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A COMPREHENSIVE MULTIPLATFORM METABOLOMIC ANALYSIS REVEALS ALTERATIONS OF 2-HYDROXYBUTYRIC ACID AMONG WOMEN WITH DEEP ENDOMETRIOSIS RELATED TO THE PESTICIDE TRANS-NONACHLOR

Tiphaine Lefebvre [a,b,c,d,1], Manon Campas [e,1], Komodo Matta [a], Sadia Ouzia [a,f], Yann Guitton [a,f], Gauthier Duval [a,b], Stéphane Ploteau [b,g], Philippe Marchand [a], Bruno Le Bizec [a], Thomas Freour [b,h], Jean-Philippe Antignac [a], Pascal de Tullio [f,2], German Cano-Sancho [a,2,3]

[a] *Oniris, INRAE, LABERCA, Nantes, France*
[b] *CHU Nantes, Nantes Université, Service de Médecine et Biologie de la Reproduction, Gynécologie médicale, 38 bd Jean Monnet, Nantes, France*
[c] *Faculty of Medicine, Nantes Université, France*
[d] *Department of Gynecology and Obstetrics, Centre Hospitalier Départemental Vendée, 85000 la Roche sur Yon, France*
[e] *Centre for Interdisciplinary Research on Medicines (CIRM), Metabolomics Group, University of Liège, Belgium*
[f] *MetaboHUB-MELISA, MetaboHUB-ANR-11-INBS-0010, Oniris, INRAE, LABERCA, Nantes, France*
[g] *CHU Nantes, Department of Gynecology and Obstetrics, 38 bd Jean Monnet, Nantes, France*
[h] *Department of Reproductive Medicine, Dexeus University Hospital, Barcelona, Spain*

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Persistant organic pollutants, Endometriosis, Metabolomics, Meet-in-the-middle

Abstract

Background: Exposure to persistent organic pollutants (POPs) has been related to the risk of endometriosis however the mechanisms remain unclear. The objective of the present study was to characterize the metabolic profiles underpinning the associations between POPs and endometriosis risk.

Methodology: A hospital-based case-control study was conducted in France to recruit women with and without surgically confirmed deep endometriosis. Women's serum was analyzed using gas and liquid chromatography coupled to high-resolution mass spectrometry (HRMS) to measure the levels of polychlorinated biphenyls (PCBs), organochlorinated pesticides (OCPs) and per-/polyfluoroalkyl substances (PFAS). A comprehensive metabolomic non-polar fractions. A "meet-in-the-middle"

[1] TF and MC contributed equally as first authors.

[2] PT and GCS contributed equally as last authors.

[3] Corresponding author at : 101 Route de Gachet LABERCA, Oniris, Site de la Chantrerie, UMR 1329 INRAE, Nantes, France.

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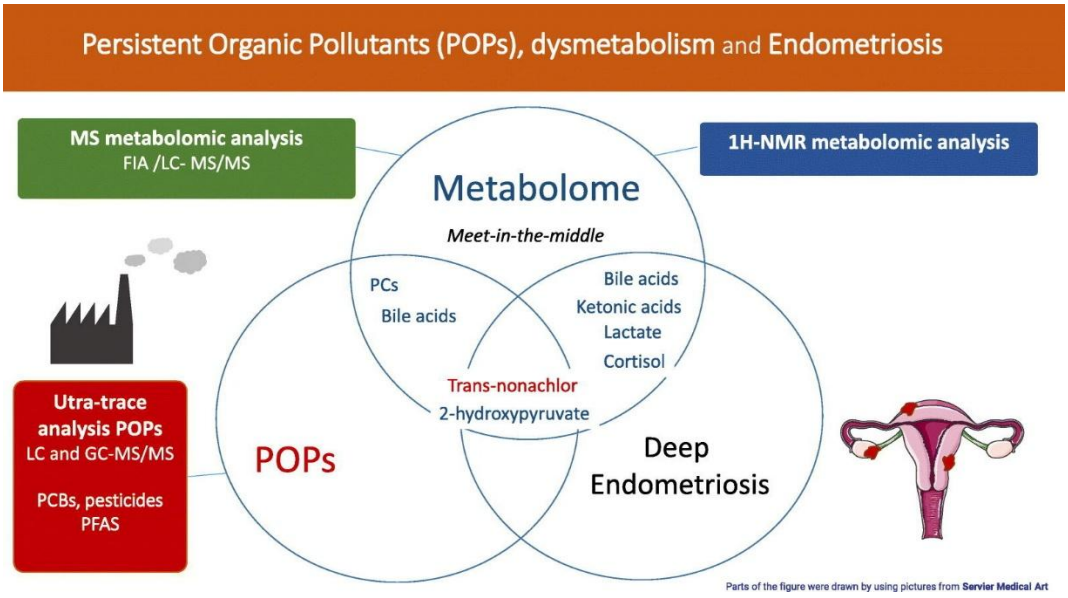
statistical framework was applied to identify the metabolites related to endometriosis and POP levels, using multivariate linear and logistic regressions adjusting for confounding variables.

Results: Fourteen PCBs, six OCPs and six PFAS were widely found in almost all serum samples. The pesticide trans-nonachlor was the POP most strongly and positively associated with deep endometriosis risk, with odds ratio (95 % confidence interval) of 2.42 (1.49;4.12), followed by PCB180 and 167. Women with endometriosis exhibited a distinctive metabolic profile, with elevated serum levels of lactate, ketone bodies and multiple amino acids and lower levels of bile acids, phosphatidylcholines (PCs), cortisol and hippuric acid. The metabolite 2-hydroxybutyrate was simultaneously associated to endometriosis risk and exposure to trans-nonachlor.

Conclusions: To the best of our knowledge, this is the first comprehensive metabolome-wide association study of endometriosis, integrating ultra-trace profiling of POPs. The results confirmed a metabolic alteration among women with deep endometriosis that could be also associated to the exposure to POPs. Further observational and experimental studies will be required to delineate the causal ordering of those associations and gain insight on the underlying mechanisms.

Graphical Abstract



Highlights

- *Trans*-nonachlor was associated with endometriosis risk
- Women with endometriosis had elevated ketone bodies, lactate and amino acids
- Women with endometriosis had decreased levels of hippuric acid and bile acids
- 2-Hydroxybutyrate was associated to endometriosis risk and trans-nonachlor
- PFAS and non-dioxin-like PCBs were correlated with bile acids and phosphatidylcholines

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1. Introduction

Humans are systematically exposed to several hundreds of synthetic chemicals, which may have particularly dire repercussions on women's reproductive health (David et al., 2021; Fuller et al., 2022). Despite that most persistent organic pollutants (POPs) have been voluntarily phase-out, banned or strongly regulated since the 1980s, POPs can still be widely found in the environment and living organisms worldwide due to their toxicological and physicochemical properties (UNEP, 2017, 2019; Wong et al., 2021). Complex mixtures of POPs can be found at trace-levels in human bodies including dioxins, polychlorinated biphenyls (PCBs), organochlorinated pesticides (OCPs) or the more emerging *per-* and polyfluoroalkyl substances (PFAS) (Long et al., 2021; Porta et al., 2021; UNEP, 2017). In the human body, POPs can disrupt the normal hormonal and metabolic function at very low doses and have been linked to a list of reproductive disorders, including altered pubertal onset (Uldbjerg et al., 2022), reduced fecundability (Kahn et al., 2021) and increased risk of endometriosis (Cano-Sancho et al., 2019; Matta et al., 2019).

Endometriosis is a steroid-dependent disease that affects about 10 % of women at childbearing age and about half of infertile women, characterized by the presence of endometrial-like tissues outside the uterus (Zondervan et al., 2020). The societal impacts of endometriosis are very serious, with an economic cost comparable to that of diabetes (approximately €9579/woman/year) (Simoens et al., 2012) and a dramatic deterioration of quality of life and productivity (De Graaff et al., 2013). Endometriosis is heterogeneous in lesion location (e.g. peritoneal, deep infiltrating, ovarian), histology, and symptoms, with each form requiring its own specific research to better understand the disease (Rogers et al., 2017).

The etiology and physiopathology of endometriosis is still poorly understood (Horne and Missmer, 2022), although there is evidence supporting the role of endocrine disrupting chemicals (Kahn et al., 2020). With regard to endometriosis, the prototypical 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been the most extensively studied POP in experimental studies, and little knowledge is currently available about most other POPs and their mixtures (Matta et al., 2021). Experimental evidence has elucidated the role of progesterone resistance and steroidogenesis, inflammation and extracellular matrix remodeling as linking molecular pathways triggered by the aryl hydrocarbon receptor (Bruner-Tran et al., 2017; Chiappini et al., 2019; Chiappini et al., 2022). Other mechanisms of toxicity of POPs involves mitochondrial dysfunction (Elmore and La Merrill, 2019), manifested by aberrant energy balance, amino acid dysmetabolism or lipid accumulation in multiple metabolic disorders (Cano-Sancho et al., 2017; Heindel et al., 2017; Nadal et al., 2017). Nevertheless, the mechanistic interplay of environmental dysmetabolism with endometriosis remains elusive.

The integration of metabolomics, the comprehensive study of metabolites (i.e. final products of biological pathways), has shown promise in the quest to identify predictive biomarkers of endometriosis to shorten the long diagnostic delays (Ortiz et al., 2021; Tomkins et al., 2022). High-Resolution Mass Spectrometry (HRMS) and Nuclear Magnetic Resonance (NMR) are leading complementary technologies to allow the comprehensive, sensitive and highly reproducible determination of metabolites in biological matrices (Letertre et al., 2021). Available metabolomic

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studies of endometriosis have revealed distinctive levels of sphingolipids, phospholipids, some amino acids (e.g. alanine, phenylalanine), organic acids (hydroxypyruvate) or other small molecules like carnitine, in biofluids from women with endometriosis (Ortiz et al., 2021; Tomkins et al., 2022). Metabolomic profiling has also gained attention in environmental-health research, showing a large potential to inform about functional associations between environmental pollutants and diseases (Ji et al., 2021; Jin et al., 2020; Wang et al., 2022).

In a preliminary study, we explored the role of the serum lipidome on the associations between POPs and endometriosis; however, the polar fraction of the metabolome was not addressed (Matta et al., 2022). Thus, the aims of this study were 1) to characterize the metabolic profiles in serum from women with and without endometriosis and 2) to analyze the relationship between the metabolic profiles with the internal levels of persistent organic pollutants. The present study extends the preliminary analysis with a complete comprehensive metabolomic profiling, integrating MS and NMR methods. We conducted different stratified analysis to account for the suspected heterogeneity underlying endometriosis sub-types and fertility status.

2. Materials and methods

2.1. STUDY POPULATION

A total of 137 women aged 18 to 45 years, referred to Gynecology- Obstetrics Department and/or the Reproductive Medicine Department of the University Hospital of Nantes, France, were prospectively recruited in 2019 as described elsewhere (Lefebvre et al., 2022; Matta et al., 2022). Our study included four distinct categories of women with and without endometriosis: 1) women with deep isolated endometriosis (n = 24) without endometrioma (noOMA), 2) women with endometriosis with endometrioma (OMA) awaiting for surgical intervention (n = 57), 3) infertile women (INF) without endometriosis or endometrioma awaiting in vitro fertilization (IVF) for female factor (i.e. polycystic ovarian syndrome PCOS, premature ovarian insufficiency, unexplained infertility) (n = 26), and 4) 20 fertile women (F) consulting for non-endometriosis related benign gynecological issues exhibiting no endometriosis-like symptoms or healthy women undergoing IVF for non-female factor infertility (i.e. male infertility or egg donation). Endometriosis diagnoses for cases were performed by laparoscopy with histological confirmation as described elsewhere (Ploteau et al., 2017) or by Magnetic Resonance Imaging (MRI). Women completed a questionnaire derived from Endometriosis Phenome Harmonisation Project (EPHect) Questionnaire about individual, socio-economic and demographic variables, assisted by a medical doctor. Clinical data (age, body mass index [BMI], infertility etiology, previous pregnancy history, medical and surgical history, medication exposure) were anonymously collected from the hospital database, in accordance with the French National Commission for information and Liberties (CNIL). Research protocol was approved by the local Ethics Committee (GNEDS) in July 2019. Written informed consent was obtained for all participants. Blood samples were collected during the visit, and serum (5 mL) was frozen and stored at – 80°until analysis.

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2.2. QUANTIFICATION OF ORGANOCHLORINE AND PER- AND POLYFLUOROALKYL SUBSTANCES IN SERUM

All analyses were performed under blind conditions following ISO17025:2005 accredited methods. Briefly, 14 PFAs were measured in serum using liquid chromatography with tandem-mass spectrometry (LC-MS/MS) as previously detailed (Mancini et al., 2020). Briefly, serum samples first underwent an alkaline digestion followed by a two-stage Solid Phase Extraction purification using polymeric Oasis® HLB and graphitized carbon (ENVI-Carb®) cartridges. The PCBs and OCPs were measured in serum using gas chromatography coupled to high- resolution mass spectrometry (GC-HRMS); details are provided elsewhere (Benoit et al., 2022; Matta et al., 2022). Serum lipid content was determined with enzymatic kits (Biolabo, Maizy, France) and calculated as the sum of phospholipids, triglycerides, total cholesterol and free cholesterol. Concentrations below the limit of detection (LOD) were assigned a value of LOD/2.

2.3. TARGETED MS METABOLOMICS: SAMPLE PREPARATION, IDENTIFICATION AND QUANTIFICATION

Targeted metabolomics profiling was performed using the MxP® Quant 500 kit (Biocrates Life Sciences AG, Innsbruck, Austria). 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 injection analysis (FIA)-MS/MS using a 5500 QTRAP® instrument (AB Sciex, Darmstadt, Germany) with an electrospray ionization source as previously detailed (Matta et al., 2022).

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

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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 derivatization 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 1 h. 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 spill-over 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 MetaboINDICATOR™ 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.

2.4. NMR METABOLOMICS: SAMPLE PREPARATION, IDENTIFICATION AND QUANTIFICATION

To prepare serum samples for proton ^{1}H NMR metabolomics, ultrafiltration (10 kDa cut-off; Amicon Ultra-0,5 centrifugal filter device) was used to separate proteins from the samples. The centrifugal filters were first washed ten times with 500 µL milli-Q water at 15557 g for 30 min to remove trace amounts of glycerol from the filter membrane. Each sample (300 µL) was then filtered at 15557 g for 80 min, 4 °C. After centrifugation, the filtrates were supplemented with 350 µL of deuterated phosphate buffer (pH 7.4), 30 µL of a 10 mM solution of calcium formate and 3 µL of a 5 mg/mL TMSP solution and finally analyzed by ^{1}H NMR. Based on the formate peak (8.46 ppm), all metabolites present on ^{1}H NMR spectra have been quantified using the Chenomx NMR Suite software (version 8.6). Water, TMSP, glycerol, acetate, ethanol, and methanol were removed from statistical analysis.

