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METABOLOMICS AND LIPIDOMICS TO IDENTIFY BIOMARKERS OF EFFECT RELATED TO EXPOSURE TO NON-DIOXIN-LIKE POLYCHLORINATED BIPHENYLS IN PIGS

Maykel Hernández-Mesa[1,*], Luca Narduzzi[1], Sadia Ouzia, Nicolas Soetart, Laetitia Jaillardon, Yann Guitton, Bruno Le Bizec, Gaud Dervilly*

Oniris, INRAE, LABERCA, 44300 Nantes, France

***Corresponding authors:**

E-mail addresses: laberca@oniris-nantes.fr; gaud.dervilly@oniris-nantes.fr (G. Dervilly) and maykelhm@ugr.es (M. Hernández-Mesa)

ABSTRACT

Recent epidemiological studies show that current levels of exposure to polychlorinated biphenyls (PCBs) remain of great concern, as there is still a link between such exposures and the development of chronic environmental diseases. In this sense, most studies have focused on the health effects caused by exposure to dioxin-like PCBs (DL-PCBs), although chemical exposure to non-dioxin-like PCB (NDL-PCB) congeners is more significant. In addition, adverse effects of PCBs have been documented in humans after accidental and massive exposure, but little is known about the effect of chronic exposure to low-dose PCB mixtures. In this work, exposure to Aroclor 1260 (i.e. a commercially available mixture of PCBs consisting primarily of NDL-PCBs congeners) in pigs is investigated as new evidence in the risk assessment of NDL-PCBs. This animal model has been selected due to the similarities with human metabolism and to support previous toxicological studies carried out with more frequently used animal models. Dietary exposure doses in the order of few ng/kg body weight (b.w.) per day were applied. As expected, exposure to Aroclor 1260 led to the bioaccumulation of NDL-PCBs in perirenal fat of pigs. Metabolomics and lipidomics have been applied to reveal biomarkers of effect related to Aroclor 1260 exposure, and by extension to NDL-PCB exposure, for 21 days. In the metabolomics analysis, 33 metabolites have been identified (level 1 and 2) as significantly altered by the Aroclor 1260 administration, while in the lipidomics analysis, 39 metabolites were putatively annotated (level 3) and associated with NDL-PCB exposure. These biomarkers are mainly related to the alteration of fatty acid metabolism, glycerophospholipids metabolism and tryptophan-kynurenine pathway.

Keywords: hazard identification, metabolomics, polychlorinated biphenyls, mass spectrometry, chemical risk analysis

[1] Present address: Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Av. Fuentenueva s/n, E-18071 Granada, Spain

1. Introduction

The Stockholm Convention sets the goal of reducing and ultimately eliminating the production and release of persistent organic pollutants (POPs), such as PCBs, into the environment due to their toxicity to human health and ecotoxicity (Xu et al., 2013). PCBs comprise a chemical class of 209 congeners consisting of a thermodynamically stable chlorine-substituted biphenyl ring. Two classes of PCBs have been classified according to their toxicological properties, dioxin-like PCBs (DL-PCBs) (n = 12), which have an analogous toxicity to dioxins, and non-dioxin-like PCBs (NDL-PCBs) (EFSA, 2005). About 1,3 million tons of PCBs were produced between 1930 and 1993 for use in various materials and applications due to their physico-chemical properties, including non-flammability, chemical stability, high boiling point, and high dielectric constants (IARC, 2016). Commercial production of PCBs was initially banned by the Toxic Substances Control Act (TSCA) in the United States in 1979 due to their risks for human health, and this prohibition has been subsequently adopted by almost all industrialized countries since the late 1980s. However, PCBs are currently present as environmental pollutants even in the most remote regions of the world (Carlsson et al., 2018)(Kim et al., 2021).

The ubiquitous presence of PCBs in the environment has made their toxic effects a public health concern for a long time because these chemicals are still detected in human samples (Weitekamp et al., 2021). Epidemiologic data suggest that body burdens of DL-PCBs and dioxins are at (or near) the point where adverse health effects may be occurring; therefore, greater efforts are required to reduce exposure to PCBs in order to prevent health (White and Birnbaum, 2009). The main sources of exposure to PCBs are diet, especially fat-containing foods, and indoor air due to the extensive use of PCBs in building materials (Grimm et al., 2015)(Lehmann et al., 2015). In 2005, the European Food Safety Agency (EFSA) indicated that more than 90% of exposure to NDL-PCBs in the general population is related to dietary exposure and estimated that the daily dietary intake of total NDL-PCBs was between 10 and 45 ng/kg b.w. per day (EFSA, 2005). Depending on the context of the study or investigation, specific congeners may be monitored. For instance, the Stockholm Convention on POPS recommends the measurement of six indicator PCBs (PCB28, PCB52, PCB101, PCB138, PCB153, and PCB180) to characterize NDL-PCB contamination. These NDL-PCBs are the most frequently detected and represent 50% of the total PCB concentration. The second French Total Diet Study has shown that mean exposure (95th percentile) to these six indicator PCBs is estimated at 2.7 (7.9) ng/kg b.w. per day in the adult population. Recently, in the French Infant Total Diet Study, the exposure levels to the six indicator PCBs were estimated between 0.87 and 3.53 ng/kg b.w. per day in children between 1 and 36 months of age (Hulin et al., 2020). In the aforementioned cases, it was observed that in some age groups the tolerable daily intake was exceeded. In this sense, tolerable daily intake s of 20 and 10 ng/kg b.w. per day have been established for total PCB exposure and exposure to the six indicator PCBs, respectively (AFSSA, 2007)(Faroon et al., 2003).

The chemical risks of PCBs are related to their persistence, bioaccumulation, and toxicity which depends on the PCB congener. Animal toxicology studies show that PCB mixtures with larger percentages of congeners with higher chlorine content and DL-PCBs carry an increased risk of liver toxicity and disturbance of thyroid function; however, similar results have been obtained for such mixtures and for PCB mixtures with lower chlorine content in immunotoxicity and neurotoxicity assays (Christensen et al., 2021). Although NDL-PCBs are present in a higher proportion in environmental PCB mixtures, risk assessments of PCBs have traditionally focused on the effects of DL-PCB congeners because they generally exert more potent toxic effects (Pikkarainen et al., 2019)(Alarcón et al., 2021). Nevertheless, government agencies such as the EFSA and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) have recently pointed out the need to address the possible adverse health effects associated with exposure to NDL-PCBs, especially in the early life stage (EFSA, 2005)(JECFA, 2016). Recent studies suggest that NDL-PCBs are primarily responsible for the developmental neurotoxicity associated with PCB exposure (Klocke and Lein, 2020). Although their role in occupational hepatotoxicity caused by higher exposure levels has been known for a long time, NDL-PCBs as well as DL-PCB congeners have also recently been associated with an environmental liver disease, specifically nonalcoholic fatty liver disease (Wahlang et al., 2019). In this framework, there is a great concern about the risks associated with environmental and dietary exposure to chemicals with endocrine disrupting properties such as PCBs, as they have recently been identified as one of the main factors to contributing to the rapid increase in the incidence of metabolic diseases such as nonalcoholic fatty liver disease (Heindel et al., 2017). In addition, it is necessary to improve knowledge about the mechanisms by which these environmental exposures induce toxic effects. Thus, later, they can be applied to relevant disease models to determine the importance of chronic environmental exposure to low chemical doses to the initiation and/or progression of disease etiologies (Armstrong and Guo, 2019).

'Omics approaches have recently emerged as interesting alternative methodologies to address the risk assessment of chemicals and involve a shift in the way toxicological studies are conducted, from identifying apical endpoints of toxicity to understanding the mechanisms of toxicity (EFSA, 2014). In this sense, the identification of effect biomarkers by 'omics contributes to reveal the mode of action of chemicals, which encompasses a sequence of plausible biological events in the organism caused by exposure to a chemical hazard and leads to an observed effect (Simon et al., 2014). Although transcriptomics has been the most widely applied 'omics approach in chemical risk assessment, the implementation of proteomics and, especially, metabolomics has experienced increasing interest in the last decade (Pielaat et al., 2013)(Hernández-Mesa et al., 2021). The metabolome is the biological layer closest to the phenotype and the exposure environment, so up- and/or down-regulated metabolites may be directly associated with the effects of chemical exposure. Investigating metabolome disturbances represents a straightforward strategy to assess the biological plausibility of chemicals and establish

their mode of action (Wishart, 2016). In recent years, metabolomics has been explored as an efficient methodology to carry out the risk assessment of a wide range of chemicals (Orešič et al., 2020)(Dai et al., 2020)(Olesti et al., 2021), including environmental contaminants such as PCBs (Shi et al., 2012)(Carrizo et al., 2017)(Pikkarainen et al., 2019)(Deng et al., 2019)(Zhang et al., 2020). In this context, metabolomics is required to not only focus on revealing the mode of action of chemicals, but also address current risk assessment challenges such as effects related to chemical co-exposures and exposures at low dose levels (Hernández-Mesa et al., 2021). Many toxicological studies for risk assessment of PCBs involved exposure doses that imply obvious toxicity; therefore, the results are only representative in human populations after accidental and massive exposure (Ulbrich and Stahlmann, 2004). Metabolomics provides the advantage of revealing early biomarkers of effect that may be related to an adverse response of the body to low-dose chemical exposure scenarios, and which manifest before visible toxicity. Consequently, metabolomics makes it possible to detect the presence or absence of an effect even when the latter goes unnoticed by other toxicological methods (Pielaat et al., 2013)(Viant et al., 2019)(Hernández-Mesa et al., 2021).

