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Metabolomics of childhood exposure to perfluoroalkyl substances: A cross-sectional study

Samantha L. Kingsley^{a,*}, Douglas I. Walker^b, Antonia M. Calafat^c, Aimin Chen^d, George D. Papandonatos^e, Yingying Xu^f, Dean P. Jones^g, Bruce P. Lanphear^{h,i}, Kurt D. Pennell^j, Joseph M. Braun^a

^aDepartment of Epidemiology, Brown University, School of Public Health, Providence, RI, USA

^bDepartment of Environmental Medicine and Public Health, Icahn School of Medicine at Mount Sinai, New York, NY, USA

^cDivision of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA, USA

^dDepartment of Environmental Health, University of Cincinnati, Cincinnati, OH, USA

^eDepartment of Biostatistics, School of Public Health, Brown University, Providence, RI, USA

^fDivision of General and Community Pediatrics, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

^gDivision of Pulmonary, Allergy, Critical Care and Sleep Medicine, Emory University, Atlanta GA, USA

^hChild and Family Research Institute, BC Children's and Women's Hospital, Vancouver, British Columbia, Canada

ⁱFaculty of Health Sciences, Simon Fraser University, Burnaby, Canada

^jSchool of Engineering, Brown University, Providence, RI, USA

Abstract

***Corresponding Author:** Samantha L. Kingsley, MPH, PhD, Postdoctoral Research Associate, Brown University School of Public Health, Box G-S121-2, Providence, RI 02912.

Author Contributions:

JMB and KDP conceived the study. AC, JMB, and YX led and supported the HOME Study and YX provided all HOME Study data. AMC measured serum PFOA concentrations. DIW, DPJ, and KDP provided metabolomics expertise and high-resolution metabolomic profiling of serum samples. SLK performed all statistical analyses with guidance from GDP and DIW. SLK drafted the manuscript, tables, and supplemental material. DIW also contributed to manuscript writing. All authors reviewed, edited, and approved the final manuscript.

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Ethics approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in this study.

Introduction: Exposure to perfluoroalkyl substances (PFAS), synthetic and persistent chemicals used in commercial and industrial processes, are associated with cardiometabolic dysfunction and related risk factors including reduced birth weight, excess adiposity, dyslipidemia, and insulin resistance. Identifying the metabolic changes induced by PFAS exposure could enhance our understanding of biological pathways underlying PFAS toxicity.

Objective: To identify metabolic alterations associated with serum concentrations of four PFAS in children using a metabolome-wide association study.

Methods: We performed untargeted metabolomic profiling by liquid chromatography with ultra-high-resolution mass spectrometry, and separately quantified serum concentrations of perfluorooctanoic acid (PFOA), perfluorooctanesulfonic acid (PFOS), perfluorononanoic acid (PFNA), and perfluorohexanesulphonic acid (PFHxS) for 114 8-year old children from Cincinnati, OH. We evaluated associations between each serum PFAS concentration and 16,097 metabolic features using linear regression adjusted for child age, sex, and race with a false discovery rate <20%. We annotated PFAS-associated metabolites and conducted pathway enrichment analyses.

Results: Serum PFAS concentrations were associated with metabolic features annotated primarily as lipids and dietary factors. Biological pathways associated with all four PFAS included arginine, proline, aspartate, asparagine, and butanoate metabolism.

Conclusions: In this cross-sectional study, childhood serum PFAS concentrations were correlated with metabolic pathways related to energy production and catabolism. Future studies should determine whether these pathways mediate associations between PFAS exposure and childhood cardiometabolic health.

Introduction

Per- and polyfluoroalkyl substances (PFAS) are synthetic chemicals used as industrial processing aids, in fire-fighting foam, and as oil and water resistant coatings in cookware, food containers, carpets, apparel, and upholstery (Agency for Toxic Substances and Disease Registry, 2015; Buck et al., 2011; DeWitt, 2015; European Food Safety Authority, 2008). Four of these PFAS, perfluorooctanoic acid (PFOA), perfluorooctanesulfonic acid (PFOS), perfluorononanoic acid (PFNA), and perfluorohexanesulphonic acid (PFHxS) have an estimated half-life ranging from 3.8 to 8.5 years in humans, bioaccumulate, and are frequently detected in human serum (Andersen et al., 2013; Jain, 2013; Kingsley et al., 2018; Lee et al., 2013; Olsen et al., 2007; Whitworth et al., 2012; Zhang et al., 2013). Despite reductions in the production of PFOA and PFOS in the United States since the early 2000's, PFOA and PFOS are environmentally and biologically stable, and human exposure still occurs (Bowman, 2015; Environmental Protection Agency, 2000; Fromme et al., 2009).