All samples were recorded at 298 K on a Bruker Avance HD spectrometer operating at 700.17 MHz for the proton signal acquisition. The instrument was equipped with a 5 mm cryoprobe with a *Z*-gradient. Calcium formate was used as an internal standard for quantification and trimethylsylil-3-propionic-acid-d_4 (TMSP) for the zero calibration. ^{1}H NMR spectra were acquired using a 1D NOESY sequence with water presaturation.

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2.5. DATA ANALYSIS

For the present study, we focused our analyses on complete data, so any observations with missing data on metabolite concentration or POPs were excluded, leaving complete datasets on specific families of chemicals with sample sizes ranging between 112 and 137, as detailed in **Fig. S1**. Additionally, we only considered chemicals with detection frequencies above 75 %. Demographic and clinical data were described using mean and standard deviation or frequency rates and percentage. Statistical comparison between reproductive phenotype groups (fertile women [F], infertile women without endometriosis [INF], deep infiltrating endometriosis without endometrioma [noOMA] and endometrioma [OMA]) was performed with Kruskal-Wallis, Mann-Whitney and Fisher's exact test. Bivariate associations between POPs were analyzed with Spearman correlation test. Principal component analysis (PCA) was used to visualize the data, identify outliers and the influence of individual variables (age, body mass index [BMI]) on MS and ^{1}H NMR metabolomic signatures. Three outliers were identified based on PCA of metabolomics profiles and removed from the final analysis. Orthogonal partial least square discriminating analysis (OPLS-DA) was used to explore the discrimination of metabolome between matrices and phenotypic groups, performed using the R Bioconductor "ropls" package considering a cross-validation with 7 segments and 20 random permutations. Then, we implemented a sequential "meet-in-the-middle" approach (Wang et al., 2022), to identify intersecting metabolites simultaneously dysregulated by the chemical exposures and the gynecological diseases, as detailed in the **Fig. 1**.

Metabolome wide association studies using multivariate linear regression models were used to identify the metabolites associated to the POP exposures, adjusted for age and BMI with Benjamini–Hochberg (BH) procedure for multiple test correction (Path 1). Multivariate logistic regression accounting for age and BMI was used to identify the metabolites associated with the gynecological phenotypes (Path 2), comparing all cases of endometriosis (noOMA+OMA) with women without endometriosis (INF + F) [Y_1], and comparing the endometriosis cases (noOMA+OMA) with only the fertile women [Y_2]. Further analyses explored differential trends between types of endometriosis (noOMA vs OMA). Lastly, the associations between POP biomarkers and the gynecological phenotypes (Path 3), were assessed using multivariate logistic regression. Statistical significance was defined as a BH adjusted *p*-value (i.e. q-value) below 0.05. Multiblock models based on sparse generalized canonical correlation discriminant analysis (sgCCDA) were used to explore the multivariate associations between exposure biomarkers, ^{1}H NMR, and MS metabolomic signatures on the endometriosis phenotype discrimination. The sgCCDA model was implemented with the Data Integration Analysis for Biomarker discovery using Latent variable approaches for Omics studies (DIABLO) workflow within the MixOmics R Bioconductor Package. All analyses and graphics were conducted using R software v 4.2.3.

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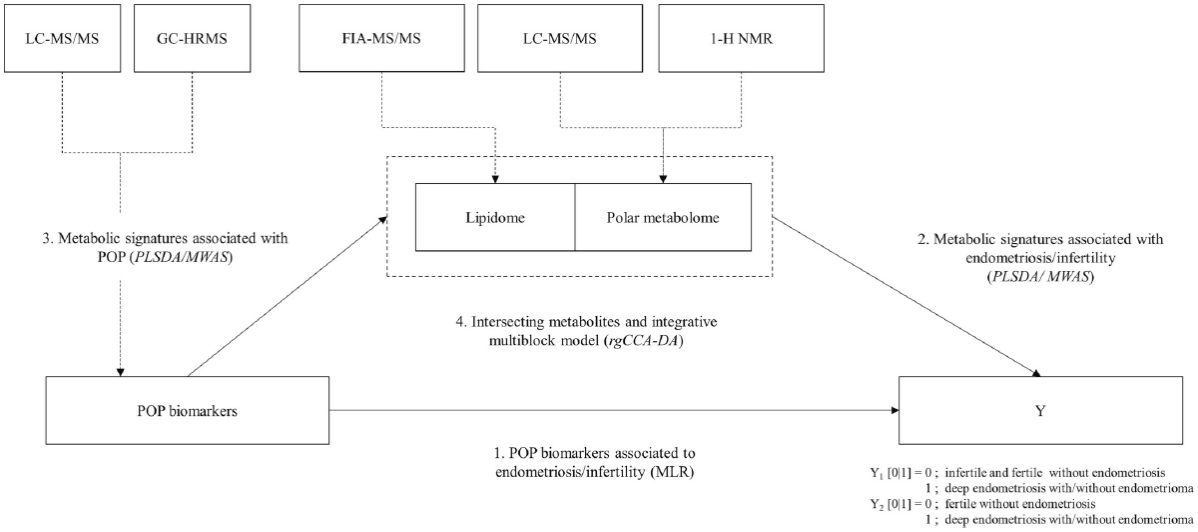

Fig. 1. Statistical workflow depicting the "meet-in-the-middle" approach implemented to identify overlapping metabolites, dysregulated by the exposure to persistent organic pollutants (POP) and the gynecological diseases (Y).

3. Results

3.1. STUDY POPULATION CHARACTERISTICS

A total of 137 women were included in this present pilot study, broadly divided into four groups for the exploratory analysis: 1) women with endometrioma (OMA, *n* = 34), 2) deep infiltrating endometriosis without endometrioma (noOMA, *n* = 57), 3) infertile women without endometriosis (INF, *n* = 26), and 4) fertile women without endometriosis (F, *n* = 20). Demographic characteristics and clinical data are presented in **Table 1**. The mean age (SD) was 34.3 (5.2) years and the mean BMI was 23.2 (3.2) kg/m^2. Variables did not differ significantly across the four groups.

Table 1
Demographic and reproductive characteristics of women included in the study (*n* = 137).

	Without Endometriosis		Deep Endometriosis		P-value
	Fertile	Infertile	Non endometrioma	Endometrioma	
	No. 20	No. 26	No. 34	No. 57	
Age	33.15 (30.50–35.60)	36.35 (33.10–39.00)	34.75 (30.25–38.00)	34.00 (29.50–37.00)	0.28
BMI	22.70 (20.55–25.00)	21.26 (19.73–22.93)	21.90 (20.36–25.66)	23.38 (20.97–25.93)	0.13
Missing	1 (5.00 %)	0 (0 %)	0 (0 %)	2 (3.51 %)	
Parity					0.23
0	9 (45.0 %)	15 (57.7 %)	22 (64.7 %)	32 (56.1 %)	
1	3 (15.0 %)	9 (34.6 %)	4 (11.8 %)	11 (19.3 %)	
≥2	6 (30.0 %)	2 (7.7 %)	7 (20.6 %)	8 (14.0 %)	
Missing	2 (10.0 %)	0 (0.0 %)	1 (2.9 %)	6 (10.5 %)	
Smoking					0.47
Active	5 (25.0 %)	4 (15.4 %)	10 (29.4 %)	15 (26.3 %)	
Never	12 (60.0 %)	16 (61.5 %)	14 (41.2 %)	22 (38.6 %)	
Past	1 (5.0 %)	6 (23.1 %)	4 (11.8 %)	8 (14.0 %)	
Missing	2 (10.0 %)	0 (0.0 %)	6 (17.6 %)	12 (21.1 %)	
Hormone contraceptive current use					0.15
No	0 (0.0 %)	26 (100.0 %)	2 (5.9 %)	6 (10.5 %)	
Yes	17 (85.0 %)	0 (0.0 %)	24 (70.6 %)	42 (73.7 %)	
Missing	3 (15.0 %)	0 (0.0 %)	8 (23.5 %)	9 (15.8 %)	

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3.2. LEVELS OF POPS AND ASSOCIATIONS WITH ENDOMETRIOSIS

Fourteen PCBs, six OCPs and six PFAS were widely found in serum from women included the present study, with frequency rates above 75 %. The distributions of congeners among main groups were summarized in **Table S1**. Fertile women exhibited lower levels of trans-nonachlor and some dioxin-like PCBs (156, 157, 167 and 189) compared to the other groups, but also the highest levels of non-dioxin like PCB52, PCB101 and the pesticide PeCBz. There were strong correlations among congeners from main chemical families (**Fig. 2** Panel A) with some exceptions. Hierarchical clustering on Spearman correlations revealed six main chemical clusters, including three clusters of PCBs, one of which was associated with the pesticide trans-nonachlor (**Fig. S2**). Non-dioxin like PCBs such as PCB28, 52 and 101 were closely related to the pesticides and PFAS clusters than the other PCBs. The first component of PCA, which accounted for 40 % of the variance of the data, also clustered these correlated POPs, with the group of fertile women (**Fig. S3**) a displaying a slight separation of the centroid from the other groups. *Trans*- nonachlor was the POP showing the highest positive associations with endometriosis risk with an OR (95 % CI) of 2.42 (1.49, 4.12) followed by PCB180 and 167, respectively (**Fig. 2**). The association trends were similar when the INF group was removed from the controls (**Fig. S4**).

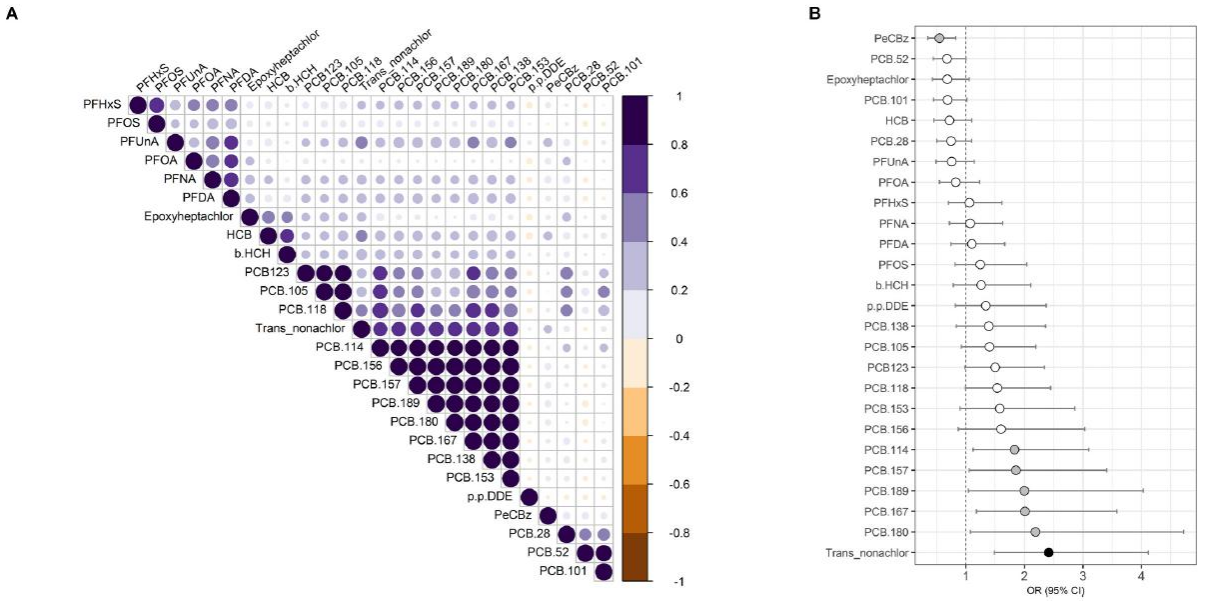

Fig. 2. Spearman correlation heatmap (Panel A) and forest plot depicting the odds ratios confidence interval (Panel B) on the associations between persistent organic pollutants and endometriosis risk from multivariate logistic regression model adjusted by age and body mass index. Statistically significant associations were highlighted with black circles if adjusted for multiple test and gray depicts the raw significant *p*-values. The control group included fertile and infertile women without endometriosis; results from the sensitivity analysis excluding infertile women can be found in **Fig. S4**.

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3.3. METABOLITES RELATED TO ENDOMETRIOSIS

3.3.1. ^{1}H NMR METABOLIC PROFILES RELATED TO ENDOMETRIOSIS

49 metabolites were identified and quantified by ^{1}H NMR. The PCA showed that the first two components retained about 47 % of total variance, with a clear overlap of women grouped by tertiles of age and BMI (**Fig. S5**). The two endometriosis groups (noOMA+OMA) were distinct from the women without endometriosis (F + INF) as seen in the PCA (**Fig. 3**A), and confirmed by PLSDA, with a R^2 of 0.5–0.6 and Root Mean Square Error of 0.3 (**Fig. S6**). 29 metabolites were positively associated with endometriosis status, five of which and five were inversely associated (**Fig. 3**C). Phenylalanine and 2-hydroxybutyrate had the strongest associations, with OR (95 % CI) of 12.45 (5.27–35.70) and 9.99 (4.51–26.52), $q < 0.001$, respectively (**Fig. 3** and **Table S2**). No differences between OMA vs noOMA or F vs INF were found (**Fig. S6**). In the sensitivity analysis removing the INF group from the controls, these metabolites remained significantly associated with endometriosis (**Fig. S7**).

3.3.2. MS METABOLITES RELATED TO ENDOMETRIOSIS

As previously described, we first conducted the exploratory analysis using PCA with the 555 MS-based metabolites and 126 functional ratios that passed the quality pre-processing filters. Neither age nor BMI showed strong discrimination; nevertheless, the centroid related to the third tertile of BMI appeared slightly isolated from tertile 1 and 2 (**Fig. S8**). The PCA (**Fig. 3**B) and PLSDA (**Fig. S9**) showed poor discrimination of observations based on disease status, with slight separation of centroids based on endometriosis status. Nevertheless, multivariate regression models showed 26 metabolites inversely associated and four metabolites positively associated with endometriosis status. Phenylalanine and asparagine were the top ranked positively associated metabolites. The top ranked inversely associated metabolites included two phosphatidylcholines (PC aaC32.2 and C36.6) followed by hippuric acid, cortisol, 3-indolepropionic acid (3-IPA), trimethylamine N-oxide (TMAO) and cortisone (**Fig. 3** and **Table S3**). The levels of metabolites were similar between endometriosis groups (OMA vs noOMA) or controls (INF vs F) without endometriosis (**Fig. S9**). 20 functional ratios were statistically associated with endometriosis status, including markers of TMAO synthesis, BA metabolism, glutaminase activity or glutathione constituents (**Fig. S10** and **Table S3**). None of those compounds nor ratios were retrieved in the sensitivity analysis removing the INF group from controls (results not shown).