The objective of this study is to identify biomarkers of effect associated with PCB exposure at dietary exposure levels. Previous animal toxicology studies applying metabolomics have typically evaluated the effects of PCB exposure using mice as animal model (Shi et al., 2012)(Petriello et al., 2018)(Deng et al., 2019)(Lim et al., 2020). Although less used for obvious reasons of infrastructure requirements and associated costs, the pig is recognized as a relevant animal model for the study of endocrine disruptors (Yang et al., 2020). In addition to a lifespan that allows for significant accumulation of environmental pollutants, it shows phylogenetic, physiological, nutritional, and pathological similarities with humans. Therefore, it is increasingly used in toxicology and biomedical research. Therefore, this work proposes a combined metabolomics-lipidomics approach to investigate PCB exposure in pigs as new piece of evidence for PCB risk assessment. To our knowledge, it is the first time that metabolomics/lipidomics has been applied to the discovery of biomarkers of effect related to PCB exposure in pig serum. In addition, an exposure dose of 20 ng/kg b.w. per day of a 'PCB cocktail', consisting primarily of NDL-PCBs (Aroclor 1260 mixture), was selected as an approach to investigate the effects on the metabolism caused by exposure to NDL-PCBs at dietary exposure levels according to the second French Total Diet Study outcomes (Sirot et al., 2012). Aroclor 1260 was selected for this study because its composition best mimics the bioaccumulation of PCBs found in human adipose tissue (Wahlang et al., 2014).

2. Material and methods

2.1 Materials and reagents

All reagents and solvents used in this study were of analytical grade unless otherwise specified. Acetonitrile (MeCN), methanol (MeOH), isopropanol (IPA), acetic acid, and ammonia were supplied

by Honeywell (Bucharest, Romania). Ultra-pure water was acquired from VWR (Fontenay-sous-Bois, France), while chloroform was purchased from Carlo Erba Reactifs (SDS, Peypin, France). Ammonium acetate salt (Emsure grade) was purchased from Merck (Darmstadt, Germany). Metabolomics isotope-labeled internal standards (L-leucine-5,5,5-d3, L-tryptophan-2,3,3-d3, indole-2,4,5,6,7-d5–3-acetic acid, and 1,14-tetradecanedioic-d24 acid) were from Sigma–Aldrich (Saint Quentin Fallavier, France) and from CDN Isotopes (Québec, Canada). Lipidomics internal standards [(LPC (15:0), PC (15:0/15:0), and TG (17:0/17:0/17:0)] were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). MSCAL6 ProteoMass LTQ/FT-Hybrid standard mixtures used for calibration of the MS instrument were obtained from Sigma–Aldrich (Saint Quentin Fallavier, France). Aroclor 1260 (certified reference material, 1000 µg/mL in isooctane) was supplied by Sigma–Aldrich (Saint Quentin Fallavier, France).

2.2 Animal experimental design

Six 4-month-old female pigs (Terrena, France) weighting 29.8 ± 2.3 kg were randomly assigned to control (n = 2 animals) and exposed (n = 4 animals) groups. The animal experiment was carried out for 32 days and consisted of three different stages (i.e. periods of acclimatization, exposure, and detoxification), as shown in **Figure 1**. During the exposure period, exposed pigs received orally a daily dose, of Aroclor 1260 (20 ng/kg b.w.) in 20 mL of sunflower oil whereas a 20 mL placebo of sunflower oil was administrated orally to the control group. Aroclor 1260 is a mixture of highly chlorinated PCBs (60% chlorine by weight) that contains 30.7% by weight of the six NDL-PCBs known as the six indicator PCBs (i.e. PCB28, PCB52, PCB101, PCB138, PCB153 and PCB180) (Rushneck et al., 2004). The exposure dose selected for this study (6.1 ng/kg b.w. per day 6 NDL-PCBs) was based on the observed P95 exposure level of the French population to the six PCB indicators in the second Total Diet Study (i.e. 7.9 ng/kg b.w. per day) (Sirot et al., 2012). This exposure level is also close to but slightly lower than the tolerable daily intake of the 6 NDL-PCBs (i.e. 10 ng/kg b.w. per day) (AFSSA, 2007)(Faroon et al., 2003).

Blood samples from control and exposed pigs were collected on days (D) 2, 4, 8, 11, 16, 19, 22, 26, 29 and 32. Animals were euthanized just after the last blood sampling point and several tissues and organs, including perirenal fat, were recovered for further investigation. The blood samples were allowed to clot at room temperature, recovering the serum part by centrifugation. Aliquots of serum samples were subsequently stored at -80°C.

The animal study was approved by the French Ethical Committee (n°6) under project agreement APAFIS#15159-2018051920446340 v2 (ONIRIS agreement E44271).

2.3 Sample preparation

The extraction of metabolites and lipids from serum samples was performed with a biphasic solvent system [(1) MeOH + water and (2) chloroform] (Peng et al., 2017). Briefly, 30 µL of serum were extracted with 190 µL of cold MeOH containing the metabolomics isotope-labeled internal standards (1 µg/mL), 390 µL of cold chloroform containing the lipidomics isotope-labeled internal standards (1 µg/mL) and 120 µL of pure water. The samples were vigorously vortexed and centrifuged at 3500 g for 20 minutes at 4 °C. For metabolomics and lipidomics analyses, 95 µL of the upper or aqueous phase (MeOH + water) and 200 µL of the chloroform phase were collected, respectively. Pooled quality control (QC) samples (i.e. a mixture of aliquots from the entire sample set) and extraction blanks (water samples) were extracted and processed as the serum samples.

2.4 UHPLC-HRMS analysis

Metabolomics and lipidomics analyses were carried out on an Ultimate® 3000 Series HPLC system coupled to a hybrid quadrupole-Orbitrap (Q-Exactive™) mass spectrometer (ThermoFisher Scientific, Bremen, Germany) equipped with a heated electrospray (H-ESI II) source. The HRMS instrument was set in dual polarity (positive/negative) acquisition mode. Metabolomics analyses were performed on a Hypersil Gold C18 column (2.1 × 100 mm, 1.9 µm particle size; Thermo Fisher Scientific) coupled with the corresponding guard column, whereas lipidomics analyses were performed on an Acquity® CSH C18 (column (2.1 × 100 mm, 1.7 µm particle size; Waters, Manchester, UK) coupled with the corresponding guard column. For metabolomics analyses, chromatographic conditions, ESI source conditions and MS tuning parameters were the same as previously reported (Peng et al., 2017). For lipidomics analyses, a previously described non-targeted UHPLC-HRMS workflow was selected (Marchand et al., 2021). For either metabolomics or lipidomics analysis, samples were randomized and divided into three batches for analysis. Data acquisition was carried out following the quality assurance (QA) plan described in the Supplementary Material.

QC samples were also submitted to data-dependent acquisition (DDA) to generate fragmentation spectra of the five most intense peaks per scan. For lipidomics, DDA experiments were replicated 3 times for each polarity, providing an exclusion list of peaks already fragmented in the previous analysis, to obtain fragmentation data of more chromatographic peaks. For metabolomics, selected reaction monitoring mode was also applied to target the features highlighted by the statistical analysis as potential biomarkers.

2.5 Data preprocessing

LC–HRMS raw data files were initially preprocessed with Xcalibur 2.2 to check the analytical performance of the method, evaluating retention time and signal intensity of internal standards. The raw (*.raw) files were converted to *.mzML format and polarity split using MSConvert (Kessner et al., 2008). The *.mzML files were subsequently uploaded to the online collaborative research resource Workflow4Metabolomics (W4M) (Guitton et al., 2017). Peak picking, grouping of chromatographic

peaks within and between samples, retention time alignment, and peak filling were applied through the XCMS R package (Smith et al., 2006) within the LC-MS workflow of the W4M platform. In general, the default parameters were applied. 'CentWaveWith-PredIsoROIs' was selected as the extraction method for peak detection, and 'PeakDensity' was used for peak grouping.

