Amino Acids (2015) 47:589–601
DOI 10.1007/s00726-014-1893-x

ORIGINAL ARTICLE

Metabolome-wide association study of phenylalanine in plasma of common marmosets

Young-Mi Go · Douglas I. Walker · Quinlyn A. Soltow · Karan Uppal · Lynn M. Wachtman · Fredrick H. Strobel · Kurt Pennell · Daniel E. L. Promislow · Dean P. Jones

Received: 14 July 2014 / Accepted: 3 December 2014 / Published online: 21 December 2014
© Springer-Verlag Wien 2014

Abstract Little systematic knowledge exists concerning the impacts of cumulative lifelong exposure, termed the exposome, on requirements for nutrients. Phenylalanine (Phe) is an essential dietary amino acid with an aromatic ring structure similar to endogenous metabolites, dietary compounds and environmental agents. Excess plasma Phe in genetic disease or nutritional deficiency of Phe has adverse health consequences. In principle, structurally similar chemicals interfering with Phe utilization could alter Phe requirement at an individual level. As a strategy to identify components of the exposome that could interfere with Phe utilization, we tested for metabolites correlating with Phe concentration in plasma of a non-human primate species, common marmosets (*Callithrix jacchus*). The results of tests for more than 5,000 chemical features detected by high-resolution metabolomics showed 17 positive correlations with Phe metabolites and other amino acids. Positive and negative correlations were also observed for 33 other chemicals, which included matches to endogenous metabolites and dietary, microbial and environmental chemicals in database searches. Chemical similarity analysis showed many of the matches had high structural similarity to Phe. Together, the results show that chemicals in marmoset plasma could impact Phe utilization. Such chemicals could contribute to early lifecycle developmental disorders when neurological development is vulnerable to Phe levels.

Keywords Amino acid · Bioinformatics · Dietary exposome · Metabolomics · Phenylalanine

Abbreviations

CAS	Chemical abstracts service
CID	PubChem compound ID
FDR	False discovery
KEGG	Kyoto encyclopedia of genes and genomes
LC–MS	Liquid chromatography–mass spectrometry
m/z	mass to charge
MWAS	Metabolome-wide association study

Electronic supplementary material The online version of this article (doi:10.1007/s00726-014-1893-x) contains supplementary material, which is available to authorized users.

Y.-M. Go · D. I. Walker · Q. A. Soltow · K. Uppal · D. P. Jones (✉)
Clinical Biomarkers Laboratory, Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, Emory University, 205 Whitehead Research Center, Atlanta, GA 30322, USA
e-mail: dpjones@emory.edu

D. I. Walker · K. Pennell
Department of Civil and Environmental Engineering, Tufts University, Medford, MA 02155, USA

Present Address:
Q. A. Soltow
ClinMet Inc., 3210 Merryfield Row, San Diego, CA 92121, USA

L. M. Wachtman
New England Primate Research Center, Harvard University, Southborough, MA 01772, USA

F. H. Strobel
Chemistry Department, Emory University, Atlanta, GA 30322, USA

D. E. L. Promislow
University of Washington, Seattle, WA 98195, USA

Phe	Phenylalanine
PPAR-γ	Peroxisome proliferator-activated receptor-γ
RT	Retention time

Introduction

Phenylalanine (Phe) is an essential amino acid that has been extensively studied due to its importance in metabolism. Information is available concerning minimum Phe requirements and utilization in protein synthesis and conversion to tyrosine (Tyr), a precursor for catecholamine synthesis and substrate for thyroid hormone and melanin synthesis. Dietary Reference Intake (DRI) values are available but uncertainties remain concerning needs at a personal level (Hsu et al. 2006). Research focused on phenylketonuria and neurodevelopmental effects of excess plasma Phe have advanced our understanding and management of genetic variations impacting Phe disposition (Blau et al. 2010; Lee et al. 2009; Okano and Nagasaka 2013). Less is known, however, about impacts of non-nutritive dietary factors, environmental factors, and microbiome-associated metabolism, collectively termed the exposome (Miller and Jones 2014; Rappaport and Smith 2010; Wild 2005), that influence Phe bioavailability and utilization.

Metabolism and regulation in biologic systems are highly dependent upon specificity of interactions of small molecules with proteins. Such specificity is a central principle underlying development of pharmaceuticals with selective agonist and antagonist properties based upon structural similarity of candidate drugs to interaction sites within protein structures. Extension of this concept to the exposome supports the hypothesis that dietary, microbiome and environmental chemicals sharing structural similarity with an essential amino acid could alter its metabolism and/or function. Available evidence indicates that the human exposome includes more than 100,000 small molecules (Jones et al. 2012) and may include more than one million when microbiome and dietary phytochemicals, lipids and complex carbohydrates are included. Thus, a considerable challenge exists to identify components of the dietary exposome relevant to nutritional requirements and health.

Phe is an aromatic amino acid containing a 6-carbon unsaturated (phenyl) ring structure. Phe is biosynthesized in plants and used to produce many phenolic, polyhydroxylated and quinone structures. The diverse biochemicals in these metabolic pathways are present in food; some are absorbed and many have structures similar to Phe. Additionally, aromatic chemicals are commonly used in manufacturing and industrial applications; thus, these compounds are present in animals due to their presence in commercial products, food and water. Positive correlations of such chemicals with Phe could indicate possible interference with Phe metabolism while negative associations could indicate interference with intestinal absorption, plasma protein binding or renal retention.

Mathematical models can account for Phe metabolism in humans with a surprisingly small number of reactions (Kaufman 1999), principally involving protein turnover, hydroxylation to Tyr and transamination to phenylpyruvate. Subsequent products of Tyr metabolism include catecholamines (dopamine, norepinephrine, epinephrine), related metabolites (tyramine, *N*-methyltyramine, p-octamine, synephrine, L-dopa, dopamine, 3-methoxytyramine) and degradation by transamination and subsequent oxidation. Thyroid hormones and metabolites are also derived from Tyr, but this involves a complex multistep sequence without direct dependence upon Phe. Tyr is also used in a series of irreversible reactions for melanin synthesis (Farishian and Whittaker 1980). Other fates include decarboxylation to phenethylamine and acetylation to *N*-acetylphenylalanine (Krishna et al. 1971; Udenfriend and Cooper 1953). Phenethylamine is metabolized to *N*-methylphenethylamine, and phenylpyruvate is metabolized to phenyllactate and phenylacetate. Phenylacetate is further metabolized to phenylacetylglutamine, which is a common urinary metabolite.

