138 and epoxyheptachlor. In turn, LC2 showed higher level of IL8 and MCP1 and the ratio of SMs to PCs, ratio DGs to TGs and ratio 12aOH.BAs to Non12aOH.BAs (Fig. 8). Whereas cytokines showed little contribution to clustering when the model was built for total cases vs controls; they contributed substantially in discriminating the OMA group from the combined group of controls with noOMA.

4. Discussion

To our knowledge, this is the first study investigating the associations between mixtures of POPs and endometriosis by integrating quantitative metabolomics and cytokine profiling in order to gain insight on the potential underlying biological mechanisms. We have also developed a comprehensive data analysis framework favouring the efficient integration of targeted biomarker data generated by multiple high-resolution MS platforms. Despite the modest sample size of the study, we identified several organochlorine pesticides (i.e. *trans*-nonachlor or PCB 114) to be positively associated with endometriosis, as well as some non-coplanar PCBs (28 and 52) which whose roles in pollutant mixtures seems to be important. These results were consistently strengthened by a pooled statistical analysis combining data from previous study conducted in the same region under the same clinical and analytical

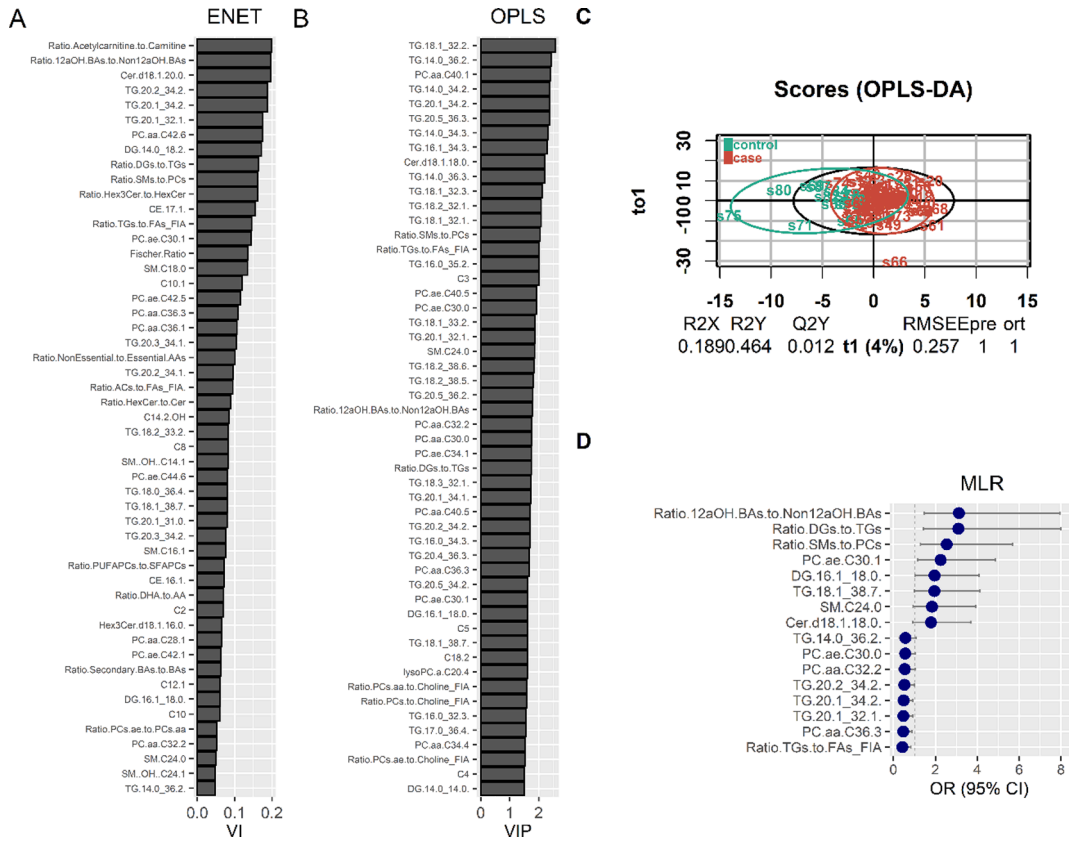

Fig. 7. Bar plot depicting the variable importance (VI) of most relevant metabolites from elastic net (ENET) logistic regression (Panel A) and variable importance on projection (VIP from orthogonal partial least square discriminating analysis (OPLS-DA, Panel B) on the discrimination of endometriosis status (Cases vs controls). Score plot from PLS-DA is shown in Panel C for cases (red) and controls (green). Forest plot in Panel D shows the odds ratios (OR) and 95% confidence intervals for the subset of overlapping metabolites selected by ENET ($\beta \neq 0$) and OPLS-DA (VIP > 1.5), estimated by Multivariate Logistic Regression (MLR), adjusted by age and body mass index. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