The data matrices generated on the W4M platform were uploaded to the NOREVA platform for data filtering, imputation of missing values, QC sample correction and normalization (Li et al., 2017). Variables (or peak features) were considered only when they were detected in 80% of the QC samples and a bias-variance tradeoff of 75% for signal correction was applied. NA values were transformed to the mean value of the 'k'-neighbors found in the datasets (KNN algorithm). Batch correction was performed using local polynomial fits, while normalization was achieved by applying the EigenMS algorithm (Karpievitch et al., 2014). Furthermore, a time 0 centering (T0-centering) was also applied as previously proposed (Narduzzi et al., 2020) to evaluate the time-trends of the variables.

2.6 Statistical analysis

Analysis of Variance (ANOVA)-Simultaneous Component Analysis (ASCA) was performed with the MetStaT package (Smilde et al., 2005) in R environment (R Development Core Team, 2008). The datasets were subsequently explored with the SIMCA-P 13.02 software (Umetrics, Umea, Sweden), applying mean centering and Unit-Variance (UV) scaling to all variables. Unsupervised Principal Component Analysis (PCA) and supervised (Orthogonal) Partial Least Squares-Discriminant Analysis [(O)PLS-DA] were investigated as discriminant models. The validation and robustness of each model were evaluated by R2X (cum), R2Y(cum) and Q2(cum) parameters, cross validation-analysis of variance (CV-ANOVA), permutation tests and misclassification test. Variable Importance in Projection (VIP) score greater than 1.5 was established as threshold. Additionally, heatmap analysis was performed using the "heatmap.plus" package (Day, 2015), using "euclidean" as distance function and "ward.D2" as clustering algorithm in R environment.

2.7 Metabolite annotation

First, the relevant features were compared with the CAMERA groups (Kuhl et al., 2012) obtained on the W4M platform to remove possible isotopes and adduct peaks. Subsequently, tentative identification was carried out by comparing the relevant features with an internal database of 500 metabolites analyzed under the same analytical conditions, applying an in-house developed script (Narduzzi et al., 2018) for matching with a tolerance threshold of 5 ppm and 30 seconds for *m/z* and retention time, respectively. Matches were confirmed by injection of the metabolite standards and comparison of MS^2 spectra; therefore, level 1 annotation was considered for these metabolites according to the confidence levels for compound annotations as recently proposed by the Compound Identification work group of the Metabolomics Society (Blaženović et al., 2018). The 'remaining' features were putatively annotated by interrogating MS^2 spectra with SIRIUS 4.0 (Dührkop et al.,

2019). Metabolites that showed a high agreement with a single molecular structure were annotated as level 2. In this sense, published literature was reviewed to consider only those molecular candidates capable of explaining the biological plausibility of exposure to PCBs. Metabolites were annotated as level 3 when only a probable structure could be assigned to the metabolite (e.g. molecules with a wide range of possible isomers were annotated as level 3). Finally, those features with no MS/MS spectra match were investigated in Metlin (Guijas et al., 2018) and HMDB (Wishart et al., 2018) to annotate metabolites based simply on their accurate mass. These metabolites were annotated as level 4. Furthermore, features with an accurate mass that could only provide a single chemical formula were also annotated as level 4. The annotation of lipids was achieved by interrogation of MS^2 spectra with MS-Dial ver. 4.24 (Tsugawa et al., 2020), and assigned as levels 3 to 4 after manual confirmation.

In addition, and to provide more confidence in the annotated metabolites, their octanol/water partition coefficient (log P) was investigated to evaluate their fit in a simple linear regression curve built with information from our in-house library. This was a tentative approach to exclude annotated metabolites that were clearly outliers in the 'log P vs retention time' trend; therefore, their annotation was probably incorrect based on the observed retention time for the related feature (Kaliszan, 1992).

The "Pathway Analysis module" included in the web-tool MetaboAnalyst 4.0 was used to identify the metabolic pathways more affected by exposure to Aroclor 1260 according to the biomarkers of effect identified (Chong et al., 2018).

2.8 Bioaccumulation of PCBs caused by Aroclor 1260 exposure

The bioaccumulation of DL-PCBs and NDL-PCBs in the pigs during the entire period of animal experimentation was investigated by respective analysis of the 12 DL-PCBs and the 6 PCB indicators (i.e. PCB28, PCB52, PCB101, PCB138, PCB153 and PCB180) in perirenal fat, which was recovered in the euthanasia of the animals. The samples were analyzed by gas chromatography (GC)-HRMS applying an analytical method already implemented in our laboratory (Vaccher et al., 2020). In order to evaluate whether the bioaccumulation of PCBs in the perirenal fat between exposed and control groups was statistically significant, F-test (for equality of variance) and T-test (for equality of means) were performed in Microsoft® Excel® 2013 included in the Microsoft Office Professional Plus 2013 software package.

3 Results

3.1 Body weight development and general observations

In general, no visual observation allowed to indicate significant differences between the control and exposed pigs. Animals in both groups were weighted on the same day that serum sampling was carried out to monitor growth and the possible impact of PCBs exposure on it. The animals weighed 30 ± 2 kg at the beginning of the experiment (D2) while they weighed 53 ± 1 kg at the end of the experiment

(D32). Weight gain was consistent for the experimentation period and the animal species according to the animal handlers (i.e. 22 ± 2 and 23.2 ± 0.8 kg for the control and exposed animals, respectively); therefore, no significant differences were observed within both groups of animals.

The mean concentration levels of DL-PCBs in the perirenal fat for control and exposed groups were not statistically different for a 95% confidence level (i.e. 0.0915 ± 0.0007 and 0.09 ± 0.02 ng DL-PCBs/kg of fat weight, respectively). On the contrary, statistical differences were observed for NDL-PCBs (p-value < 0.05), with mean concentration levels of 0.3 ± 0.1 and 1.2 ± 0.1 ng NDL-PCBs/kg of fat weight in the perirenal fat of control and exposed animals, respectively. In the latter case, the f-test indicated that the variance for both groups was statistically equivalent. These results are in accordance with our expectations, as Aroclor mixtures mainly consist of NDL-PCB congeners (98%) (Klocke and Lein, 2020).

3.2 LC–HRMS data

Serum samples were analyzed applying traditional non-targeted LC-HRMS workflows, resulting in four datasets: two datasets from metabolomics analysis of serum samples under ESI+ and ESI- conditions, and another two datasets from lipidomics analysis applying both ionization conditions. After data deconvolution, 1813 and 1731 features were obtained for metabolomics analysis in positive and negative ionization mode, respectively. In the case of lipidomics, 3624 and 1450 features were detected in ESI+ and ESI- mode, respectively.

After data pre-processing, metabolomics datasets (ESI+ and ESI- mode) consisted of 725 and 1731 variables, respectively, while 3561 and 1439 variables were contained in lipidomics datasets (ESI+ and ESI- mode, respectively). In general, the number of variables in the datasets corresponds to the number of features detected by XCMS deconvolution, except for the metabolomics ESI+ dataset. In this case, less than half of the detected features remained as variables after data pre-processing. It was directly related to the fact of an observed depletion of signal intensity during batch-to-batch data acquisition.

3.3 General data exploration

In our experimental design, two groups of animals (i.e. control and exposed pigs) and three experimental stages (i.e. periods of acclimatization, exposure, and detoxification) were established. In total, ten blood samples were collected per animal throughout the investigation period as indicated in Figure 1, and all of these samples were included in further metabolomics studies. Since the animals in both groups were under the same experimental conditions during the acclimatization and detoxification stages, the samples from the datasets were divided into four different observation classes for initial data exploration (i.e. ‘acclimatization’, ‘detoxification’, ‘exposed’ and ‘control’ groups). Neither non-supervised (i.e. PCA) nor supervised (i.e. PLS-DA) multivariate analysis

provided separation of the four groups. However, preliminary results showed that statistical separation of groups was possible when data from the 'detoxification' group were included in 'exposed' and 'control' groups according to the animal to which the serum sample belonged to. In this context, three classes of samples belonging to 'acclimatization' (n = 12 observations), 'exposed' (n = 32) and 'control' (n = 16) groups were established for further statistical exploration of the data.

Furthermore, ASCA analysis indicated that the intrinsic biological difference of each animal in our experiment was one of the main sources of variance in all metabolomics and lipidomics datasets ('subject' factor, **Table 1**). In contrast, 'exposure' (or not) to Aroclor 1260 was not a factor by itself that explains the variance observed in the datasets. However ASCA analysis also highlighted that the interaction between the 'subject' and 'exposure' factors was significant, indicating that there was a subject-specific effect of the treatment.