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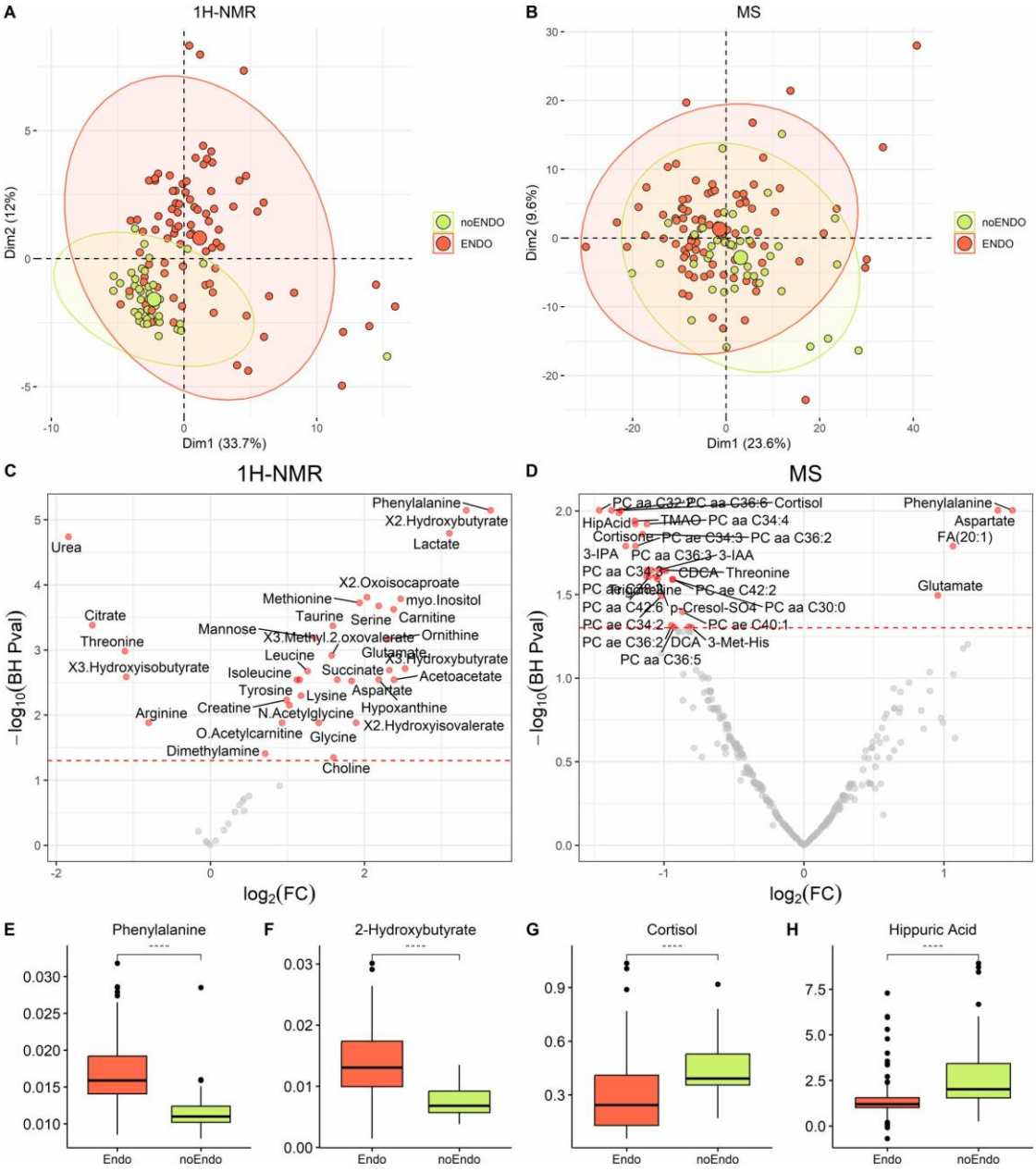

Fig. 3. Metabolic signatures associated to endometriosis. The projection of observations on the first two components from principal component analysis colored by endometriosis status are depicted in panel A for ^{1}H NMR data and panel B for MS data. Volcano plots of ^{1}H NMR (Panel C) and MS data (Panel D) summarize the statistically significant metabolites associated with endometriosis risk adjusted for age and body mass index. Univariate comparisons (Mann-Whitney test) among women groups are depicted for selected metabolites from ^{1}H NMR such as phenylalanine (Panel E) and 2-hydroxybutyrate (Panel F) or MS including cortisol (Panel G) and hippuric acid (Panel H).

3.4. METABOLITES RELATED TO POP EXPOSURES

In order to conduct an efficient differential metabolic analysis, we conducted an MWAS on six POP-group variables based on the hierarchical clustering detailed in Section 3.2 and **Fig. S2**. In addition, a separate analysis was conducted with trans-nonachlor because its unique profile suggested in the dendrogram (**Fig. S2**) and its significant associations with endometriosis risk.

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3.4.1. MS METABOLITES RELATED TO POPS

The levels of PFAS and the sum of three OCPs (HCB, β-HCH, epoxyheptachlor) in cluster 6 were differentially associated with MS metabolites. Globally we found a positive association between the levels of these POP families with a specific lipid signature rich in PCs (**Fig. 4**A-D). Among positively associated compounds with PFAS we also found the bile acids, chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA). The finding from functional ratios were consistent, showing the sum of PCs among the most strongly related lipids, but also the marker of xanthine synthesis and sum of steroid hormones among the top scored markers (**Fig. 4**B). In turn, the levels of PFAS were inversely associated with a list of polar metabolites, including the polyamines spermine and spermidine or the amino acid glycine.

3.4.2. ^{1}H NMR METABOLITES RELATED TO POP LEVELS

Differential metabolic analysis of ^{1}H NMR data revealed some metabolites inversely related with PFAS and levels of some dioxin-like PCBs (PCB105, PCB118, PCB123) identified in cluster 2, notably glycine and proline, respectively (**Fig. S11A** and **S11**B). The levels of non-dioxin like PCBs, grouped in the fourth cluster, were inversely associated with 14 metabolites, with glycine having the largest estimate (**Fig. S11**C). *Trans*-nonachlor was positively and significantly associated with 2-hydroxyoyruvate and six other metabolites (**Fig. 4**E and F).

3.5. METABOLITES INTERSECTING EXPOSURE TO POPS AND ENDOMETRIOSIS

A subset of metabolites associated with endometriosis risk was also found to be associated with PFAS levels (22 metabolites, **Fig. 4**A-B), non-dioxin-like (NDL) PCBs (11 metabolites) and pesticides (16 metabolites, **Fig. 4**C-D). Choline, glutamate, glycine, hypoxanthine, isoleucine, lactate, leucine, lysine, ornithine, phenylalanine and serine were found at higher concentrations among women with endometriosis, who also presented lower levels of NDL-PCBs. In turn, women with endometriosis and lower levels OCPs presented lower levels of cortisol and ten PCs. Serine, lysine, glycine, creatinine and asparagine were found at highest concentrations among women with endometriosis, and women with lower levels of PFAS (**Fig. 4**A). A subset of four ^{1}H NMR metabolites and 18 MS metabolites related with PFAS levels were also associated with endometriosis risk, including five amino acids positively related with endometriosis and inversely with PFAS levels. Two bile acids (DCA and CDCA), the sum of steroid hormones, p-cresol-SO4 and ten PCs were inversely associated with endometriosis risk and positively associated with PFAS levels. The pesticide trans-nonachlor, strongly associated with endometriosis, was associated ($q < 0.1$) with elevated levels of 2-hydroxybutyrate, acetone and myo-inositol, and lower levels of citrate (**Fig. 4**E-F). The associations were strengthened when the group of women was excluded from the analysis (**Fig. S12**).

The complementary multiblock analysis highlighted the relationships between metabolites from both analytical platforms (MS and ^{1}H NMR) with POP biomarkers to maximize the discrimination function of endometriosis status. The results elucidated two main clusters of variables as shown in the canonical correlation network plot in **Fig. 5**A. The first cluster was generated around acetone, 2-hydroxybutyrate and 3-hydroxybutyrate and was positively associated with some PCBs (e.g. PCB153, 157, 167 and 138). The second included lactate and pyruvate as the central node, and was mainly related to PCB28 and

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PCB52. PCB52, 101 and 28 were among the top ranked POPs, with the highest concentrations among women without endometriosis as shown by the model loadings (**Fig. 5**B). Mannose and 2-hydroxybutyrate was the top ranked ^{1}H NMR metabolite whereas C18.1 and FA(20:1) were the two top ranked MS metabolites in the first component (**Fig. 5**C and D).

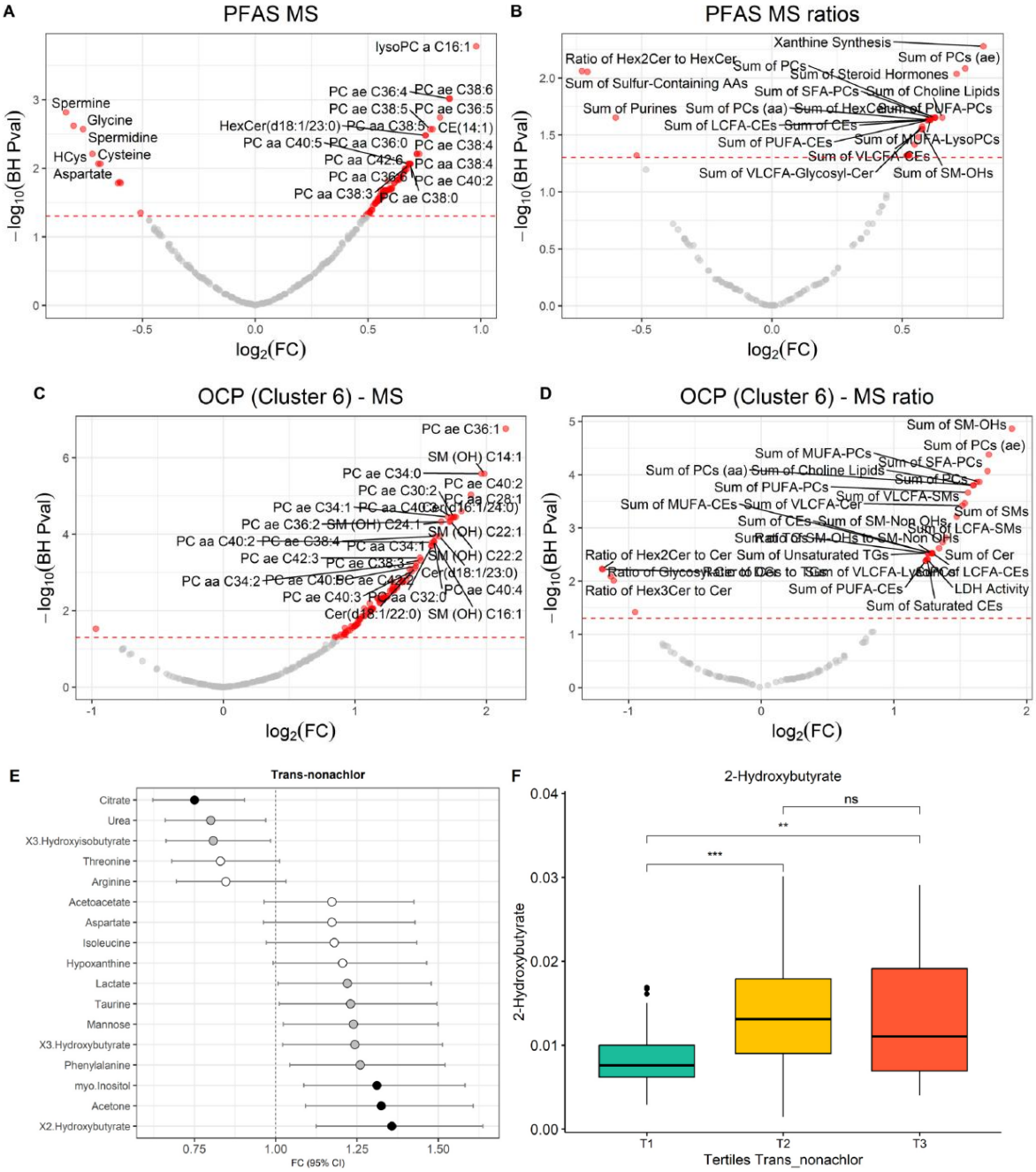

Fig. 4. Volcano plots of differential MS metabolomics analysis for per/polyfluorinated substances cluster levels (Panel A and B) and the cluster of three organochlorinated pesticides (epoxyheptchlor, hexachlorobenzene and β-hexachlorocyclohexane; Panel C and D). Panel E depicts in a forest plot the odds ratios confidence interval on the associations between trans-nonachlor and top ranked ^{1}H NMR metabolites from multivariate linear regression model adjusted by age and body mass index. Black circle depict significant associations after multiple test correction at $q < 0.1$, and gray circles at $q < 0.15$. The levels of 2-hydroxybutyrate by tertiles of trans-nonachlor are shown in Panel F compared with Mann-Whitney tests. In this analysis, the control group included fertile and infertile women without endometriosis; results from the sensitivity analysis excluding infertile women can be found in **Fig. S12**.

