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Metabolome-Wide Association Study of the Relationship Between Insecticide Exposure and First Trimester Serum Metabolite Levels in North Thailand Women

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Abstract

Metabolome-Wide Association Study of the Relationship Between Insecticide Exposure and First Trimester Serum Metabolite Levels in North Thailand Women

By Jonathan Batross

Intro: Organophosphate (OP) insecticides are a common group of neurotoxic insecticides. Their main target is acetylcholinesterase which are in charge of muscle contractions. While OP insecticides are made to kill pests and insects, they can also affect humans. Thailand is a country highly dependent on agricultural work and thus has a high usage of OP insecticides. Women who are pregnant frequently work in these fields, and thus can expose the fetus to these insecticides, ultimately causing neurodevelopmental issues among the newborns and children.
Methods: This study is a subset of the Study of Asian Women and Offspring's Development and Environmental Exposures (SAWASDEE) study which followed 322 women-child pairs from pregnancy until three years old. We used high-resolution metabolomics (HRM) to assess serum samples collected on 50 of these women at the first trimester. To assess the levels of OP insecticide exposure, we measured the stable metabolite 3,5,6-trichloro-2-pyridinol (TCPY) in urine samples. Following an untargeted metabolome-wide association study (MWAS) workflow, we used liquid chromatography-mass spectroscopy (LCMS) instruments to conduct metabolic profiling on our samples, and then we used in house bioinformatics software in Python and R to identify significant metabolites and pathways associated with OP exposures. We used 2 multiple linear regression models which included recruitment site, age, ethnicity, if their husband smoked, the use of medication, and use of fertilizer and pesticides. One model also controlled for creatinine adjust TCPY levels.
Results: We found 37 significant pathways that occurred in at least 2 of our models. Most of these metabolic pathways were linked to the production of reactive oxygen species (ROS) which indicates cellular damage. These pathways include: Tryptophan metabolism, fatty acid oxidation and peroxisome metabolism, drug metabolism using CYP450, Glutathione metabolism, and Vitamin B3.
Discussion: This work is meant to pioneer and further add research of the maternal metabolome during pregnancy and OP insecticide exposure. This research is especially important because this exposure is occurring when the fetus is most at risk of neural development disruption. It can also lead to targeted interventions which would lower the health burden for this large population.

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Introduction

Organophosphates (OP) are a group of commonly neurotoxic insecticides that include compounds like chlorpyrifos, diazinon, and malathion (Harley et al. 2011). Two of these chemicals, chlorpyrifos and diazinon, have been phased out by manufacturers in 2001, but there are still approximately 73 million pounds used per year in the US alone (Harley et al. 2011). OP insecticides inhibit acetylcholinesterase (AChE) (Jusko et al. 2019) by biding to the site where the acetylcholine binds and acts as a non-competitive inhibitor, eventually leading to severe reactions such as paralysis or death by exhaustion.(Naksen et al. 2015) Multiple animal studies (Spyker and Avery 1977; Srivastava and Raizada 1996) have shown that higher OP insecticide exposure has led to reduction in fetal growth in rodents. There have also been findings (Rauch et al. 2012; Sagiv et al. 2019; Silver et al. 2018; Srivastava and Raizada 1996) of associations of higher OP metabolites found in serum and reduction in prenatal development. Despite these epidemiological observations and limited mechanistic studies, there is a gap in current literature between the biological pathways and linkages between prenatal exposure to OP insecticides through the maternal metabolome.

High-resolution metabolomics (HRM) is a high-throughput analytical method capable of quantifying and identifying a large number of metabolites from exogenous and endogenous sources(Kim et al. 2016). This method is useful for studying effects of environmental exposures on the human metabolome (Rappaport 2011). Other environmental metabolomics studies (Bonvallot et al. 2013; Liang et al. 2019) have shown the great potential of HRM as a powerful tool to improve internal exposure estimation to complex environmental mixtures. In this study, we conducted HRM to examine the association between OP exposures and perturbations in the maternal metabolome during pregnancy and its potential effects on prenatal development.

To better elucidate this biological link, we used the Study of Asian Women and Offspring's Development and Environmental Exposures (SAWASDEE), a longitudinal birth cohort based in the Northern Thailand districts of Fang and Chom Thong. The study followed women through pregnancy, collected multiple, time-resolved biological samples, and evaluated neural development until 3 years of age. Thailand is considered one of the world's largest food exporters and because of this, there is a lot of agricultural work and insecticide use. Different crops require different levels and duration of spraying with OP insecticides. This mix of exposures in pregnant women and other factors make this cohort a great candidate for environmental HRM application.

We built an initial metabolomics analysis using bioinformatics techniques to identify biological perturbations in metabolites and potential biological pathways associated with OP insecticide usage on maternal metabolome. We expect to observe signs of oxidative stress due to the perturbations caused by OP insecticide exposure. This work is meant to pioneer and further add research of the maternal metabolome during pregnancy and OP insecticide exposure. This research is especially important because this exposure is occurring when the fetus is most at risk of neural development disruption. It can also lead to targeted interventions which would lower the health burden for this large population.

Methods.

Study Population

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), who are studying neurodevelopmental impacts of prenatal insecticide exposure. Estimates show that 98% of pregnant women in Thailand received prenatal care in 2016 (*World Development Indicators 2017* 2017). Because of the strong agricultural workforce of Thailand, the generalizability to other low/middle income countries (LMIC), and the study team's previous work in the Chiang Mai Province, this region was chosen. These previous studies in the region found elevated insecticide exposure in women and children in this area (Fiedler et al. 2015; Naksen et al. 2015, 2017; Onchoi et al. 2020).

Participant Recruitment

The study population included pregnant women who were agricultural workers or lived within 50 meters from an agricultural field; had a Thai identification card which allowed them to receive antenatal clinic access; resided in their region for at least 5 months and planned to live there 3 years after deliver; spoke the Thai language 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 didn't use illegal drugs; were less than 16 weeks of gestation.; singleton pregnancy; and lastly agrees 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 women were enrolled into the study. 50 of those participants had urine and serum samples taken for our first trimester analysis. The study was reviewed and approved by Emory and Chiang Mai Universities IRB, Rutgers University relied on Emory University's IRB and Chulalongkorn University relied upon Chang Mai University's IRB.

Exposure Assessment

Urine samples were composited using equal volumes to create early, mid, and late pregnancy samples that roughly correspond to trimester. All 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 organophosphate insecticides chlorpyrifos and chlorpyrifos methyl using a modification of a previously validated method (Olsson et al. 2004). Briefly, samples are spiked with stable isotopic analogues of the target analytes then are enzymatically digested using purified β-glucuronidase and sulfatase enzymes (derived from *H. pomatia*) to liberate bound metabolites. The hydrolysates are centrifuged and transferred to autosampler vials. To facilitate on-line solid phase extraction, samples are injected into a column switching system for concentration of the target analytes on a Strata RP on-line SPE column (2.1 x 20 mm). The on-line extraction column is washed with the acetonitrile:Milli-Q water (10:90, V/V) solution to remove undesired matrix interferences. The target analytes are then eluted from the on-line extraction column to a Poroshell 120 EC-C18 analytical column (3.0 x 100 mm, 2.7 um) for chromatographic separation. The target analytes are measured using negative mode electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) with isotope dilution quantification. During mass spectrometric analysis, the target analytes are monitored using the multiple reaction monitoring (MRM) mode. One quantitation ion and one confirmation ion are monitored for the native analytes, and one quantitation ion is monitored for the labeled analogues. Concentrations of the target analytes are 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 control) 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 parameter of the method. The limits of detection (LOD) were 0.31 ng/mL (TCPY, 3PBA) and 125 ng/mL (cDCCA, tDCCA) and the relative recoveries ranged from 90-99%. For statistical analysis, the LOD divided by the square root 2 was imputed for all values below the LOD.