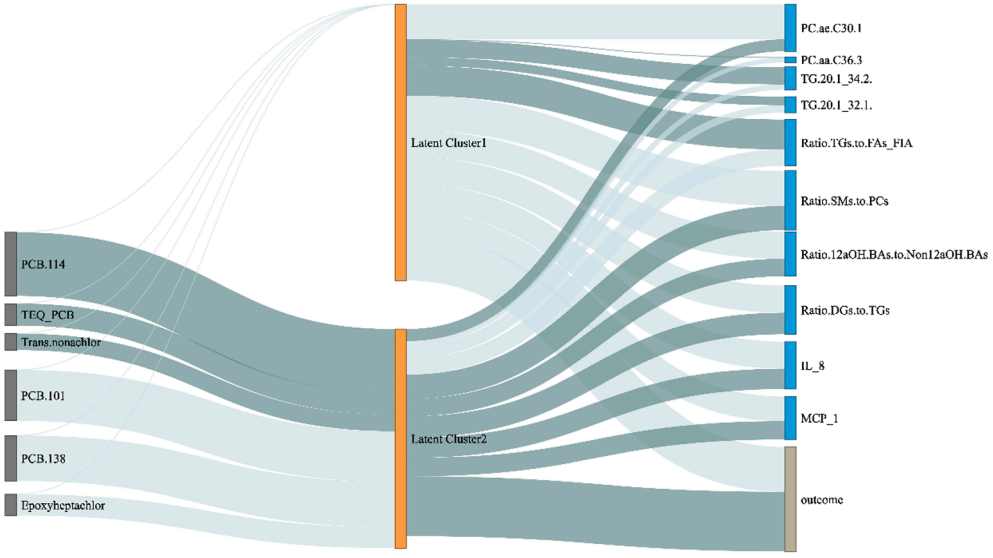

Fig. 8. Sankey diagram of the secondary structural integrated analysis of POPs exposure and individual lipid metabolites and cytokines in serum for the identification of latent variable clusters associated with severe cases of endometriosis (OMA). Dark grey bands represent positive associations and light grey bands indicate negative associations, with the width proportional to the size of the association. POPs exposures and cytokines are positively associated to Latent Cluster 2, which contributes positively towards outcome (OMA).

settings. Mixture analysis with BKMR showed that the overall mixture of selected POPs (PCBs and pesticides) had a positive trend on cumulative effects and highlighted the contribution of *trans*-nonachlor within the mixture regardless the concentrations of the others. Conversely, the mixture analysis suggested interactive synergy between *trans*-nonachlor and PCB 118 or PCB 167, strengthening the associations with endometriosis risk only at higher concentrations of the pesticide. We have also elucidated a differential metabolome between cases of endometriosis and controls, identifying a handful of lipids and functional metabolite ratios related to this discrimination including the ratio of 12-alpha-hydroxylated bile acids to non-12-alpha-hydroxylated bile acids (ratio 12aOH BAs to Non-12aOH BAs), the ratio of diglycerides to

triglycerides (Ratio DGs to TGs) and the ratio sphingomyelins to phosphatidylcholines (Ratio SMs to PCs). In turn, the integrative structural analysis revealed that patients with OMA, had a profile characterised by higher concentrations of POPs (*trans*-nonachlor, PCB 114 and TEQ-PCBs), inflammation markers (IL-8 and MCP-1) and a metabolic profile with dysregulated functions of bile acid balance and lipase activity.

