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Metabolome-wide association study of the relationship between chlorpyrifos exposure and first trimester serum metabolite levels in pregnant Thai farmworkers

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

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.114319.
Abstract

Introduction: Organophosphate (OP) insecticides, including chlorpyrifos, have been linked with numerous harmful health effects on maternal and child health. Limited data are available on the biological mechanisms and endogenous pathways underlying the toxicity of chlorpyrifos exposures on pregnancy and birth outcomes. In this study, we measured a urinary chlorpyrifos metabolite and used high-resolution metabolomics (HRM) to identify biological perturbations associated with chlorpyrifos exposure among pregnant women in Thailand, who are disparately exposed to high levels of OP insecticides.

Methods: This study included 50 participants from the Study of Asian Women and their Offspring’s Development and Environmental Exposures (SAW ASDEE). We used liquid chromatography-high resolution mass spectrometry to conduct metabolic profiling on first trimester serum samples collected from participants to evaluate metabolic perturbations in relation to chlorpyrifos exposures. We measured 3,5,6-trichloro-2-pyridinol (TCPy), a specific metabolite of chlorpyrifos and chlorpyrifos-methyl, in first trimester urine samples to assess the levels of exposures. Following an untargeted metabolome-wide association study workflow, we used generalized linear models, pathway enrichment analyses, and chemical annotation to identify significant metabolites and pathways associated with urinary TCPy levels.

Results: In the 50 SAWASDEE participants, the median urinary TCPy level was 4.36 μg TCPy/g creatinine. In total, 691 unique metabolic features were found significantly associated with TCPy levels (p < 0.05) after controlling for confounding factors. Pathway analysis of metabolic features associated with TCPy indicated perturbations in 24 metabolic pathways, most closely linked to the production of reactive oxygen species and cellular damage. These pathways include tryptophan metabolism, fatty acid oxidation and peroxisome metabolism, cytochromes P450 metabolism, glutathione metabolism, and vitamin B3 metabolism. We confirmed the chemical identities of 25 metabolites associated with TCPy levels, including glutathione, cystine, arachidic acid, itaconate, and nicotinamide adenine dinucleotide.

Discussion: The metabolic perturbations associated with TCPy levels were related to oxidative stress, cellular damage and repair, and systemic inflammation, which could ultimately contribute to health outcomes, including neurodevelopmental deficits in the child. These findings support the future development of sensitive biomarkers to investigate the metabolic underpinnings related to pesticide exposure during pregnancy and to understand its link to adverse outcomes in children.

Keywords
Thailand; Farmworker; Birth cohort; Metabolomics; Organophosphate; Biomarkers

1. Introduction

Organophosphate (OP) insecticides are a group of commonly used neurotoxic pesticides that include compounds such as chlorpyrifos, diazinon, and malathion (Harley et al., 2011). Although chlorpyrifos was phased out in the United States for residential use by manufacturers in 2001, millions of pounds of chlorpyrifos have been widely used per
year in agricultural settings in the United States until 2021 when U.S. Environmental Protection Agency announced the decision to end the use of chlorpyrifos on all food products nationwide (Hites, 2021). While the global production and use of chlorpyrifos has declined over the years, it is still commonly used in agriculture in many developing countries (Foong et al., 2020), including Thailand, an agricultural country and one of the world’s major food exporters, which relies heavily on the annual use of over 100,000 tons of pesticides to protect crops and increase yields (Panuwet et al., 2012a; Rohitrattana et al., 2014a). With chlorpyrifos’ high production and wide range of uses, humans are frequently exposed to chlorpyrifos through contaminated food products.

The toxicology of chlorpyrifos and the associated adverse health effects, including neurological effects, endocrine disruption, and cardiovascular diseases, have been characterized previously (Eaton et al., 2008; ur Rahman et al., 2021; Smegal, 2000). Similar to many other OPs, chlorpyrifos can inhibit acetylcholinesterase (AChE) (Colović et al., 2013) by covalently attaching to its binding site and preventing the breakdown of acetylcholine, eventually leading to acute toxic effects and severe reactions such as excessive salivation, seizures, paralysis, or death (Adeyinka et al., 2022; Costa, 2006; Naksen et al., 2015). Exposures to chlorpyrifos have also been linked with adverse pregnancy and birth outcomes (Mink et al., 2012; Taheri et al., 2022). Specifically, multiple animal studies have shown that higher OP insecticide exposure can lead to reduction in fetal growth and neurodevelopmental deficits in rodents likely via pathways not associated with AChE inhibition (Aldridge et al., 2003, 2005a, 2005b; Slotkin et al., 2002; Spyker and Avery, 1977; Srivastava and Raizada, 1996; Xu et al., 2020). Furthermore, prenatal exposure to OP insecticides, such as chlorpyrifos, in humans has been linked with a variety of postnatal outcomes, including adverse birth and neurodevelopmental outcomes (Berkowitz et al., 2004; Bouchard et al., 2011; Engel et al., 2007, 2011, 2016; Eskenazi et al., 2004, 2007, 2010; Furlong et al., 2014, 2017a, 2017b; Harley et al., 2016; Marks et al., 2010; Rauh et al., 2006, 2011, 2012; Whyatt et al., 2004; Young et al., 2005). Despite these epidemiological observations and animal studies, the detailed biological mechanisms and endogenous pathways underlying the toxicity of chlorpyrifos exposures on pregnancy and birth outcomes are complex and remain largely unknown, thus limiting the development of effective interventions.