To gain an understanding of possible interactions of other chemicals with Phe, this study was designed to test the hypothesis that plasma contains chemicals that are structurally similar and correlate with Phe concentration. Hereafter, we use "metabolite" as a generic term that includes all known and unknown chemicals detected in plasma regardless of origin or function. To minimize dietary and environmental variables likely to confound interpretation of Phe correlations in humans, analyses were performed in *Callithrix jacchus* (common marmoset), a non-human primate species useful for biomedical research (Tardif et al. 2003). Fifty marmosets were selected to provide a balanced composition of males and females and age distribution from 2 to 16 years. High-resolution metabolomics was used to measure a broad array of metabolites, and data were analyzed with a workflow designed to test for correlated metabolites in concentration and structural similarities to Phe (Fig. 1). Results of a metabolome-wide association study (MWAS) of Phe showed positive correlations for expected metabolites, phenylpyruvate, phenyllactate and Tyr. Positive correlations also occurred with essential amino acids and some other metabolites. Importantly, both positive and negative correlations were observed with other metabolites. Metabolomics database searches showed that these included matches to endogenous metabolites as well as dietary, microbial and environmental agents; structural similarity analysis showed that many of these had >70 % structural similarity to Phe. Together, the correlations and structural similarity imply that variations in

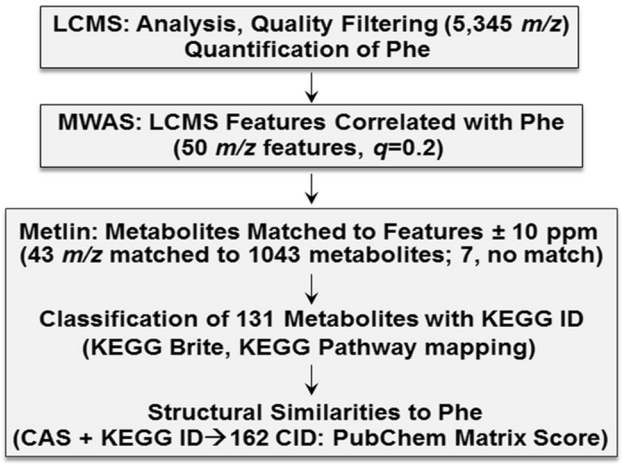

Fig. 1 Workflow to test for dietary, microbiome and environmental metabolite correlations with Phe. Fifty marmoset plasma samples were analyzed by liquid chromatography-mass spectrometry (LCMS) resulting in selection of 5,345 *m/z* features for Phe analysis. Following metabolome-wide association study (MWAS) of 5,345 LCMS features shows 50 features were correlated with Phe ($q \leq 0.2$). These features were then used with the Metlin metabolomics database to obtain metabolites that matched the accurate mass within 10 ppm. The Kyoto Encyclopedia of Genes and Genomes (KEGG) identifiers from the Metlin search were then used to classify metabolites with KEGG Brite and KEGG Pathway mapping. KEGG and CAS identifiers from Metlin were converted to CID and used with PubChem Matrix Score Service to obtain structural similarity scores

such metabolites could impact biologic functions related to Phe. Such variations could impact embryogenesis or early development and warrant detailed investigation.

Materials and methods

Animals

Common marmosets (*Callithrix jacchus*) (50 total animals; 26 males, 24 females) ranging in age from 2 to 16 years were housed at the New England Primate Research Center (Southborough, MA, USA) and maintained in accordance with the Guide for Care and Use of Laboratory Animals as described by protocols (Wachtman et al. 2011). The facility is AAALAC-accredited. Colony animals were maintained under an animal holding and breeding protocol approved by Harvard Medical School's Standing Committee on Animals. The animals received commercial marmoset food (New World Primate Chow 8791, Harlan Teklad, Indianapolis, IN, USA) and were supplemented daily with a combination of fresh fruits, vegetables, seeds, eggs and/or mealworms. Water was provided ad libitum in polycarbonate water bottles. Husbandry of this marmoset colony has been previously described (Wachtman et al. 2011). Blood samples were obtained during the quarterly physical examinations after sedation with 0.2 mL of ketamine given intramuscularly. Blood was collected in EDTA-containing evacuated tubes; plasma was separated, frozen, shipped on dry ice and maintained at −80 °C until analysis.

Liquid chromatography–mass spectrometry (LC–MS)

To prepare samples for mass spectral analyses, 50 μL of plasma was added to 100 μL of acetonitrile and 2.5 μL of a mixture of 14 stable isotope standards (Soltow et al. 2013). After mixing and incubation at 4 °C for 30 min, precipitated proteins were pelleted via centrifugation for 10 min at the maximal setting on a microcentrifuge at 4 °C. Supernatants were transferred to autosampler vials and analyzed using an autosampler maintained at 4 °C. Samples were analyzed in triplicate by liquid chromatography–Fourier transform mass spectrometry (Accela- LTQ Velos Orbitrap; *m/z* range from 85 to 850) with 10 uL injection volume using a dual chromatography setup (anion exchange and C18) and a formic acid/acetonitrile gradient (Soltow et al. 2013). Electrospray ionization was used in the positive ion mode. Data were extracted using apLCMS (Yu et al. 2009) with modifications by xMSanalyzer (Uppal et al. 2013) as *m/z* features, where an *m/z* feature is defined by *m/z* (mass-to-charge ratio), RT (retention time) and ion intensity (integrated ion intensity for the chromatographic peak). Identities of many of the *m/z* features are known from previous research using ion dissociation patterns by tandem mass spectrometry (MS/MS), coelution with authentic standards and cross-platform validation. Possible identities of other *m/z* features were obtained using the Metlin Mass Spectrometry Database (Smith et al. 2005). Where feasible, metabolite identities were confirmed via MS/MS and matching fragmentation patterns to those of known standards. For Phe and some metabolites, quantification was obtained relative to authentic standards using a method of additions and/or calibration relative to NIST Standard Reference Material 1950 (Phinney et al. 2013; Simon-Manso et al. 2013). Concentrations are expressed as mean ± standard deviation. For metabolites without confirmed identities, data are given as mean ion intensity ± standard deviation.

Biostatistics and bioinformatics

Bioinformatics and biostatistical analyses included ANOVA and two-sample *t* tests, as appropriate, for group characteristics and Phe concentrations. Pearson correlation with Phe and associated *t* test data were used with the Benjamini/Hochberg false discovery rate (FDR) method to correct for multiple comparisons (Benjamini and Hochberg 1995). Raw *p* values were used in a Manhattan plot ($-\log_{10} p$ vs metabolic feature) to visualize the calculated significance for individual metabolite correlations and

identified the FDR 0.05 and 0.2 thresholds with horizontal lines (Fig. 3). Because this study was developed to discover potentially important associations with Phe, we used $q = 0.2$ (where the q value is the FDR adjusted p value) as a reference cut-off to minimize type 2 statistical errors (i.e., failure to reject a false null hypothesis). At $q = 0.2$, 80 % of values are expected to be correct and 20 % are expected to be false discovery. For analysis of differences due to sex and age, the number of individuals was too small for any metabolite to be significant at $q = 0.2$. To address this limitation, we used a pre-selection procedure, Pearson's $|r| > 0.3$ with Phe, similar to the fold-change filtering used in gene expression studies. This filtering eliminated metabolites with weak correlations, which could be present due to analytical artifacts but limits the interpretation of sex and age comparisons.