The mixture profile of POPs combined historical chemicals like PCBs and OCPs with more emerging classes like PFAS, has never been explored before in relation with endometriosis risk. Serum concentrations of the seven PFAS compounds and six NDL PCBs were comparable to those found in similar populations of French women (Koual et al. 2019; Ploteau et al. 2017), as well as those reported in a recent study on a 2014–2016 French population for PFAS (Fillol et al. 2021). The POPs found to be most associated with endometriosis are consistent with our previous findings (Ploteau et al. 2017; Cano-Sancho et al. 2019). In a previous *meta*-analysis, organochlorine pesticides as a family were associated with endometriosis risk with an OR (95% CI) of 1.23 (1.13–1.36), whereas PCBs had an even stronger association with an OR (95% CI) of 1.70 (1.20–2.39) (Cano-Sancho et al. 2019). PCBs are often studied either individually, grouped based on their aryl hydrocarbon receptor affinity as DL or NDL, or as a sum of TEQ (Pauwels et al. 2001). For instance, there has been experimental evidence that DL but not NDL PCBs are linked to the endometriosis through pro-inflammatory interactions (Huang et al. 2017). Other studies have considered the estrogenic potential of PCBs as rationale for biologically-informed grouping, finding higher risk of endometriosis among women with the highest concentrations of anti-estrogenic PCB summed (Buck Louis et al. 2005). Only one recent study similarly used BKMR to analyse the adipose to serum ratio (ASR) of PCB and OCP mixtures, finding positive associations between the ASR for OCPs and estrogenic PCBs and endometriosis (Pollack et al. 2021).

Our results further support the interest of analysing POP mixtures on endometriosis risk. For instance, BKMR allowed us to characterize the cumulative patterns of mixtures of PCBs and pesticides, accounting for interactive effects. On this regard, synergy between *trans*-nonachlor and PCB 118 or PCB 167 were suggested (Fig. S5). The results from BKMR also showed that considering the co-exposure may result in different exposure–response associations to those from single pollutant models. For instance, the null associations of PCB 138 (Fig. 4) tended to be negative in BKMR, especially when the rest of mixture was set to the lowest 25th percentile (Fig. S4), yet did not reach significance. The flexibility of BKMR elucidated non-monotonic inverted U-shaped effects of *trans*-nanochlor within the EndoxOmics study. The negative associations for some PCBs (i.e. PCB 28 or PCB 52) remain still unclear, though we have noticed similar patterns in our previous EndoTox study among congeners with low chlorination. It is possible that the inconsistent results from previous studies on POPs can be explained not only by the heterogeneous nature of this group of chemicals, but also the potential presence of interactions between PCBs and other concomitant exposures may be possible. On this regard, our study also included PFAS, a family of POPs with specific physiochemical and toxicological characteristics (e.g. amphiphilic) that remains barely explored in relation with endometriosis risk. Our results did not show relevant associations of PFAS alone nor in mixtures with historical POPs; however, one previous study found positive associations between serum levels of perfluorooctanoic acid (PFOA; OR = 1.89 [95% CI = 1.17–3.06]), perfluorononanoic acid (2.20 [1.02–4.75]) and endometriosis risk (Louis et al. 2012). Future studies should consider this group of chemicals in endometriosis research due to the relevance in terms of population exposure (e.g. present in all samples from this study at relevant concentrations) and toxicological effects. We should also highlight the fact that our study was conducted with serum biomarkers of POPs which may be an analytical challenge as certain underlying biological process may alter the circulation of serum lipids (Cano-Sancho et al. 2018). In our case, we did not notice differences of circulating levels of lipids between groups, minimising any potential bias due to normalisation of biomarkers (Cano-Sancho et al. 2020). We recommend future studies to consider the analysis of POPs and endogenous metabolic markers within adipose tissue, as the stores of lipophilic contaminants in adipose tissue may more stable and accurately depict the long-term body burdens of POPs exposures (Mustieles and Arrebola 2020).

Another highlight is the targeted metabolic profiling that allowed the identification of a handful of lipids and functional ratios to be differentially regulated between endometriosis cases and controls. Specifically, women with endometriosis presented larger ratios of 12-α OH to non-12-α OH bile acids. This was especially true for the endometrioma subgroup. Bile acids are molecules with both hydrophobic and hydrophilic components which act as signalling molecules involved in lipid metabolism but also enterohepatic absorption-excretion cycle of POPs (Jandacek and Tso 2007). The 12-α-hydroxylation of BAs is mainly regulated by a liver-specific enzyme (Vlahcevic et al. 2000), and the imbalance of ratio of 12-α OH to non-12-α OH BAs has been suggested as a mechanism linking dyslipidaemia with insulin resistance, throughout the Akt-FoxO1 pathway and modulation of levels of triacylglycerols (Haeusler et al. 2013). In female mice, short term exposure to TCDD tended to decrease the levels of BA in serum and liver, including the levels 12-OH BAs throughout the suppression of 12α-hydroxylase Cyp8b1 mediated by the AhR activation (Csanaky et al. 2018). We have also found the ratio of glycerolipids diacylglycerols (DGs) to triacylglycerols (TGs), as well as of TGs to fatty acids (FAs) to be significantly different between endometriosis cases and controls. For both ratios, we found that women with endometriosis had increased lipase activity. This altered lipid profile has been found to be linked to both endometriosis and implantation status for women seeking *in vitro* fertilisation treatments (Domínguez et al. 2017; Matorras et al. 2020). We have also found larger ratios of sphingomyelins (SMs) and phosphatidylcholines (PCs) and absolute levels of some PCs (e.g PC ae C30:1) associated with endometriosis risk. Sphingomyelins (SMs) and phosphatidylcholines (PCs) have been identified as potential biomarkers for ovarian endometriosis in previous studies (Vouk et al. 2012). It is hypothesised that elevated levels of SMs and PCs may contribute to the suppression of apoptosis and alter lipid-related signalling pathways. In turn, the ratio of SMs to PCs is an indicator of SM synthesis from ceramides and was found here to be a potential predictor of endometriosis.