To overcome the masking effect of the inter-individual variability, T0-centering was applied to all datasets to address inter-individual differences and highlight differences between groups. Using metabolomics data acquired under ESI- conditions as an example, **Figure 2** shows how the groups are clearly separated after T0-centering when PCA is performed, while the differences between them are masked before T0-centering. After T0-centering, it can be observed how the individual variability for D2 and D4 is reduced; thus, leading to the grouping of samples from the acclimation period (**Figure 2.b**). A subsequent representation of the appropriate principal components on the score plot of the PCA model (**Figure 2.c**) visually highlights the differences between samples based on animal biology and the presence or absence of exposure to Aroclor 1260. In this case, the PCA model consisted of eight principal components and while the first principal component of the model (*y*-axis) remarks the biological differences existing in the animals of each group, the fourth principal component (*x*-axis) highlights the differences in the samples due to exposure to Aroclor 1260. A similar pattern was observed for the other datasets as indicated in Supplementary Material (**Figures S1-S3**). Applying this approach, and in addition to the separation of the different groups, clustering of the samples of singular individuals was also observed.

Significant differences in features in the datasets for the three group classes were shown in clustering heatmap. **Figure 3** shows the differences observed for features detected in lipidomics analysis under ESI+ conditions. This preliminary non-supervised analysis allowed to confirm the clustering of samples from 'control', 'exposed' and 'acclimatization' groups, respectively. Furthermore, samples from the same individual also clustered together except for one of the subjects from the 'exposed' group, confirming the importance of the biological status of each subject in our datasets. In addition, the time factor demonstrated to not have any relevance in our datasets since no clustering from samples from the sampling day was observed.

3.4 Discriminant models to highlight biomarkers of effect

Since the previous results showed differences in the groups due to exposure to Aroclor 1260, PLS-DA models were built to highlight relevant features that could represent biomarkers of effect associated with said chemical exposure. The three groups considered in our datasets were separated in all cases (**Figure 4** and **Figure S4**). CV-ANOVA showed that the four PLS-DA models are statistically significant (p-value < 0.05), while the values of the R2Y(cum) and Q2Y(cum) were always ≥ 0.649 and ≥ 0.405, respectively, demonstrating the robustness of the models (**Table 2**). Permutation tests consisting of 100 permutations were also carried out for each PLS-DA model and for the 'control' and 'exposed' groups, confirming that the models are not the result of a random factor and that they offer a valid and robust discrimination between control and exposed populations. Furthermore, a misclassification test was performed on each PLS-DA model obtaining a classification accuracy greater than 91.7%, with two of the four models correctly assigning the classes to all samples (**Table 2**).

Subsequently, VIP-plots of each PLS-DA model, including all components (2 or 3 components according to the PLS-DA model) were investigated to highlight the relevant features that differentiated the classes in the statistical models. Features with VIP values > 1.5 in any of the model components were retained as possible biomarkers related to exposure to Aroclor 1260. In total, 129 and 276 features were retained from metabolomics datasets (ESI+ and ESI- conditions, respectively), while 589 and 240 features were retained from lipidomics datasets (ESI+ and ESI- conditions, respectively). These features were considered of interest for our study since they represented the variables responsible for the separation of the groups in the PLS-DA projection. These relevant features were investigated against CAMERA groups to remove isotopes or adducts and considering only protonated ions for subsequent metabolite annotation. Features that CAMERA noted as adducts or isotopes were removed from the list of relevant features when their related protonated ions showed VIP values < 1. If their VIP values were greater than 1 for any of the model components, the adducts and isotopic features were replaced by the protonated ion feature in the list of relevant features. The number of aforementioned features was reduced by 11.6 and 33.6 % after relevant features selection, specifically referring to the 'metabolomics ESI+' and 'lipidomics ESI+' datasets, respectively.

Finally, OPLS-DA models were built to confirm that the selected features differentiated the 'control' and 'exposed' groups and to generate S-line plots to further establish whether the annotated metabolites were down- or up-regulated in pigs exposed to Aroclor 1260 (**Figures S5-S6**). The CV-ANOVA of the four OPLS-DA models indicated that they are statistically significant (p-value < 0.05), while R2Y(cum) (> 0.94) and Q2(cum) (> 0.887) parameters showed that the data fit well to the models as well as their high degree of classification (**Table S1**).

3.5 Metabolite annotation

Table 3 shows all the metabolites annotated as level 1 or 2, while the relevant features found by metabolomics and annotated either as level 3 or 4 are included in **Tables S2-S4**. Lipids were only annotated as level 3 as the maximum confidence level for the annotation due to the wide range of isomeric lipids present in nature and the little structural information from our experiments for their unequivocal annotation at a higher level of confidence (**Table S5-S7**).

Finally, in the 'metabolomics' datasets, 9, 24, 18, and 105 metabolites were annotated as level 1, 2, 3 and 4, respectively. Although metabolite annotation is time-consuming and, in general, most of the relevant of features remain unidentified when performing metabolomics studies, a great effort was made to annotate as many metabolites as possible. As consequence, up to 44.7 % of the features observed as relevant in Section 3.4 were annotated at any of the annotation levels considered in this study. In the case of 'lipidomics' datasets, up to 39 and 55 lipids were putatively annotated with an annotation confidence level of 3 and 4, respectively. The annotated lipids represented only 16.1% of the features highlighted as relevant variables in the discriminant models discussed in the previous section. This highlights the main drawback of metabolomics and lipidomics approaches which is metabolite annotation.

Biomarkers of effect, previously identified or putatively annotated, were investigated using the MetaboAnalyst 4.0 pathway analysis (Chong et al., 2018), showing that lipid metabolism was significantly affected by exposure to Aroclor 1260 (**Figure 5**). In this sense, for example, lipid-lipid correlation analysis has shown relevant negative correlations between lysophophatidylcholines (LPCs) [i.e. LPC (16:0) and LPC (18:0)] and phosphatidylcholines (PCs) [i.e. PC (35:2) and PC (37:4)] for exposed animals, which have not been observed for control animals (**Figure S7**).

4. Discussion

The pig was selected as an animal model due to the comparable physiology of pigs to that of humans, making it an ideal model to address chemical risk assessment for human health (Goldansaz et al., 2017). Both groups of animals showed similar bioaccumulation of DL-PCBs related to unknown environmental and dietary exposures, while significant bioaccumulation of NDL-PCBs caused by exposure to Aroclor 1260 was observed in the perirenal fat of the exposed animals compared to the control group. Therefore, and in the framework of this study, the possible disturbances observed in the metabolism of the pigs caused by exposure to Aroclor 1260 are attributed to NDL-PCBs.

The general data exploration highlighted that the inter-individual variability masks the effect of the exposure to low doses of NDL-PCBs. This fact reflects one of the main risks of toxicological metabolomics studies involving low doses of exposure. Chemical exposure cannot show a clear impact on the metabolism because the subject variability masks the effect of the treatment. There are some strategies to overcome this limitation, but given the limited number of samples, we selected the most basic approach: T0-centering. This method makes it possible to follow the fate of the variables over

time. Thus, if the fate of the variables varies in the different groups (control vs. exposed), it means that there is a difference in their metabolism between them. The results clearly show that this approach highlighted several features with a different fate between the groups, indicating a change in their metabolism due to the exposure to Aroclor 1260.

Our study is a first approach to evaluate the consequences of exposure to NDL-PCBs in pigs at realistic exposure levels (in the order of few ng/kg b.w. per day), at which no observable toxicity is expected. There was great uncertainty at the time of planning the animal experimentation as to whether the metabolism of pigs would be altered by such low levels of exposure to NDL-PCBs or if these alterations would have any toxicological relevance, while the selection of a greater number of animals for this first approach was not exempt of greater economical and ethical costs. In this sense, current ethical standards in animal experimentation require replacing, refining and reducing the use of animals in scientific research and testing as much as possible (3R principles) (Scholz et al., 2013). In this context where only six animals were included in the animal experiment. We preferred to unbalance the experiment towards the exposed group to reduce the odds of missing biomarkers (reduce the false negative ratio). Certain limitations can be attributed to the present study due to the low number of animals included in the experimentation which might undermine the validity of the biomarkers found. Therefore, as discussed below, the main results obtained in our study have been compared to previous toxicological and epidemiological studies that include a greater number of individuals under study to give a biological explanation of the biomarkers, strengthening their validity. Nevertheless, we are aware that a complete validation will require further experimentation to confirm or discard these biomarkers. Indeed, taken singularly, none of the metabolic markers identified in this experiment are unique to Aroclor 1260 exposure. The strength of this experimentation is the fact that, through a multi-marker approach, it was possible to identify a metabolic profile uncommon in young pigs, which is generally associated with long-term disease development. This study demonstrates that such risks of disease development are associated with environmental exposure to chemicals as NDL-PCBs at low doses, as discussed below. Linoleic acid metabolism, glycerophospholipid metabolism, and arachidonic acid metabolism were the metabolic pathways more impacted by this chemical exposure.