Creatinine was measured by diluting urine samples 1000-fold with water after spiking with its isotopically labeled analogue. Diluted samples were analyzed by liquid chromatography electrospray ionization coupled with tandem mass spectrometry. For creatinine, two ion transition were monitored (m/z 113.9 -> m/z 44.2 and m/z 113.9 ->86) and only one ion transition was monitored for labeled creatinine (m/z 116.9 -> m/z 47.2) (Kwon et al. 2012). Quantification was achieved using an isotope calibration. Quality control/assurance included the concurrent measurement of calibrants, blanks and quality control materials and semi-annual certification by the GEQUAS program. The LOD was 5 mg/dL with a relative standard deviation of 5%. Specific gravity was measured using an automated refractometer using water for standardization.

Equation 1: Creatinine corrected values

$$Creatinine\ corrected\ concentration\ (\frac{\mu g}{g}creat) = \left(\frac{Concentration\ \left(\frac{ng}{mL}\right)}{creatinine\ \left(\frac{mg}{dL}\right)}\right) * 100$$

High Resolution Metabolomics

HRM analyses were conducted on the serum samples using established protocols (Go et al. 2015; Liang et al. 2018). Each sample was treated with two volumes of acetonitrile and analyzed in triplicate using liquid chromatography-high-resolution mass spectrometry (LC–HRMS) techniques (Dionex Ultimate 3000; ThermoScientific QExactive). Two technical columns, hydrophilic interaction liquid chromatography (HILIC) with positive ESI and C18 hydrophobic reversed-phase chromatography with negative ESI, were used to enhance the coverage of metabolic feature detection. Two quality control pooled reference plasma samples, including NIST 1950 (Simón-Manso et al. 2013) and pooled human plasma purchased from Equitech Bio were included at the beginning and end of each analytical batch for normalization, control for background noise, batch evaluation, and post hoc quantification. Following instrument analyses of all samples, raw data files were converted to .mzML files using ProteoWizard (Chambers et al. 2012) and extracted using apLCMS with modifications by xMSanalyzer (Uppal et al. 2013; Yu et al. 2009). Detected signals (referred to as metabolic features) were uniquely defined by their mass-to-charge ratio (*m/z*), retention time, and ion intensity. Only metabolic features detected in >10% of the serum samples with median coefficient of variation (CV) among technical replicates <30% and Pearson correlation >0.7 were included in further analyses. Following quality assessment, the median intensity was taken across replicate samples and these intensities were natural log transformed for analysis.

Statistical Analysis.

We analyzed the associations between TCPY levels and metabolic features using multivariable linear regression models adjusted for creatinine adjusted TCPY levels (one model with this variable and one model without), recruitment site, age, ethnicity, husband smoking status, use of

medications other than prenatal multivitamins, and use of fertilizer and insecticides at work since pregnancy using R (Version 4.0.4). The models used the equations of:

Equation 2a: Multilinear Regression Model without Creatinine Adjusted TCPY Levels.

$$\boldsymbol{Y_{ij} = \beta_{1j} TCPY_i + \beta_{2j} Site_i + \beta_{3j} Age_i + \beta_{4j} Ethnicity_i + \beta_{5j} Hus\_smoke_i + \beta_{6j} Meds_i + \beta_{7j} UseFP_i}$$

Equation 2b: Multilinear Regression Model with Creatinine Adjusted TCPY Levels.

$$\boldsymbol{Y_{ij} = \beta_{1j} TCPY_i + \beta_{2j} Creat_i + \beta_{3j} Site_i + \beta_{4j} Age_i + \beta_{5j} Ethnicity_i + \beta_{6j} Hus\_smoke_i + \beta_{7j} Meds_i + \beta_{8j} UseFP_i}$$

where Y_{ij} refers to the intensity (i.e., relative concentration) of metabolic feature *j* for participate *i*. Separate models were conducted for each metabolic feature, from each ionization mode (urine C18 negative ESI, and urine HILIC positive ESI). Multiple comparison correction was conducted using the Benjamini-Hochberg false discovery rate ($FDR_{B\text{-}H}$) procedure, a widely used procedure in Metabolome Wide Association Studies (MWAS) study, at a 5% false positive threshold.

Metabolic pathway enrichment analysis and metabolite annotation

We conducted pathway enrichment analysis utilizing mummichog (Version 1.0.10, Python Version 3.39), a novel bioinformatics platform that infers and categorizes functional biological activity directly from mass spectrometry output, without prior metabolite validation (Li et al. 2013). An adjusted p-value for each pathway was calculated from resampling the reference input file in mummichog using a gamma distribution, which penalizes pathways with fewer reference

hits, and assigning greater significance to pathways with more reference hits (Li et al. 2013). We applied two strategies to select eligible metabolic features for pathway analysis: (i) at raw p-values < 0.05 (less conservative); (ii) at raw p-values <0.005 (more conservative). For the both approaches, to compensate for false discoveries, we excluded pathways identified by mummichog with a p-value higher than 0.05 and those containing less than 4 significant metabolic features that were matched with known compounds by *m/z*. We conducted pathway analysis separately for each of the model, one with and without log adjusted creatinine levels, and by ionization mode.

Next, we identified overlapping significant metabolic features and pathways between models. These overlapped metabolic features were then annotated by matching the m/z value for commonly formed adducts to the METLIN, ChemSpider, Human Metabolome Database, and Kyoto Encyclopedia of Genes and Genomes databases, using a mass error threshold of 10 ppm (Uppal et al. 2017). Finally, we confirmed a select number of annotated metabolites by comparison of m/z, retention time and ion dissociation patterns to authentic chemical reference standards analyzed in our lab using the identical method and instrument parameters via tandem mass spectrometry.

Results

The characteristics of these woman can be found more in depth in Table 1. The median age was 24.8 (SD = 5.73) years. There were 22 (44%) women from Chom Thong and 28 (56%) women from Fang. Chom Thong had a higher percentage of Thai people (68.2% vs 14.3%) where Fang had a higher percentage of hill tribes (4.5% vs 53.6%). The majority of the women didn't use

other medications (18%) and weren't exposed to their husband's smoking (38%). However, most women did use fertilizer and insecticides (52%).