In addition, inflammatory cytokines were found to be more associated with OMA compared with combined group of controls and noOMA than in overall endometriosis risk (compared to controls). The integrative analysis elucidated a shared signature between POPs (dioxin-like PCBs TEQs, PCB 114 and *trans*-nonachlor) and endogenous markers of dysregulation of bile acid metabolism, lipolytic activity and inflammation were upregulated in severe endometriosis cases with endometrioma. These findings support the main underlying mechanisms elucidated in animal and *in vitro* studies, mainly reported for the prototypical toxicant TCDD (Matta et al. 2019). The contribution of each individual chemical from a complex mixture of endocrine disrupting chemicals becomes elusive in current observational research methods; however, it is likely that complex interactions may occur, especially in light of reported synergistic interactions between endogenous oestrogen (and oestrogenic chemicals) on endometriosis progression. We have also elucidated a lipotoxic profile pattern among patients with OMA and higher levels of POPs that could also suggest an interplay between POPs and lipid metabolism or adipose tissue dysfunction.

These results, however, should be taken with caution due to a number of methodological limitations, including modest statistical power due to the small sample size of this pilot study and the cross-sectional design. We attempted to address the first issue by pooling the present exposure data from this study with exposure data from our previous EndoTox study conducted in the same clinical setting and the same analytical platform (Cano-Sancho et al. 2018; Ploteau et al. 2016; Ploteau et al. 2017). Concerning the cross-sectional design, we acknowledge that a risk of reverse causation and disease progression

bias may exist. However, the stable and persistent nature of POPs supports the use of single samples (e.g. at diagnosis) as surrogate of exposures during long periods previous the collection. In any case, considering the body of evidence on endometriosis and POPs is built on cross-sectional designs (Cano-Sancho et al. 2019), future studies on endometriosis should consider prospective designs and nested case-studies to minimise the risk of bias. We should also acknowledge the statistical limitations inherent to multipollutant modelling with highly correlated data structures. This issue impairs the model performance and may easily lead to wrongly attribute the association to those exposures in the mixture with lower measurement error, despite using advanced methods as BKMR (Lazarevic et al. 2020). Nonetheless, the consistent findings across the different variable selection methods used in this study strengthened the robustness of findings yet experimental research will be required for confirmation. Conversely, the lack of statistical association for specific chemicals and/or mixture should be taken with caution in the light of these mentioned limits. Another important note is that the population controls used in this study may not be completely representative of the general population at risk for endometriosis (Upson 2020). Clinical recruitment may present different exposure and metabolic profiles than the general population, preventing the generalisation of results. Additionally, though controls comprised mostly women who presented no endometriosis symptoms, for most controls, there was no surgical or histological confirmation of absence of endometriosis due to ethical reasons. Considering that we focused only on severe stages of deep endometriosis with and without endometriosis, we believe that asymptomatic and fertile women, minimize the control selection bias. Lastly, as with all studies based on epidemiological data, finding may be subject to confounding bias. For instance, we were unable to adjust by contraceptive use due to the large proportion of missing data (37%). Nonetheless, experimental evidence suggest that interactions between estrogen and dioxin-like compounds may boost the progression of endometriotic lesions (Wang et al. 2010; Yu et al. 2008).