PFAS are of particular concern given their biological persistence in humans and the adverse health effects associated with exposure. Early life exposure to PFAS may be particularly important since children are often more susceptible to the effects of environmental chemical exposures (Braun, 2016). Higher serum PFAS concentrations during gestation are associated with cardiometabolic risk factors including reduced birth weight, reduced birth length, and increased adiposity, but not insulin resistance in childhood (Braun et al., 2016; Fleisch et al., 2017; Johnson et al., 2014; Maisonet et al., 2012; Mora et al., 2017). During childhood,

higher serum PFAS concentrations are associated with cardiometabolic risk factors, including, increased adiposity, later onset of puberty, dyslipidemia, and alterations in glucose homeostasis (Alderete et al., 2019; Lopez-Espinosa et al., 2011; Nelson et al., 2009; Rappazzo et al., 2017). Several of these risk factors have been associated with alterations in the serum metabolome of both children and adults, but it is not clear whether the effects of PFAS on cardiometabolic endpoints are mediated by metabolome alterations (Guasch-Ferre et al., 2016; Llewellyn et al., 2015; Park et al., 2012; Perng et al., 2014; Ruiz-Canela et al., 2017).

Three human studies have identified metabolic alterations associated with PFAS exposure. Serum or plasma PFAS levels have been associated with metabolomics alterations, including, fatty acid metabolism and the tricarboxylic acid (TCA) cycle pathways among adult men, lipids and amino acids among overweight and obese Hispanic children, and lipids, fatty acid metabolism, and an unnamed metabolite in older adults (Alderete et al., 2019; Salihovic et al., 2018; Wang et al., 2017). An *in vitro* study found that PFOA altered lipid and amino acid metabolism (Peng et al., 2013). Several experimental studies in animals reported that PFAS exposure caused metabolic changes related to lipid and amino acid pathways (Lankadurai et al., 2013; Lee et al., 2017; Tan et al., 2013; Yu et al., 2016; Zheng et al., 2017).

We are unaware of any studies examining metabolomic responses to PFAS in a general population of children. Thus, we performed a metabolome-wide association study (MWAS) using untargeted ultra-high-resolution mass spectrometry to identify metabolic alterations associated with serum PFAS concentrations in 8-year old children.

Methods

Study Participants

We used data from the Health Outcomes and Measures of the Environment (HOME) Study. Pregnant women enrolled in the HOME Study, a prospective pregnancy and birth cohort based in Cincinnati, Ohio from March 2003 - January 2006. Detailed information about participant recruitment and eligibility criteria has been described previously (Braun et al., 2017). Briefly, 37% of 1,263 eligible women agreed to participate. Of these, 468 women enrolled, and 410 live-born children were delivered and followed-up between birth and age 8 years. For the present study, we randomly selected 115 of the 230 singletons (50%) who returned for the 8-year clinic visit. To ensure an adequate range of PFOA concentrations, we selected children in equal proportions of children from each tercile of age 8-year serum PFOA concentrations.

All women provided written informed consent for themselves and their children before enrolling in the study. The study protocol was approved by the institutional review board (IRB) of Cincinnati Children's Hospital Medical Center (CCHMC). The Centers for Disease Control and Prevention (CDC) and Brown University deferred to the CCHMC IRB as the IRB of record.

Serum PFAS Measurements

We collected venous blood samples from children at the 8-year clinic visit and measured serum concentrations of PFOA, PFOS, PFNA and PFHxS at the CDC laboratory using a modified version of a previously described analytic chemistry method (Kato et al., 2011; Kato et al., 2014; Kuklenyik et al., 2005). Briefly, online solid phase extraction coupled to high performance liquid chromatography-isotope dilution with tandem mass spectrometry was used to quantify PFAS concentrations. The limits of detection (LOD) are 0.1 ng/mL (PFOA and PFHxS), 0.2 ng/mL (PFOS), and 0.082 ng/mL (PFNA); all serum PFAS concentrations were greater than the LODs in our study participants.