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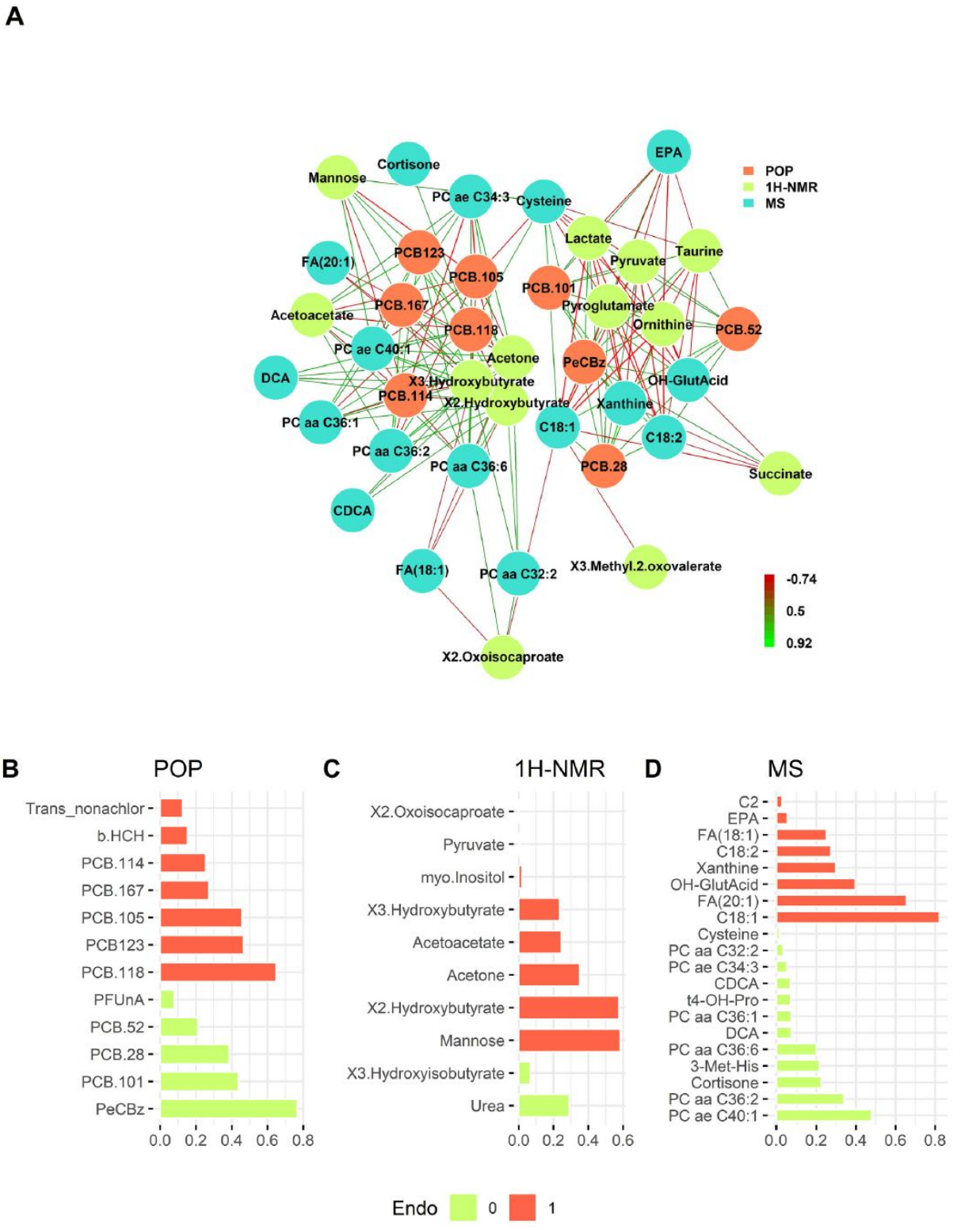

Fig. 5. Correlation network depicting a shared signature between exposure and metabolic biomarkers that maximize the discrimination of endometriosis (Panel A) from the integrative multiblock model using sparse generalized canonical correlation analysis to identify considering two latent components. Loadings from the first component depicts the contribution of each variable to the overall model, represented with barplots for the block of persistent organic pollutants (POP, Panel B), ^{1}H NMR metabolomics (Panel C) and MS metabolomics (Panel D). The color key identifies the endometriosis group with the highest concentration of chemical (endometriosis in red [1] and without endometriosis in green [0]). In this analysis, the control group included fertile and infertile women without endometriosis.

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4. Discussion

The present study provides a first comprehensive metabolome characterization of women at childbearing age with different gynecological conditions, combining MS and ^{1}H NMR instrumentation. These results showed that women with endometriosis have a distinctive metabolome from women without, with minor differences among endometriosis subtypes. We also found similar metabolic profiles among women without endometriosis, both fertile and infertile. Different metabolic changes were simultaneously related to POPs (PFAS, NDL-PCBs and some pesticides) and/or endometriosis risk. The pesticide trans-nonachlor was positively associated with endometriosis risk and the metabolite 2-hydroxybutyrate.

4.1. METABOLIC DYSREGULATION RELATED TO ENDOMETRIOSIS

Our results suggest an alteration of energy metabolism among women with deep endometriosis, characterized by elevated serum levels of lactate and ketone bodies and lower citrate levels (**Fig. 6**). These findings are consistent with previous studies which found higher levels of lactate in serum (Dutta et al., 2012; Jana et al., 2013) and follicular fluid (Castiglione Morelli et al., 2019; Karaer et al., 2019; Marianna et al., 2017) in women with endometriosis. Ketone bodies such as 2-hydroxybutyrate or 3-hydroxybutyrate have also been found elevated in the serum f women with endometriosis in previous observational studies (Dutta et al., 2012; Jana et al., 2013). An experimental study with non-human primates found that the endometrium from animals with endometriosis exhibited decreased mitochondrial respiration, suggesting mitochondrial dysfunction or metabolic shifts from oxidative phosphorylation (Atkins et al., 2019). The authors pointed towards ROS production and Warburg-like effects as two potential mechanisms responsible for mitochondrial dysfunction. The Warburg effect, well-documented in cancer cells, is a metabolic switch that allow the utilization of glucose and glutamine as energetic substrates under virtually anaerobic conditions (Warburg et al., 1927), hence bypassing mitochondrial respiration and boosting alternative pathways including the utilization of lipids. This phenomena was also observed in ectopic stromal cells from women with endometriosis (Kasvandik et al., 2016) and the Transforming Growth Factor-β might play central role in that metabolic switch (Young et al., 2014). Carnitine is an essential cofactor in this metabolic switch, allowing fatty acids entering the mitochondria towards the β-oxidation pathway (Console et al., 2020). In the present study, carnitine, acylcarnitine and one free fatty acid (20:1) were found elevated among women with deep endometriosis. Previous studies have reported similar raised levels of fatty acids in serum from endometriosis patients (Maignien et al., 2020), whereas levels of carnitines were found depleted in peritoneal fluid from endometriomas (Vouk et al., 2016).

A generalized downregulation of 14 PCs was observed among the group of women with deep endometriosis, particularly PC aa C32.2 and C36.6. Previous case-control studies have reported divergent results: lowered concentrations of PCs in peritoneal fluid (Vouk et al., 2016) and endometrial tissue (Li et al., 2018), whereas upregulation of PCs were found in follicular fluid from endometriomas (Cordeiro et al., 2015) and plasma from patients with ovarian endometriosis (Vouk et al., 2012).

Women with deep endometriosis presented a unique amino acid signature. The elevated serum levels of phenylalanine and tyrosine and depleted levels of hippuric acid were consistent with previous

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observations of mild endometriosis cases (Dutta et al., 2018). A higher level of other amino acids such as glycine, methionine and the branched-chain leucine and isoleucine, was observed in the present study, consistent with the previous findings reflecting the high catabolic state of endometriotic tissues (Ortiz et al., 2021).

The bile acid metabolism of women with endometriosis also showed lower levels of deoxycholic acid (DCA), chenodeoxycholic acid (CDCA) and some relate sums of bile acids compared to women without endometriosis (**Fig. S9**), consistent with a previous study that found a decrease of primary bile acids in serum from endometriosis cases (Ghazi et al., 2016). The authors attributed the alteration of bile acid metabolism as potential cause of hyperestrogenism among women with endometriosis, a hypothesis that we could not confirm due to the lack of accurate measurement of steroidal metabolites within the target panel.

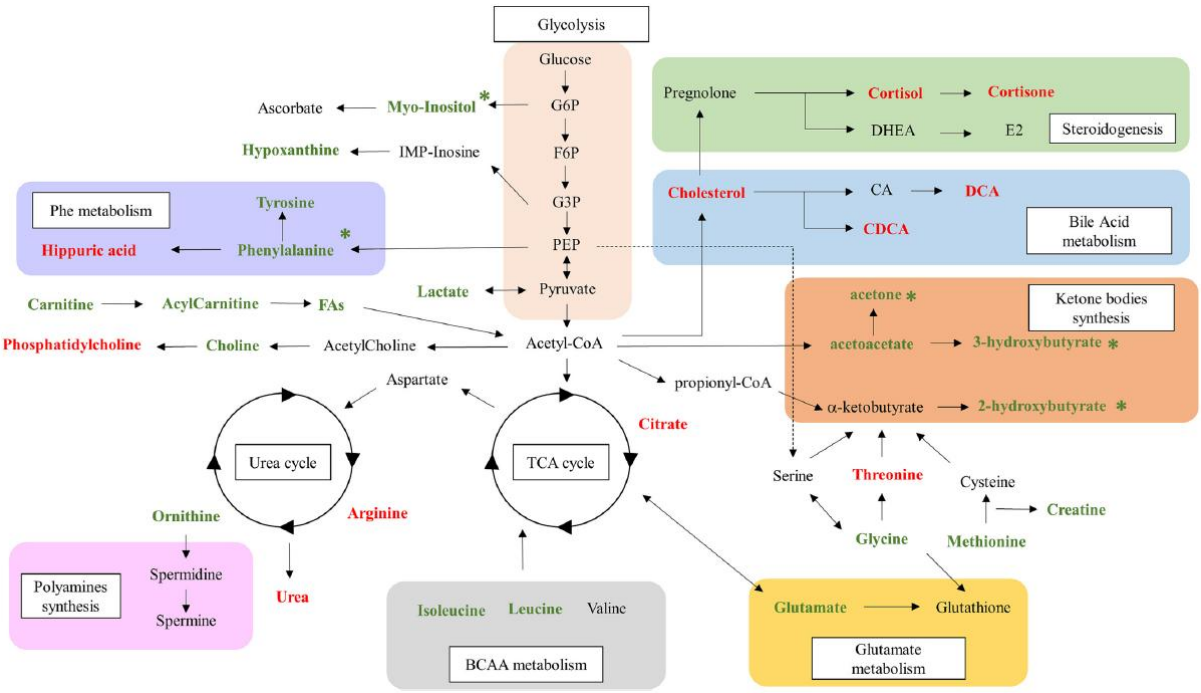

Fig. 6. Metabolic pathway network map depicting the most significantly altered metabolites in deep endometriosis (elevated highlighted in green and decreased in red). Those metabolites also related to the exposure of the pesticide trans-nonachlor are identified with an asterisk.

4.2. METABOLIC DYSREGULATION RELATED TO POPS

From the MWAS analysis of POP groups, we found positive associations between the levels PFAS or the sum of three OCPs (HCB, β-HCH, epoxyheptachlor) and a number of PCs. A growing body of evidence supports the effects of PFAS on the disruption of glycerophospholipid metabolism with increased levels of PCs in both human epidemiological (India-Aldana et al., 2023) and experimental studies (Chen et al., 2022). We also found a positive associations between PFAS levels and concentrations of bile acids DCA and CDCA in agreement with the current state-of-the-science

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supporting liver injury due to the exposure to this class of synthetic compounds (India-Aldana et al., 2023; Rhee et al., 2023; Yoo et al., 2023).

4.3. METABOLITES BRIDGING THE EXPOSURE TO POPS AND ENDOMETRIOSIS RISK

Trans-nonachlor was identified as the xenobiotic most strongly associated with endometriosis, also exhibiting positive associations with ketone bodies acetone, 2- and 3-hydroxybutyrate. *Trans*-nonachlor is one of the congeners of the technical chlordane mixture and one of the most abundant OCPs found in biological samples worldwide despite being banned in the 1980s. The French biomonitoring program (Esteban 2014–216) detected trans-nonaclor in almost all serum samples (99.6 %) from adults aged 18–74 years. Experimental studies have shown that trans-nonachlor can alter the lipid metabolism by promoting lipogenesis in rat primary hepatocytes (Howell et al., 2018) and male Sprague Dawley Rats liver (McDevitt et al., 2023); however, there are no studies on its effects on the endometrium or endometriotic lesions. *Trans*-nonachlor interacts agonistically with the estrogen receptor alpha and beta (Klotz et al., 1996; Lemaire et al., 2006) and pregnane X receptor, which are both involved in steroid metabolism (Jacobs et al., 2015). In addition, we found trans-nonachlor to be highly correlated to a mixture of PCBs with established aryl hydrocarbon receptor agonistic activity, the main mechanism currently linking POPs and endometriosis (Matta et al., 2021). These results suggest that the pesticide could play a role on endometriosis progression through direct and indirect metabolic pathways.

4.4. LIMITATIONS AND STRENGTHS

The present study has several limitations. The sample size is relatively small despite being in the upper tier of present publications on endometriosis metabolomics (most studies $n < 50$) (Ortiz et al., 2021; Tomkins et al., 2022). In addition, our analyses were limited to severe cases of deep endometriosis with and without endometrioma, thus limiting the generalizability to mild cases of endometriosis. Moreover, although the women comprising the control population did not display symptoms of endometriosis, the presence of endometriosis cannot not be ruled out as endometriosis may also be asymptomatic. Finally, the retrospective nature of case-control studies precludes us from drawing causal conclusions. Hence, further observational studies with a prospective design will be required to validate these findings complemented with experimental studies to gain insight on the underlying causal relationships and molecular mechanisms.

Some strengths can be highlighted in the present study. To the best of our knowledge, this study is the first to combine ^{1}H NMR and four HRMS platforms to ensure a comprehensive characterization of endometriosis metabolome, including a variety of polar metabolites, lipids and also xenobiotic biomarkers. Previous metabolomic studies on endometriosis have focused on single analytical platforms with a limited number of metabolic families, and, in most cases, have used non-targeted methods which are not quantitative (Ortiz et al., 2021). In this regard, the use of a high-throughput quantitative method ensures an efficient balance between coverage and robust characterization of chemical identities and abundances to improve the statistical analysis and interpretation. We should also highlight the proper confirmation of deep endometriosis cases using gold standard methods such as laparoscopy, and the diagnostic details on severity and localization of lesions. Finally, we have

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applied a comprehensive statistical workflow based on the "meet-in-the- middle" framework including advanced multiblock models to better understand the associations' patterns between POP biomarkers, metabolic signatures, and endometriosis status.

5. Conclusions

The results of the present study confirmed that women with deep endometriosis present a distinctive metabolome, supporting the use of HRMS and NMR to gain insight into the physiopathology of the disease and biomarker discovery to develop predictive diagnostic tools. To the best of our knowledge, this is the first comprehensive metabolome-wide association study on severe endometriosis including accurate ultra-trace profiling of POPs. The exposure to the pesticide trans-nonachlor was positively associated to endometriosis risk and alteration of several metabolites such as 2-hydroxybutyrate. Considering the high societal and economic costs associated with endometriosis, further research is urged to validate these findings and extend the examination with other environmental chemicals to support preventive and protective strategies.