Database searches and computational analysis

Except as indicated, accurate mass *m/z* were searched for metabolite matches using the Metlin database (http://metlin.scripps.edu/index.php) with the mass tolerance set to 10 ppm for matches to H^+, Na^+, NH_4^+, K^+, $-H_2O + H^+$, $-2H_2O + H^+$, acetonitrile + H^+, acetonitrile + Na^+, or $2Na^+ - H^+$ and with exclusion of matches to peptides. Data were manually searched for Phe metabolites. Manual searches were also performed for ^{13}C forms, and for other common ion forms, including adducts consisting of Na-formate clusters and NaCl. Data were downloaded as .csv files, and KEGG (Kyoto Encyclopedia of Genes and Genomes) and CAS (Chemical Abstract Service) identifiers were used for subsequent database analyses. KEGG identifiers were used with KEGG Compound database (http://www.genome.jp/kegg/compound/) to obtain chemical classifications (Brite Mapping) and associated metabolic pathways (Pathway Mapping). For structural similarity studies, KEGG identifiers and CAS identifiers (from Metlin) were separately converted to PubChem compound ID (CID) using Hyperlink Management System (http://biodb.jp/). The CID lists were consolidated to eliminate redundancies, and CID were used with PubChem Score Matrix Service (http://pubchem.ncbi.nlm.nih.gov/score_matrix) to obtain scores for 3-dimensional structural similarity to Phe, optimized by feature overlap (Bolton et al. 2008). Multiple types of structural similarity scores are available (Willett et al. 1998); the PubChem score uses a scale from 0 to 100, with 100 being identical. Similarity is measured using the Tanimoto equation (Willett et al. 1998) and the PubChem dictionary-based binary fingerprint, which consists of series of chemical substructure keys denoting the presence or absence of a particular substructure.

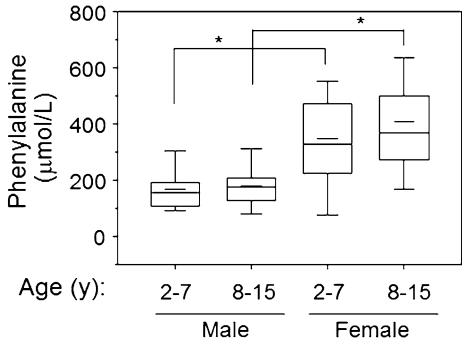

Fig. 2 Phe concentrations in marmoset plasma. There was no anticipation of sex or age differences in Phe concentration, so study design was to use an approximately balanced population of young females ($n = 12$), older females ($n = 12$), young males ($n = 12$) and older males ($n = 14$) and analyze data without consideration of sex or age effects. Statistical analyses showed differences ($*p < 0.05$) in Phe concentration between respective age groups of females and males

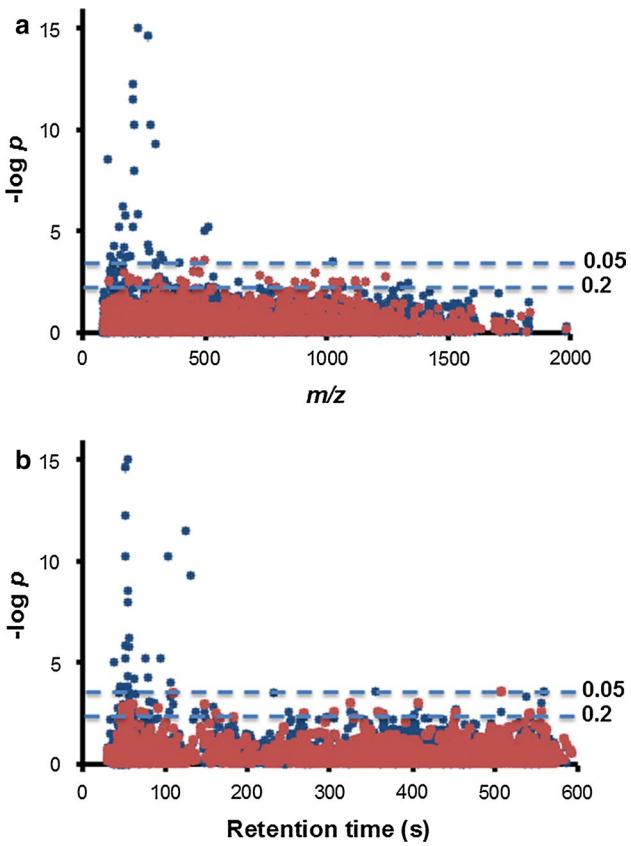

Fig. 3 Metabolome-Wide Association Study (MWAS) of metabolites in marmoset plasma correlated with Phe. **a** Type 1 Manhattan plot showing the negative log p ($-\log p$) for correlation of each metabolite (*m/z* feature) as a function of the *m/z* (mass/charge). **b** Type 2 Manhattan plot showing $-\log p$ for each metabolite as a function of chromatographic retention time. False discovery rate (FDR) thresholds are shown by *broken lines*. Positive correlations are shown in *blue* and negative correlations are shown in *red*

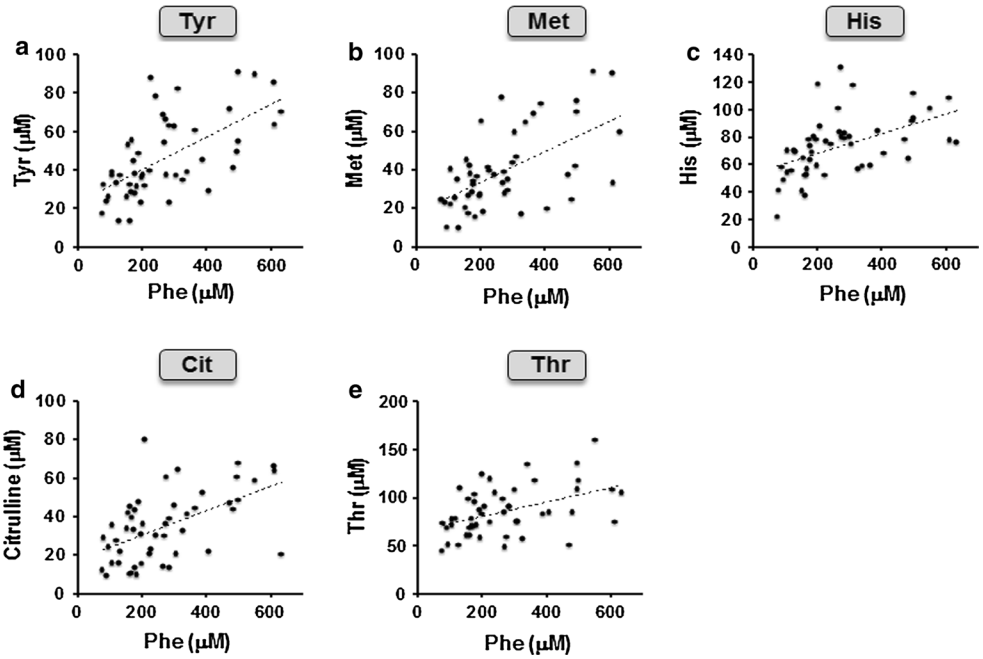

Fig. 4 Correlations of amino acids in marmoset plasma with plasma phenylalanine. Intensity values for ions (identified by *m/z* and chromatographic retention time) for individual marmoset plasma samples plotted as a function of the Phe concentration in the sample. Data for Tyr (**a**, $r = 0.62$), Met (**b**, $r = 0.59$), His (**c**, $r = 0.50$) and citrulline (**d**, $r = 0.53$) were significant at FDR 0.05. Thr was significant at FDR 0.2 (**e**, $r = 0.45$). Pearson correlation and p for correlation are provided in Online Resource 1