High-Resolution Metabolomics

We conducted untargeted, high-resolution metabolomic profiling of serum collected at age 8 years from 115 participants using established methods (Soltow et al., 2013). Briefly, serum samples were removed from storage at -80°C and thawed on ice. Next, 65 μL of serum was added to 130 μL of acetonitrile containing a mixture of stable isotope standards, vortexed, and allowed to equilibrate for 30 minutes. Samples were analyzed using liquid chromatography and Fourier transform high-resolution mass spectrometry (Dionex Ultimate 3000, Q-Exactive HF, Thermo Scientific) (Johnson et al., 2010). For each sample, 10 μL aliquots were analyzed in triplicate using hydrophilic interaction liquid chromatography (HILIC) with electrospray ionization (ESI) source operated in positive mode and reversed phase chromatography (RPC) with ESI operated in negative mode, which is also referred to as C18-negative mode. This use of complementary chromatography phases and ionization polarity has been shown to improve the detection of endogenous and exogenous chemicals (Liu et al., 2016). The high-resolution mass spectrometer was operated in full scan mode at 120,000 resolution and mass-to-charge ratio (m/z) range 85–1275. Raw data files were extracted and aligned using apLCMS (Yu et al., 2013) with modifications by xMSanalyzer (Uppal et al., 2013). Uniquely detected ions, referred to as m/z features, consisted of m/z , retention time, and ion abundance.

We implemented several quality control measures prior to data analysis. We excluded one sample that did not meet quality control standards resulting in an analytic sample of $N=114$. All m/z features were batch corrected using ComBat (Johnson et al., 2007). We averaged ion abundances across triplicates and removed those with a coefficient of variation $\geq 100\%$ or $\geq 20\%$ non-detected intensities for all study participant's samples. The ion abundances of the remaining 9,899 and 6,198 m/z features for HILIC-positive mode and C18-negative mode, respectively, were $\log_2$ -transformed for all statistical analyses.

Statistical Analyses

We performed a MWAS using a series of linear regression models to estimate the association between serum PFAS concentration (continuous, ng/mL) and each m/z feature. We adjusted these models for child age (continuous, years), sex (male vs. female), and race (non-Hispanic white vs. Other). We controlled the false discovery rate ($\text{FDR} < 20\%$) using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

We first matched m/z features associated with any of the four serum PFAS concentrations to a library of approximately 500 metabolites with identities previously confirmed by comparison of m/z , retention time and ion dissociation patterns (MS^2) to authentic reference standards (Uppal et al., 2016). Additional features not matching these metabolites were annotated with the Human Metabolome Database (HMDB) (Wishart et al., 2018), LipidMaps (Fahy et al., 2007), the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2017; Kanehisa and Goto, 2000; Kanehisa et al., 2016), and the Norman Network databases PFASTRIER (Trier et al., 2015) and SFISHFLUORO (KEMI Swedish Chemical Agency, 2015). Identities were assigned to m/z features using evidence scoring provided in *xMSannotator* for matching to commonly detected adducts formed in positive and negative ESI at ± 5 ppm (Uppal et al., 2017). Chemical identity confidence was assigned using the Metabolomics Standards Initiative recommendation, where level 1 is a compound identified by comparison to an authentic reference standard, level 2 is annotated compound by matching isotopic patterns (when available) and matching m/z , level 3 is a putatively annotated compound class, and level 4 is an unknown compound (Sumner et al., 2007).

We used a two-step approach to identify biological pathways associated with each PFAS. In the first step, we conducted a network-based metabolome-wide correlation analysis, where we estimated all pairwise Spearman correlation coefficients between the entire set of m/z features ($n=29,607$) and m/z features identified in the PFAS-MWAS using the R package MetabNet (Uppal et al., 2015). For the second step, m/z features with Spearman $r \geq 0.5$ and Benjamini-Hochberg FDR $\leq 20\%$ were tested for metabolic pathway enrichment using Mummichog (Li et al., 2013). We conducted the pathway enrichment analyses with the human_mfn network (Li et al., 2010), mass accuracy of 5, and 10,000 permutations. The two-step approach and pathway enrichment analyses were performed separately for HILIC-positive and C18-negative and for each of the four PFAS. Finally, we calculated the R^2 between individual PFAS and metabolites that were associated with each other to evaluate the predictive ability of our models.

Results

On average, children were 8.1 years old ($SD=0.6$) and had a household income of \$76,000 per year ($SD=52,000$). The children were 57% female and 55% non-Hispanic white. The characteristics of the participants included in this study ($N=115$) were not materially different than the characteristics of the entire cohort ($N=389$) at baseline (Supplemental Table 2).