PCB exposure has previously been associated with glucose and lipid metabolic disorders in the liver, which can lead to chronic systemic metabolic disorders such as obesity, type 2 diabetes, fatty liver disease, cardiovascular disease, and cancer (Shan et al., 2020). Serum lipids have also been shown to be disturbed by PCB exposure, causing dysregulation of cholesterol synthesis and degradation mechanisms (Hennig et al., 2005). Among the lipids tentatively annotated in this work, glycerophospholipids and specifically glycerophosphocolines and glycerophosphoethanolamines, which are involved in lipid metabolism and regulation, are the main classes of lipids disturbed by exposure to NDL-PCBs. Previous research has already shown disturbances in glycerophospholipid

levels in serum and plasma samples from humans exposed to POPs, including PCBs (Carrizo et al., 2017)(Walker et al., 2019). Glycerophospholipids are involved in the formation of the cellular membranes of all organisms and organelles within cells, as well as in cell signaling systems and as an anchor for proteins in cell membranes. They are also involved in the transport of triacylglycerols and cholesterol in the body (Blanco and Blanco, 2017)(Carrizo et al., 2017)(Triebl, 2019). Important metabolome alterations, mainly related to glycerophospholipid levels in serum, have recently been reported in rat offspring after in utero and lactational exposure to PCB 180, which is a NDL-PCB congener and one of the most abundant in the environment (Pikkarainen et al., 2019). Furthermore, a generalized increase in glycerophospholipid levels has also been observed in rat pheochromocytoma PC12 cells exposed to PCB 153, which is also a NDL-PCB congener (Wang et al., 2019).

Within the group of glycerophosphocolines, and as observed in our study, it has been found that LPCs are the main biomarkers of effect in the serum of mice exposed to diethylhexylphthalate (DEHP) and Aroclor 1254 at doses higher than environmental exposure levels (Zhang et al., 2012). Either in mice (Zhang et al., 2012) or in pigs (as shown in this work), animals exposed to Aroclor mixtures show increased concentration levels of LPC (16:0) and LPC (18:0) in serum in comparison to control individuals. Increased plasma levels of LPCs are related to cardiovascular diseases, diabetes, ovarian cancer, and renal failure (Law et al., 2019). These findings indicating an impact on LPC levels associated with NDL-PCB exposure are in line with previous studies. They have linked cardiometabolic diseases and exposure to endocrine disrupting compounds, such as PCBs, where LPC metabolites have been suggested as mediators in those events (Salihovic et al., 2016). LPCs result from the cleavage of PCs through the action of phospholipase A2 (PLA2) and/or by the transfer of fatty acids to free cholesterol through lecithin-cholesterol acyltransferase. LPCs can be converted back into PCs by the action of the enzyme lysophosphatidylcholine acyltransferase in the presence of Acyl-CoA (Law et al., 2019). These metabolic processes are part of the Lands' cycle, which in addition to the Kennedy pathway and the phosphatidylethanolamine N-methyltransferase pathway constitute the synthesis pathways of PCs (Moessinger et al., 2014). In this framework, the negative correlations between LPC (16:0) and LPC (18:0) with PC (35:2) and PC (37:4) for exposed animals indicate a probable disturbance of the Lands' cycle. This hypothesis is also supported by the identification at level 1 of arachidonic acid as a biomarker of effect of exposure to NDL-PCBs. Furthermore, in the present work, several sphingomyelins, which belong to the sphingolipid class, have also been determined as potential effect biomarkers of Aroclor 1260 exposure. Sphingomyelins also participate in PLA2 activity (Rodriguez-Cuenca et al., 2017), which reinforces our hypothesis on the alteration of Lands' cycle caused by exposure to NDL-PCBs at environmental dose levels.

As mentioned above, PLA2s hydrolyze the sn-2 ester bond of cellular phospholipids, producing LPCs and free fatty acids, frequently arachidonic acid, which is the precursor to the eicosanoid family of potent inflammatory mediators (Balsinde et al., 2002). Activation of PLA2s and increased arachidonic

acid levels caused by exposure to NDL-PCBs have also been previously reported in rat cells and human platelets (Brant and Caruso, 2006)(Forsell et al., 2005). Increased levels of arachidonic acid are associated with inflammatory processes that, even at low-grade levels, can induce metabolic and cardiovascular diseases (Sonnweber et al., 2018). Free arachidonic acid also induces oxidative stress, which is a relevant factor in the development of hepatic steatosis (Sonnweber et al., 2018). Hepatic steatosis is an hepatic disorder that can lead to the development of nonalcoholic fatty liver disease, which has previously been associated with exposure to NDL-PCBs (Wahlang et al., 2019). The hepatotoxicity of Aroclor 1260 mixture and the link between exposure to it and nonalcoholic fatty liver disease progression have been previously documented (Armstrong and Guo, 2019). Although it is not yet clear, oxidative stress may be the key link between nonalcoholic fatty liver disease and cardiovascular disease (Polimeni et al., 2015). The alteration of the linolenic acid pathway represents another evidence of oxidative stress caused by exposure to PCBs. Bioactive oxidized linoleic acid metabolites and diols of linoleate epoxides have previously been linked to oxidative stress and inflammatory disorders (Deng et al., 2019).

In addition, several ether-linked phosphatidylcholines and ether-linked phosphatidylethanolamine were found as biomarkers of effect in the serum of pigs exposed to Aroclor 1260. In contrast to LPCs, a general decrease of ether lipid levels was observed in the serum of exposed animals compared to the control animals. Recently, ether lipids have been proposed as potential biomarkers of various diseases, linking decreased ether lipid synthesis with multiple neurological and metabolic abnormalities (Dean and Lodhi, 2018). However, it is not yet clear whether they are simply by-products of disease processes or whether they contribute to disease pathogenesis. Decreased levels of ether-phospholipids in the liver have also been observed in rats exposed to different doses of a DL-PCB, specifically PCB 126, and which have been related to hepatic disorders (Kania-Korwel et al., 2017). In this sense, although DL-PCBs and NDL-PCBs have been shown to have different mechanisms of action in liver diseases such as nonalcoholic fatty liver disease, they share common effects (Wahlang et al., 2019).

Although all these results show a significant impact on lipid metabolism caused by exposure to NDL-PCBs, other identified biomarkers of effect indicate an alteration of other metabolic pathways. Several metabolites from the kynurenine pathway of tryptophan metabolism have been identified as effect biomarkers of Aroclor 1260 exposure, namely L-tryptophan, kynurenine, quinaldic acid and N'-formylkynurenine. Previous *in vivo* and *in vitro* studies have shown an impact of Aroclor 1254 mixture and PCB3, which is a NDL-PCB congener, on tryptophan metabolism (Khan and Thomas, 2004)(Zhang et al., 2012)(Zhang et al., 2021). Similar evidences have been reported for exposure to DL-PCBs (Mesnage et al., 2018). The disturbance of tryptophan-kynurenine pathway is related to inflammation, oxidative stress and immune activation in cardiovascular diseases (Wang et al., 2015). This finding agrees with the previous discussion about abnormal lipid metabolism caused by exposure to Aroclor 1260, which is another metabolic indicator of the pathogenesis of cardiovascular disease.

Indeed, there are several pieces of evidence linking chemical exposure to PCBs and the development of cardiovascular diseases (Perkins et al., 2016). In this sense, this work provides new evidence that current environmental exposures to NDL-PCBs can cause health effects similar to those previously observed in toxicological studies at higher exposure doses but likely to be observed after a longer period of exposure. Therefore, although levels of exposure to PCBs have been reduced in recent decades (Lehmann et al., 2015), the observed metabolic changes caused by exposure to Aroclor 1260 suggest that actual exposure scenarios to NDL-PCBs contribute to the onset and progression of environmental diseases, namely cardiovascular disease.

5 Concluding remarks

This study provides new information on biomarkers of effect in serum samples associated with exposure to Aroclor 1260 at dietary dose levels (i.e. 6.1 ng of six NDL-PCBs/kg b.w. per day). By extension, these biomarkers of effect have been related to exposure to NDL-PCBs, which have been shown to bioaccumulate in perirenal fat. In addition, the investigation of the pig as an animal model for the hazard identification of NDL-PCBs gives new evidence for the human health risk assessment of NDL-PCBs. Our no hypothesis-driven approach has demonstrated to be suitable to highlight the biomarkers of effect related to exposure to NDL-PCBs at levels of environmental exposure. Several glycerophosphocholines, some fatty acids, including arachidonic acid and linolenic acid, tryptophan, kynurenine and some of its metabolites, have been found as probable biomarkers of effect of said chemical exposure. These metabolites are mainly associated with glycerophospholipids metabolism, fatty acid metabolism and tryptophan-kynurenine pathway; thus, these metabolic pathways have been identified as the main pathways impacted by exposure to NLD-PCBs at low dose levels. Such metabolic alterations induce chronic oxidative stress and inflammation that are important factors in cardiovascular disease. These observations agree with other toxicological and epidemiological studies and suggest that exposure to current low levels of NDL-PCBs may still cause adverse health effects.