High-resolution metabolomics (HRM), a type of omics-based technology, is an innovative high-throughput analytical technique capable of quantifying and identifying a large number of metabolites from exogenous and endogenous sources (Kim et al., 2016), and has emerged as a promising platform useful for studying the effects of environmental exposures on human health (Rappaport, 2011). Numerous environmental metabolomics studies have demonstrated HRM as an effective tool to augment internal exposure estimation and biochemical pathway elucidation associated with exposures to complex environmental chemical mixtures (Bonvallot et al., 2013; Liang et al., 2018, 2019; Ladva et al., 2018; Li et al., 2021; Zhang et al., 2021; Tan et al., 2022; Hood et al., 2022a; Tang et al., 2022). To address these knowledge gaps and elucidate the mechanistic evidence underlying the toxicity of chlorpyrifos on maternal and child health, we designed and conducted this proof-of-concept study, where we measured chlorpyrifos metabolites and conducted novel HRM analyses to identify biological perturbations associated with chlorpyrifos exposures.
among a subset of 50 farmworker women from an established birth cohort in Thailand who are disproportionately exposed to high chlorpyrifos levels. We hypothesized that maternal exposures to chlorpyrifos would be associated with altered maternal metabolome, with perturbations centering around endogenous endocrine disruption, inflammation, and oxidative stress-related pathways.

2. Methods

2.1. Study population

In this analysis, study participants were from the Study of Asian Women and their Offspring’s Development and Environmental Exposures (SAWASDEE), a longitudinal birth cohort based in the Northern Thailand districts of Fang and Chom Thong (Baumert et al., 2022). The study followed participants through pregnancy, collected multiple, time-resolved biological samples for biomarker measurements, and evaluated child neurodevelopment until 3 years of age. The SAWASDEE study is a collaboration between researchers from Emory (Atlanta, GA, USA), Rutgers (Piscataway, NJ), Chiang Mai (Chiang Mai, TH), and Chulalongkorn Universities (Bangkok, TH) (Baumert et al., 2022). We chose this region for the investigation because of the strong agricultural industry of Thailand, the generalizability to other low/middle income countries (LMIC), and the study team’s previous work in the Chiang Mai Province. Our previous studies among others in the region found elevated insecticide exposure in mothers and children in Thailand (Naksen et al., 2015; Fiedler et al., 2015; Kongtip et al., 2014; Kunstadter et al., 2001; Panuwet et al., 2009; Petchuay et al., 2006; Rohitrattana et al., 2014b; Woskie et al., 2017). Full details on the SAWASDEE study have been published previously (Baumert et al., 2022).

2.2. Participant recruitment

The study population included pregnant women who were agricultural workers or lived within 50 m from an agricultural field; had a Thai identification card which allowed them to receive antenatal clinic access; resided in the region for at least 5 months and planned to live there 3 years after delivery; spoke Thai at home; were generally healthy (i.e. no major medical conditions like hypertension, diabetes, or HIV); consumed fewer than two alcoholic beverages per day and did not use illegal drugs; were less than 16 weeks of gestation at intake; had a singleton pregnancy; and agreed to participate with informed consent. Participant enrollment started in July 2017 and was completed in June 2019; 1298 women were screened and 322 of those participants were enrolled into the study (Baumert et al., 2022). For this proof-of-concept study, we included 50 of these participants who had urine and serum samples taken during the first trimester. Specifically, we randomly selected 25 participants within the highest quartile of the TCPy levels and 25 within the lowest quartile of the TCPy levels, while balancing the number of the participants from each study site and demographic characteristics. Convenience samples were taken at routine antenatal visits. Non-fasting serum and urine samples were collected and stored in coolers until being taken back to the laboratory at the end of the day and processed. These samples were stored at −80 °C freezer until analysis (Baumert et al., 2022). The study was reviewed and approved by Emory and Chiang Mai Universities Institutional Review Boards (IRB); Rutgers University relied upon Emory’s IRB and Chulalongkorn University relied upon Chang Mai’s IRB.
2.3. Chlorpyrifos exposure assessment