Results

Plasma Phe and other amino acids in marmosets

There was no significant difference in age between male and female marmosets (8.0 ± 3.9 years and 7.8 ± 4.0 years, respectively; $p = 0.8$). Mean plasma Phe concentration of 50 common marmoset was 270 ± 150 μmol/L. To test for differences in Phe according to sex and age, we performed ANOVA with four groups: males <8 years (mean age 4.5 ± 1.8 years), males ≥8 years (mean age 11.1 ± 2.3 years), females <8 years (mean age 4.5 ± 1.8 years) and females ≥8 years (mean age 11.1 ± 2.5 years). Results (Fig. 2) showed that differences were present at $p < 0.05$. Post-hoc tests showed no significant difference between males <8 years compared to ≥8 years (168 ± 61, 181 ± 62 μmol/L, respectively) or between females <8 years compared to ≥8 years (349 ± 142, 406 ± 159 μmol/L, respectively). Differences between males and females were present for younger groups (males vs females), older groups (males vs females) and when all males were compared to all females (175 ± 61, 378 ± 150 μmol/L, respectively, $p < 0.05$).

Metabolome-wide association study (MWAS) of Phe

High-resolution mass spectrometry data obtained for all age groups of males and females were filtered to exclude metabolites not present in at least 50 % of the samples each for males and females, leaving 5,345 ions (*m/z* with retention time and intensity; hereafter termed "metabolites") for analysis (Fig. 1). The resulting MWAS, illustrated by a Manhattan plot of the −log p of metabolite correlation with Phe concentration as a function of the *m/z* (Fig. 3a) or as a function of retention time (Fig. 3b), showed correlating metabolites with a broad range of *m/z* and retention time. With an FDR cutoff of $q = 0.05$, 29 metabolites were correlated with Phe (Online Resource 1). These included the Phe metabolites, phenylpyruvate, phenyllactate, *N*-acetyl-Phe and Tyr, as well as other amino acids (Met, His, citrulline). Each of these identifications was confirmed by MS/MS relative to authentic standards. At a less stringent cutoff of $q = 0.2$, 50 metabolites were correlated with Phe, including another essential amino acid, Thr (Online Resource 1). Plots of the 5 amino acids (Tyr, Met, His, Thr and citrulline) (Fig. 4), along with four other amino acids (Pro, Lys, Gln, Leu/Ile) that were not associated with Phe at an FDR threshold of 0.05 (Online Resource 2, a–d), show that less stringent FDR criteria could facilitate the discovery of other biologically important associations of Phe such as those with dietary or environmental agents.

The fifty *m/z* that correlated with Phe at $q < 0.2$ were used to search the Metlin metabolomics database for matches to common ions (H^+, Na^+, NH_4^+, K^+, $-H_2O + H^+$, $-2H_2O + H^+$, $2Na^+ - H^+$) with 10 ppm tolerance. Matches included 11 forms of Phe and metabolites and seven other amino acids (each with confirmed identification), accounting for 36 % of the metabolites correlated with Phe (Fig. 5). The concentration of each of these was positively correlated with Phe (Table 1); the other 64 % included 48 % positively correlated and 16 % negatively correlated metabolites. The remaining 32 metabolites

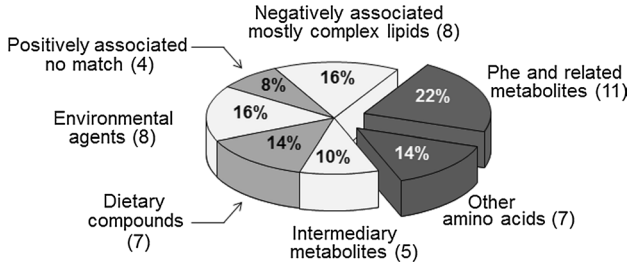

Fig. 5 Classification of 50 plasma metabolites correlated with plasma Phe at FDR <0.2. Seventeen of the features correlated with Phe had confirmed identities as Phe, metabolites of Phe or other amino acids. Other features were manually annotated by searching the Metlin database and selecting likely matches. Where multiple matches were present, a single count was used, i.e., multiple matches for a single *m/z* feature to multiple dietary compounds were given a single count. Most of the correlations were positive, so only these are shown as separate segments. Negatively associated features mostly matched complex lipids (*blue symbols* in Fig. 3b with retention times >200 s). The detailed information of 50 metabolites is provided in Online Resource 1

(64 % of total) were used to search for dipeptides, tripeptides and tetrapeptides, which could possibly contain Phe and correlate due to common transporter activities. Four matches were obtained, with only two (*m/z* 166.0849, 539 s; 174.0539, 110 s) containing aromatic amino acids (Phe, Tyr, Trp); thus, the 32 metabolites mostly were not peptides varying with Phe. Other matches for positively correlating metabolites included five intermediary metabolites, seven dietary compounds, eight environmental agents and four without matches (Table 1; Fig. 4). The remaining eight negatively correlated metabolites mostly matched to complex lipids, with an exception being *m/z* 174.0539 at 110 s, a match to phenylglycine, dopaquinone or other natural product; two negatively correlated metabolites did not have matches in Metlin (Table 1).

Correlations of selected database matches with Phe among all the marmosets are shown in Online Resource 2. Note that these are not confirmed identities but rather correlating ions detected in plasma (Online Resource 2, e–j). The correlations are similar to those for confirmed amino acids (Online Resource 2, a–d). Some of these metabolites (Online Resource 2, f) co-elute with Phe and may be unrecognized Phe ions formed upon ionization. Others, however, have different retention times (Online Resource 2, g–j), indicating that they are derived from other chemicals.