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CRediT authorship contribution statement

Tiphaine Lefebvre: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Manon Campas:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Komodo Matta:** Writing – review & editing, Formal analysis, Data curation. **Sadia Ouzia:** Writing – review & editing, Formal analysis, Data curation. **Yann Guitton:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Data curation. **Gauthier Duval:** Writing – review & editing, Formal analysis, Data curation. **Stephane Ploteau:** ´ Writing – review & editing, Resources, Funding acquisition. **Philippe Marchand:** Writing – review & editing, Supervision, Formal analysis, Data curation. **Bruno Le Bizec:** Writing – review & editing, Resources, Funding acquisition. **Thomas Freour:** Writing – review & editing, Supervision, Resources. **Jean- Philippe Antignac:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Pascal de Tullio:** Writing – review & editing, Supervision, Resources, Funding

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acquisition, Formal analysis, Data curation, Conceptualization. **German Cano-Sancho:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supplementary data

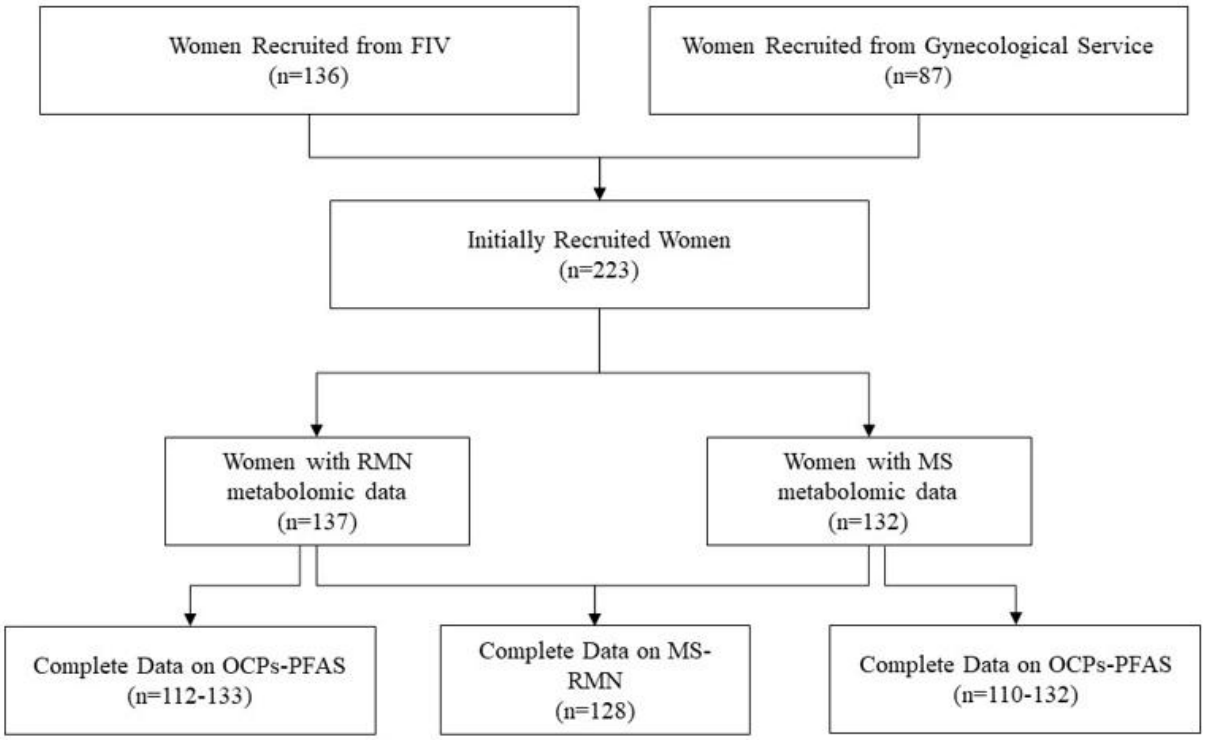
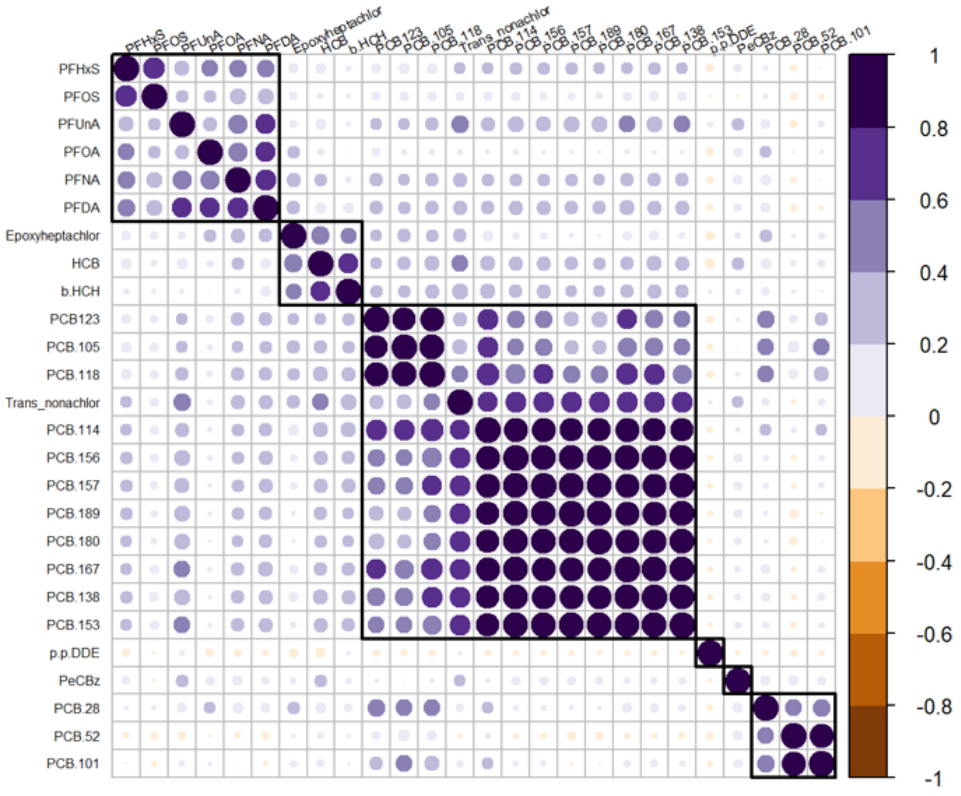


Fig. S1. Workflow of study population and datasets



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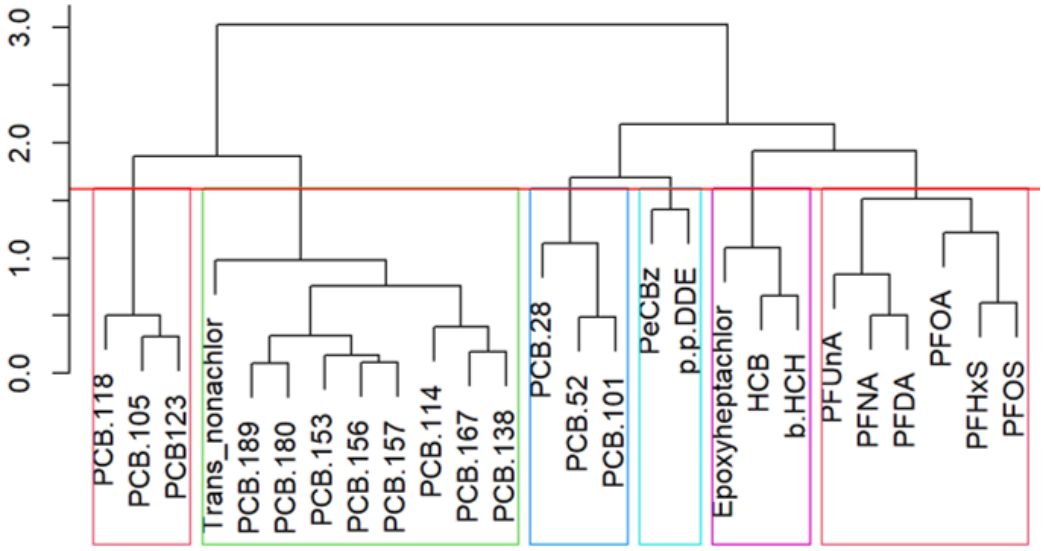

Fig. S2. Spearman correlation heatmap and dendogram representing the hierarchical clustering of persistent organic pollutants.

Cluster 1, PFAS : PFHxS, PFOS, PFOA, PFNA, PFDA, PFUnA.
Cluster 2, DL-PCBs mono-ortho-substituted: **PCB105, PCB118, PCB123**.
Cluster 3, mix PCBs: **PCB114**, **PCB156**, **PCB157**, **PCB167, PCB189**, PCB138, PCB153, PCB180, trans-nonachlor.
Cluster 4, NDL-PCBs: PCB28, PCB52, PCB101.
Cluster 5, PeCBzDDE: PeCBz, *p,p'*DDE
Cluster 6, OCPs: HCB, □-HCH, epoxyheptachlor.

*Dioxin-like PCBs highlighted in bold

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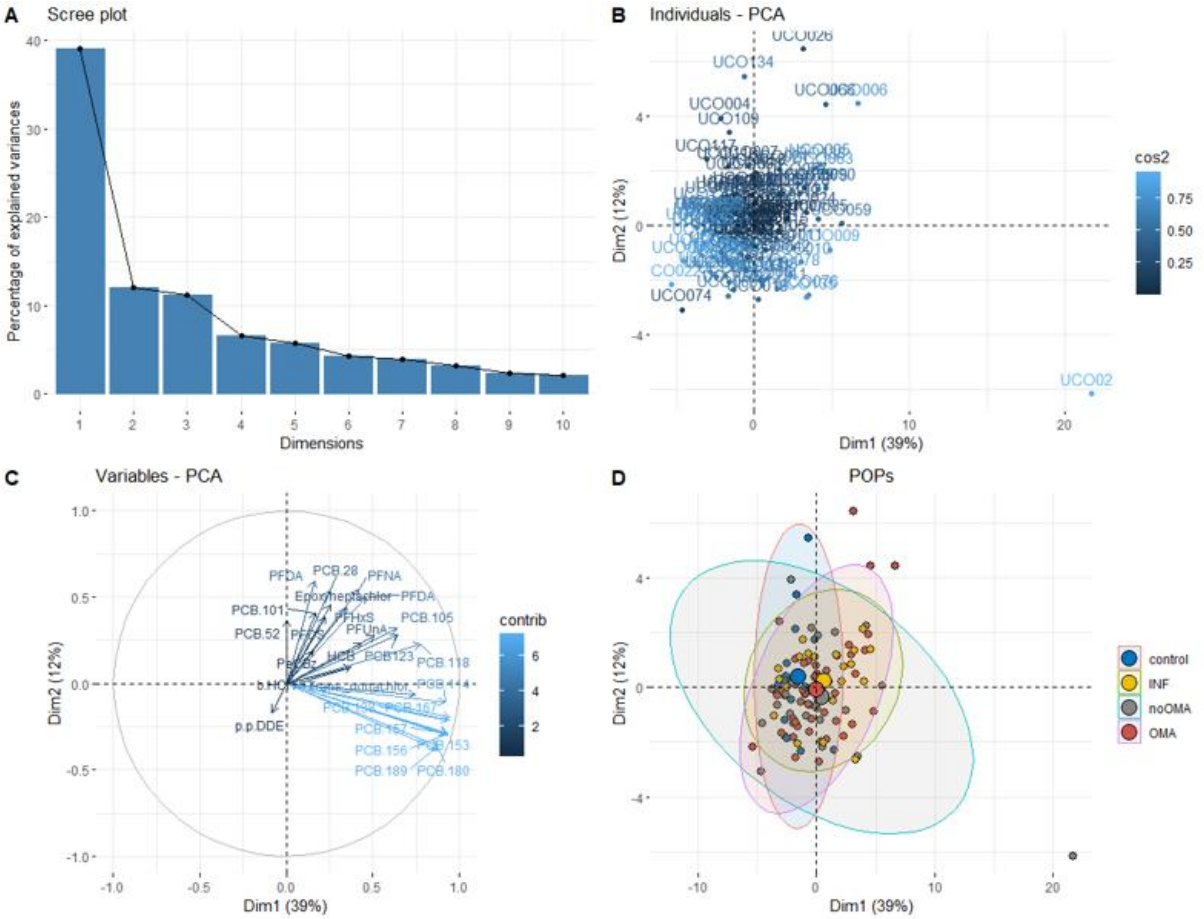

Fig. S3. Principal component analysis results of persistent organic pollutant data depicted by the scree plot of variance (Panel A), the projection of individuals on the first two components (Panel B), the projection of compounds (Panel C) and the projection of individuals colored by the female group identifier (Control: fertile women without endometriosis, INF: infertile women without endometriosis, noOMA: deep endometriosis without endometrioma, OMA: deep endometriosis with endometrioma).

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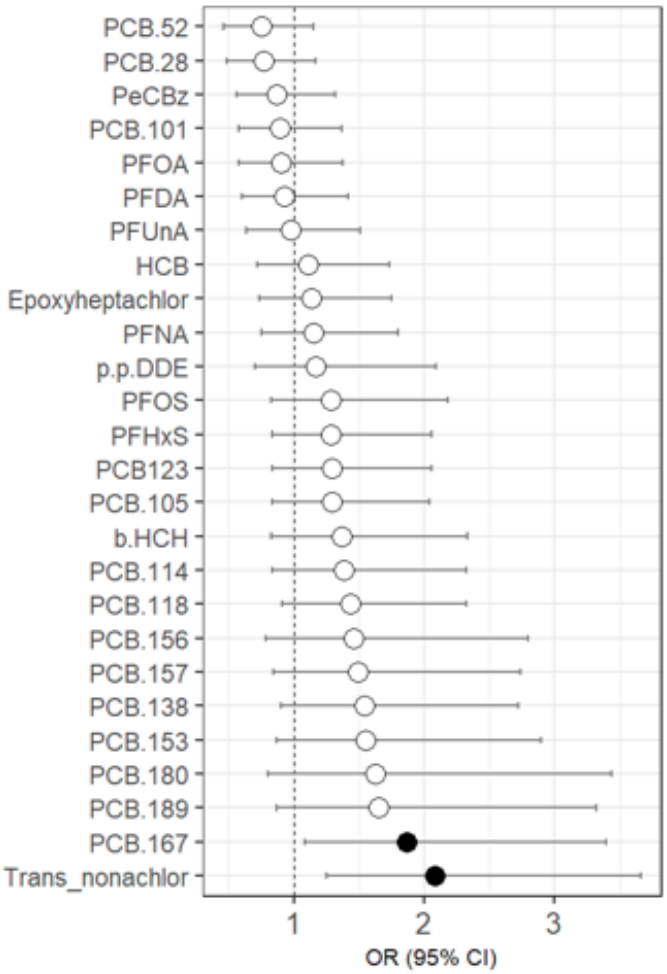

Fig. S4. Forest plot depicting the odds ratios confidence interval on the associations between persistent organic pollutants and endometriosis risk (all cases combined) from multivariate logistic regression model adjusted by age and body mass index. Circles highlighted in black the statistically significant raw p-values. In this analysis the group infertile without endometriosis was excluded from the referent group. None of the chemicals was significant after multiple-test correction.

