USE OF NON-TARGETED METABOLOMIC PROFILING TO STUDY POSSIBLE METABOLIC EFFECTS OF *p,p'*-DDE IN A POPULATION-BASED STUDY

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Introduction

Metabolomics is defined as the global analysis of the metabolome and has, since its introduction, stimulated a large number of publications in a broad range of research fields [1]. Recent advances in mass spectrometry technology and bioinformatics have enabled the application of metabolomics in the context of large clinical and epidemiological studies allowing researchers to study individual metabolite profiles and how they relate to various phenotypes in humans.

Persistent organic pollutants (POPs), such as the organochlorine pesticides, are known toxicants and have been linked to numerous adverse effects in humans [2]. DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane) is one of the most well-known amongst the organochlorine pesticides and large quantities of DDT have been and still are distributed worldwide [3] . DDT is easily metabolized into two major breakdown products including *p,p'*-DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene) and *p,p'*-DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethane). In contrast to the parent compound, *p,p'*-DDE is extremely persistent and is frequently detected in the general population in countries all over the world [4] .

Several metabolites in human circulation (e.g., fatty acids, amino acids, glycolipids) play a key role in human health and are important in development of human diseases [5]. However, whether POPs affect the levels and profiles of metabolites in human circulation remains unclear. In this study we investigated the effect of *p,p'*-DDE levels on the metabolome by determining both circulating levels of *p,p'*-DDE and global metabolite profiles in 1,016 serum samples from the "Prospective Investigation of the Vasculature in Uppsala Seniors" (PIVUS) study.

Materials and methods

Study population and blood sample collection

The participants in the PIVUS study were randomly selected from the general population in the community of Uppsala, Sweden. Invitation letters were sent between April 2001 and June 2004 and within two months of each of the participants 70th birthday. The target sample population was 2, 025 participants out of which 1, 016 participated. Serum and plasma were collected in the morning after overnight fast. After the samples were collected (1-2 mL vials), the vials were placed in freezers (-20 °C) until used for chemical analysis. The study was approved by the Ethics Committee of the University of Uppsala and the participants gave written informed consent.

p,p'-DDE analysis

The sample extraction and clean-up method for *p,p'*-DDE analysis is described in detail by Salihovic et al. (2012). In short, plasma sample were extracted using solid phase extraction with Oasis® HLB SPE (Waters, Milford, MA,

USA) cartridges. Further cleanup was performed using small activated multilayer silica gel columns. The extracts were injected on a 6890N gas chromatograph (GC) (Agilent Technologies, Atlanta, GA, USA) and separated on a 30m x 0.25 i.d. x 0.25 µm DB-5 column (SGE Analytical Science, Victoria, AUS). Measurements were performed on a Micromass Autospec Ultima (Waters, Mildford, MA, USA) mass spectrometer, monitoring the two most abundant ions of the chlorine cluster of the most abundant fragments in addition to one ion for the ^{13}C-labeled internal and recovery standard.

Metabolomics profiling

Non-targeted metabolite profiling was performed using methods previously described by Broeckling et al. (2012). Briefly, serum samples were thawed and 100 µL of serum was transferred to a 96-well plate and 400 µL methanol was added to precipitate proteins. Separation and data acquisition was performed on Acquity UPLC coupled to a Xevo G2 Q-TOFMS (Waters Corporation, Milford, USA) with an atmospheric electrospray interface operating in positive ion mode. Non-consecutive duplicate sample aliquots of 1 µL were injected onto an Acquity UPLC BEH C8 (1.8 µM, 1.0 x 100 mm) analytical column held at 50°C using a gradient of water, methanol, and formic acid. Mass analysis was performed in the full scan MS mode (m/z 50-1200) at 6 V and fragmentation scans were collected in the MS^E mode using a collision ramp of 15-30V.

Data processing

Raw data were processed using XCMS software [8]. Data processing was performed according to the work-flow described by Ganna et al. (2013). In short, metabolic feature detection, alignment, grouping, imputation and normalization were performed separately for each study. Each feature is characterized by a specific mass-to-charge ratio (m/z) and retention time and a single metabolite is normally represented by more than one feature. Indiscriminant (id) MS and idMS/MS (or MS^E mode) spectra were generated for all features and those with highly similar spectra and similar retention time were deemed to be from the same metabolite. Features were then taken further into the metabolite annotation and identification procedure.