KEGG brite and KEGG pathway mapping of positively and negatively correlated metabolites

An alternative search strategy using KEGG Compound mapping provided similar results. Metlin database searching of 50 significant *m/z* features resulted in 309 tentative matches to chemicals with KEGG identifiers (Online Resource 1). A search of these chemicals in KEGG Brite Mapping (http://www.genome.jp/kegg/compound/) resulted in 24 phytochemicals, 22 chemicals with biological roles, 20 lipids, 13 carcinogens, eight pesticides, three animal drugs, three natural toxins, one endocrine disrupting chemical and five other chemicals. These features represented numerous KEGG Pathways including metabolic pathways, 40; microbial metabolism, 38; biosynthesis of secondary metabolites, 28; xenobiotic metabolism (chlorocyclohexane and chlorobenzene, polycyclic aromatic hydrocarbons, naphthalene, toluene, etc.), 31; plant and plant metabolites, 23; complete listing provided as Online Resource 3). KEGG Pathway Maps showed that metabolites with positive correlation with Phe (Online Resource 4a) were present in different pathways than those with negative correlation (Online Resource 4b). Positively associated matches were widely distributed among amino acid, secondary metabolism and xenobiotic metabolism pathways. Negatively associated matches were enriched in lipid metabolism pathways, with some other matches among xenobiotic and secondary metabolism pathways.

Structural similarity to Phe of putative metabolites correlated with Phe

To examine whether the dietary compounds, environmental agents and other chemicals obtained from the Metlin searches (50 correlated *m/z* features) had structures similar to Phe, we used PubChem Score Matrix Service (http://pubchem.ncbi.nlm.nih.gov/score_matrix). KEGG and CAS identities from Metlin were converted to CID using Hyperlink Management System (http://biodb.jp/), and CID were searched in PubChem for 3-dimensional structural similarity to Phe, optimized by feature overlap. The results were filtered for similarity score greater than that for amino acid Pro (≥61 out of 100) and are provided in Table 2. Phe metabolites and other amino acids had scores between 61 and 100, with citrulline and Tyr having the highest scores (89 and 88, respectively). Phenylpyruvate, phenyllactate and His also had relatively high scores (80, 77, and 77, respectively). Noteworthy endogenous metabolites with high similarity scores included multiple positional isomers of hydroxyprogesterone (*m/z* 331.2245, 356 s, similarity score 81), metabolites of the aromatic amino acids, Tyr and Trp, and sphingomyelin (*m/z* 731.6027, 407 s, similarity score 72). Dietary compounds with high similarity scores included myricetin (similarity score 70) and other plant alkaloids. Environmental agents with high similarity scores included herbicides, herbicide metabolites and polycyclic aromatic hydrocarbon (PAH) metabolites (Table 2).

As a control for the above similarity score strategy, we randomly selected 50 metabolites out of 5,345 *m/z* from the

Table 1 Metabolites correlating with Phe in marmoset plasma. From 5,345 features measured by LCMS, fifty were significantly associated with Phe at FDR 0.2

Metabolite or database match	*m/z*	Retention time (s)	Average intensity	Pearson's *r*
Phe ions and metabolites (11)				
Phe ($2Na^+–H^+$)	210.0487	53	1,102,072	0.82
^{13}C Phe (H^+)	167.0884	62	1,691,766	0.94
Phe (–formic acid)	120.0798	53	1,898,265	0.95
Phe ($2Na^+–H^++Na^{35}Cl$)	268.0072	53	231,328	0.86
Phenylpyruvate	209.0170	126	107,875	0.84
Phenyllactate	211.0327	104	42,509	0.80
^{13}C Phe ($2Na^+–H^+$)	211.0524	56	96,420	0.73
Tyr (formate + H^+)	226.0436	54	275,771	0.62
Tyr (H^+)	182.0799	58	1,309,966	0.62
Acetylphenylalanine	208.0953	96	61,760	0.59
Phe ($2Na^+–H^++Na^{37}Cl$)	270.0044	56	94,786	0.58
Other amino acids (7)				
Met	150.0568	79	908,949	0.59
Citrulline	176.1018	63	199,055	0.53
His	200.0392	50	787,508	0.51
Pro	116.0698	54	3,629,362	0.51
Lys	191.0755	48	185,576	0.51
His	178.0575	51	281,228	0.50
Thr	120.0646	88	439,500	0.45
Intermediary metabolite matches in Metlin database (5)				
Hydroxyprogesterone, trihydroxypregnenone	331.2245	356	387,606	0.53
Isocaproic acid	158.1165	55	7,027,342	0.51
Ethylaminobenzoate, Methyl methylanthranilate, Cinnamic acid	166.0849	539	62,742	0.51
Lipid, phytochemicals, sterols	119.0676	81	186,484	0.46
Thioguanosine triphosphate	539.9760	49	238,755	0.43
Dietary metabolite matches in Metlin database (7)				
Natural products	168.0907	58	103,276	0.72
Furano[2″,3″:6,7]aurone (flavanoid); Paraquat	301.0248	133	58,314	0.80
Many natural products (also matches to environmental compound)	278.0586	107	36,553	0.62
Benzylcinnamate	131.0482	79	104,159	0.54
Multiple plant products	1026.2788	45	177,305	0.50
Gliotoxin, pentahydroxyflavone	327.0495	48	59,708	0.52
Myricetin, other phenolic natural products	301.0363	58	158,302	0.49
Environmental chemical matches in Metlin database (8)				
Glufosinate	226.0226	55	145,617	0.87
Dicamba or dichloroacetic acid	283.9816	53	238,498	0.79
Dihydrostilbene	103.0534	55	347,523	0.72
Clorsulon, trichlormethiazide	401.8943	57	131,606	0.49
Dibutylphthalate (+Na)	301.1391	66	613,157	0.48
Dichlorohydroquinone, phosphate	142.9471	60	196,121	0.46
Diethyltetrahydrofuran, octenol, octanal, others	129.1264	232	260,695	0.49
Cyclohexanecarboxylic acid, others	129.0901	159	132,274	0.45
Positively associated metabolites without Metlin database match (4)				
No match	517.8113	54	156,652	0.61
No match	501.8374	40	131,400	0.60
No match	123.5245	108	51,525	0.51
No match	164.9289	59	178,144	0.46

Table 1 continued

Metabolite or database match	*m/z*	Retention time (s)	Average intensity	Pearson's *r*
Negatively associated metabolites (8)				
Glycolipid	480.6125	57	271,303	−0.45
DAT; glycolipid	955.7424	49	634,386	−0.45
SM, TG, other lipids	731.6027	407	270,179	−0.45
TG	457.3169	558	78,980	−0.45
No match	478.6162	57	277,903	−0.46
Phenylglycine	174.0539	110	330,375	−0.46
No match	462.6420	57	461,080	−0.49
Vit D and steroids	499.3154	560	657,392	−0.58

These included eighteen with confirmed identification as Phe metabolites and amino acids. Others included matches in Metlin metabolomics database to endogenous metabolites, dietary compounds and environmental agents, some of which are summarized here and in Fig. 5. Plots of amino acids as a function of plasma Phe are shown in Fig. 4. Additional Metlin matches are provided in Online Resource 1

original LC-MS data and obtained CID as described above to determine similarity scores. A similar number of total CID was obtained (112 matches for randomly selected 50 metabolites compared to 125 matches for the 50 metabolites correlated with Phe). The maximum Pearson *r* correlation of these randomly selected metabolites with Phe was 0.27, and the median was 0.0043. Similarity scores ranged from 0 to 83 with a median of 59 and mean of 56. In comparison, values for the metabolites correlated with Phe ranged from 0 to 100. After removing multiple ion forms of Phe, respective median and mean values were 68 and 66. These results are summarized in a histogram of Phe-correlated metabolites as a function of similarity score, with an overlay of those obtained for the randomly selected metabolites shown in Fig. 6. The results show that the workflow (Fig. 1) allows selection of metabolites with characteristics expected for dietary or environmental chemicals that could impact Phe utilization, especially for similarity scores >80.