In the model containing TCPY only, there were 651 total metabolites found significantly associated with our exposure (275 in the C18 column and 376 in the HILIC). In the model adjusting for creatinine adjust TCPY levels, the number of total significant metabolites is 691 (324 in the C18 column, 367 in the HILIC column). Overall, for the respective columns, there were 31,995 metabolic features detected after data filtering in the C18 column and 38,210 metabolic features detected in the HILIC column. Manhattan plots (Figs 6-9) show the distribution of significant features above FDR corrected levels of 0.2 (black), 0.1 (blue), and 0.05 (red), respectively.

When performing pathway analysis, there were 37 pathways found significantly associated with OP exposures in either model (Figure 5). For our analysis, we focused on a subset of 24 pathways which appeared in more than one column or model. Notably, Lysine metabolism, purine metabolism, and aspartate and asparagine metabolism were found significant over all models and columns (N=3). Along with those three pathways there were an addition 18 that appeared in both 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; Tyrosine metabolism (N=18). The remaining 3 were only found in a single model.

We found, for these pathways, 5 distinct metabolites for glutathione metabolism; 6 distinct metabolites for fatty acid oxidation, peroxisome metabolism; 11 distinct metabolites for drug metabolism – cytochrome P450; and 22 distinct metabolites for tryptophan metabolism (Table 2). We then matched the samples with authentic reference standards, verified by tandem mass spectrometry, to confirm the chemical identity of metabolic features that were both associated with the TCPY levels and enriched within OP exposure-relevant metabolic pathways. In total, we identified 32 metabolites with level 1 evidence, including arachidic acid, glutathione, and itaconate.

Lastly, we performed a sensitivity analysis using a cut-off p-value of 0.005 for a more conservative pathway list. We found 3 pathways: Leukotriene metabolism, sialic acid metabolism, and carnitine shuttle for the HILIC column; we also found 6 pathways: Tyrosine metabolism, Drug metabolism – cytochrome P450, tryptophan metabolism, purine metabolism, pentose phosphate metabolism, and aspartate and asparagine metabolism, in the C18 column.

Discussion

Through this untargeted MWAS, we have elucidated some of the important or significant pathways that are connected with OP insecticide exposure and the disruption of the maternal metabolome. These pathways can provide important insight and help improve the gaps in the literature about the impacts of OP insecticide exposure, in low dose long-term exposure and high

dose short-term exposure, to pregnant woman in LMICs. OP insecticide exposure during this crucial period can have everlasting developmental impacts on the fetus and infants.

The following pathways will be the focus for the paper, however, there are other pathways that could also be interesting to look at. Fatty acid oxidation and peroxisome metabolism is linked to OP insecticide exposure as it has been found to cause abnormal lipid metabolism (Howell III et al. 2016). The same paper found that this exposure caused increased lipid accumulation as well. This can lead to oxidative stress in the breaking down of fatty acids in the mitochondrial to make energy as this energy generation process had been found to be a generator of reactive oxygen species (ROS) (Howell III et al. 2016). More commonly, people with more fatty acids also contain other toxins stored in those fat cells leading to more ROS and stress. Tryptophan metabolism is an essential amino acid which has been shown in previous studies to have decreased when exposed to OP insecticides (Du et al. 2014; Hasanoğlu Özkan et al. 2021). This exemplifies that OP insecticide exposure disturbs amino acid structure and metabolism. Other amino acids such as Urea cycle/amino group, lysine, and purine show up in our MWAS output showing that multiple amino acid groups could be affected by the OP insecticide exposure. Glutathione metabolism is an important marker of toxicity through glutathione S transferase (GST) and reduced glutathione (GSH). Glutathione also protects against free radical damage. Higher levels of GSH indicates that there are xenobiotics in the body and assist in their excretion (Chatterjee et al. 2021; Tang et al. 2021). Glutathione metabolism also shows that there is the presence of ROS in the body due to extracellular GSH consumption and an increase in oxidized glutathione levels (Ledda et al. 2021). Drug metabolism – cytochrome p450 AChE is metabolized by enzymes like cytochrome P450 (CYP450). AChE is the main target for these OP

insecticides and CYP450 has been found to have its activity inhibited by exposure as well. (Christen et al. 2019; Hernández-Toledano et al. 2020) This pathway being identified as significant corresponds to previous studies of OP insecticide exposure and could also indicate DNA damage of certain cells. (Vega et al. 2009). AChE inhibition has been found in honey bees which are not the main targets for these insecticides (Williamson et al. 2013).

OP insecticides mainly target AChE receptors mainly found in the central and peripheral nervous system, neuromuscular junctions, and red blood cells (Alejo-González et al. 2018). The liver, which is involved in human detoxification, comes up frequently in our pathway analysis. Some examples would be fatty-acid, peroxisome metabolism, tryptophan metabolism, glutathione metabolism, and cytochrome P450 drug metabolism. As mentioned previously, fatty acid oxidation and peroxisome metabolism are linked to generating ROS. Peroxisomes contain large number of ROS-producing enzymes such as acyl-CoA oxidases (Wanders et al. 2016). Some of the overlapping features that showed up included hexadecanoyl-CoA, stearoyl-CoA, cluponadonyl CoA, and octadecenoyl-CoA which are part of β-oxidation of fatty acid chains. However, the peroxisomes also have a large network of antioxidants that protect organelles from oxidative damage. Tryptophan metabolism has been shown to be disturbed by OP insecticide exposure mainly in the liver. Tryptophan fluorescence is used to monitor changes in amino acids in the presence or absence of foreign molecules. A previous study looking at TCPY shows that there are significant changes in tryptophan and tyrosine (another pathway which showed up in our analysis) with the presence of TCPY which is a metabolite of OP insecticide exposure (Dahiya et al. 2017). It was also found that in baby chicks, the inhibition of tryptophan cause by OP insecticides reduced the nicotinamide adenine dinucleotide (NAD/NAD+) levels of the baby

chicks (Henderson and Kitos 1982). Lowering the availability of energy products to the embryo cause complications such as congenital malformations and miscarriages. In coordination with this, vitamin B3 which includes nicotinamide, a part of NAD, was a significant pathway identified by our analysis. In mice, it was found that a lack of dietary vitamin B3 and tryptophan increase a frequency of multiple birth defects as well (Cuny et al. 2020).

Other pathways show a significance connected to oxidative stress by increased of xenobiotic compounds. In the case of this study that xenobiotic compound would be in the form of OP insecticide exposure. Glutathione metabolism showed a positive association with exposure to OP insecticides. During pathway analysis reduced glutathione (GSH) showed to positively associated with an increased of TCPY. Meaning that as the level of TCPY increased, there was a higher level of GSH (β= 0.016). Impairment of these antioxidant enzymes can lead to elevated levels of oxidative stress (Chatterjee et al. 2021). Based on Chatterjee et al. and Tang et al. it seems that there is a dose response to GSH with an early spike in GSH and then a decrease in GSH levels. Because these samples were taken in early pregnancy, there might be a decrease in GSH levels showing an increase in ROS overtime that isn't contained by GSH. Lastly, drug metabolism through cytochrome p450 is another pathway that is connected to the liver. A previous study looking at different types of OP insecticides exposure shows that they all inhibited CYP450's enzymatic ability (Abdou et al. 2020). They also showed that the OP insecticides inhibited the CYP450s in a non-competitive manner.