All urine samples were randomized using a Fisher-Yates shuffling algorithm prior to analysis to reduce any potential batch effects (Finney, 1948; Knuth, 1960). Samples were analyzed for 3,5,6-trichloro-2-pyridinol (TCPy), a specific metabolite of chlorpyrifos and chlorpyrifos-methyl, using a modification of a previously validated method (Olsson et al., 2004). Briefly, samples were spiked with a stable isotopic analogue of the target analyte then were enzymatically digested using purified β-glucuronidase and sulfatase enzymes (derived from H. pomatia) to liberate glucuronide-bound metabolites. The hydrolysates were centrifuged and transferred to autosampler vials. Samples were injected into a liquid chromatography (LC) column switching system for extraction of the target analytes using a Strata RP on-line solid phase extraction (SPE) column (2.1 × 20 mm). The on-line SPE column was washed with the acetonitrile:Milli-Q water (10:90, V/V) solution to remove undesired matrix interferences. The target analytes were then eluted from the on-line SPE column to a Poroshell 120 EC-C18 analytical column (3.0 × 100 mm, 2.7 μm) for separation. The target analytes were measured using negative mode electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) with isotope dilution quantification. During mass spectrometric analysis, the target analytes were monitored using the multiple reaction monitoring mode. One quantitation ion and one confirmation ion were monitored for the native analyte, and one quantitation ion was monitored for the labeled analogue. Concentrations of TCPy were determined from the relative response (per volume of sample injected) of native to labeled standards in the samples, using an equation derived from a matrix-matched standard calibration curve. For each analytical run of 44 unknown samples, 2 blank samples (negative controls) and four positive quality control samples at 2 different levels were analyzed concurrently. Successful participation in the German External Quality Assessment Scheme (GEQUAS) served as an additional quality assurance measure of the method. The linearity was <3% error on the slope, the accuracy was indistinguishable from 100%, and relative standard deviation was <10%. The limit of detection (LOD) was 0.31 ng/mL (TCPy) and the average relative recovery was 95%. For statistical analysis, the value equal to the LOD divided by the square root 2 was imputed for all values below the LOD. Creatinine was measured directly using diluted urine samples (1000-fold) without sample extraction using LC-MS/MS (Park et al., 2008; Kwon et al., 2012). The LOD was 5 mg/dL with a relative standard deviation of 5%.

2.4. High resolution metabolomics

We used established protocols (Liang et al., 2018; Go et al., 2015) to conduct HRM analyses on first-trimester maternal serum samples. Briefly, we used liquid chromatography-high-resolution mass spectrometry (LC–HRMS) (Dionex Ultimate 3000 and ThermoScientific QExactive) to conduct the HRM profiling on each sample. In each extraction, two volumes of acetonitrile were used to treat each sample and samples were analyzed in triplicate. To enhance the coverage of metabolic feature detection, we used two chromatographic columns for metabolite separation, which included the C18 hydrophobic reversed-phase liquid chromatographic column coupled with negative ESI, as well as the hydrophilic interaction liquid chromatographic (HILIC) column coupled with positive ESI. At the beginning and end of each analytical batch, we used two quality control
pooled reference plasma samples for normalization, control for background noise, batch evaluation, and post hoc quantification, which included a standard reference material (SRM) obtained from the National Institute of Standards and Technology (SRM, 1950) (Simón-Manso et al., 2013) and pooled human plasma purchased from Equitech Bio. After obtaining the data files from the instrument analyses of all samples, we used ProteoWizard (Chambers et al., 2012) to convert these raw data to.mzML files. For metabolic feature extraction, we used innovative algorithms, apLCMS with modifications by xMSanalyzer (Uppal et al., 2013; Yu et al., 2009), where the detected metabolic features were uniquely defined by their mass-to-charge ratio (m/z), retention time, and ion intensity. In the data filtering process, we only included metabolic features detected in >10% of the serum samples with median coefficient of variation (CV) among technical replicates <30% and Pearson correlation >0.7 to remove the noise signals and improve the quality of the metabolomics data (Chang et al., 2021; Tan et al., 2022; Tang et al., 2022). Following quality assessment and assurance, we took the median intensity across replicate samples and then natural log-transformed these metabolic feature intensities for the data analysis.

2.4.1. Statistical analysis—We analyzed the associations between TCPy levels and metabolic features using generalized linear regression (GLM) models (one using creatinine adjusted TCPy levels in the model (Barr et al., 2005) and the other adding creatinine as a covariate in the MW AS model), adjusting for recruitment site, age, ethnicity, husband smoking status, use of medications other than prenatal multivitamins, and use of fertilizer and insecticides at work since pregnancy. The GLM models used:

Equation 1a: GLM Model with Creatinine Adjusted TCPy Levels.

\[
Y_{ij} = \beta_1 TCPy_{ij} Creai + \beta_2 Site_i + \beta_3 Age_i + \beta_4 Ethnicity_i + \beta_5 Hus\_smoke_i + \beta_6 Medsi + \beta_7 UseFP_i
\]

Equation 1b: GLM Model without Creatinine Adjusted TCPy Levels.

\[
Y_{ij} = \beta_1 TCPy_{ij} + \beta_2 Site_i + \beta_3 Age_i + \beta_4 Ethnicity_i + \beta_5 Hus\_smoke_i + \beta_6 Medsi + \beta_7 UseFP_i + \beta_8 Creai
\]

where \( Y_{ij} \) refers to the intensity (i.e., relative concentration) of metabolic feature \( j \) for participant \( i \). Separate models were conducted for each metabolic feature, from each type of chromatography (serum C18 negative ESI, and serum HILIC positive ESI). Multiple comparison correction was conducted using the Benjamini-Hochberg false discovery rate (FDR_{B-H}) procedure (Benjamini and Hochberg, 1995), a widely used procedure in Metabolome Wide Association Studies (MWAS) study, at a 5% false positive threshold. Results were presented using Manhattan plots, with the retention time of each metabolic feature on the x-axis against the \(-\log_{10}(p)\) for \( \beta_1 \) from each equation above on the y-axis. All analyses were completed in R version 4.1.2.
2.5. Metabolic pathway enrichment analysis