5. Conclusion

In summary, the present study is the first of its kind to integrate metabolomics and cytokine profiling in observational research of endometriosis to gain insight on the underlying modes of actions of POPs. This study proved the high exploratory potential of integrating multiple MS platforms simultaneously covering a comprehensive panel of endogenous and exposure biomarkers with major applications in etiological research. Using high-throughput targeted metabolomics provided a good compromise, taking advantage of the accuracy and selectivity of targeted approaches with the high analytical capacity provided by non-targeted approaches. The results, strengthened by a pooled analysis, showed positive associations between some POPs (i.e. *trans*-nonachlor OR (95% CI) 3.38 (2.06–5.98), $p < 0.0001$ and PCB 114 OR (95% CI) 1.83 (1.17–2.93), $p = 0.009$) and endometriosis risk. The integrative multiblock analysis revealed a latent cluster of women with higher risk of OMA, presenting higher concentrations of *trans*-nonachlor, PCB 114 and toxic equivalents from dioxin-like PCBs, as well as an increased inflammatory profile and a metabolic pattern characterised by dysregulation of bile acid homeostasis and lipase activity. Results suggest the role of certain POPs in promoting pro-inflammatory metabolic conditions which may be involved in the development of severe endometriosis. Our results support that further studies are required to confirm these findings, conducted on a larger and population scale designed prospectively.

CRediT authorship contribution statement

Komodo Matta: Formal analysis. **Tiphaine Lefebvre:** . **Evelyne Vigneau:** Methodology, Software, Validation. **Véronique Cariou:** Methodology, Software, Validation. **Philippe Marchand:** . **Yann Guitton:** . **Anne-Lise Royer:** . **Stéphane Ploteau:** Resources. **Bruno Le Bizec:** Resources. **Jean-Philippe Antignac:** Resources, Conceptualization. **German Cano-Sancho:** Resources, Conceptualization, Methodology, Validation.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

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References

Akins, J.R., Waldrep, K., Bernert, J.T., 1989. The estimation of total serum lipids by a completely enzymatic 'summation' method. Clin. Chim. Acta 184 (3), 219–226.
Becker, C.M., Laufer, M.R., Stratton, P., Hummelshoj, L., Missmer, S.A., Zondervan, K.T., et al., 2014. World endometriosis research foundation endometriosis phenome and biobanking harmonisation project: I. Surgical phenotype data collection in endometriosis research. Fertil. Steril. 102, 1213–1222.
Bobb JF, Valeri L, Claus Henn B, Christiani DC, Wright RO, Mazumdar M, et al. 2015. Bayesian kernel machine regression for estimating the health effects of multi-pollutant mixtures. Biostatistics 16:493-508.
Buck Louis G, Weiner J, Whitcomb B, Sperrazza R, Schisterman E, Lobdell D, et al. 2005. Environmental pcb exposure and risk of endometriosis. Hum. Reprod. 20:279-285.
Cano-Sancho G, Labrune L, Ploteau S, Marchand P, Le Bizec B, Antignac JP. 2018. The challenging use and interpretation of circulating biomarkers of exposure to persistent organic pollutants in environmental health: Comparison of lipid adjustment approaches in a case study related to endometriosis. Chemosphere 200: 388-396.
Cano-Sancho, G., Ploteau, S., Matta, K., Adoamnei, E., Louis, G.B., Mendiola, J., Darai, E., Squifflet, J., Le Bizec, B., Antignac, J.-P., 2019. Human epidemiological evidence about the associations between exposure to organochlorine chemicals and endometriosis: Systematic review and meta-analysis. Environ. Int. 123, 209–223.
Cano-Sancho, G., Marchand, P., Le Bizec, B., Antignac, J.-P., 2020. The challenging use and interpretation of blood biomarkers of exposure related to lipophilic endocrine disrupting chemicals in environmental health studies. Mol. Cell. Endocrinol. 499, 110606. https://doi.org/10.1016/j.mce:2019.110606.
Cariou, V., Qannari, E.M., Rutledge, D.N., Vigneau, E., 2018. Comdim: From multiblock data analysis to path modeling. Food Qual. Prefer. 67, 27–34.
Cordeiro, F.B., Cataldi, T.R., Perkel, K.J., do Vale Teixeira da Costa, L., Rochetti, R.C., Stevanato, J., Eberlin, M.N., Zylbersztejn, D.S., Cedenho, A.P., Turco, E.G.L., 2015. Lipidomics analysis of follicular fluid by esi-ms reveals potential biomarkers for ovarian endometriosis. J. Assist. Reprod. Genet. 32 (12), 1817–1825.
Csanaky IL, Lickteig AJ, Klaassen CD. 2018. Aryl hydrocarbon receptor (ahr) mediated short-term effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (tcdd) on bile acid homeostasis in mice. Toxicol. Appl. Pharmacol. 343:48-61.
Diamanti-Kandarakis E, Bourguignon J-P, Giudice LC, Hauser R, Prins GS, Soto AM, et al. 2009. Endocrine-disrupting chemicals: An endocrine society scientific statement. Endocrine Rev. 30:293-342.
Domínguez F, Ferrando M, Díaz-Gimeno P, Quintana F, Fernández G, Castells I, et al. 2017. Lipidomic profiling of endometrial fluid in women with ovarian endometriosis†. Biol. Reprod. 96:772-779.
Fassbender A, Rahmioglu N, Vitonis AF, Viganò P, Giudice LC, D'Hooghe TM, et al. 2014. World endometriosis research foundation endometriosis phenome and biobanking harmonisation project: Iv. Tissue collection, processing, and storage in endometriosis research. Fertil. Steril. 102:1244-1253.
Fillol, C., Oleko, A., Saoudi, A., Zeghnoun, A., Balicco, A., Gane, J., et al., 2021. Exposure of the french population to bisphenols, phthalates, parabens, glycol ethers, brominated flame retardants, and perfluorinated compounds in 2014–2016: Results from the esteban study. Environ. Int. 147, 106340.
Giudice, L.C., 2010. Endometriosis. N. Engl. J. Med. 362 (25), 2389–2398.