To date, there are no reported studies which focus on metabolomics analysis on the prenatal/maternal metabolome in relation to exposure to OP insecticides. This group of people

are highly exposed to OP insecticides uses due to living in a highly agricultural reliant country such as Thailand, and because of this there needs to be more studies done in this area. OP insecticides are the largest group of insecticides used across the world and with emerging omics-based and metabolomics-based research being recognized and done, further research is needed. It is known that OP insecticides have effects of neural development in mammals such as mice and many of those papers have been used in this paper; however, there is still a lack of human data or field data to elucidate this relationship further.

Despite these promising findings, there are several potential limitations in this study that warrants attention. First, this was a pilot analysis among a subset of 50 participants from the SWASDEES birth cohort. Because of the relatively small sample size, we were unable to control for several potential confounding factors in the MWAS statistical modeling, including education, frequency of crop used, and poverty level. We plan to conduct follow-up analysis using a larger sample size in this cohort to validate our findings in this pilot analysis. Secondly, we were examining serum samples collected during the first trimester serum samples and thus the results may not be representative of metabolic patterns across different time periods through the pregnancy. Future analysis shall compare perturbations in maternal metabolome using longitudinal samples collected from different pregnancy periods. The models used to look at both TCPY levels and creatinine adjusted TCPY levels many similar pathways. Using creatinine as an adjustment for urine dilution is a common practice (Barr et al. 2005). However, because of the variation in pregnant women physiology and fluctuations during pregnancy, this creatinine adjusted TCPY levels might be skewed. However, as this is preliminary or pilot data, this

shows the merit for further and more extensive research in the field of OP insecticides exposure and pregnant women metabolome.

Conclusions

The SAWASDEE birth cohort study provides information essential for risk assessment paradigms addressing the risk of prenatal insecticide exposure and neurodevelopment. This MWAS provides crucial information on potentially impactful metabolic pathways and biological clues to the effects of OP insecticides on the maternal metabolome and fetus. This pilot study will hopefully begin the elucidation of these pathways and bridge the current gap in knowledge.

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Table 1. Demographics

	Chom Thong (N=22)	Fang (N=28)	Total (N=50)
Age			
Mean (SD)	26.7 (5.73)	23.4 (5.26)	24.8 (5.67)
Median [Min, Max]	26.5 [18.0, 38.0]	22.5 [18.0, 38.0]	24.5 [18.0, 38.0]
Ethnicity			
Karen	3 (13.6%)	1 (3.6%)	4 (8.0%)
Thai	15 (68.2%)	4 (14.3%)	19 (38.0%)
Hill Tribes**	1 (4.5%)	15 (53.6%)	16 (32%)
Other	3 (13.6%)	8 (28.6%)	11 (22%)
Husband Smoke*			
Yes	7 (31.8%)	12 (42.9%)	19 (38.0%)
No	15 (68.2%)	15 (53.6%)	30 (60.0%)
Use Medications			
Yes	5 (22.7%)	4 (14.3%)	9 (18.0%)
No	17 (77.3%)	24 (85.7%)	41 (82.0%)
TCPY levels (ng/ml)			
Median [Min, Max]	9.44 [0.220, 65.2]	1.43 [0.475, 50.0]	2.39 [0.220, 65.2]
Missing	0 (0%)	1 (3.6%)	1 (2.0%)
TCPY adjusted Creatnine (ug/gcreat)			
Median [Min, Max]	6.57 [0.52, 186.65]	4.21 [2.22, 69.55]	4.36 [2.81, 5.76]
Missing	0 (0%)	1 (3.6%)	1 (2.0%)
Use Fert/Pest			
Yes	12 (54.5%)	14 (50.0%)	26 (52.0%)
No	6 (27.3%)	10 (35.7%)	16 (32.0%)
Missing	4 (18.2%)	4 (14.3%)	8 (16.0%)

*None of the woman in the study reported smoking

** includes Thai Yai, Lahu, and Pa Long

Table 2. Confirmed Metabolites

M/z	RT (s)	Validated Metabolite	Adduct Form	beta	Columns
134.047	25.6	ADENINE	M-H	0.010	C18
145.014	21.0	ALPHA-KETOGLUTARIC ACID	M-H	0.017	C18
311.295	292.2	ARACHIDIC ACID	M-H	-0.026	C18
239.016	27.9	CYSTINE	M-H	-0.027	C18
377.086	20.4	LACTOSE	M+Cl	0.020	C18
377.086	20.4	SUCROSE	M+Cl	0.020	C18
377.086	20.4	MELIBIOSE	M+Cl	0.020	C18
377.086	20.4	MALTOSE	M+Cl	0.020	C18
377.086	20.4	CELLOBIOSE	M+Cl	0.020	C18
377.086	20.4	PALATINOSE	M+Cl	0.020	C18
129.019	21.6	2-METHYLMALEATE	M-H	-0.012	C18
129.019	22.2	ITACONATE	M-H	-0.012	C18
178.087	36.6	1-METHYL-6,7-DIHYDROXY-1,2,3,4-TETRAHYDROISOQUINOLINE	M-H	-0.014	C18
114.019	22.2	MALEAMATE	M-H	-0.014	C18
137.071	44.0	1-METHYLNICOTINAMIDE	M+	-0.046	HILIC
152.057	49.8	GUANINE	M+H	0.027	HILIC
664.117	293.2	NAD	M+H	-0.040	HILIC
117.092	30.5	TRANS-CYCLOHEXANE-1,2-DIOL	M+H	0.001	HILIC
583.256	27.7	BILIVERDIN	M+H	0.047	HILIC
244.080	59.2	ACETYL-GALACTOSAMINE	M+Na	-0.001	HILIC
208.097	30.3	ACETYL-PHENYLALANINE	M+H	-0.006	HILIC
186.017	278	PHOSPHO-SERINE	M+H	-0.019	HILIC
189.160	107.4	NEPSILON, NEPSILON-TRIMETHYLLYSINE	M+H	0.012	HILIC
178.054	33.6	FORMYL-METHIONYL PEPTIDE	M+H	0.090	HILIC
219.113	29.9	ACETYLSEROTONIN	M+H	-0.008	HILIC
284.099	54.7	GUANOSINE	M+H	0.025	HILIC
308.092	252	GLUTATHIONE	M+H	0.013	HILIC
217.069	47.5	METHYL BETA-GALACTOSIDE	M+Na	0.066	HILIC
189.124	76.0	NALPHA-ACETYL-LYSINE	M+H	-0.019	HILIC
332.076	122.2	DAMP	M+H	-0.020	HILIC
147.092	28.8	DIMETHYLBENZIMIDAZOLE	M+H	-0.007	HILIC
377.146	44.7	RIBOFLAVIN	M+H	0.021	HILIC