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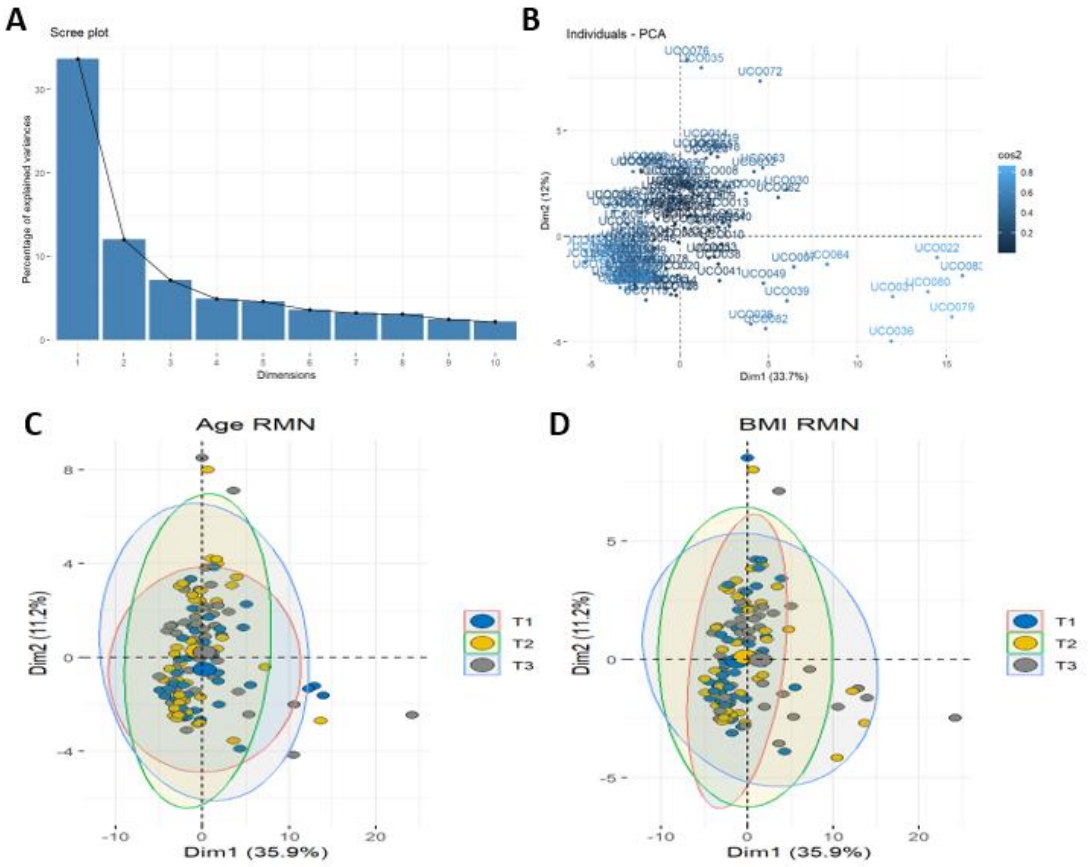

Fig. S5. Principal component analysis results of ^{1}H-NMR metabolomic data depicted by the scree plot of variance (Panel A), the projection of individuals on the first two components (Panel B), and the projection of individuals colored by tertiles (T1-3) of age (Panel C) and tertiles(T1-3) of body mass index (Panel D).

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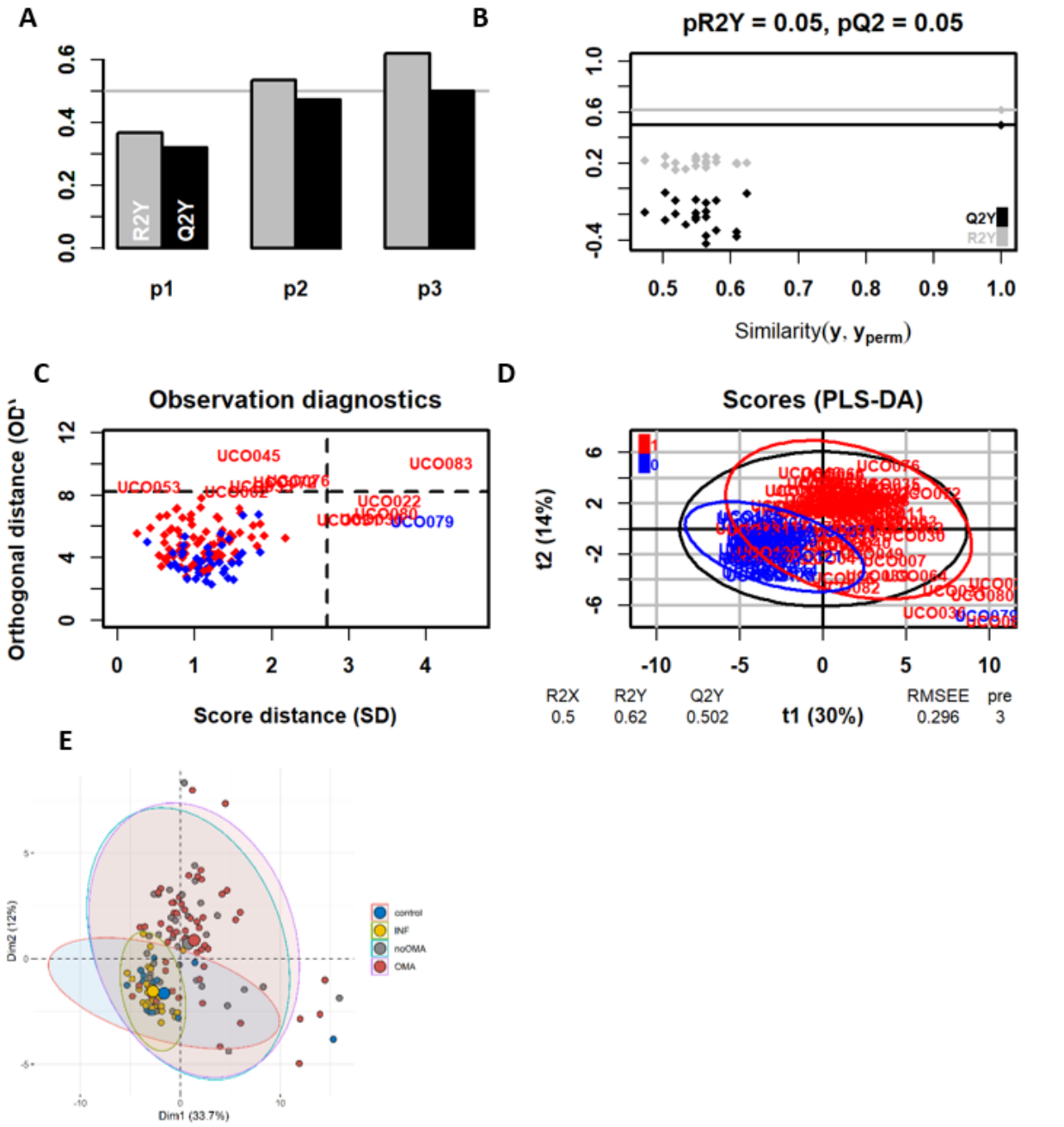

Fig. S6. Results from the partial least square discrimination analysis of ^{1}H-NMR metabolomic data. In this analysis the group infertile without endometriosis was combined in the referent group. Panel E depicts the observations in the first 2 principal components colored by endometriosis subgroup (OMA [endometrioma] and noOMA [no endometrioma]), fertile women (control) or infertile (INF).

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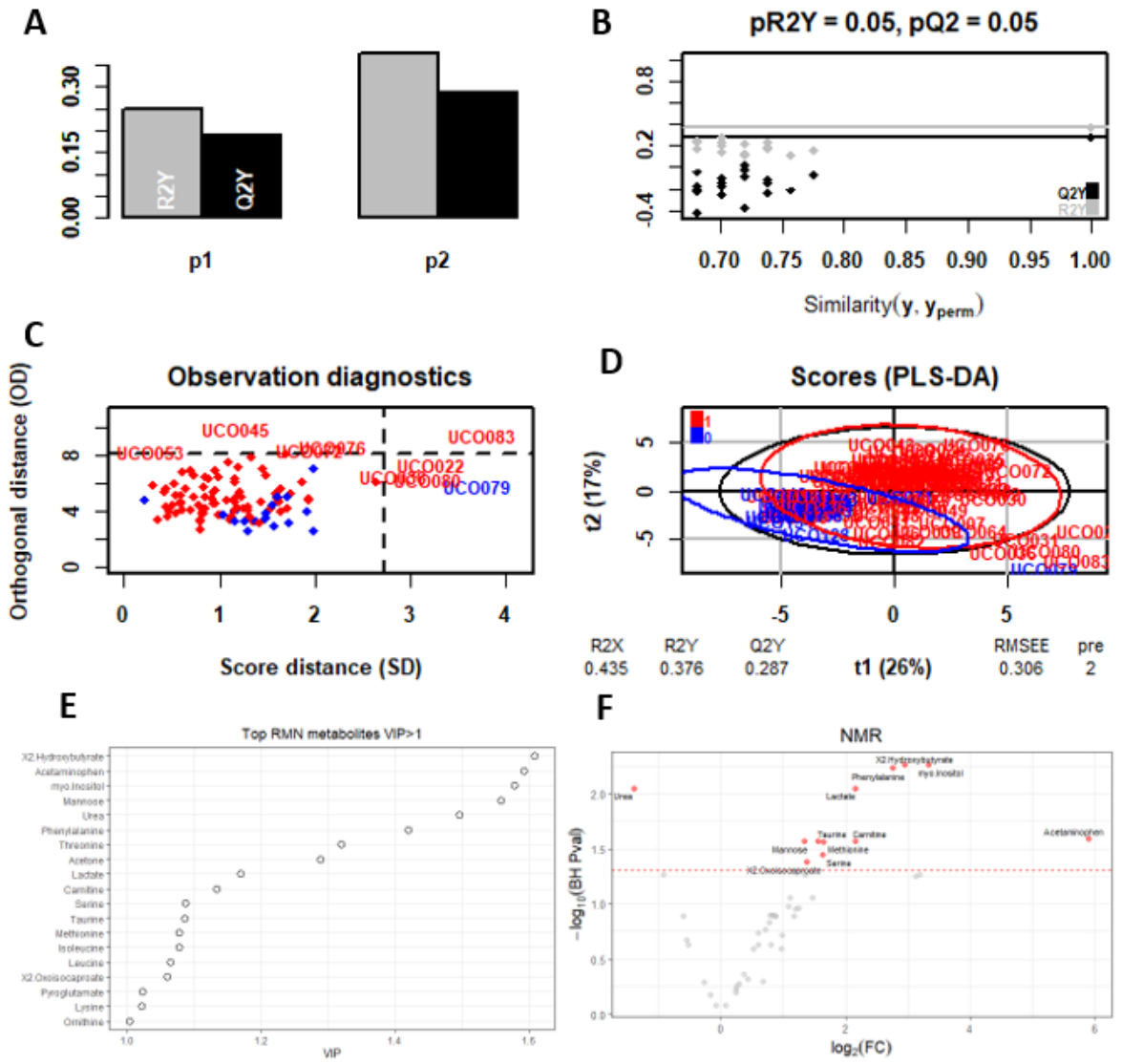

Fig. S7. Results from the partial least square discrimination analysis and multivariate regression analysis of ^{1}H-NMR metabolomic data. In this analysis the group infertile without endometriosis was excluded from the referent group.

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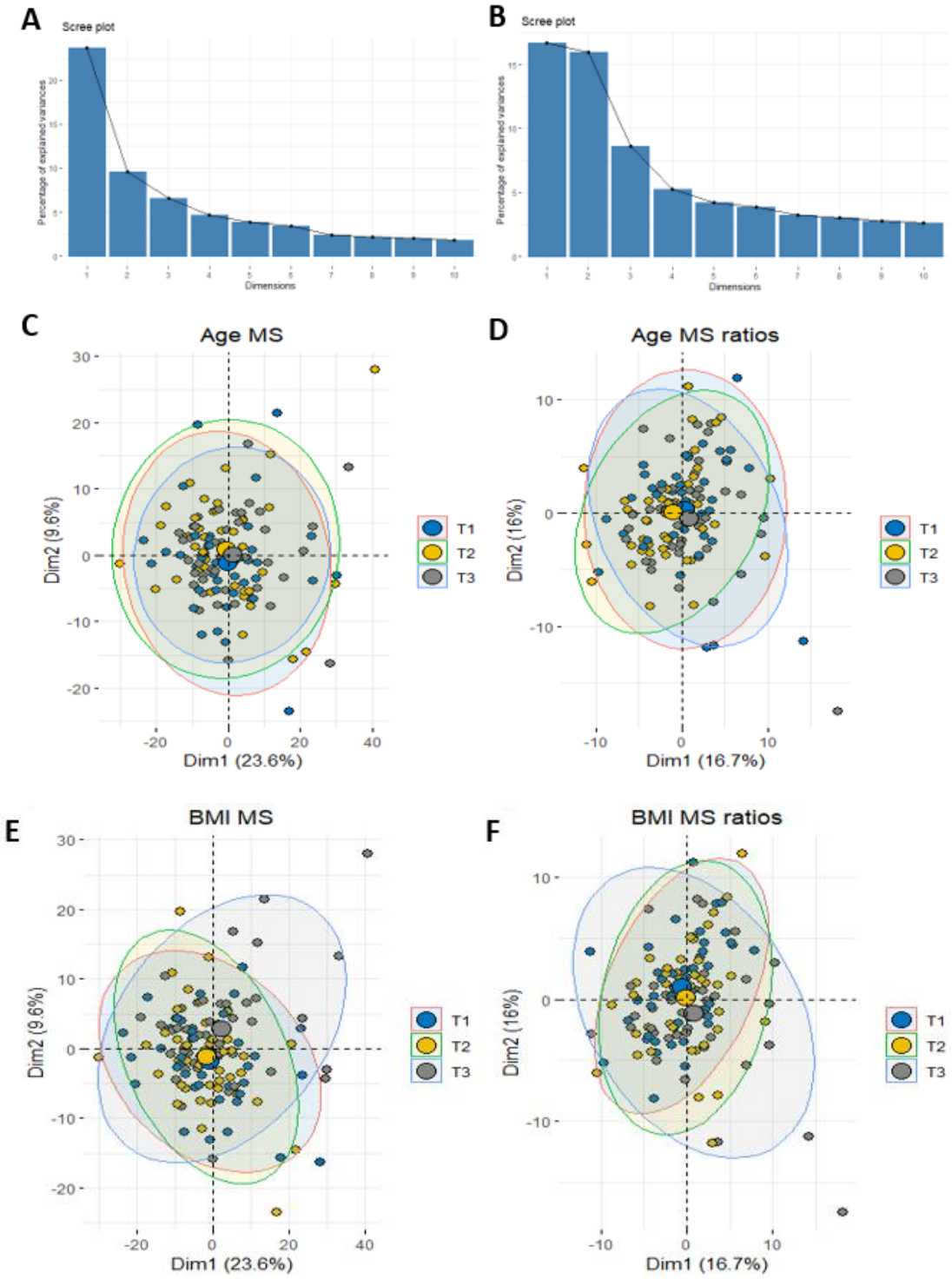

Fig. S8. Principal component analysis results of mass spectrometry metabolomic data depicted by the scree plot of variance of metabolites (Panel A) and functional ratios (Panel B), and the projection of individuals colored by tertiles (T1-3) of age (Panel C and D) and tertiles (T1-3) of body mass index (Panel D and F) for metabolites and ratios, respectively.