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Figure captions

Figure 1. Stages of animal experimentation in this study, indicating the sampling days.

Figure 2. Score plots for PCA models built with the 'metabolomics ESI-' dataset before (a) and after (b, c) T0-centering. Number of principal components of each model: 7 (a) and 8 (b, c). The first and second principal components are represented in the score plots (a) and (b), while the first and fourth principal components are represented in the score plot (c). Group classes: *red circles* refer to samples from the acclimatization period, *green circles* indicate control samples from the exposure and the detoxification stages, and *blue circles* represent samples from exposed pigs collected in the periods of exposure and detoxification. Samples from the groups of control and exposed animals are indicated by (A,B) and (C-F), respectively.

Figure 3. Clustering heatmap and hierarchical analysis resulted from the NDL-PCB-related lipidomics study of serum samples analyzed by LC-HRMS under ESI+ conditions. In red / blue, the group of lipids with an increase / decrease in their concentration in serum associated with exposure to Aroclor 1260.

Figure 4. Evaluation of the PLS-DA model for the 'lipidomics ESI-' dataset: (a) PLS-DA score plot, (b) permutation tests for 'control' and 'exposed' groups. Group classes: *red circles* refer to samples from the acclimatization period, *green circles* indicate control samples from the exposure and the detoxification stages, and *blue circles* represent samples from exposed pigs collected in the periods of exposure and detoxification. Samples from the groups of control and exposed animal are indicated by (A,B) and (C-F), respectively.

Figure 5. Significantly disturbed metabolic pathways identified from pathway analysis by using the web service of MetaboAnalyst 4.0.

Table 1. Analysis of Variance (ANOVA)-Simultaneous Component Analysis (ASCA) of each of the datasets generated in this work. The confidence level was established at 95%.

Factor	**Subject**	**Time**	**Exposure**	**Subject × Time**	**Subject × Exposure**	**Time × Exposure**	**Subject × Time × Exposure**
Dataset	***p-value***						
Metabolomics (ESI+)	< 0.05	0.08	1.00	0.99	< 0.05	0.33	0.91
Metabolomics (ESI-)	< 0.05	0.30	1.00	0.94	< 0.05	0.38	0.82
Lipidomics (ESI+)	< 0.05	0.10	1.00	1.00	< 0.05	0.41	0.97
Lipidomics (ESI-)	< 0.05	< 0.05	1.00	1.00	< 0.05	0.52	0.98

Table 2. PLS-DA statistics of the models differentiating serum samples from pigs exposed (or not) to Aroclor 1260, as well as serum samples from the acclimatization period.

Dataset	**Number of components**	**CV-ANOVA (*p*-value)**	**R2X (cum)**	**R2Y (cum)**	**Q2 (cum)**	**Classification accuracy**
Metabolomics ESI+	3	1.025×10^{-16}	0.176	0.836	0.639	100 %
Metabolomics ESI-	2	1.436×10^{-19}	0.101	0.746	0.462	98.3 %
Lipidomics ESI+	2	1.534×10^{-17}	0.145	0.649	0.405	91.7 %
Lipidomics ESI-	2	2.597×10^{-19}	0.129	0.690	0.506	100 %

Table 3. Metabolites annotated (with confidence level 1 or 2) as possible biomarkers of exposure to Aroclor 1260 and previously found as relevant features in metabolomics datasets. Notes: [a] measured *m/z* of protonated ions; [b] *m/z* related to $[M+H-NH_3]^+$ ion; [c] *m/z* related to $[M-H-H_2O]^-$ ion. In red/blue, the group of metabolites with an increase/decrease in their concentration in serum associated with exposure to Aroclor 1260.

m/z[a]	RT (min)	Ionization mode	Putative annotation	Confidence level of annotation	Variation of metabolite concentration levels in serum as a consequence of Aroclor 1260 exposure
114.0661	0.67	ESI+	creatinine	Level 1	
218.1385	1.1	ESI+	propionyl-L-carnitine	Level 1	
190.1185	0.67	ESI+	L-homocitrulline	Level 2	
214.2164	11.81	ESI+	tridecanamide	Level 2	
225.0519	1.56	ESI-	3-nitro-L-tyrosine	Level 1	
303.2331	13.24	ESI-	arachidonic acid	Level 1	
87.0087[c]	0.77	ESI-	glyceric acid	Level 2	
89.0244	0.82	ESI-	glyceraldehyde	Level 2	
156.0667	6.97	ESI-	N-tiglylglycine	Level 2	
188.0705[b]	2.38	ESI+	L-tryptophan	Level 1	
141.0051	0.56	ESI+	phosphono carbamimidate	Level 2	
231.1450	0.67	ESI+	L-alaninamide, L-alanyl-L-alanyl-	Level 2	
338.0867	4.56	ESI+	3-indoleformate glucuronide	Level 2	
230.1749	7.26	ESI+	N-decanoylglycine	Level 2	
432.3107	8.08	ESI+	3-hydroxy-5-cholenoylglycine	Level 2	
357.2785	9.04	ESI+	chola-4,6-dien-24-oic acid	Level 2	
467.3164	15.77	ESI+	L-eicosanoyl-glycero-3-phosphate	Level 2	
207.0775	1.42	ESI-	kynurenine	Level 1	
197.0432	1.28	ESI-	vanillylmandelic acid	Level 1	
201.1133	5.39	ESI-	sebacic acid	Level 1	
171.1391	9.91	ESI-	capric acid	Level 1	
129.0194[c]	0.79	ESI-	2-hydroxyglutaric acid	Level 2	
145.0143	0.86	ESI-	2-oxoglutaric acid	Level 2	
130.0874	0.96	ESI-	DL-leucine	Level 2	
292.1403	0.96	ESI-	N-isopropyl-2'-deoxyadenosine	Level 2	
117.0558	2.29	ESI-	3-hydroxy-2-methyl-butanoic acid	Level 2	
188.0929	5.97	ESI-	N-lactyl-valine	Level 2	
172.0405	6.38	ESI-	quinaldic acid	Level 2	
211.0977	7.25	ESI-	3,4-methyleneazelaic acid	Level 2	
228.1605	7.27	ESI-	N-decanoylglycine	Level 2	
242.1763	7.86	ESI-	11-acetamidoundecanoic acid	Level 2	
243.1602	8.75	ESI-	brassylic acid	Level 2	
191.1079	9.2	ESI-	6-phenylcaproic acid	Level 2	