Figure 1: TCPY Model HILIC Significant Pathways

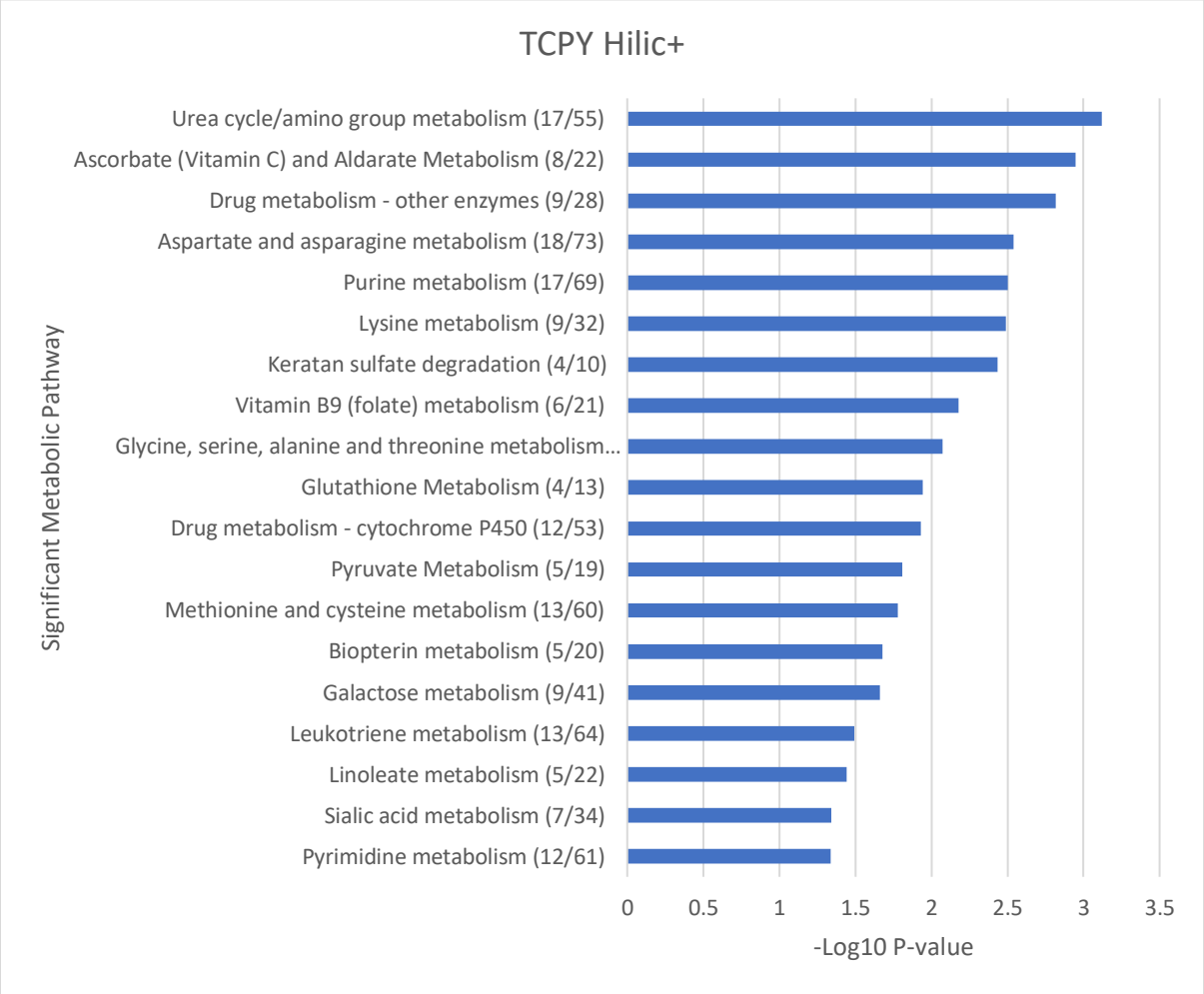


Figure 2: TCPY Model C18 Significant Pathways

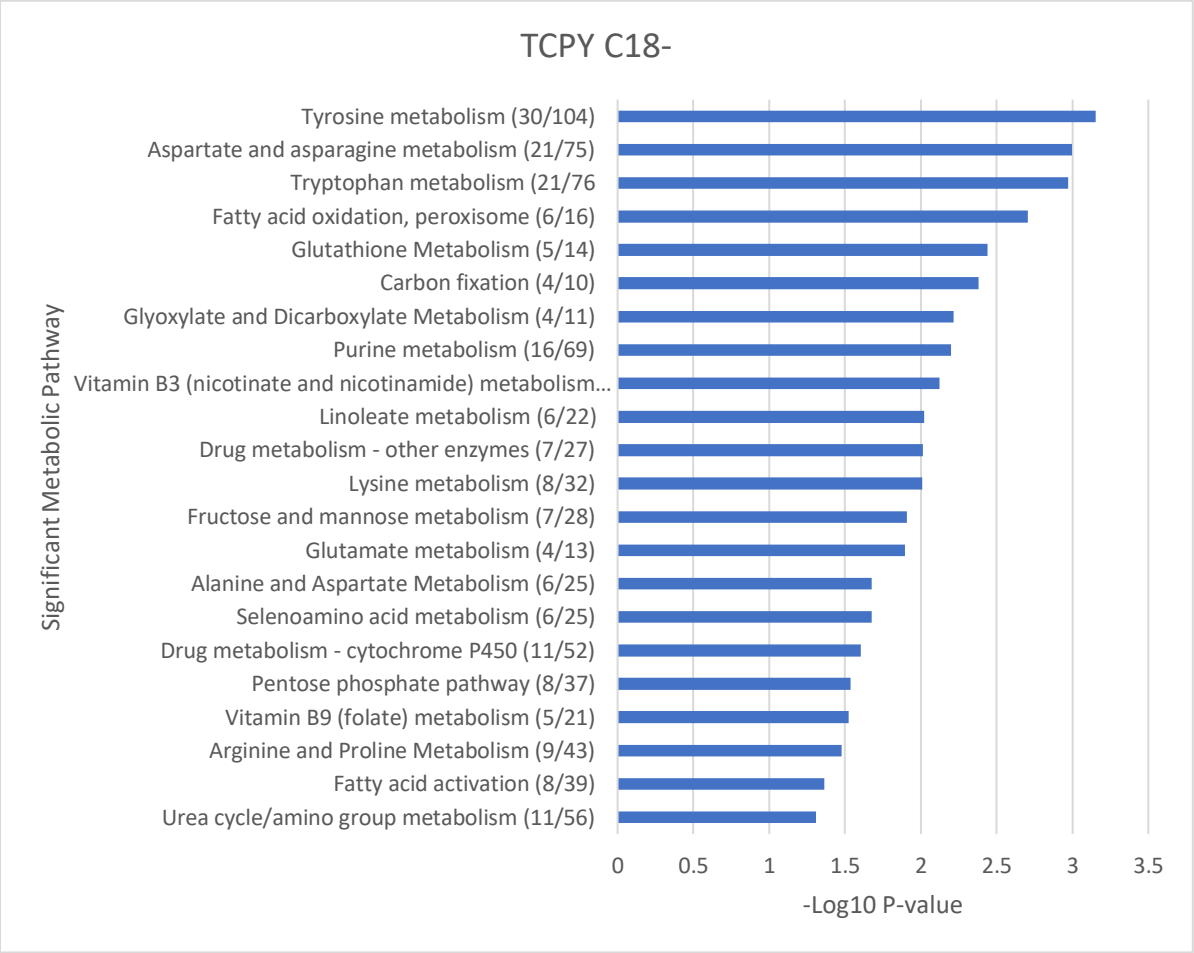


Figure 3: TCPY + Creatinine adjusted TCPY Model HILIC+ Significant Pathways

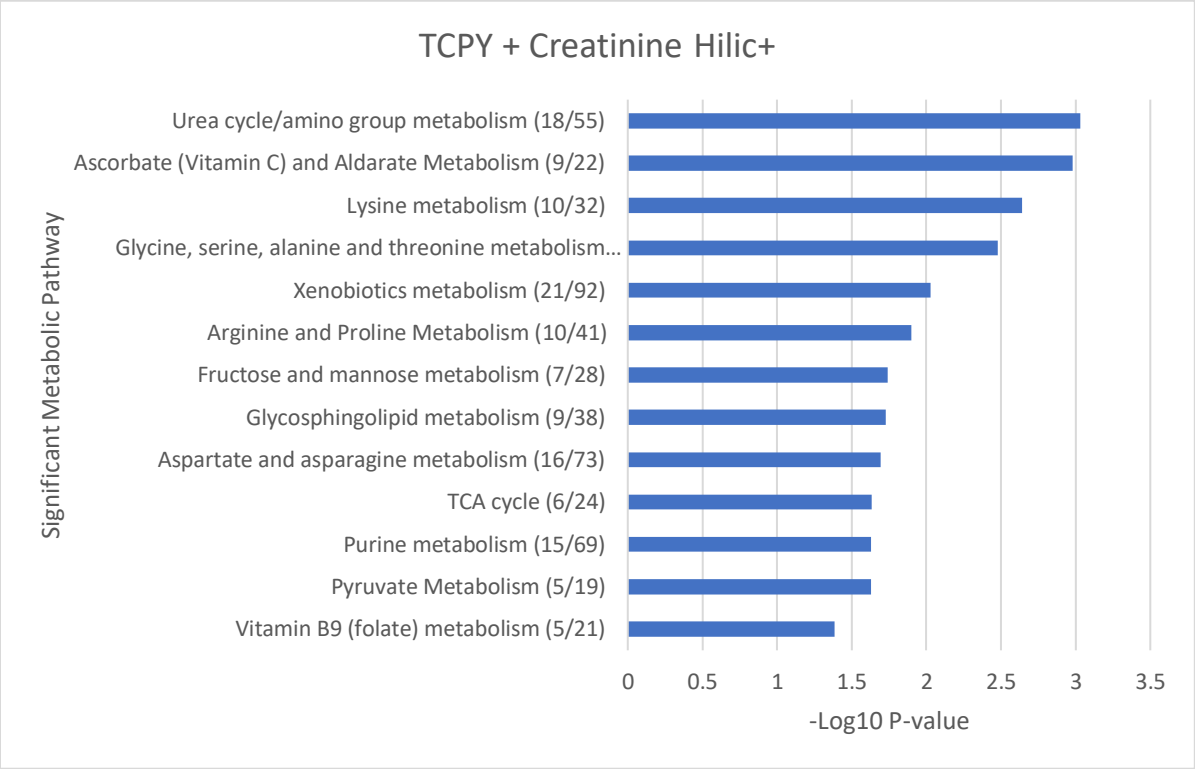


Figure 4: TCPY + Creatinine adjusted TCPY Model C18 Significant Pathways

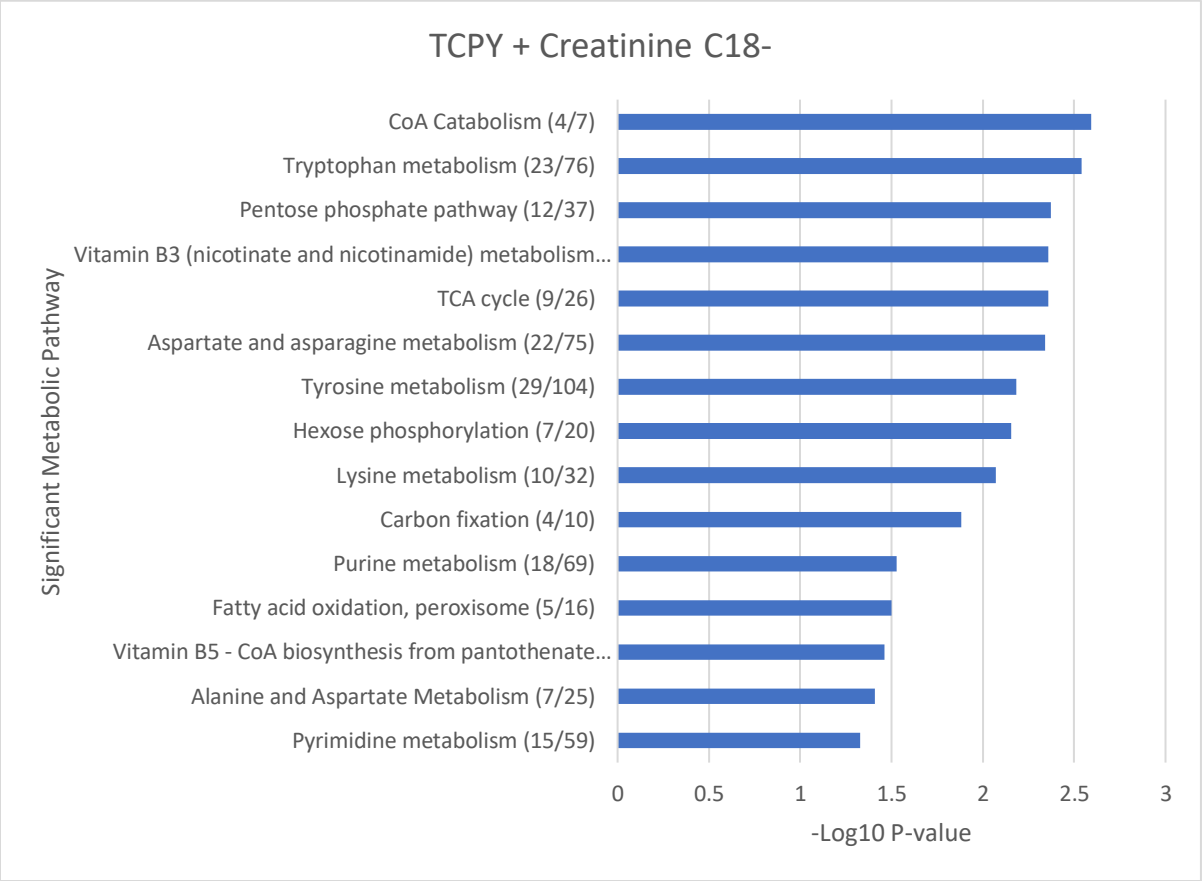


Figure 5: Heat Map of Significant Pathways Across All Models

Pathway Name	Number of metabolites in pathway	Number of overlapping features	HILIC		C18 Negative		Total P-value < 0.05