Gore, A.C., Chappell, V.A., Fenton, S.E., Flaws, J.A., Nadal, A., Prins, G.S., et al., 2015. Edc-2: The endocrine society's second scientific statement on endocrine-disrupting chemicals. Endocr. Rev. 36, E1–E150.

Green, M.P., Harvey, A.J., Finger, B.J., Tarulli, G.A., 2021. Endocrine disrupting chemicals: Impacts on human fertility and fecundity during the peri-conception period. Environ. Res. 194, 110694. https://doi.org/10.1016/j.envres.2020.110694.

Grümmer R. 2006. Animal models in endometriosis research. Hum. Reprod. Update 12: 641-649.

Haeusler RA, Astiarraga B, Camastra S, Accili D, Ferrannini E. 2013. Human insulin resistance is associated with increased plasma levels of 12α-hydroxylated bile acids. Diabetes 62:4184-4191.

Huang, Q., Chen, Y., Chen, Q., Zhang, H., Lin, Y.i., Zhu, M., Dong, S., 2017. Dioxin-like rather than non-dioxin-like pcbs promote the development of endometriosis through stimulation of endocrine-inflammation interactions. Arch. Toxicol. 91 (4), 1915–1924.

Jain, P., Vineis, P., Liquet, B., Vlaanderen, J., Bodinier, B., van Veldhoven, K., Kogevinas, M., Athersuch, T.J., Font-Ribera, L., Villanueva, C.M., Vermeulen, R., Chadeau-Hyam, M., 2018. A multivariate approach to investigate the combined biological effects of multiple exposures. J. Epidemiol. Community Health 72 (7), 564–571.

JANDACEK, R., TSO, P., 2007. Enterohepatic circulation of organochlorine compounds: A site for nutritional intervention. J. Nutr. Biochem. 18 (3), 163–167.

Jin, R., McConnell, R., Catherine, C., Xu, S., Walker, D.I., Stratakis, N., Jones, D.P., Miller, G.W., Peng, C., Conti, D.V., Vos, M.B., Chatzi, L., 2020. Perfluoroalkyl substances and severity of nonalcoholic fatty liver in children: An untargeted metabolomics approach. Environ. Int. 134, 105220. https://doi.org/10.1016/j.envint.2019.105220.

Kahn, L.G., Philippat, C., Nakayama, S.F., Slama, R., Trasande, L., 2020. Endocrine-disrupting chemicals: Implications for human health. Lancet Diabetes Endocrinol. 8 (8), 703–718.

Koual, M., Cano-Sancho, G., Bats, A.-S., Tomkiewicz, C., Kaddouch-Amar, Y., Douay-Hauser, N., Ngo, C., Bonsang, H., Deloménie, M., Lecuru, F., Le Bizec, B., Marchand, P., Botton, J., Barouki, R., Antignac, J.-P., Coumoul, X., 2019. Associations between persistent organic pollutants and risk of breast cancer metastasis. Environ. Int. 132, 105028. https://doi.org/10.1016/j.envint.2019.105028.

Kvaskoff, M., Mu, F., Terry, K.L., Harris, H.R., Poole, E.M., Farland, L., Missmer, S.A., 2015. Endometriosis: A high-risk population for major chronic diseases? Hum. Reprod. Update 21 (4), 500–516.