Pathway enrichment analysis was conducted using mummichog (Version 1.0.10) (Li et al., 2013), an innovative bioinformatics platform widely used in metabolomics analysis (Li et al., 2013; Tian et al., 2022). Briefly, mummichog bypasses the metabolite validation step and predicts functional biological activity directly (Li et al., 2013), where for each biological pathway, an adjusted p-value would be calculated by resampling the reference input file in mummichog via a gamma distribution. To select eligible metabolic features for pathway analysis, we applied the following three strategies: (i) at raw p-values < 0.05 (less conservative); (ii) at raw p-values <0.005 (more conservative; iii) at multiple testing corrected p-value at 0.05 using Benjamini-Hochberg method (most stringent). For all three approaches, to compensate for false discoveries, we excluded pathways identified by mummichog with a p > 0.05 and those containing fewer than four significant metabolic features that were matched with known compounds by m/z. We conducted pathway analysis separately for each model, one using creatinine adjusted TCPy levels in the model and the other adding creatinine as a covariate in the MWAS model.

2.6. Metabolite annotation and confirmation

We conducted the metabolite annotation on the features significantly associated with TCPy levels and also enriched in a relevant pathway. Using a mass error threshold of 10 ppm, we annotated these metabolites by matching the accurate mass m/z values to common adducts using established databases, including METLIN, ChemSpider, Human Metabolome Database (HMDB), and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Uppal et al., 2017). Finally, we compared the m/z, retention time, and ion dissociation patterns of these annotated metabolites to authentic chemical standards analyzed in our lab using the identical method and instrument parameters via tandem mass spectrometry to confirm their identities with Metabolomics Standards Initiative Level One criteria (Morrison et al., 2007).

3. Results

Among the 50 SAWASDEE study participants included in the present study, demographic data for participants in this study are presented in Table 1. Twenty-two (44%) women were from Chom Thong and 28 (56%) were from Fang. Chom Thong had a higher percentage of women who identified solely as Thai (68.2%) than indigenous hill tribes (18.2%) whereas Fang had a higher percentage of women from indigenous hill tribes (57.1%) than from Thai (14.3%). The median age at intake was 24.8 (SD = 5.73) years. Most of the women did not use other medications (82%) and were not exposed to secondhand smoke via their partner’s smoking (60%). When comparing with demographic information in original SAWASDEE cohort, maternal age and smoking status is similar, while our subset population has lower percentage of women who identified as Thai (38.0%) (Table S1). However, the majority of the women did use fertilizer and insecticides (52%). The correlation between pesticide uses and urinary TCPy levels is not significant for most of pesticide use categories (Fig. S1). The median urinary TCPy level was 2.39 ng/ml (4.73 μg/g creatinine). The geometric mean of raw and creatinine adjusted urinary TCPy were 3.71 ± 5.10 ng/ml and 5.37 ± 3.08 μg/g, respectively. Generally, participants from Chom Thong had a higher exposure level and detection rate for both raw and creatinine adjusted TCPy (geometric mean of
raw and creatinine adjusted urinary TCPy being 4.45 ± 5.63 ng/ml and 6.70 ± 3.69 μg/g, respectively), compared to geometric mean of 3.16 ± 4.73 ng/ml and 4.62 ± 2.66 μg/g among participants in Fang (Table S2). For comparison in data derived from the National Health and Nutrition Examination Survey (2013–2014), the median level of urinary TCPy in the general US population of non-Hispanic Asian women of reproductive age (18–40 years; n = 43) was 0.86 ng/mL (1.09 ng/g creatinine), and for all US women of reproductive age (n = 377), was 1.01 ng/mL (0.90 ng/g creatinine).

3.1. MWAS model

After data quality assurance and quality control, 31,995 and 38,210 metabolic features detected in HILIC + ESI and C18 -ESI, respectively, were included for final analyses. In the MWAS models without adjusting for creatinine, 651 metabolic features were significantly associated with TCPy levels (p < 0.05), with 376 and 275 features detected from the HILIC and C18 column, respectively. We observed a slightly higher number of significant features in the MWAS model using creatinine adjusted TCPy levels (691 significant metabolic features, with 367 and 324 features detected from the HILIC and C18 columns, respectively). After false positive discovery rate correction, 18 unique metabolic features remained significantly associated with TCPy levels (adjusted p < 0.2 via Benjamini-Hochberg procedure, Fig. 1).

3.2. Pathway enrichment analysis

Using significant metabolic features detected in the two chromatographic columns (raw p-value<0.05) as input in the pathway enrichment analysis, 37 metabolic pathways were significantly associated with TCPy levels in at least one or more MWAS model (Fig. 2 & Fig. 3), where 24 metabolic pathways were consistently significant in more than two MWAS models/chromatography columns. Most of these metabolic pathways are closely linked to oxidative stress, cellular damage and repair, and systemic inflammation. Notably, lysine metabolism, purine metabolism, and aspartate and asparagine metabolism were significant in all MWAS models and columns. In addition to these three pathways, there were 18 metabolic pathways that appeared in both MWAS models, which include vitamin B9 (folate) metabolism, urea cycle/amino group metabolism, carbon fixation, keratan sulfate degradation, pyruvate metabolism, fatty acid oxidation, peroxisome, alanine and aspartate metabolism, fructose and mannose metabolism, drug metabolism, vitamin B3 (nicotinate and nicotinamide) metabolism, ascorbate (vitamin C) and aldarate metabolism, arginine and proline metabolism, cytochrome p450 metabolism, pentose phosphate pathway, pyrimidine metabolism, glycine, serine, alanine and threonine metabolism, tryptophan metabolism, and tyrosine metabolism.