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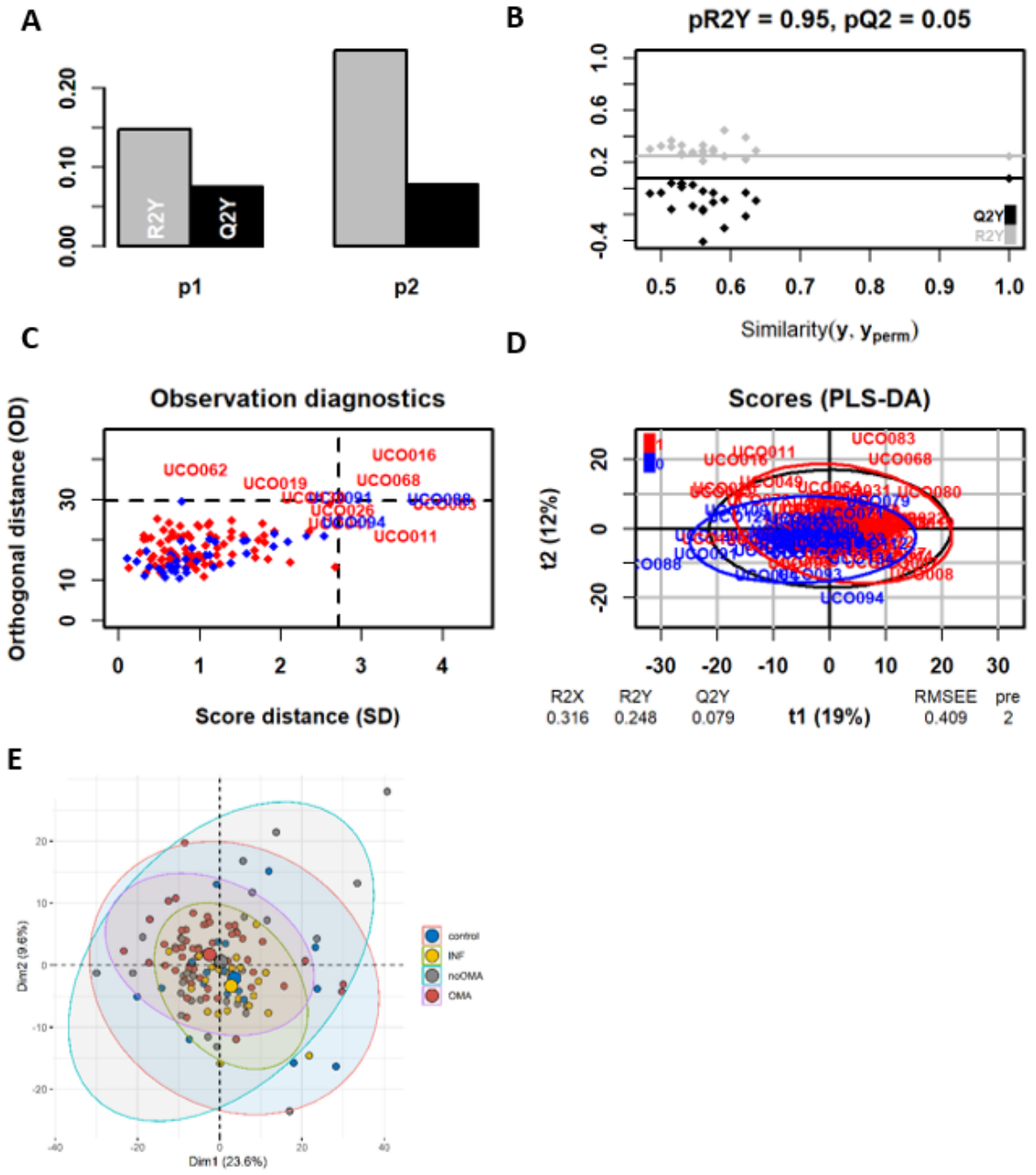

Figure S9. Results from the partial least square discrimination analysis of mass spectrometry metabolomic data (A-D). In this analysis the group infertile without endometriosis was combined in the referent group. Panel E depicts the observations in the first 2 principal components colored by endometriosis subgroup (OMA [endometrioma] and noOMA [no endometrioma]), fertile women (control) or infertile (INF).

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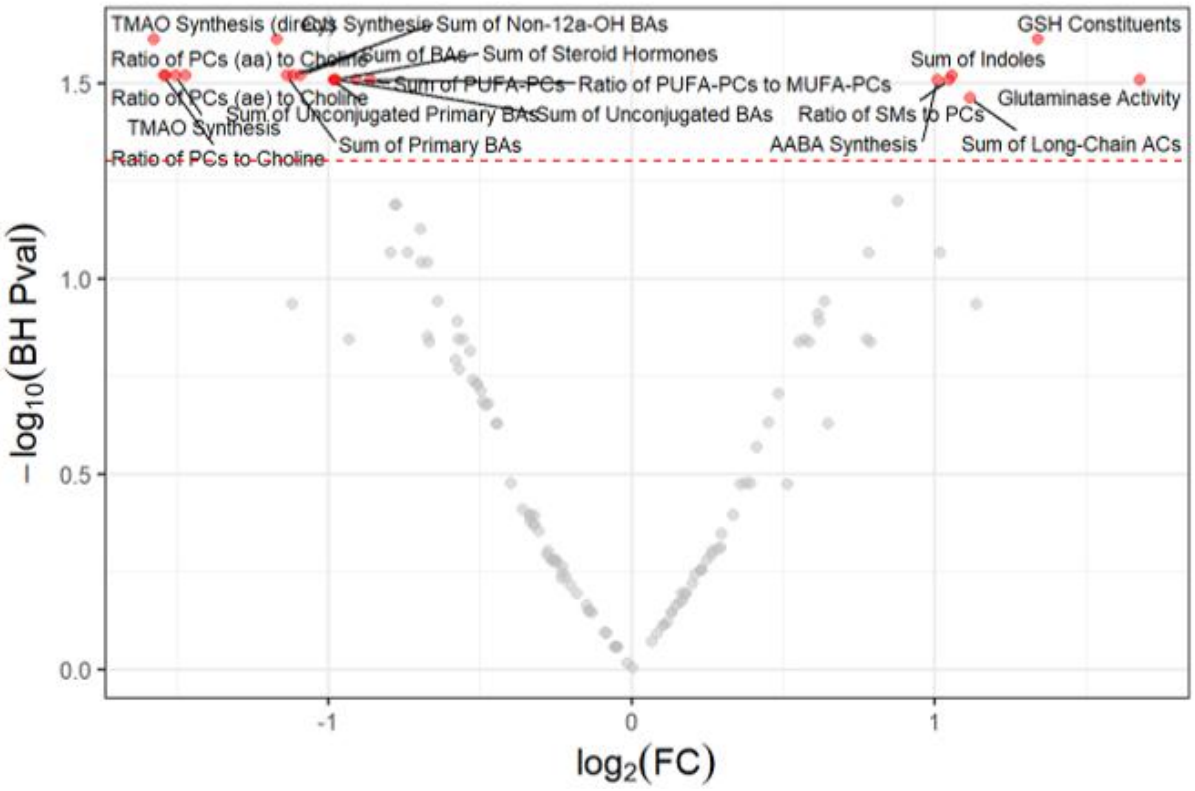

Fig. S10. Volcano plots of mass spectrometry based functional ratios summarizing the statistically significant metabolites associated with endometriosis risk adjusted for age and body mass index. In this analysis the group infertile without endometriosis was combined in the referent group.

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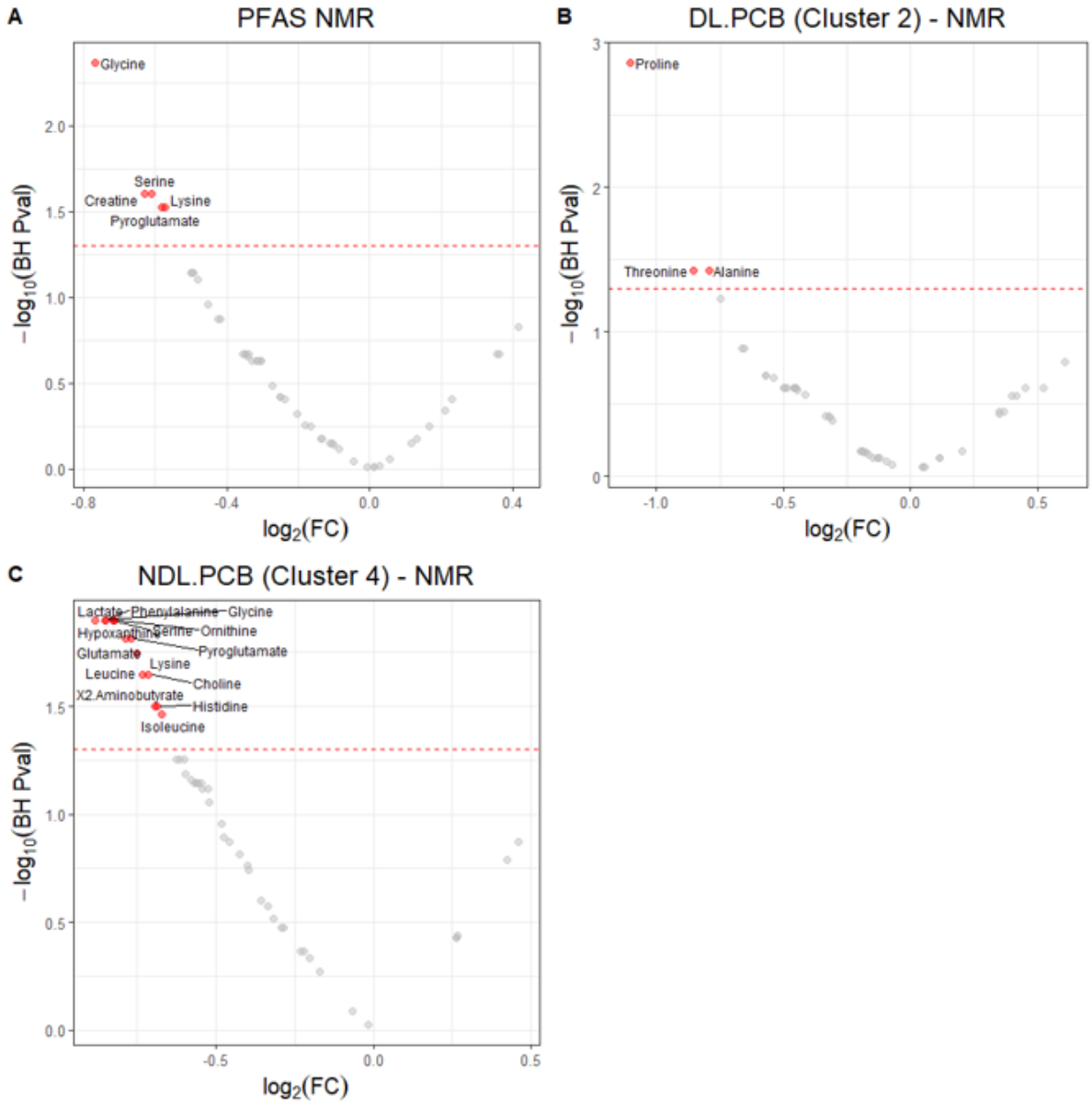

Fig. S11. Volcano plots depicting the associations between 1H-NMR metabolites and the levels of PFAS (A), dioxin-like PCBs (B) and non-dioxin like PCBs (C) from the metabolome-wide association study.

Cluster 1, PFAS : PFHxS, PFOS, PFOA, PFNA, PFDA, PFUnA.
Cluster 2, DL-PCBs mono-ortho-substituted: **PCB105, PCB118, PCB123**.
Cluster 4, NDL-PCBs: PCB28, PCB52, PCB101.

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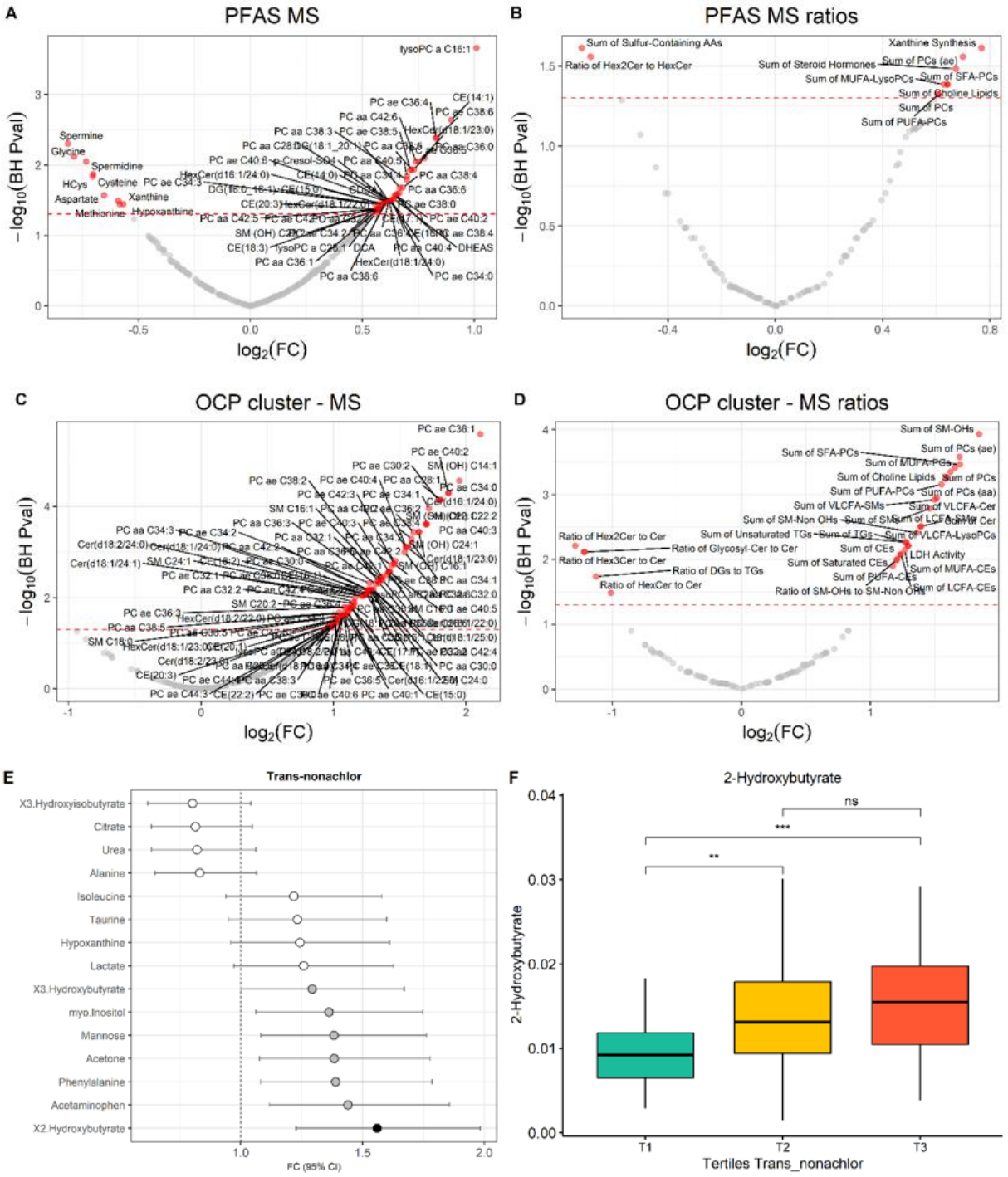

Fig. S12. Volcano plots of differential MS metabolomics analysis for per/polyfluorinated substances cluster levels (Panel A and B) and the cluster of three organochlorinated pesticides (epoxyheptchlor, hexachlorobenzene and β-hexachlorocyclohexane; Panel C and D). Panel E depicts in a forest plot the odds ratios confidence interval on the associations between trans-nonachlor and top ranked ^{1}H-NMR metabolites from multivariate linear regression model adjusted by age and body mass index. Black circle depict significant associations after multiple test correction at q <0.05, and gray circles at q <0.1. The levels of 2-hydroxybutyrate by tertiles of trans-nonachlor are shown in Panel F compared with Mann-Whitney tests. In this analysis, the control group excluded infertile women.