Kvaskoff M, Mahamat-Saleh Y, Farland LV, Shigesi N, Terry KL, Harris HR, et al. 2021. Endometriosis and cancer: A systematic review and meta-analysis. Hum. Reprod. Update 27:393-420.

Lazarevic N, Knibbs LD, Sly PD, Barnett AG. 2020. Performance of variable and function selection methods for estimating the nonlinear health effects of correlated chemical mixtures: A simulation study. Statist. Med. 39:3947-3967.

Lenters, V., Vermeulen, R., Portengen, L., 2018. Performance of variable selection methods for assessing the health effects of correlated exposures in case-control studies. 75 (7), 522–529.

Li B, Tang J, Yang Q, Li S, Cui X, Li Y, et al. 2017. Noreva: Normalization and evaluation of ms-based metabolomics data. Nucl. Acids Res. 45:W162-W170.

Louis GMB, Peterson CM, Chen Z, Hediger ML, Croughan MS, Sundaram R, et al. 2012. Perfluorochemicals and endometriosis: The endo study. Epidemiology 23:799-805.

Mancini, F.R., Cano-Sancho, G., Gambaretti, J., Marchand, P., Boutron-Ruault, M.-C., Severi, G., Arveux, P., Antignac, J.-P., Kvaskoff, M., 2020. Perfluorinated alkylated substances serum concentration and breast cancer risk: Evidence from a nested case-control study in the french e3n cohort. Int. J. Cancer 146 (4), 917–928.

Matorras, R., Martinez-Arranz, I., Arretxe, E., Iruarrizaga-Lejarreta, M., Corral, B., Ibañez-Perez, J., Exposito, A., Prieto, B., Elortza, F., Alonso, C., 2020. The lipidome of endometrial fluid differs between implantative and non-implantative ivf cycles. J. Assist. Reprod. Genet. 37 (2), 385–394.

Matta, K., Ploteau, S., Coumoul, X., Koual, M., Le Bizec, B., Antignac, J.-P., Cano-Sancho, G., 2019. Associations between exposure to organochlorine chemicals and endometriosis in experimental studies: A systematic review protocol. Environ. Int. 124, 400–407.

Matta, K., Koual, M., Ploteau, S., Coumoul, X., Audouze, K., Le Bizec, B., Antignac, J.-P., Cano-Sancho, G., 2021. Associations between exposure to organochlorine chemicals and endometriosis: A systematic review of experimental studies and integration of epidemiological evidence. Environ. Health Perspect. 129 (7), 076003. https://doi.org/10.1289/EHP8421.

Mustieles, V., Arrebola, J.P., 2020. How polluted is your fat? What the study of adipose tissue can contribute to environmental epidemiology. J. Epidemiol. Community Health 74 (5), 401–407.

Pauwels A, Schepens PJ, D'Hooghe T, Delbeke L, Dhont M, Brouwer A, et al. 2001. The risk of endometriosis and exposure to dioxins and polychlorinated biphenyls: A case-control study of infertile women. Hum Reprod 16:2050-2055.

Peng C, Wang J, Asante I, Louie S, Jin R, Chatzi L, et al. 2019. A latent unknown clustering integrating multi-omics data (lucid) with phenotypic traits. Bioinformatics 36:842-850.

Ploteau, S., Antignac, J.-P., Volteau, C., Marchand, P., Vénisseau, A., Vacher, V., Le Bizec, B., 2016. Distribution of persistent organic pollutants in serum, omental, and parietal adipose tissue of french women with deep infiltrating endometriosis and circulating versus stored ratio as new marker of exposure. Environ. Int. 97, 125–136.

Ploteau, S., Cano-Sancho, G., Volteau, C., Legrand, A., Vénisseau, A., Vacher, V., Marchand, P., Le Bizec, B., Antignac, J.-P., 2017. Associations between internal exposure levels of persistent organic pollutants in adipose tissue and deep infiltrating endometriosis with or without concurrent ovarian endometrioma. Environ. Int. 108, 195–203.

Pollack, A.Z., Krall, J.R., Kannan, K., Buck Louis, G.M., 2021. Adipose to serum ratio and mixtures of persistent organic pollutants in relation to endometriosis: Findings from the endo study. Environ. Res. 195, 110732. https://doi.org/10.1016/j.envres.2021.110732.