3.3. Metabolite annotation and confirmation

Among the features enriched in the pathways associated with TCPy levels, we annotated distinct metabolic features in glutathione metabolism (n = 5), fatty acid oxidation and peroxisome metabolism (n = 6), cytochrome P450 metabolism (n = 11), and tryptophan metabolism (n = 22). We then matched the samples with authentic reference standards, verified by MS/MS, to confirm the chemical identity of metabolic features that were both associated with the TCPy levels and enriched within relevant metabolic pathways. In total,
we successfully identified 25 metabolites with Level One evidence (Table 2), which include glutathione, cystine, arachidic acid, itaconate, and nicotinamide adenine dinucleotide.

### 3.4. Sensitivity analysis

Several sets of sensitivity analyses were performed to test the robustness of our results to confounding adjustment. Specifically, we included additional variables in the MWAS model, including body mass index of the mother during 1st visit, and the results were consistent with the main analysis. We also observed similar number of significant features and pathways when excluding each of the covariates in the statistical models. In addition, we performed the pathway enrichment analysis using a more stringent cut-off, with a p-value at 0.005. We found a consistent suite of oxidative stress and inflammation related pathways associated with TCPy levels. Specifically, we found three pathways, including leukotriene metabolism, sialic acid metabolism, and carnitine shuttle, in the HILIC column analysis; we also found 6 pathways, including tyrosine metabolism, cytochrome p450 metabolism, tryptophan metabolism, purine metabolism, pentose phosphate metabolism, and aspartate and asparagine metabolism, in the C18 column analysis. Furthermore, we used two different methods to adjust for urine dilution (i.e., using creatinine adjusted TCPy levels in the model or adding creatinine as a covariate in the MWAS model) and compared the results using exposure pathway enrichment. All significant metabolic pathways found in the main analysis were also observed in the sensitivity analysis, indicating our normalization approaches were consistent.

### 4. Discussion

Chlorpyrifos insecticide exposure during pregnancy can have irreversible developmental impacts on infants. As one of the world’s largest food exporters, half the population in Thailand engages in agriculturally related work that involves extensive use of OP insecticides (Panuwet et al., 2012b; Laohaudomchok et al., 2021). Hence, understanding the potential health outcomes and biological pathways leading to these exposure-related diseases is crucial. Nonetheless, there are very few studies evaluating these pathways in humans thereby highlighting the need for similar studies, especially in underserved or disenfranchised populations that are disparately exposed to high levels of pesticides during critical windows of development (Yang et al., 2020). Using both targeted exposure assessment of urinary TCPy levels and untargeted HRM, we detected consistent perturbations in numerous metabolic pathways associated with urinary TCPy levels among a subset of 50 participants of the SAWASDEE birth cohort. We also verified metabolites that were closely linked and connected in several inflammation, oxidative stress, and cellular damage and repair related pathways. Taken together, we believe these findings point to several potential biological mechanisms of chlorpyrifos toxicity and provide important insight in filling the gaps in the literature about the metabolic impacts of chlorpyrifos exposure in agricultural workers in LMICs. To our knowledge, this constitutes the first untargeted MWAS study examining the biological perturbation associated with prenatal chlorpyrifos exposure in the maternal serum metabolome.
Among the pathways and metabolites significantly associated with maternal urinary TCPy levels, many are related to hepatic functions and closely involved in detoxification. These perturbed biological pathways include fatty-acid oxidation and peroxisome metabolism, tryptophan metabolism, vitamin B3 metabolism, glutathione metabolism, and cytochrome P450 metabolism, and many toxicological and epidemiologic studies have examined their relationship with inflammatory process or oxidative stress. Specifically, fatty acid oxidation and peroxisome metabolism have been linked to OP insecticide exposure as they can cause abnormal lipid metabolism and increase lipid accumulation, where the enhancing oxidative stress resulting from mitochondrial catabolism of fatty acids for energy would in turn generate reactive oxygen species (ROS) (Howell et al., 2016). More commonly, people with more fatty acids tend to have other bioaccumulated lipophilic toxicants which can lead to more ROS and oxidative stress. In particular, peroxisomes contain large number of ROS-producing enzymes such as acyl-CoA oxidases (Wanders et al., 2015), and they also have a large network of antioxidants that protect organelles from oxidative damage. Consistently in this study, we detected and annotated 6 distinct metabolic features that are significantly associated with urinary TCPy levels in fatty acid oxidation and peroxisome metabolism, including hexadecanoyl-CoA, cluponadonyl CoA, stearoyl-CoA, and octadecenoyl-CoA which are part of the β-oxidation of fatty acid chains.

We found that perturbations in tryptophan metabolism and vitamin B3 metabolism, the pathways closely related to oxidative stress and systemic inflammation, were consistently associated with urinary TCPy levels. The metabolism of tryptophan, an essential amino acid, perturbed when exposed to OP insecticides (Du et al., 2014; Hasanoglu Özkan et al., 2021). Previous studies have found significant changes in tryptophan metabolism with the presence of TCPy (Dahiya et al., 2017), and the OP-induced tryptophan inhibition reduced nicotinamide adenine dinucleotide (NAD/NAD+) levels in baby chicks (Henderson and Kitos, 1982). The decreasing transmission of these energy-producing biomolecules (i.e., NAD/NAD+) to the embryo can cause complications such as congenital malformations and fetal death. In coordination with this, vitamin B3, which includes nicotinamide—an essential precursor of NAD—was a top significant pathway identified in our study. The lack of dietary vitamin B3 and tryptophan has been found to increase the frequency of multiple birth defects in mice (Cuny et al., 2020). Consistently, we also confirmed NAD with Level One evidence in our analysis, where NAD intensity was negatively associated with TCPy levels.