Among study participants, average serum PFOA, PFOS, PFNA, and PFHxS concentrations were 2.6 ng/mL (standard deviation [SD]=1.0), 4.4 ($SD=3.2$), 0.9 ($SD=0.7$), and 2.1 ($SD=2.7$), respectively (Table 1). The median PFAS concentrations were slightly lower than the average concentrations (Supplemental Table 1). Spearman correlations between the four PFAS concentrations were weak to moderate, ranging from 0.2 to 0.6 (Supplemental Table 3).

In the C18-negative mode, 17, 63, 47, and 29 m/z features were associated with serum PFOA, PFOS, PFNA, and PFHxS concentrations after covariate adjustment and correction

for multiple comparisons, respectively (Figure 1). Annotation of these m/z features indicated that all four PFAS were significantly associated with several lipids and dietary factors (Supplemental Tables 4–7). The most significant features associated with PFOA were other PFAS, including the monoisotopic and ^{13}C isotope for both PFOA and PFOS (Supplemental Table 4). Similarly, serum concentrations of PFOS, PFNA, and PFHxS were significantly associated with PFOS, PFNA, and PFHxS m/z features, respectively. The identity of these metabolites was confirmed by comparison of m/z , retention time, and MSMS to PFOA, PFOS, PFNA, and PFHxS standards. Interestingly, exogenous compounds associated with PFOA, PFOS, and PFHxS included some annotated fungicides and insecticides. In univariate models, R^2 values ranged from -0.9% - 65% with a median of -0.5% .

In the HILIC-positive mode, 18, 253, 76, and 39 m/z features were associated with serum PFOA, PFOS, PFNA, and PFHxS concentrations, respectively, after adjusting for covariates and multiple comparisons (Figure 2). Several lipids and dietary factors were associated with all four PFAS concentrations (Supplemental Tables 4–7). Interestingly 10 different annotated pesticides were associated with PFOS, as well as five PFAS, polychlorinated biphenyl-21 (PCB), and a plasticizer (Supplemental Table 5). Additional annotated features associated with PFNA included amino acids and five PFAS (Supplemental Table 6). Serum PFHxS concentrations were also associated with three PFAS, two herbicides, and an insecticide (Supplemental Table 7). In univariate models, R^2 values ranged from -0.9% - 49% with a median of -0.5% .

The network-based metabolome-wide correlation analysis identified additional m/z features associated with the significant m/z features from each PFAS-MWAS in C18-negative and HILIC-positive modes. To improve detection of relationships among metabolites, we used all of these m/z features associated with each PFAS for pathway enrichment analyses. Eleven and 9 pathways were enriched for all four PFAS in C18-negative and HILIC-positive modes, respectively (Figure 3 and Figure 4). These included arginine, proline, aspartate, asparagine, butanoate, glycine, serine, alanine, and threonine metabolism (Table 2 and Figures 5 and 6). Some pathways were only associated with one of the four PFAS (Figures 5 and 6). For example, in C18-negative mode tyrosine, galactose, Vitamin A (retinol), and lysine metabolism pathways were enriched for PFOA, PFOS, PFNA, and PFHxS, respectively. De novo fatty acid biosynthesis, TCA cycle, pyrimidine, and purine metabolism pathways were enriched in HILIC-positive mode for PFOA, PFOS, PFNA, and PFHxS, respectively.

Discussion

In this study, we found that serum PFOA, PFOS, PFNA, and PFHxS concentrations were associated with several PFAS, lipids, and dietary factors in the serum of 8-year old children. Pathways associated with these metabolites were related to several aspects of energy production and catabolism. Specifically, the network of metabolites associated with serum PFAS concentrations mapped to several pathways related to amino acid and lipid metabolism.