			TCPy	Creatinine	TCPy	Creatinine	
Lysine metabolism	9	32					4
Purine metabolism	17	59					4
Aspartate and asparagine metabolism	19	74					4
Vitamin B9 (folate) metabolism	5	21					3
Urea cycle/amino group metabolism	15	56					3
Carbon fixation	4	10					2
Keratan sulfate degradation	4	10					2
Glutathione Metabolism	5	14					2
Pyruvate Metabolism	5	19					2
Fatty acid oxidation, peroxisome	6	16					2
Linoleate metabolism	6	22					2
Alanine and Aspartate Metabolism	7	25					2
Fructose and mannose metabolism	7	28					2
Drug metabolism - other enzymes	8	28					2
TCA cycle	8	25					2
Vitamin B3 (nicotinate and nicotinamide) metabolism	8	26					2
Ascorbate (Vitamin C) and Aldarate Metabolism	9	22					2
Arginine and Proline Metabolism	10	42					2
Drug metabolism - cytochrome P450	11	52					2
Pentose phosphate pathway	11	37					2
Pyrimidine metabolism	14	60					2
Glycine, serine, alanine and threonine metabolism	15	61					2
Tryptophan metabolism	22	76					2
Tyrosine metabolism	30	104					2

Figure 6: Manhattan Plot of TCPY Model HILIC Metabolites

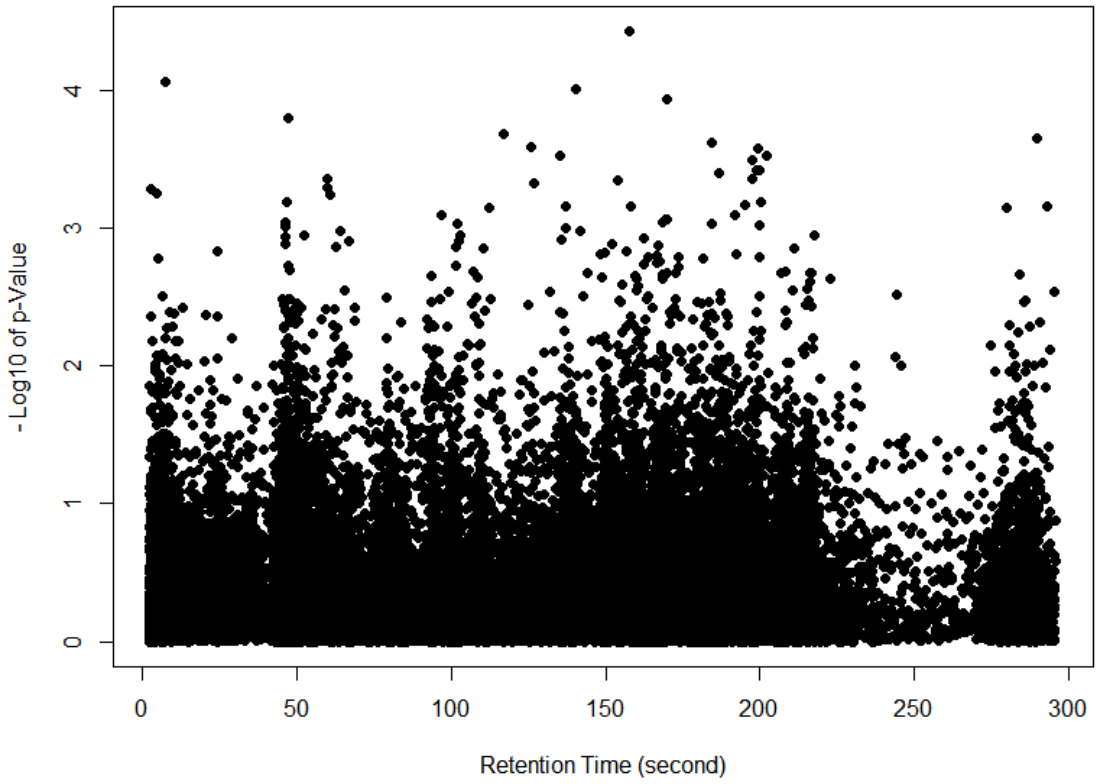
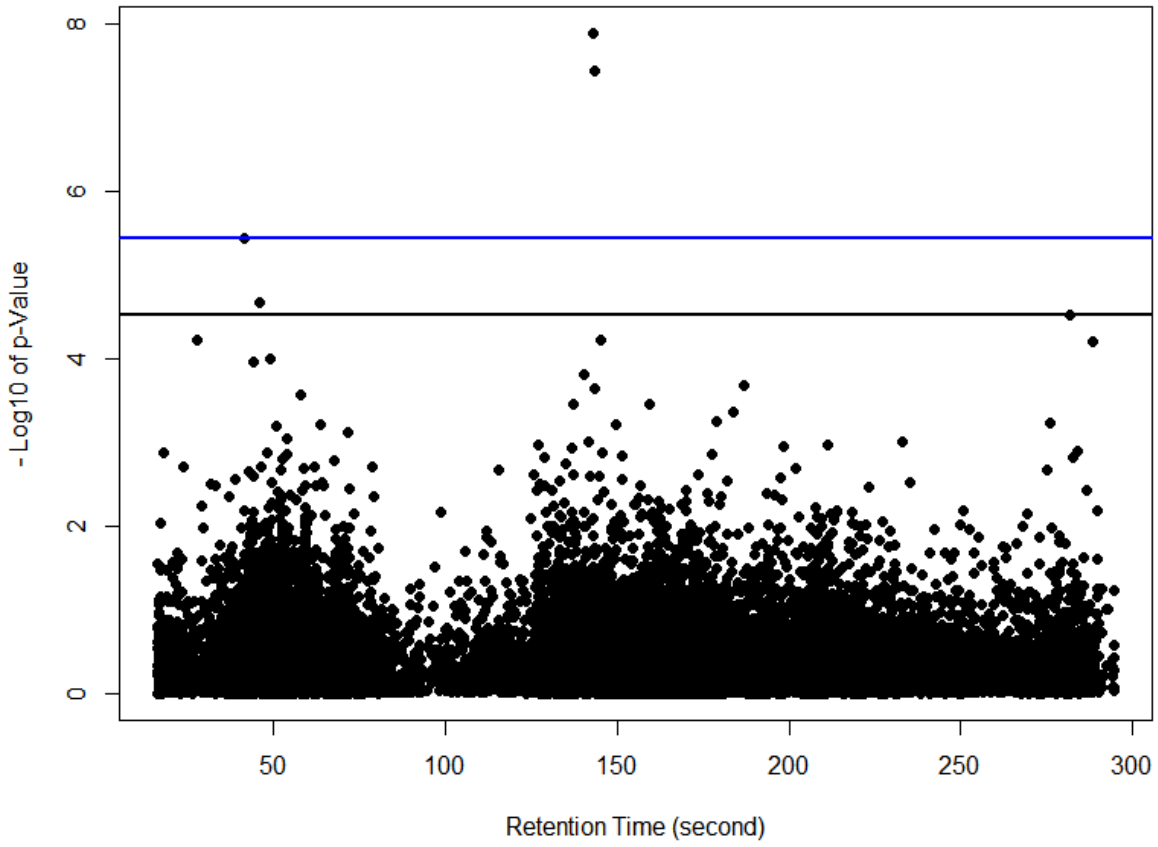