Metabolite annotation

Metabolite identification and quantification was performed by using our own in-house reference library or by turning to publicly available information databases such as the Human Metabolome Database (HMDB) and Metabolite and Tandem MS Database (METLIN). A combination of spectral searching and manual spectral interpretation was used for metabolite annotation and results are presented in accordance with the Metabolomics Standard Initiative (MSI). Four levels of confidence were considered, in accordance with the Metabolomics Standard Initiative (MSI). This approach enabled the successful identification of 136 metabolites in serum samples from participants in the PIVUS study. The metabolites detected come from diverse compound classes including amino acids and derivatives, peptides, steroids and steroid derivatives, alcohols and polyols, and various compounds related to the lipid metabolism such as fatty acids, glycerophospholipids, sphingolipids, and glycerolipids.

Statistical analysis

Linear regression models were applied to assess the association of each metabolite with DDE levels adjusting for age and sex. Only metabolites with a significant *p-value* after Bonferroni multiple-testing adjustment were reported.

Results and discussion

Valid measurements of *p,p'*-DDE were obtained for 992 participants in the PIVUS study. The detection rate was 99% and the concentrations ranged from 2.13 ng/g lipid to 4260.4 ng/g lipid. The median concentration was 309 ng/g lipid which is similar to the median concentrations in previous studies from Sweden and Norway [10, 11] but two times lower than those reported in the Belgium and U.S. [12, 13].

To examine the link between *p,p'*-DDE and metabolites we used both a targeted and an untargeted approach. In the first approach, we included levels of *p,p'*-DDE and 136 identified metabolites in the analysis. As shown in Table 1, circulating levels of *p,p'*-DDE were found to be negatively associated with three phosphocholines and one phosphoethanolamine and positively associated with oleamide and flavone. In the second approach, which is a more explorative approach, we analyzed associations included levels of *p,p'*-DDE levels and all 8,185 metabolic features detected in the study. The results from the second approach showed that higher levels of *p,p'*-DDE were negatively associated with 40 metabolic features. The majority of these features (36) were identified as 1-Linoleoyl-glycero-3-phosphocholine (LPC18:2) while three features were identified as 1-Palmitoyl-glycero-3-phosphocholine (LPC16:0), 1-Palmitoleoyl-glycero-3-phosphocholine (LPC 16:1), and 1-Stearoyl-glycero-3-phosphocholine (LPC 18:0).
In summary, we performed non-targeted metabolomics in a large number of participants to study the effects of *p,p'*-DDE on human metabolic profiles. We found levels of *p,p'*-DDE to be related to a number of phospholipid metabolites involved in key metabolic process such as cell signaling, energy regulation, and membrane composition. These findings suggest that *p,p'*-DDE may affect human lipid metabolism and which might have human health implications.

Table 1. Metabolites significantly associated with higher levels of DDE

Metabolites	Abbrev.	Metabolic pathway	*p,p'*-DDE	
			β (95% CI)	*p-value*
Oleamide		Phospholipid biosynthesis	0.06 (0.03, 0.08)	0.00019
Flavone		Phenylpropanoid biosynthesis	0.15 (0.08, 0.23)	0.00008
1-Vaccenoyl-glycero-3-phosphocholine	LPC (18:1)	Phospholipid biosynthesis	-0.34 (-0.52, -0.16)	0.00017
1-Linoleoyl-glycero-3-phosphocholine	LPC (18:2)	Phospholipid biosynthesis	-0.38 (-0.53, -0.22)	2.54E-06
1-Eicosadienoyl-glycero-3-phosphocholine	LPC (20:2)	Phospholipid biosynthesis	-0.24 (-0.38, -0.11)	0.00031
1-Pentadecanoyl-2-oleoyl-sn-glycero-3-phosphocholine, 1-Palmitoleoyl-2-arachidonyl-sn-glycero-3-phosphoethanolamine	PC (33:1), PE(36:1)	Phospholipid biosynthesis	-0.19 (-0.3, -0.09)	0.00031

Acknowledgements

This study was supported by the Swedish Research Council (VR) and the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS).

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