Figure 1

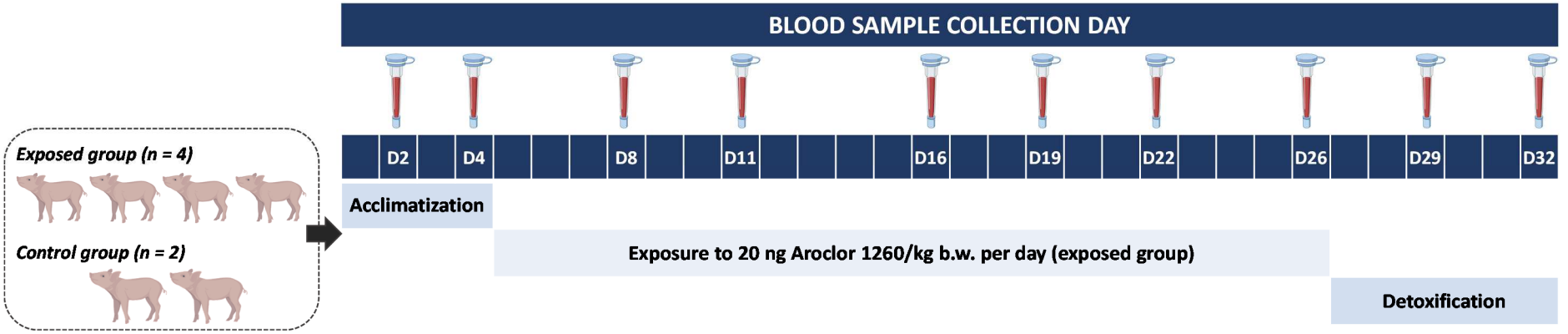
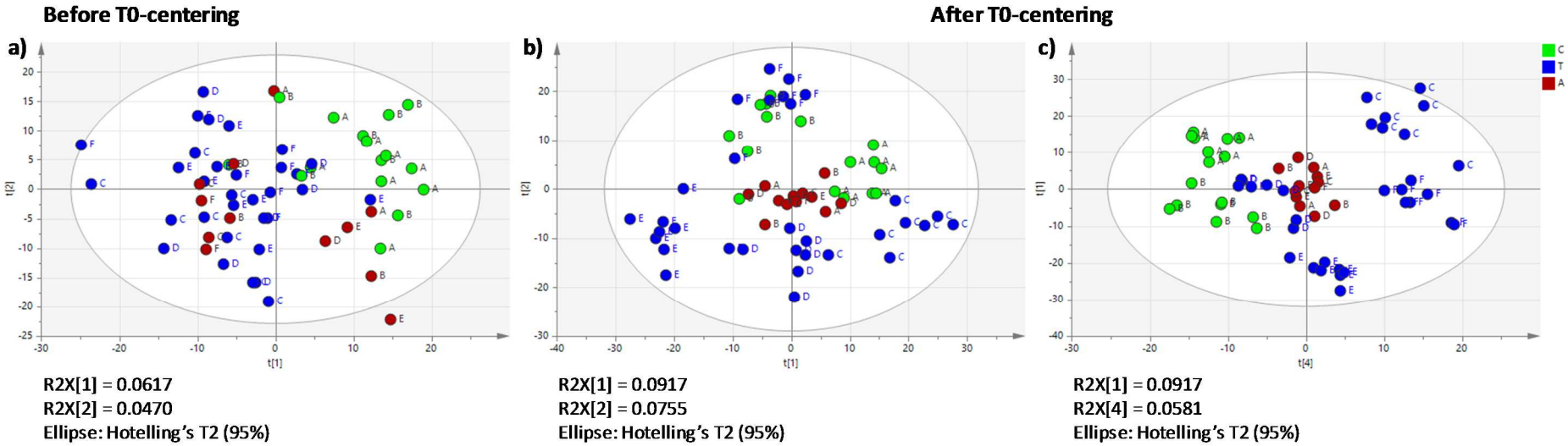

BLOOD SAMPLE COLLECTION DAY
Exposed group (n = 4)
Control group (n = 2)
D2
D4
D8
D11
D16
D19
D22
D26
D29
D32
Acclimatization
Exposure to 20 ng Aroclor 1260/kg b.w. per day (exposed group)
Detoxification

Figure 2



R2X[1] = 0.0617
R2X[2] = 0.0470
Ellipse: Hotelling's T2 (95%)

R2X[1] = 0.0917
R2X[2] = 0.0755
Ellipse: Hotelling's T2 (95%)

R2X[1] = 0.0917
R2X[4] = 0.0581
Ellipse: Hotelling's T2 (95%)