We also observed significant associations between TCPy levels and features enriched in glutathione metabolism, including an inverse association with the tripeptide glutathione ($\beta = 0.016$), which is an important biomolecule for eliminating circulating toxicants in humans. Glutathione also protects against free radical damage (Chatterjee et al., 2021; Tang et al., 2021). Consistently, perturbations in glutathione metabolism indicate the presence of ROS in the body due to extracellular reduced glutathione consumption and an increase in oxidized glutathione levels (Ledda et al., 2021). Impairment of these antioxidant enzymes can lead to elevated levels of oxidative stress (Chatterjee et al., 2021) potentially in a dose-response fashion with an early spike in GSH and then a decline in levels (Chatterjee et al., 2021; Tang et al., 2021). More interestingly, we observed perturbations in cytochrome P450 (CYP450) metabolism significantly associated with TCPy levels. Catalyzed by CYP450, OP insecticides including chlorpyrifos, would convert to oxon derivatives, which then bind

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to the acetylcholinesterase and inhibit neurotransmission (Christen et al., 2019; Hernández-Toledano et al., 2020). The significant perturbations in the CYP450 pathway observed in our study are consistent with previous studies of OP insecticides exposures and could indicate the occurrence of DNA damage of cells (Vega et al., 2009). Several other amino acids such as urea cycle amine, lysine, and purine were also found to be significantly associated with TCPy levels, suggesting that multiple amino acid groups can be altered by chlorpyrifos exposure as well.

Despite these promising findings related to the biochemical mechanisms of chlorpyrifos effects, there are several potential limitations in this study that warrant attention. First, this was a proof-of-concept analysis among a subset of 50 participants from the SAWASDEES birth cohort. Although we controlled for several important covariates in the MWAS models, because of the relatively small sample size, we were unable to adjust for additional potential confounding factors, including education and poverty level due to insufficient statistical power. Similarly, the use of non-fasting blood samples may have had an impact on the metabolomics results, and we did not adjust for dietary and nutrition variables due to the limited statistical power. To minimize the potential impact of non-fasting state, we followed an established metabolomics profiling protocol by using internal references and pool standards, which has been shown to successfully analyze many non-fasting samples previously (Chen et al., 2020; Gaskins et al., 2021; Hood et al., 2022b). Although we balanced the number of the participants from each study site and demographic characteristics including maternal age and smoking status when selecting study subjects for this proof-of-concept study, we did observe lower percentage of women who identified as Thai in the subset population, compared to the parent cohort. To address these potential limitations, we plan to conduct follow-up analyses using a larger sample size in this cohort to validate our findings in this analysis. Secondly, we were examining serum samples collected during the first trimester and thus the results may not be representative of metabolic patterns across different time periods through the pregnancy. Future analyses will compare perturbations in maternal metabolome using longitudinal samples collected from different pregnancy periods. Using creatinine as an adjustment for urine dilution is a common practice for urinary exposure biomarkers (Barr et al., 2005). However, because of the variation in pregnant-people physiology and fluctuations during pregnancy, creatinine adjustment may introduce more error into our models. Nevertheless, some reassurance was provided because both models with creatinine adjusted and unadjusted concentrations produced similar results. Lastly, since TCPy can be found in the environment, its elimination via urine may not represent direct chlorpyrifos and chlorpyrifos-methyl exposure, and the inclusion of environmentally derived TCPy, which would introduce non-differential exposure misclassification, may bias the results toward the null. Nevertheless, this may be less of a concern since we observed robust and significant metabolic signals associated with TCPy. Furthermore, we observed non-significant correlations between most pesticide uses categories and urinary TCPy levels, which revealed potential bias in self-reporting and necessitated the use of sensitive biomarkers in future studies to improve accuracy of internal exposure assessment. Additionally, given that our study was relatively small and designed to be exploratory, we ultimately decided on less stringent criteria for statistical significance (i.e., conduct the pathway analysis on features that are significant at raw p values < 0.05)
to decrease the likelihood of false negatives. Therefore, considering the elevated chance of false positive findings, results should be interpreted with caution and requires validation in much larger cohorts.

5. Conclusions

Using the highly sensitive HRM analytical platform, we demonstrated that urinary TCPy levels were associated with perturbations in key biological pathways closely linked to inflammation, oxidative stress, and endocrine implications. We also confirmed the chemical identities of 25 metabolites associated with prenatal exposures to chlorpyrifos, including glutathione, cystine, arachidic acid, itaconate, and nicotinamide adenine dinucleotide. These findings support future hypothesis-testing investigations on potential molecular mechanisms underlying the impact of maternal chlorpyrifos exposure on adverse pregnancy and birth outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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Data availability

Data will be made available on request.