Despite differences in species, study design, and life-stage, our results are largely consistent with prior studies examining metabolic responses to PFAS exposure. Among overweight and obese Hispanic children from Los Angeles, plasma PFAS concentrations were associated with lipid and amino acid metabolism pathways (Alderete et al., 2019). There was substantial overlap in the enriched pathways associated with PFAS in both the prior and present study, including glycosphingolipids, linoleate, aspartate, asparagine, tyrosine, arginine, proline, glycine, serine, alanine, threonine, histidine, beta-alanine, glutamate, Vitamin B3 (nicotinate and nicotinamide), and urea cycle/amino group metabolism. Our pathway enrichment results were also consistent with a cross-sectional study of PFAS exposure and metabolomic alterations in adult males from China, in which PFOA and PFOS concentrations were associated with fatty acid metabolism, glutathione, ascorbate, purine, and TCA cycle metabolism (Wang et al., 2017). Among 70-year-old adults from Sweden, a group of PFAS was associated with glycerophospholipid, linoleate, and purine metabolism pathways, which is similar to our results (Salihovic et al., 2018). Two other studies in mice reported that PFOA caused alterations in amino acid, fatty acid, and energy metabolism pathways, including, arginine, proline, glutathione, tyrosine, histidine, tryptophan, alanine, aspartate, glutamate, cysteine, methionine, linoleate, glycine, serine, threonine, glutamate, pyruvate, glycolysis, glycerophospholipid, glyoxylate, dicarboxylate, galactose, fructose, and the TCA cycle (Tan et al., 2013; Yu et al., 2016). In human liver cells, PFOA exposure caused changes to amino acid and lipid metabolism pathways (Peng et al., 2013), consistent with our results observing that PFOA was associated with purine, leucine, and tryptophan metabolism pathways.

The results of our study, in addition with prior findings suggest connections between PFAS and alterations in the TCA cycle. Consistent with findings from epidemiologic studies and mouse models, we observed that serum PFAS concentrations were associated with several amino acids involved in the TCA cycle, an important aspect of energy production (Alderete et al., 2019; Tan et al., 2013; Wang et al., 2017; Yu et al., 2016). This complements the findings of previous studies observing cross-sectional associations between PFAS and glucose utilization (Cardenas et al., 2017; Liu et al., 2018).

Findings from our pathway enrichment analyses suggest that lipid metabolism is associated with serum PFAS concentrations. In fact the chemical structure of PFAS is similar to that of fatty acids, suggesting that PFAS could alter lipid metabolism (Fletcher et al., 2013). Moreover, prior epidemiologic studies have consistently reported a positive relationship between serum PFOA and cholesterol concentrations in children, adolescents, and adults (Eriksen et al., 2013; Frisbee et al., 2010; Liu et al., 2018; Steenland et al., 2009).

Our study has several strengths and limitations. We used ultra-high resolution mass spectrometry to identify a wide range of exogenous and endogenous metabolites. Moreover, we used an unbiased and untargeted approach to discover metabolic signals associated with serum concentrations of four different PFAS in children, which is an advantage over targeting specific metabolic pathways (e.g., glucose-insulin homeostasis or specific lipids) or focusing on a single PFAS. Using this approach, we found both endogenous and exogenous metabolites were associated with serum PFAS concentrations. Exogenous compounds do not reflect the biological pathways impacted by PFAS exposure. However, using an untargeted

metabolomics approach allowed us to identify potential mixtures of compounds that may be correlated with PFAS exposures. This is particularly important given the growing interest in studying the health effects of chemical mixtures (Braun Joseph et al., 2016).

The most notable limitation of this study is its cross-sectional design, which prevents us from inferring the temporality of these results. Moreover, it is possible that metabolic alterations related to cardiometabolic function could impact PFAS pharmacokinetics and features of the metabolome (Verner et al., 2016). Additional limitations of our study could be improved upon in future studies. The participants included in this pilot study were selected from HOME Study participants who completed the 8-year follow up visit. Thus, selection bias due to loss-to-follow is a potential source of bias. However, participant characteristics were not markedly different between the full HOME Study sample and the participants included in the present study. The modest sample size of 114 limited our statistical power to detect small effect sizes in the face of multiple comparisons and univariate regression models revealed that individual PFAS were generally not good predictors of metabolite intensities. To address these issues, future studies could use longitudinal measures of the metabolome with larger sample sizes and other data dimension reduction methods (e.g., Partial Least Squares Regression) to infer the directionality of these associations, increase statistical power, and better predict metabolite intensities with PFAS concentrations.

The results of this cross-sectional study, which require replication in other populations, provide new insights into the potential biological responses associated with PFAS exposure. Specifically, serum concentrations of four PFAS were associated with a common set of biological pathways related to energy production and catabolism. Future longitudinal cohort studies could provide useful information to determine whether these biological pathways mediate associations between PFAS exposure and adverse childhood cardiometabolic outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

Manhattan plot of negative $\log_{10}$ -transformed p-values for the association between serum m/z features and serum PFAS concentrations in C18-negative mode among HOME Study children at age 8 years

*P-values are adjusted for age, sex, and race. The dotted line is the p-value corresponding to false discover rate threshold of 0.2.