The remaining plasma was used to perform ion dissociation (MS/MS) studies of the unidentified metabolites to support chemical identification. The low abundance of some of the ions and the co-elution of ions with others within the ion selection window limited our ability to unambiguously confirm identities. Of those ions with acceptable fragmentation data, *m/z* 331.2245, 356 s had product ions consistent with identification as a hydroxyprogesterone isomer (Table 2, Online Resource 5). MS/MS analysis and comparison to authentic reference standards for 17- and 21-hydroxyprogesterone revealed a fragmentation pattern that contained the main product ions present in both; however, due to the low abundance of the parent ion in the marmoset sample it was not possible to differentiate between two isomers. The ion with *m/z* identical to Phe (*m/z* 166.0849), but eluting at 539 s, had a fragmentation pattern sharing some product ions with Phe and also having other dissimilar product ions (Online Resource 6). Even in the absence of definitive identification, the results are sufficient to conclude that the structure has a ring structure similar if not identical to Phe.

Phe concentration did not differ according to age (Fig. 2), but substantial differences were present in classes of metabolites correlated with Phe for 2- to 7-year-old marmosets compared to 8- to 15-year-old marmosets (Fig. 7). Younger marmosets had many more positively and negatively correlated metabolites [younger (+R, 144; −R, 80), older (+R, 52; −R, 32)] suggesting more tightly regulated metabolic processes. Notable differences occurred in amino acid correlations with Phe with age; significant correlations were present in young marmosets for Tyr, Met, Glu, Lys, His, Pro, Thr and citrulline, but only Tyr had significant correlation in older marmosets. In young marmosets, Phe had significant positive correlation with steroids and significant negative correlations with arachidonic and linoleneic acid. These correlations were not present in older marmosets (Fig. 7).

Discussion

In this study a non-human primate species (common marmoset) was maintained under controlled environmental conditions with relatively constant diet to test the feasibility of using MWAS to identify dietary, microbiome and environmental metabolites that vary in a manner which could indicate effects on dietary utilization. Phe was selected as the essential nutrient for study because Phe has (1) few metabolic products, (2) similar structure to a large number of dietary and environmental chemicals and (3) known adverse effects from insufficiency or excess plasma concentration. The mean plasma concentration in marmosets (270 ± 150 μmol/L) was substantially greater than adult human (mean 57–87 μmol/L, Human Metabolome Database) or mouse concentrations [50–52 μmol/L; (Solverson et al. 2012)]. This could reflect species differences

Table 2 Similarity scores for Phe and selected metabolites correlated with Phe in marmoset plasma

Name	Similarity score	Input mass	Adduct	Comment
Phe metabolites and amino acids				
Phe	100	166.0849	$[M + H]^+$	
Citrulline	89	176.1018	$[M + H]^+$	
Tyrosine	88	226.0436	$[M + 2Na–H]^+$	
Phenylpyruvic acid	80	209.0170	$[M + 2Na–H]^+$	
Histidine	77	178.0575	$[M + Na]^+$	
Phenyllactic acid	77	211.0327	$[M + 2Na–H]^+$	
Lysine	70	191.0755	$[M + 2Na–H]^+$	
Threonine	67	120.0646	$[M + H]^+$	
Methionine	66	150.0568	$[M + H]^+$	
Proline	61	116.0698	$[M + H]^+$	
Endogenous metabolites				
Dopamine quinone	81	174.0539	$[M + Na]^+$	Tyr metabolite
Hydroxyprogesterone	81	331.2245	$[M + H]^+$	Many isobaric steroids
SM(d18:1/18:0)	72	731.6027	$[M + H]^+$	Complex sphingolipid
5-Hydroxyindoleacetic acid	70	174.0539	$[M + H–H_2O]^+$	Trp metabolite
Deoxycorticosterone	66	331.2245	$[M + H]^+$	Many isobaric steroids
Quinolinic acid (Gliotoxin)	64	327.0495	$[M + H]^+$	Trp metabolite
L-2-Amino-4-(hydroxymethylphosphinyl)butanoate	62	226.0226	$[M + 2Na–H]^+$	Glu metabolism
Dietary compounds				
4-Quinolinecarboxylic acid	83	174.0539	$[M + H]^+$	Alkaloid
6-Methyl-5-hepten-2-ol	78	129.1264	$[M + H]^+$	Flavoring agent
3-Isopropyl-3-butenoic acid	76	129.0901	$[M + H]^+$	Flavoring agent
Myricetin	70	301.0363	$[M + H–H_2O]^+$	Flavonoid
Taxifolin	67	327.0495	$[M + Na]^+$	Fruit metabolite
Homostachydrine	64	158.1165	$[M + H]^+$	Alfalfa
Lentiginosine	62	158.1165	$[M + H]^+$	Alkaloid
Environmental agents				
Deisopropylatrazine	82	174.0539	$[M + H]^+$	Herbicide metabolite
2-Nitronaphthalene	81	174.0539	$[M + H]^+$	PAH metabolite
Diisobutyl phthalate	77	301.1391	$[M + Na]^+$	Plasticizer
Phosphinothricin (glufosinate)	76	226.0226	$[M + 2Na–H]^+$	Herbicide
N-Hydroxy-2-acetamidofluorene	75	278.0586	$[M + K]^+$	PAH metabolite
2,6-Dichlorohydroquinone	71	142.9471	$[M + H–2H_2O]^+$	Environmental chemical
Flonicamid	69	268.0072	$[M + K]^+$	Insecticide
Paraquat	68	301.0248	$[M + 2Na–H]^+$	Herbicide
Drugs, other				
N-(Acetyloxy)benzenamine	80	174.0539	$[M + Na]^+$	KEGG cmpd, no information
Trichlormethiazide	78	401.8943	$[M + Na]^+$	Drug
Floxuridine (fluorodeoxyuridine)	71	211.0524	$[M + H–2H_2O]^+$	Drug
1-Oxa-2-oxo-3-methylcycloheptane	69	129.0901	$[M + H]^+$	KEGG cmpd, no information
5-Fluorouridine	63	301.0248	$[M + K]^+$	Drug
Alcophosphamide	63	301.0248	$[M + Na]^+$	Drug metabolite
Cinoxacin	62	301.0248	$[M + K]^+$	Antimicrobial