References


Fig. 1. Manhattan Plots of four TCPy Models in HILIC and C18 columns.
Top left is the TCPy model in HILIC column; top right is the TCPy model in C18 column; bottom left is the TCPy + Creatinine covariate model in HILIC column; and bottom right is the TCPy + Creatinine covariate model in C18 column. X-axis denotes the retention time (in seconds), Y-axis denotes the negative log10 of the p-values calculated from the MWAS models. The threshold lines in colors represent the different cut-off value of FDR corrected P-value: red = 0.05, blue = 0.1, and black = 0.2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
Fig. 2. Significant metabolic pathways in TCPy models in HILIC and C18 columns. Top left is the TCPy model in HILIC column; top right is the TCPy model in C18 column; bottom left is the TCPy + Creatinine covariate model in HILIC column; and bottom right is the TCPy + Creatinine covariate model in C18 column.
Fig. 3. Heat Map of Significant Pathways Across All TCPy Models.
Cells are shaded according to the magnitude of p-values generated by mummichog for each pathway. Pathways significantly associated with more than two TCPy models were presented. The pathways were ordered according to the total number of the significant associations (p-value<0.05). Abbreviations: TCPy: 3,5,6-trichloro-2-pyridinol.
Table 1

Demographics characteristics and urinary TCPy levels of a subset of study participants overall (n = 50) and by residential site, from the Study of Asian Women and their Offspring’s Development and Environmental Exposures cohort (2017–2019).

<table>
<thead>
<tr>
<th></th>
<th>Chom Thong (N = 22)</th>
<th>Fang (N = 28)</th>
<th>Total (N = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>26.7 (5.73)</td>
<td>23.4 (5.26)</td>
<td>24.8 (5.67)</td>
</tr>
<tr>
<td>Median [Min, Max]</td>
<td>26.5 [18.0, 38.0]</td>
<td>22.5 [18.0, 38.0]</td>
<td>24.5 [18.0, 38.0]</td>
</tr>
<tr>
<td><strong>BMI of mother at visit 1</strong> (kg/m², mean ± SD)</td>
<td>22.8 ± 3.8</td>
<td>23.2 ± 4.9</td>
<td>22.9 ± 4.1</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thai</td>
<td>15 (68.2%)</td>
<td>4 (14.3%)</td>
<td>19 (38%)</td>
</tr>
<tr>
<td>Hill Tribes b</td>
<td>4 (18.2%)</td>
<td>16 (57.1%)</td>
<td>20 (40%)</td>
</tr>
<tr>
<td>Other</td>
<td>3 (13.6%)</td>
<td>8 (28.6%)</td>
<td>11 (22%)</td>
</tr>
<tr>
<td><strong>Smoking partner a</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7 (31.8%)</td>
<td>12 (42.9%)</td>
<td>19 (38.0%)</td>
</tr>
<tr>
<td>No</td>
<td>15 (68.2%)</td>
<td>15 (53.6%)</td>
<td>30 (60.0%)</td>
</tr>
<tr>
<td><strong>Medication Use</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5 (22.7%)</td>
<td>4 (14.3%)</td>
<td>9 (18.0%)</td>
</tr>
<tr>
<td>No</td>
<td>17 (77.3%)</td>
<td>24 (85.7%)</td>
<td>41 (82.0%)</td>
</tr>
<tr>
<td><strong>TCPy levels (ng/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median [Min, Max]</td>
<td>9.44 [0.220, 65.2]</td>
<td>1.43 [0.475, 50.0]</td>
<td>2.39 [0.220, 65.2]</td>
</tr>
<tr>
<td>Missing</td>
<td>0 (0%)</td>
<td>3 (11.0%)</td>
<td>3 (6.0%)</td>
</tr>
<tr>
<td><strong>TCPy levels (ug/g creat)</strong></td>
<td>8.87 [0.52, 69.55]</td>
<td>3.94 [0.96, 186.65]</td>
<td>4.73 [0.52, 186.65]</td>
</tr>
<tr>
<td>Missing</td>
<td>0 (0%)</td>
<td>3 (11.0%)</td>
<td>3 (6.0%)</td>
</tr>
<tr>
<td><strong>Fertilizer/Pesticide Use</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>12 (54.5%)</td>
<td>14 (50.0%)</td>
<td>26 (52.0%)</td>
</tr>
<tr>
<td>No</td>
<td>6 (27.3%)</td>
<td>10 (35.7%)</td>
<td>16 (32.0%)</td>
</tr>
<tr>
<td>Missing</td>
<td>4 (18.2%)</td>
<td>4 (14.3%)</td>
<td>8 (16.0%)</td>
</tr>
</tbody>
</table>

TCPy: 3,5,6-trichloro-2-pyridinol, metabolite of chlorpyrifos and chlorpyrifos-methyl.

a None of the study participants reported smoking.

b Includes Thai Yai, Lahu, and Pa Long.
Table 2

Chemical identity of the metabolites significantly associated with TCPy levels (raw p < 0.05), the SAWSDEE study (2017–2019).