Rahmioglu, N., Fassbender, A., Vitonis, A.F., Tworoger, S.S., Hummelshoj, L., D'Hooghe, T.M., et al., 2014. World endometriosis research foundation endometriosis phenome and biobanking harmonization project: Iii. Fluid biospecimen collection, processing, and storage in endometriosis research. Fertil. Steril. 102, 1233–1243.

Rivière, G., Sirot, V., Tard, A., Jean, J., Marchand, P., Veyrand, B., Le Bizec, B., Leblanc, J.C., 2014. Food risk assessment for perfluoroalkyl acids and brominated flame retardants in the french population: Results from the second french total diet study. Sci. Total Environ. 491-492, 176–183.

Shafrir, A.L., Farland, L.V., Shah, D.K., Harris, H.R., Kvaskoff, M., Zondervan, K., Missmer, S.A., 2018. Risk for and consequences of endometriosis: A critical epidemiologic review. Best Pract. Res. Clin. Obstetr. Gynaecol. 51, 1–15.

Stratakis, N., V. Conti, D., Jin, R., Margetaki, K., Valvi, D., Siskos, A.P., Maitre, L., Garcia, E., Varo, N., Zhao, Y., Roumeliotaki, T., Vafeiadi, M., Urquiza, J., Fernández-Barrés, S., Heude, B., Basagana, X., Casas, M., Fossati, S., Gražulevičienė, R., Andrušaitytė, S., Uppal, K., McEachan, R.R.C., Papadopoulou, E., Robinson, O., Haug, L.S., Wright, J., Vos, M.B., Keun, H.C., Vrijheid, M., Berhane, K.T., McConnell, R., Chatzi, L., 2020. Prenatal exposure to perfluoroalkyl substances associated with increased susceptibility to liver injury in children. Hepatology (Baltimore, MD) 72 (5), 1758–1770.

Terza, J.V., Basu, A., Rathouz, P.J., 2008. Two-stage residual inclusion estimation: Addressing endogeneity in health econometric modeling. J. Health Econom. 27 (3), 531–543.

UNEP. 2017. Stockholm convention global monitoring plan on persistent organic pollutants second monitoring report.

Upson, K., 2020. Environmental risk factors for endometriosis: A critical evaluation of studies and recommendations from the epidemiologic perspective. Curr. Epidemiol. Reports 7 (3), 149–170.

Vermeulen, R., Schymanski, E.L., Barabási, A.-L., Miller, G.W., 2020. The exposome and health: Where chemistry meets biology. Science 367 (6476), 392–396.

Vigneau, E., Chen, M., Qannari, E., 2015. Clustvarlv: An r package for the clustering of variables around latent variables. R Journal 7. 7 (2), 134. https://doi.org/10.32614/RJ-2015-026.

Vitonis, A.F., Vincent, K., Rahmioglu, N., Fassbender, A., Buck Louis, G.M., Hummelshoj, L., et al., 2014. World endometriosis research foundation endometriosis phenome and biobanking harmonization project: Ii. Clinical and covariate phenotype data collection in endometriosis research. Fertil. Steril. 102, 1223–1232.

Vlahcevic, Z.R., Eggertsen, G., Björkhem, I., Hylemon, P.B., Redford, K., Pandak, W.M., 2000. Regulation of sterol 12alpha-hydroxylase and cholic acid biosynthesis in the rat. Gastroenterology 118, 599–607.

Vouk K, Hevir N, Ribić-Pucelj M, Haarpaintner G, Scherb H, Osredkar J, et al. 2012. Discovery of phosphatidylcholines and sphingomyelins as biomarkers for ovarian endometriosis. Hum. Reprod. 27:2955-2965.

Walker, D.I., Valvi, D., Rothman, N., Lan, Q., Miller, G.W., Jones, D.P., 2019. The metabolome: A key measure for exposome research in epidemiology. Curr. Epidemiol. Reports 6 (2), 93–103.

Wang, Y., Yu, J., Luo, X., Wang, X., Li, M., Wang, L., Li, D., 2010. Abnormal regulation of chemokine teck and its receptor ccr9 in the endometriotic milieu is involved in pathogenesis of endometriosis by way of enhancing invasiveness of endometrial stromal cells. Cell Mol. Immunol. 7 (1), 51–60.

Yu, J., Wang, Y., Zhou, W.-H., Wang, L., He, Y.-Y., Li, D.-J., 2008. Combination of estrogen and dioxin is involved in the pathogenesis of endometriosis by promoting chemokine secretion and invasion of endometrial stromal cells. Hum. Reprod. 23 (7), 1614–1626.