Metabolites with available KEGG identifier or CAS identifier from Metlin search were analyzed for 3-D structural similarity with Phe using PubChem Score Matrix Service. Similarity scores are from 0 to 100 with 100 denoting identity

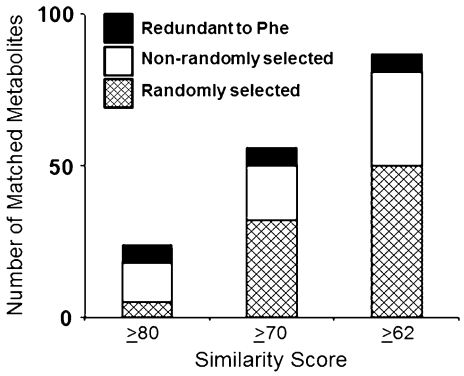

Fig. 6 Histogram of metabolites correlated with Phe in marmoset plasma as a function of structural similarity score. PubChem Compound identifiers (CID) were available for 125 metabolites matching the 50 *m/z* features correlated with Phe; these were used to calculate chemical similarity scores using the PubChem Score Matrix Service (http://pubchem.ncbi.nlm.nih.gov/score_matrix). CID were similarly derived for 50 randomly selected *m/z* features not correlated with Phe, and the 112 resulting metabolites were used to calculate similarity scores. For each similarity score threshold indicated, the total number of metabolite matches is indicated by the height of the *bar*, with the hatched component of the *bar* representing the number derived from the randomly selected metabolites, the *black* component representing those due to redundant detection of Phe and the *white* component representing the number of non-randomly detected metabolites correlated with Phe

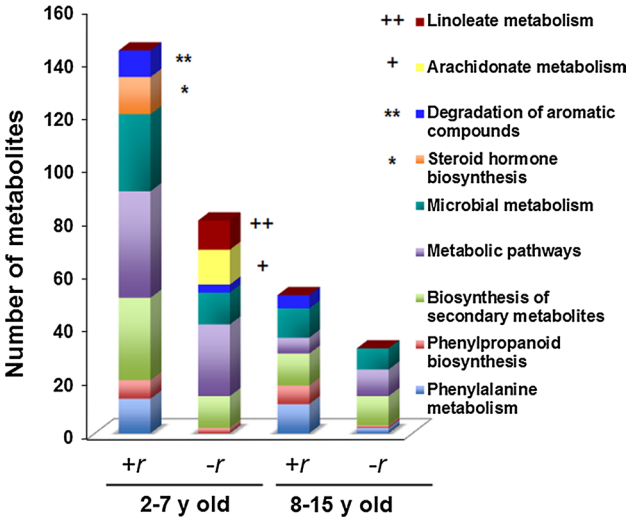

Fig. 7 KEGG Pathway Mapping of plasma metabolites associated with Phe in younger (2- to 7-year-old) and older (8- to 15-year-old) marmosets. Metabolites were separated according to positive (+*r*) and negative (−*r*) associations according to Pearson correlation. Mass spectrometry features were matched to metabolites within 10 ppm using the Metlin metabolomics database. Respective KEGG identifiers were used with the KEGG Pathway Mapping function to obtain pathway classifications. Results show differences in the classes of metabolites correlated with Phe in younger and older marmosets. The analysis is imprecise because of inclusion of multiple isobaric chemical species matching to a single *m/z* feature and some features not being represented due to lack of metabolite match and/or KEGG identifier. *Symbols* (*, +) are used to facilitate distinction of pathways differing between positive and negative associations in younger marmosets

in dietary Phe intake and/or disposition. Recommended plasma level for Phe in children with phenylketonuria is 120–360 μmol/L (Anastasoaie et al. 2008); meta analysis of intelligence quotient (IQ) as a function of blood Phe in children with phenylketonuria showed 1.3–4 % decline in IQ with each 100 μmol/L increase in Phe (Waisbren et al. 2007).

The high Phe concentration in females of marmosets (2.2-fold higher than males) was interesting (Fig. 2) and unanticipated. Previous research showed minor sex difference in plasma Phe in adult humans in response to Phe load (Stegink et al. 1981) and no noteworthy differences in fasting levels in humans or mice (Solverson et al. 2012; Stegink et al. 1981). Additional studies will be needed to address whether this difference is due to unique physiology, dietary requirements or environmental influences. The finding suggests that marmosets may be useful for neurodevelopmental studies of dietary Phe. Correlation of metabolites with Phe independently in males and females supported the overall conclusion that metabolites are present in plasma which interact with Phe. Importantly, the results also suggested that some metabolites have effects in one sex but not the other. Schumacher et al. (2008) showed that high Phe activated peroxisome proliferator-activated receptor-γ (PPAR-γ) and changed gene expression in manner comparable to the PPAR-γ agonist, rosiglitazone. Associated inhibition of cell proliferation led to the conclusion that neurodevelopmental effects of Phe in phenylketonuria may occur due to interruption of PPAR-γ signaling. Studies in adult rat adipose tissue showed sex differences in PPAR-γ activity (Kadowaki et al. 2007). Although speculative, the combination of these observations suggests that dietary, microbiome and/or environmental agents with structures similar to Phe could have biologic consequences related to Phe by impacting PPAR-γ signaling.

The present study used a high-resolution metabolomics method to measure a broad spectrum of endogenous and exogenously derived plasma metabolites. The method relies upon the mass resolution and mass accuracy of Fourier-transform mass spectrometers (Johnson et al. 2010; Soltow et al. 2013) and advanced computational algorithms for data extraction (Uppal et al. 2013). Of the more than 5,000 metabolites measured, 514 correlated with Phe at $p < 0.05$, but only 29 were correlated after implementing false discovery rate (FDR) correction of 0.05, while 50 were correlated at FDR of 0.2. Database searches of the 50 metabolites included 17 matches to Phe, metabolites of Phe and other amino acids that are expected to vary with Phe levels. Thus, the results confirm the utility of MWAS with

metabolomic database searches to identify specific metabolites related to target molecules. The approach is not without pitfalls, however, because targeted analyses of amino acids correlated with Phe showed significance (Fig. 4, Online Resource 2) but were discarded as non-significant when included within MWAS using traditionally accepted false discovery rate criteria. Prior research showed correlations of amino acids due to common transport mechanisms; dimethylglycine was correlated with glycine betaine while glycine betaine correlated with Pro as results of transport by three carriers, SLC6A12, SLC6A20 and SLC36A2 (Deo et al. 2010). Thus, the results show that a relatively non-stringent FDR cut-off of 0.2 is necessary to minimize the exclusion of important correlations with Phe.