<table>
<thead>
<tr>
<th>m/z</th>
<th>RT (s)</th>
<th>Confirmed Metabolite</th>
<th>Adduct Form</th>
<th>Probability (p)</th>
<th>Chromatography Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>134.047</td>
<td>25.6</td>
<td>adenine</td>
<td>M-H</td>
<td>0.010</td>
<td>C18</td>
</tr>
<tr>
<td>145.014</td>
<td>21.0</td>
<td>alpha-ketoglutaric acid</td>
<td>M-H</td>
<td>0.017</td>
<td>C18</td>
</tr>
<tr>
<td>311.295</td>
<td>292.2</td>
<td>arachidonic acid</td>
<td>M-H</td>
<td>-0.026</td>
<td>C18</td>
</tr>
<tr>
<td>239.016</td>
<td>27.9</td>
<td>cystine</td>
<td>M-H</td>
<td>-0.027</td>
<td>C18</td>
</tr>
<tr>
<td>129.019</td>
<td>21.6</td>
<td>2-methylmalate/taconate</td>
<td>M-H</td>
<td>-0.012</td>
<td>C18</td>
</tr>
<tr>
<td>178.087</td>
<td>36.6</td>
<td>1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline</td>
<td>M-H</td>
<td>-0.014</td>
<td>C18</td>
</tr>
<tr>
<td>114.019</td>
<td>22.2</td>
<td>melemate</td>
<td>M-H</td>
<td>-0.014</td>
<td>C18</td>
</tr>
<tr>
<td>137.071</td>
<td>44.0</td>
<td>1-methylnicotinamide</td>
<td>M+</td>
<td>-0.046</td>
<td>HILIC</td>
</tr>
<tr>
<td>152.057</td>
<td>49.8</td>
<td>guanine</td>
<td>M+ H</td>
<td>0.027</td>
<td>HILIC</td>
</tr>
<tr>
<td>664.117</td>
<td>293.2</td>
<td>nicotinamide adenine dinucleotide</td>
<td>M+ H</td>
<td>-0.040</td>
<td>HILIC</td>
</tr>
<tr>
<td>117.092</td>
<td>30.5</td>
<td>trans-cyclohexane-1,2-diol</td>
<td>M+ H</td>
<td>0.001</td>
<td>HILIC</td>
</tr>
<tr>
<td>583.256</td>
<td>27.7</td>
<td>biliverdin</td>
<td>M+ H</td>
<td>0.047</td>
<td>HILIC</td>
</tr>
<tr>
<td>244.080</td>
<td>59.2</td>
<td>acetyl-galactosamine</td>
<td>M+ Na</td>
<td>-0.001</td>
<td>HILIC</td>
</tr>
<tr>
<td>208.097</td>
<td>30.3</td>
<td>acetyl-phenylalanine</td>
<td>M+ H</td>
<td>-0.006</td>
<td>HILIC</td>
</tr>
<tr>
<td>186.017</td>
<td>278</td>
<td>phospho-serine</td>
<td>M+ H</td>
<td>-0.019</td>
<td>HILIC</td>
</tr>
<tr>
<td>189.160</td>
<td>107.4</td>
<td>nepisolin, nepisolin-trimethyllysine</td>
<td>M+ H</td>
<td>0.012</td>
<td>HILIC</td>
</tr>
<tr>
<td>178.054</td>
<td>33.6</td>
<td>formyl-methionyl peptide</td>
<td>M+ H</td>
<td>0.090</td>
<td>HILIC</td>
</tr>
<tr>
<td>219.113</td>
<td>29.9</td>
<td>acetylseryotonin</td>
<td>M+ H</td>
<td>-0.008</td>
<td>HILIC</td>
</tr>
<tr>
<td>284.099</td>
<td>54.7</td>
<td>guanosine</td>
<td>M+ H</td>
<td>0.025</td>
<td>HILIC</td>
</tr>
<tr>
<td>308.092</td>
<td>252</td>
<td>glutathione</td>
<td>M+ H</td>
<td>0.013</td>
<td>HILIC</td>
</tr>
<tr>
<td>217.069</td>
<td>47.5</td>
<td>methyl beta-galactoside</td>
<td>M+ Na</td>
<td>0.066</td>
<td>HILIC</td>
</tr>
<tr>
<td>189.124</td>
<td>76.0</td>
<td>nalpha-acetyl-lysine</td>
<td>M+ H</td>
<td>-0.019</td>
<td>HILIC</td>
</tr>
<tr>
<td>332.076</td>
<td>122.2</td>
<td>damp</td>
<td>M+ H</td>
<td>-0.020</td>
<td>HILIC</td>
</tr>
<tr>
<td>147.092</td>
<td>28.8</td>
<td>dimethylbenzimidazole</td>
<td>M+ H</td>
<td>-0.007</td>
<td>HILIC</td>
</tr>
<tr>
<td>377.146</td>
<td>44.7</td>
<td>riboflavin</td>
<td>M+ H</td>
<td>0.021</td>
<td>HILIC</td>
</tr>
</tbody>
</table>

Note: Chemical identity of metabolic features was confirmed by matching peaks via accurate mass to charge ratio and retention time to authentic reference standards under the same conditions using tandem mass spectrometry.
Abbreviations: m/z: mass to charge ratio; RT: retention time; HILIC: hydrophilic interaction chromatography column with positive electrospray ionization mode; C18: hydrophobic chromatography column with negative electrospray ionization mode; TCPy: stable metabolite 3,5,6-trichloro-2-pyridinol of organophosphate insecticide.

The beta coefficient represents the change in log-transformed metabolite intensity per 1-unit increase in urinary TCPy level.