Figure 2:

Manhattan plot of negative $\log_{10}$ -transformed p-values for the association between serum m/z features and serum PFAS concentration in HILIC-positive mode among HOME Study children at age 8 years

*P-values are adjusted for age, sex, and race. The dotted line is the p-value corresponding to false discover rate threshold of 0.2.

Figure 3:
Venn diagram of number of enriched pathways associated with serum PFOA, PFOS, PFHxS, or PFNA concentrations in C18-negative mode

Figure 4:
Venn diagram of number of enriched pathways for m/z features associated with serum PFOA, PFOS, PFHxS, or PFNA concentrations in HILIC-positive mode

Figure 5:

Enriched pathways associated with serum PFOA, PFOS, PFHxS, or PFNA concentrations in C18-negative mode among HOME Study children, Cincinnati, OH

Note: The size of the dot represents the number of overlapping metabolites in the pathway.

The position of the dot is determined by the $-\log p$ -value obtained from 10,000 permutations.

Figure 6: Enriched pathways associated with serum PFOA, PFOS, PFHxS, or PFNA concentrations in HILIC-positive mode among HOME Study children, Cincinnati, OH

Note: The size of the dot represents the number of overlapping metabolites in the pathway. The position of the dot is determined by the $-\log p$ -value obtained from 10,000 permutations.

Table 1:

Characteristics of HOME Study children at age 8 years (n=115)

Characteristic	Mean $\pm$ standard deviation or N (%)
Serum PFOA (ng/mL)	2.6 $\pm$ 1.0
Serum PFOS (ng/mL)	4.4 $\pm$ 3.2
Serum PFNA (ng/mL)	0.9 $\pm$ 0.7
Serum PFHxS (ng/mL)	2.1 $\pm$ 2.7
Child age (years)	8.1 $\pm$ 0.6
Child sex (Female)	66 (57.4)
Child race	
White, non-Hispanic	62 (55.4)
Other	50 (44.6)
Household Income (\$10,000/Yr)	7.6 $\pm$ 5.

Table 2:

List of enriched pathways associated with all four PFAS in C18-negative mode or HILIC-positive mode (Values are permutation p-values).

Pathway	C18-negative					HILIC-positive				
	PFOA	PFOS	PFNA	PFHxS	PFOA	PFOS	PFNA	PFHxS	PFOA	PFHxS
Arginine and Proline Metabolism	0.003	0.006	0.001	<0.001	0.001	0.003	0.002	0.001	0.001	0.001
Aspartate and asparagine metabolism	0.040	0.008	0.041	<0.001	0.001	0.003	0.002	0.041	0.001	0.041
Beta-Alanine metabolism	0.001	0.004	<0.001	<0.001	0.001	0.003	0.002	0.016	0.001	0.016
Butanoate metabolism	0.009	0.001	0.004	0.002	0.040	0.004	0.029	0.016	0.001	0.016
Glutamate metabolism	0.001	0.002	<0.001	<0.001	0.001	0.003	0.002	0.016	0.001	0.016
Glycerophospholipid metabolism	0.001	0.003	<0.001	<0.001	0.001	0.003	0.002	0.016	0.001	0.016
Glycine, serine, alanine and threonine metabolism	0.020	0.012	0.002	<0.001	0.004	0.003	0.002	0.003	0.001	0.003
Glycosphingolipid metabolism	0.005	0.025	0.001	<0.001	0.001	0.003	0.002	0.003	0.001	0.003
Glyoxylate and Dicarboxylate Metabolism	0.010	0.018	0.003	<0.001	0.001	0.003	0.002	0.003	0.001	0.003
Histidine metabolism	0.002	0.001	<0.001	0.030	0.001	0.003	0.002	0.003	0.001	0.003
Linoleate metabolism	0.002	0.001	<0.001	0.030	0.001	0.003	0.002	0.003	0.001	0.003
Methionine and cysteine metabolism	0.002	0.001	<0.001	0.030	0.001	0.003	0.002	0.003	0.001	0.003
Tyrosine metabolism	0.002	0.001	<0.001	0.030	0.001	0.003	0.002	0.003	0.001	0.003
Urea cycle/amino group metabolism	0.002	0.001	<0.001	0.030	0.001	0.003	0.002	0.003	0.001	0.003
Vitamin B1 (thiamin) metabolism	0.003	0.013	0.024	0.003	0.001	0.003	0.002	0.003	0.001	0.003
Vitamin B3 (nicotinate and nicotinamide) metabolism	0.003	0.013	0.024	0.003	0.001	0.003	0.002	0.003	0.001	0.003