Figure 3

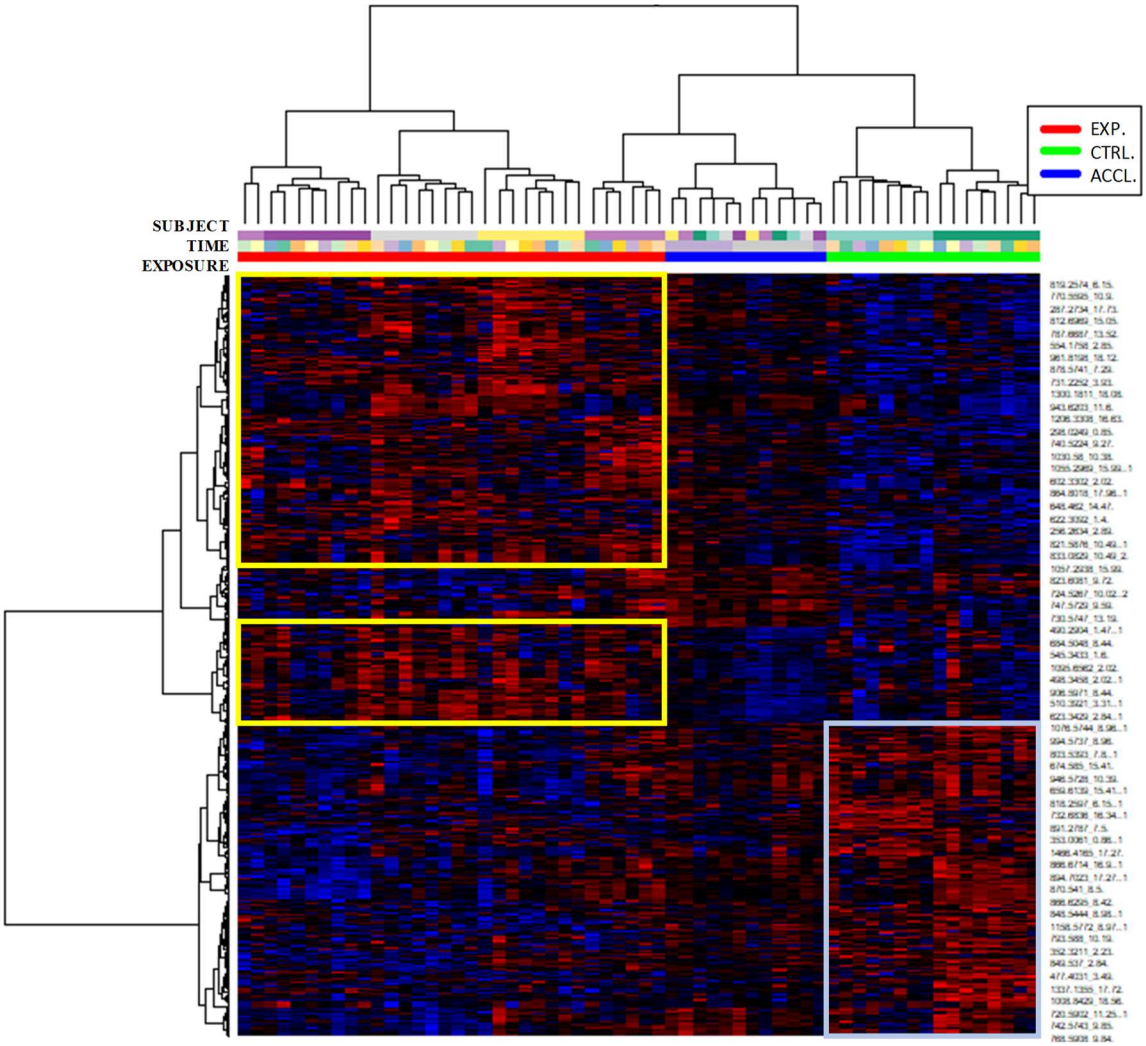


Figure 4

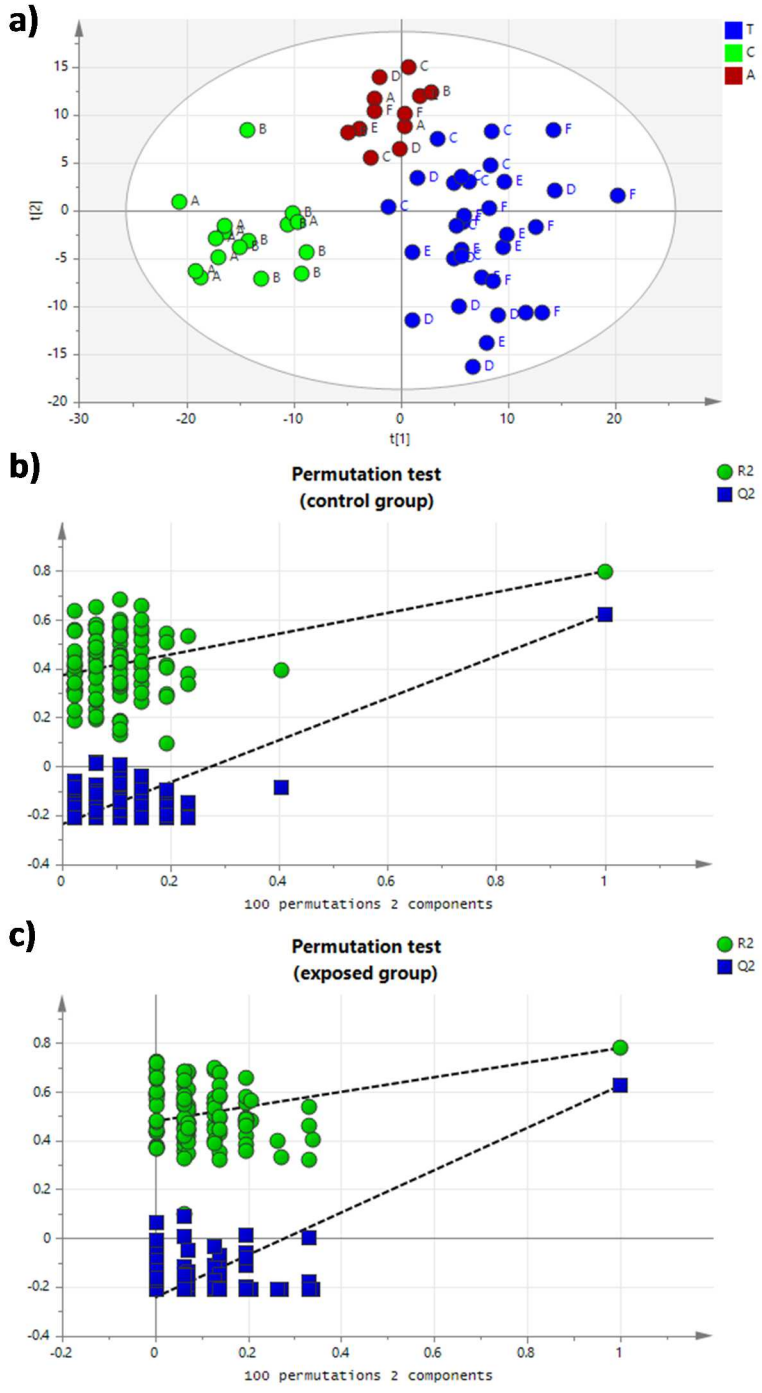
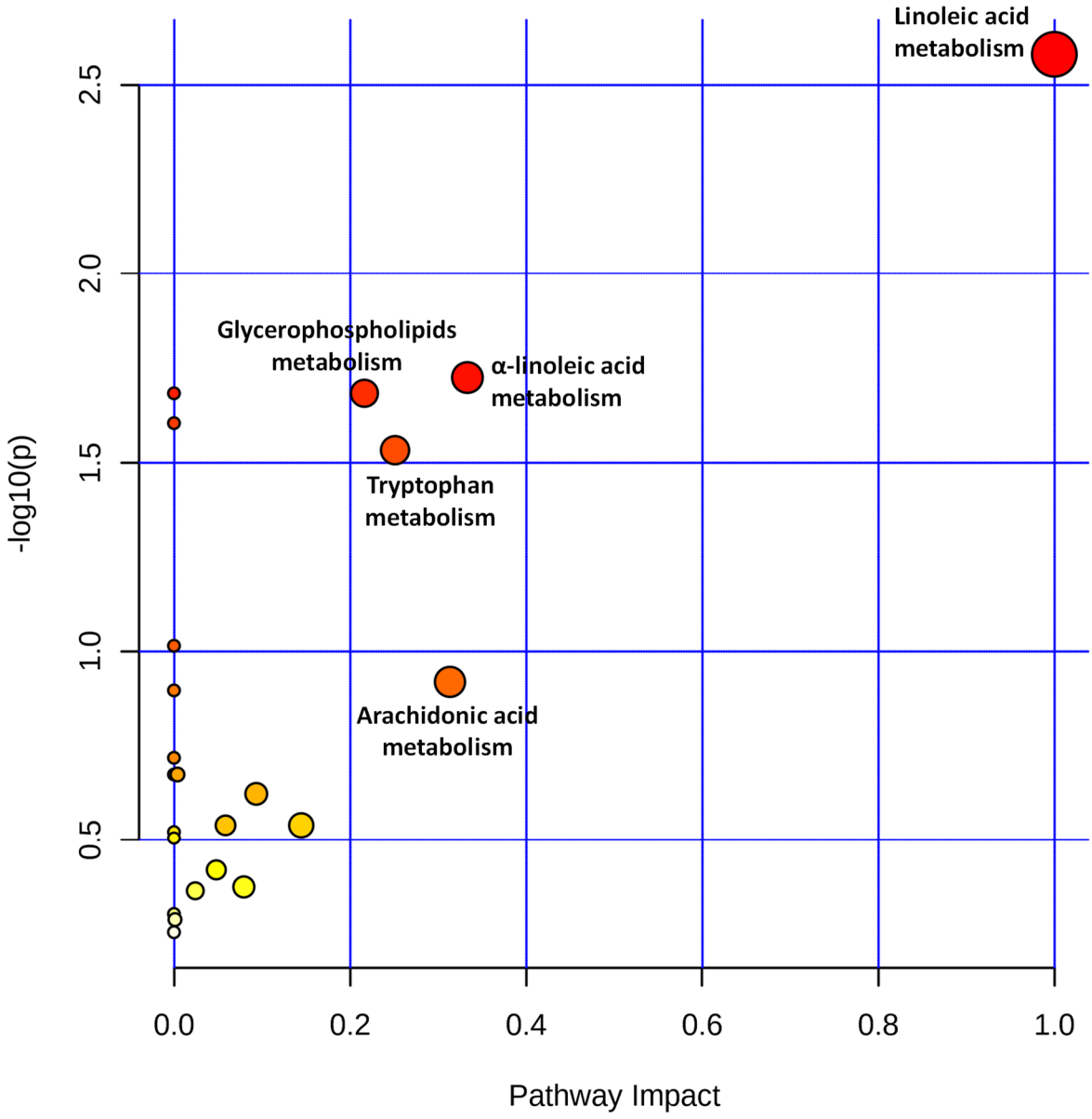
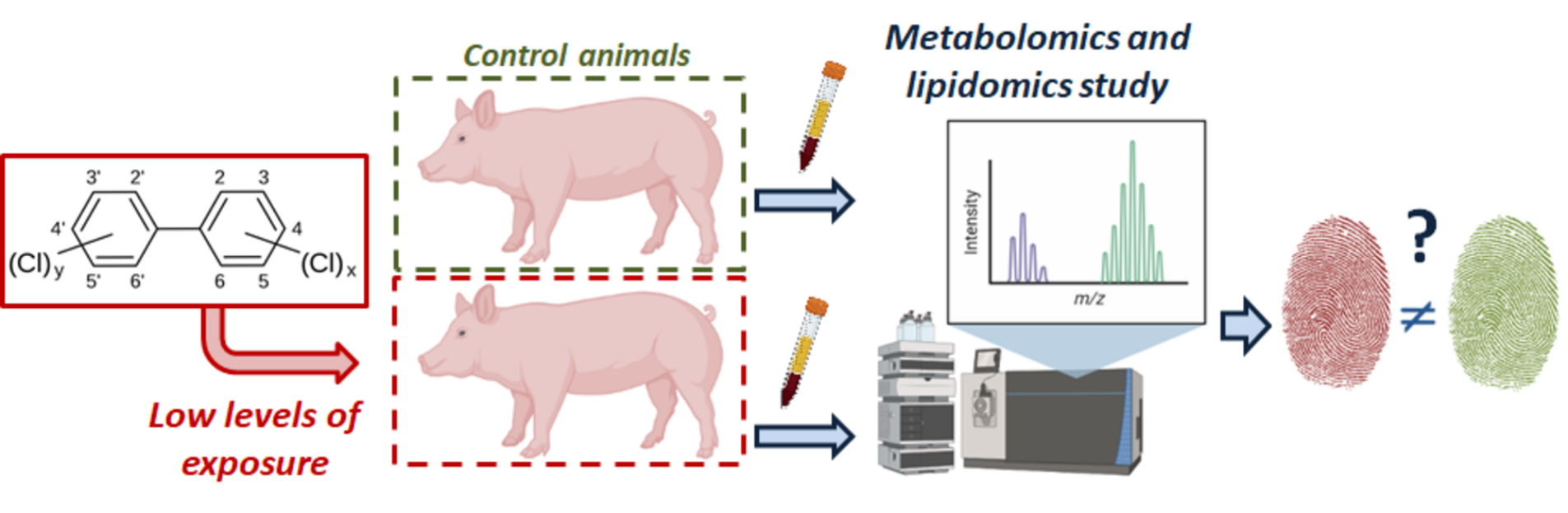

a)
T
C
A
t[1]
t[2]
b)
Permutation test
(control group)
R2
Q2
100 permutations 2 components
c)
Permutation test
(exposed group)
R2
Q2
100 permutations 2 components

Figure 5


Linoleic acid metabolism
Glycerophospholipids metabolism
α-linoleic acid metabolism
Tryptophan metabolism
Arachidonic acid metabolism
-log10(p)
2.5
2.0
1.5
1.0
0.5
0.0
0.2
0.4
0.6
0.8
1.0
Pathway Impact


Control animals
Metabolomics and lipidomics study
3' 2' 2 3
4' 4
(Cl)y
(Cl)x
5' 6' 6 5
Low levels of exposure
Intensity
m/z
?
≠