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Table S1. Distributions of POPs depicted by the median and interquartile range.

	LOD	Fertile n=16	Infertile n=22	No Endometrioma n=27	Endometrioma n=50	*p-value
Per/polyfluorinated substances (ng/g serum)						
PFHxS	0.01	0.69 (0.45 - 0.92)	1.03 (0.86 - 1.47)	0.86 (0.58 - 1.33)	0.92 (0.72 - 1.21)	0.07
PFOS	0.01	1.81 (1.28 - 2.08)	2.59 (1.93 - 4.83)	2.21 (1.56 - 3.36)	2.46 (1.68 - 3.49)	0.03
PFOA	0.01	0.95 (0.77 - 1.69)	1.45 (1.20 - 2.14)	1.24 (0.82 - 1.78)	1.22 (0.81 - 1.59)	0.13
PFNA	0.01	0.46 (0.33 - 0.52)	0.61 (0.42 - 0.74)	0.49 (0.37 - 0.67)	0.49 (0.41 - 0.65)	0.25
PFDA	0.01	0.20 (0.17 - 0.24)	0.26 (0.19 - 0.37)	0.22 (0.18 - 0.32)	0.22 (0.18 - 0.31)	0.30
PFUnA	0.01	0.14 (0.09 - 0.18)	0.19 (0.14 - 0.24)	0.15 (0.10 - 0.19)	0.13 (0.10 - 0.18)	0.05
Dioxin-like Polychlorinated biphenyls (pg/g lipid)						
PCB 105	25	720.37 (629.82 - 868.05)	838.04 (611.95 - 1148.21)	910.02 (555.40 - 1114.26)	871.47 (680.18 - 1253.58)	0.45
PCB 114	25	129.44 (104.57 - 172.57)	203.81 (120.67 - 255.99)	199.20 (131.27 - 248.26)	187.26 (128.32 - 255.43)	0.10
PCB 118	25	3257.45 (3028.20 - 4685.22)	4073.11 (2799.58 - 6107.40)	4460.22 (2487.05 - 5412.60)	4199.50 (2942.10 - 6207.49)	0.46
PCB 123	25	60.16 (37.36 - 64.88)	53.56 (34.04 - 87.36)	66.44 (43.32 - 91.16)	60.81 (44.82 - 86.64)	0.55
PCB 156	25	1072.49 (828.08 - 1520.51)	2005.00 (1180.48 - 2574.25)	1692.98 (1023.87 - 2121.82)	1647.59 (1198.79 - 2196.64)	0.04
PCB 157	25	243.06 (181.91 - 302.75)	421.95 (255.07 - 569.34)	363.13 (251.83 - 454.57)	363.41 (268.18 - 484.29)	0.03
PCB 167	25	516.60 (394.35 - 619.56)	802.11 (551.07 - 962.61)	680.40 (423.20 - 892.79)	724.44 (522.72 - 989.83)	0.03
PCB 189	25	144.27 (105.57 - 194.25)	279.52 (176.58 - 367.79)	257.49 (123.88 - 327.83)	225.72 (149.12 - 306.07)	0.04
Non-Dioxin-like Polychlorinated biphenyls (ng/g lipid)						
PCB 28	0.05	0.56 (0.39 - 1.33)	0.56 (0.33 - 1.03)	0.54 (0.42 - 0.74)	0.47 (0.33 - 0.70)	0.61
PCB 52	0.05	0.21 (0.14 - 0.37)	0.17 (0.13 - 0.25)	0.16 (0.12 - 0.21)	0.13 (0.10 - 0.21)	0.04
PCB 101	0.05	0.19 (0.14 - 0.25)	0.18 (0.15 - 0.24)	0.14 (0.11 - 0.22)	0.16 (0.10 - 0.20)	0.05
PCB 138	0.05	7.71 (6.71 - 9.78)	11.52 (7.82 - 15.34)	10.36 (6.60 - 13.21)	10.67 (8.14 - 14.70)	0.14
PCB 153	0.05	14.95 (12.41 - 21.99)	26.30 (17.03 - 32.66)	20.42 (14.35 - 29.47)	22.79 (16.71 - 30.27)	0.10
PCB 180	0.05	9.29 (7.59 - 12.35)	18.29 (12.35 - 22.62)	16.78 (8.47 - 20.53)	15.03 (9.97 - 21.82)	0.07
Organochlorinated pesticides (ng/g lipid)						
PeCBz	0.01	0.81 (0.41 - 1.34)	0.93 (0.50 - 1.36)	0.44 (0.25 - 0.64)	0.47 (0.28 - 0.79)	0.03
HCB	1	43.60 (35.72 - 55.90)	51.38 (42.54 - 63.92)	38.67 (32.06 - 51.52)	43.97 (34.07 - 54.49)	0.04
b.HCH	0.2	17.92 (14.19 - 23.51)	22.11 (16.00 - 35.47)	20.58 (13.63 - 39.82)	22.93 (16.21 - 38.78)	0.26
Epoxyheptachlor	0.2	10.73 (7.62 - 13.13)	9.06 (7.78 - 11.46)	6.91 (6.19 - 10.33)	9.37 (6.40 - 12.24)	0.25
Trans-nonachlor	0.2	1.65 (0.73 - 3.86)	5.10 (0.73 - 12.53)	4.90 (3.53 - 6.74)	6.38 (5.14 - 9.56)	0.01
p,p.DDE	4	112.74 (97.87 - 172.63)	161.53 (126.46 - 245.99)	145.07 (92.10 - 248.37)	168.46 (121.18 - 219.99)	0.15

* The concentrations were compared between groups using the Kruskall-Wallis test.

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Table S2. Summary of top associations between metabolites measured with ^{1}H-NMR and endometriosis risk depicted by the fold-change (FC) and 95% confidence interval (CI) from the regression analysis, and the variable importance in projection (VIP) from the partial least square discriminant analysis (PLSDA).

Metabolites	FC	CI Lower bound	CI Upper bound	p-value	q-value	PLSDA VIP
Phenylalanine	12.45	5.27	35.69	<0.0001	<0.01	1.40
2-Hydroxybutyrate	9.99	4.51	26.52	<0.0001	<0.01	1.48
Lactate	8.59	3.96	22.44	<0.0001	<0.01	1.33
Urea	0.28	0.16	0.45	<0.0001	<0.01	1.53
2.Oxoisocaproate	4.09	2.27	8.23	<0.0001	<0.01	1.19
myo.Inositol	5.54	2.71	13.20	<0.0001	<0.01	1.21
Methionine	3.83	2.14	7.56	<0.0001	<0.01	1.08
Serine	4.55	2.36	9.96	<0.0001	<0.01	1.11
Carnitine	5.20	2.53	12.37	<0.0001	<0.01	1.03
Citrate	0.35	0.20	0.57	0.0001	<0.01	1.23
Taurine	3.01	1.80	5.46	0.0001	<0.01	0.96
Mannose	2.56	1.62	4.35	0.0002	<0.01	1.37
Ornithine	4.94	2.33	12.51	0.0002	<0.01	1.07
Threonine	0.46	0.30	0.69	0.0003	<0.01	1.64
3.Methyl.2.oxovalerate	2.98	1.70	5.70	0.0004	<0.01	1.03
3.Hydroxybutyrate	5.77	2.43	18.47	0.0006	<0.01	1.14
Glutamate	5.01	2.18	14.14	0.0007	<0.01	0.99
Leucine	2.40	1.50	4.19	0.0008	<0.01	1.04
3.Hydroxyisobutyrate	0.47	0.29	0.71	0.0010	<0.01	0.96
Acetoacetate	5.21	2.20	16.54	0.0013	<0.01	1.10
Isoleucine	2.23	1.41	3.78	0.0013	<0.01	1.04
Hypoxanthine	4.55	2.08	13.68	0.0013	<0.01	0.94
Succinate	3.14	1.66	6.76	0.0014	<0.01	0.86
Tyrosine	2.18	1.39	3.64	0.0014	<0.01	1.09
Aspartate	3.56	1.75	8.44	0.0016	<0.01	0.88
Lysine	2.26	1.38	4.03	0.0027	0.01	0.98
Creatine	1.98	1.29	3.23	0.0033	0.01	0.75
N.Acetylglycine	2.04	1.29	3.43	0.0041	0.01	0.80
O.Acetylcarnitine	1.91	1.22	3.18	0.0082	0.01	1.16
Arginine	0.57	0.37	0.85	0.0083	0.01	0.65
2.Hydroxyisovalerate	3.71	1.51	10.80	0.0087	0.01	0.51
Glycine	2.65	1.39	5.88	0.0088	0.01	0.89
Dimethylamine	1.64	1.08	2.61	0.0269	0.04	0.84
Choline	3.03	1.32	9.94	0.0318	0.04	0.92

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Table S3. Summary of top associations between metabolites measured with mass spectrometry metabolites and endometriosis risk depicted by the fold-change (FC) and 95% confidence interval (CI) from the regression analysis, and the variable importance in projection (VIP) from the partial least square discriminant analysis (PLSDA).

Metabolites	FC	CI Lower bound	CI Upper bound	p-value	q-value	PLSDA VIP
3-Met-His	0.57	0.38	0.84	0.0049	0.05	2.05
PC aa C36:5	0.53	0.33	0.80	0.0047	0.05	1.50
Glutamate	1.94	1.29	3.06	0.0025	0.03	2.20
PC ae C42:2	0.52	0.34	0.78	0.0019	0.03	1.63
PC aa C30:0	0.52	0.34	0.77	0.0019	0.03	1.58
PC aa C42:6	0.46	0.28	0.73	0.0016	0.02	1.74
Trigonelline	0.48	0.29	0.73	0.0016	0.02	1.75
CDCA	0.50	0.32	0.75	0.0013	0.02	1.52
PC aa C34:3	0.46	0.28	0.71	0.0011	0.02	1.64
PC ae C34:3	0.43	0.26	0.69	0.0007	0.02	1.87
FA(20:1)	2.09	1.39	3.28	0.0007	0.02	1.94
PC aa C36:2	0.45	0.28	0.69	0.0004	0.01	1.68
PC aa C34:4	0.46	0.29	0.69	0.0003	0.01	1.66
Cortisone	0.43	0.27	0.67	0.0003	0.01	2.01
TMAO	0.43	0.27	0.66	0.0003	0.01	1.95
Hippuric Acid	0.40	0.24	0.63	0.0002	0.01	2.23
Phenylalanine	2.81	1.70	4.98	0.0002	0.01	2.08
Cortisol	0.40	0.25	0.62	0.0001	0.01	2.06
PC aa C36:6	0.39	0.23	0.60	0.0001	0.01	2.06
PC aa C32:2	0.36	0.21	0.58	0.0001	0.01	1.99

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Table S4. Summary of top associations between functional ratios of metabolites measured with mass spectrometry and endometriosis risk depicted by the fold-change (FC) and 95% confidence interval (CI) from the regression analysis, and the variable importance in projection (VIP) from the partial least square discriminant analysis (PLSDA).

Metabolite ratios	FC	CI Lower bound	CI Upper bound	p-value	q-value	PLSDA VIP
TMAO Synthesis (direct)	0.34	0.18	0.57	0.0002	0.02	1.86
Cys Synthesis	0.44	0.27	0.68	0.0005	0.02	1.80
GSH Constituents	2.53	1.55	4.52	0.0006	0.02	1.29
Sum of Primary BAs	0.45	0.27	0.71	0.0011	0.03	1.30
Sum of Non-12a-OH BAs	0.46	0.28	0.72	0.0016	0.03	1.34
TMAO Synthesis	0.35	0.18	0.64	0.0018	0.03	1.70
Sum of Indoles	2.08	1.33	3.43	0.0021	0.03	0.22
Ratio of PCs (aa) to Choline	0.34	0.16	0.64	0.0023	0.03	1.52
Ratio of PCs to Choline	0.34	0.16	0.64	0.0023	0.03	1.52
Ratio of PCs (ae) to Choline	0.36	0.18	0.66	0.0024	0.03	1.54
Sum of BAs	0.47	0.28	0.74	0.0026	0.03	1.25
AABA Synthesis	2.01	1.30	3.28	0.0031	0.03	1.69
Sum of Unconjugated Primary BAs	0.51	0.31	0.78	0.0037	0.03	1.22
Sum of Steroid Hormones	0.51	0.31	0.78	0.0037	0.03	0.85
Ratio of PUFA-PCs to MUFA-PCs	0.55	0.36	0.82	0.0041	0.03	1.75
Sum of PUFA-PCs	0.53	0.34	0.80	0.0043	0.03	1.29
Glutaminase Activity	3.20	1.55	7.62	0.0043	0.03	1.14
Ratio of SMs to PCs	2.07	1.29	3.53	0.0045	0.03	1.28
Sum of Unconjugated BAs	0.51	0.31	0.79	0.0047	0.03	1.22
Sum of Long-Chain ACs	2.17	1.31	3.93	0.0055	0.03	1.26

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