Figure 7: Manhattan Plot of TCPY Model C18 Metabolites



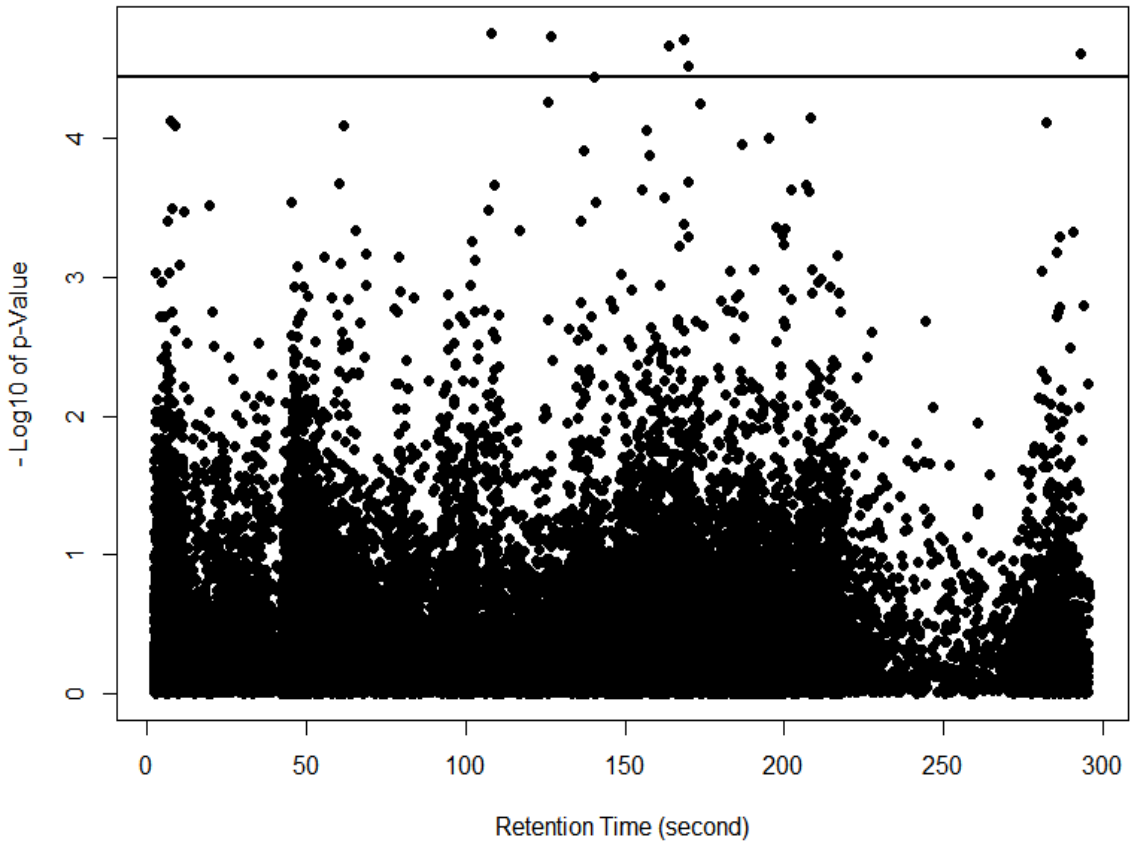
Where P-value FDR Corrected:

Red = 0.05

Blue = 0.1

Black = 0.2

Figure 8: Manhattan Plot of TCPY + Creatinine adjusted TCPY levels Model HILIC Metabolites



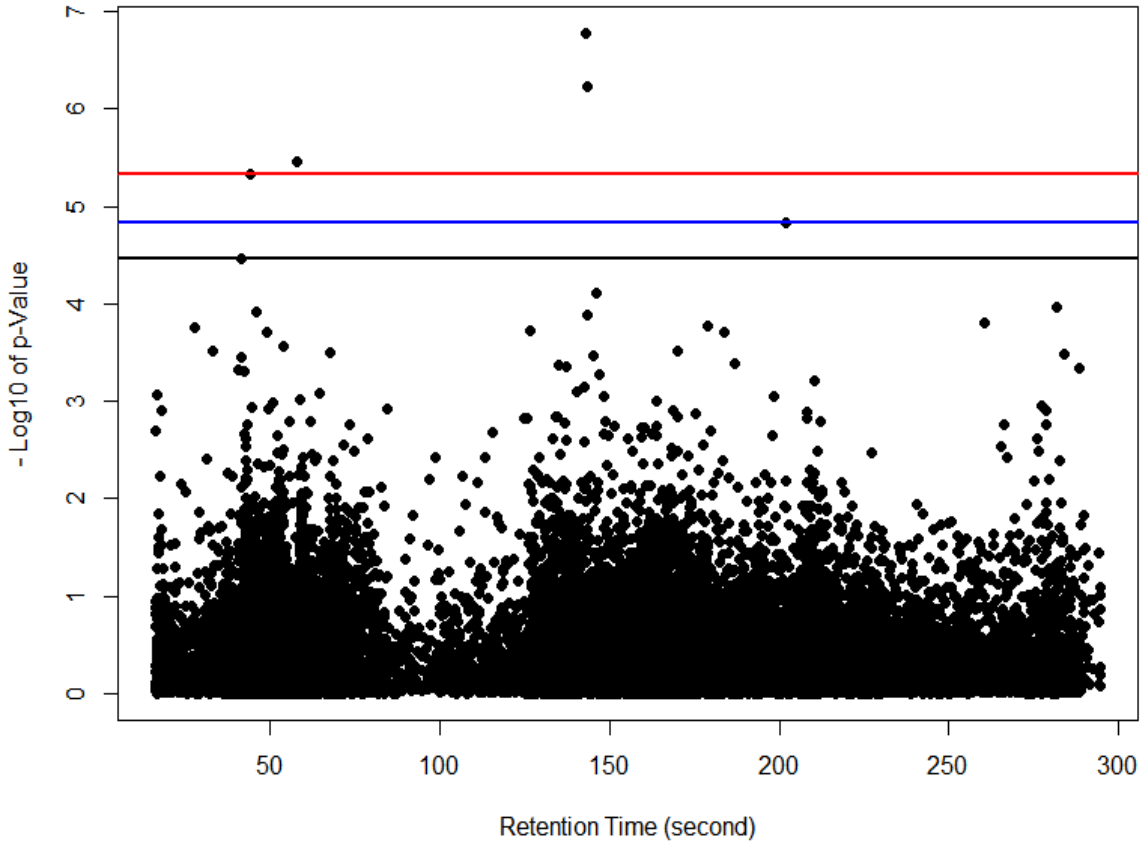
Where P-value FDR Corrected:

Red = 0.05

Blue = 0.1

Black = 0.2

Figure 9: Manhattan Plot of TCPY + Creatinine adjusted TCPY levels Model C18 Metabolites



Where P-value FDR Corrected:

Red = 0.05

Blue = 0.1

Black = 0.2