An alternative approach commonly used in gene expression analysis is to impose filtering criteria to eliminate signals of limited interest prior to statistical testing. In effect, such processing reduces the statistical penalty for performing large numbers of comparisons. The use of MWAS applied to detect correlations with a specific metabolite filter of Pearson $|r| > 0.3$ provided a rationale to exclude metabolites of low interest. Specifically, by ordering metabolites according to decreasing r, the resulting cascade plot (Online Resource 7) showed that greater than 80 % of metabolites correlate with other metabolites with $|r| < 0.25$ (dark blue, Online Resource 7). Multiple factors contribute to such correlations, including co-variation due to handling of samples during collection, extraction and analysis. Additionally, several types of biologic variation, such as changes in intestinal absorption, water consumption, hepatic function and renal function, can result in systematic co-variation of metabolites. Use of a $|r| > 0.3$ selection criterion eliminates metabolites with weak correlations due to analytic artifacts or non-specific biologic effects. In the present study, use of $|r| > 0.3$ filter prior to statistical testing increased the number of metabolites at $q = 0.2$ from 50 to 399 (Online Resource 7). Although the potential bias introduced by such pre-selection methods must be recognized, the approach protects against discarding metabolites with weak but important biologic interactions.

Subsets of 50 to 399 metabolites, as obtained with this strategy, can be readily searched for accurate mass matches to metabolites in Metlin and other metabolomics databases. These data can then be used for pathway enrichment studies to identify pathways associated with Phe. The present study showed that metabolites positively correlated with Phe tended to be present in different pathways than negatively associated metabolites [see black dots (metabolites) in metabolic pathways, Online Resource 4a, 4b). The database matches to the 50 metabolites correlated with Phe at $q = 0.2$ (without filtering) were used to calculate structural similarity scores using structural biology methods based upon the Tanimoto equation (Willett et al. 1998). This analysis showed that despite the relatively large number of database matches, the similarity scores for these other endogenous metabolites and dietary, microbiome and environmental chemicals were higher ($n = 24$) than those obtained randomly ($n = 5$) at similarity score ≥ 80 (Fig. 6). At similarity score ≥ 70, randomly selected metabolites included 57 % as many as correlated with Phe. The potential importance of correlated chemicals with structural similarity to Phe is illustrated by the match to *N*-hydroxyl-2-acetamidofluorene, a polycyclic aromatic hydrocarbon (similarity score 75, Table 2). Previous studies show that depletion of Phe and Tyr increased demand for cigarette smoking (Hitsman et al. 2008). This suggests that environmental or dietary chemicals disrupting Phe metabolism could impact behaviors such as smoking. These observations emphasize a need for studies to characterize unidentified target chemicals which could alter Phe availability.

Separate analyses of Phe correlations in younger and older marmosets (Fig. 7) showed that strong correlations with other amino acids were present in younger marmosets but were not present in older marmosets. Amino acid homeostasis has been extensively studied in aging (Banay-Schwartz et al. 1993; Droge and Kinscherf 2008; Fujita and Volpi 2006; Fukagawa et al. 1988). Accumulating evidence, including data from methionine restriction studies (Miller et al. 2005; Wanders et al. 2014), points to a central role for essential amino acids as determinants of longevity (Collino et al. 2013). Essential amino acids impact anabolic metabolism through the mammalian target of rapamycin (mTOR) system, an evolutionarily conserved system with a central role in healthy aging and longevity (Johnson et al. 2013). The substantial shift from a pattern of correlated amino acids in young marmosets to a non-correlated pattern in older marmosets suggests that the common marmoset may provide a useful non-human primate model for study of diet and aging. This complements findings of oxidative stress marker studies, which also support the utility of this species for aging research (Roede et al. 2013).

In conclusion, the present study shows that MWAS of an essential nutrient by high-resolution metabolomics provides insight into the range of dietary, microbiome and environmental chemicals, which could impact nutrient utilization in health and disease. The study outlines a framework for use of targeted MWAS in human health research to identify dietary, microbiome and environmental chemicals contributing to health outcomes. Specifically, the results show correlations of unidentified chemicals with Phe, an amino acid with known adverse outcomes linked to disrupted metabolism in development. The findings point to a need for systematic study of human exposures impacting essential amino acid utilization during pregnancy and

early development to identify relevant exposures that may be linked to adverse outcomes such as autism and childhood cancers.

Acknowledgments The authors gratefully acknowledge the technical expertise of Yongliang Liang and ViLinh Tran for mass spectrometry analyses. This study was supported by NIH grants, AG038746 (D.J., D. P., L.W.), ES023485 (D.J, Y.G.), ES009047 (D.J.), HL113451 (D.J.) and ES019776 (D.J.).

Conflict of interest Authors have no conflicts of interest.

References

Anastasoaie V, Kurzius L, Forbes P, Waisbren S (2008) Stability of blood phenylalanine levels and IQ in children with phenylketonuria. Mol Genet Metab 95:17–20

Banay-Schwartz M, Palkovits M, Lajtha A (1993) Heterogeneous distribution of functionally important amino acids in brain areas of adult and aging humans. Neurochem Res 18:417–423

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B 57:289–300

Blau N, van Spronsen FJ, Levy HL (2010) Phenylketonuria. Lancet 376:1417–1427

Bolton EE, Wang Y, Thiessen PA, Bryant SH (2008) PubChem: integrated platform of small molecules and biological activities. In: Ralph AW, David CS (eds) Annual Reports in Computational Chemistry, vol 4. Elsevier, pp 217–241

Collino S et al (2013) Metabolic signatures of extreme longevity in northern Italian centenarians reveal a complex remodeling of lipids, amino acids, and gut microbiota metabolism. PLoS One 8:e56564

Deo RC et al (2010) Interpreting metabolomic profiles using unbiased pathway models. PLoS Comput Biol 6:e1000692

Droge W, Kinscherf R (2008) Aberrant insulin receptor signaling and amino acid homeostasis as a major cause of oxidative stress in aging. Antioxid Redox Signal 10:661–678

Farishian RA, Whittaker JR (1980) Phenylalanine lowers melanin synthesis in mammalian melanocytes by reducing tyrosine uptake: implications for pigment reduction in phenylketonuria. J Invest Dermatol 74:85–89

Fujita S, Volpi E (2006) Amino acids and muscle loss with aging. J Nutr 136:277S–280S

Fukagawa NK, Minaker KL, Rowe JW, Matthews DE, Bier DM, Young VR (1988) Glucose and amino acid metabolism in aging man: differential effects of insulin. Metabolism 37:371–377

Hitsman B et al (2008) Effects of acute tyrosine/phenylalanine depletion on the selective processing of smoking-related cues and the relative value of cigarettes in smokers. Psychopharmacology 196:611–621

Hsu JW, Goonewardene LA, Rafii M, Ball RO, Pencharz PB (2006) Aromatic amino acid requirements in healthy men measured by indicator amino acid oxidation. Am J Clin Nutr 83:82–88

Johnson JM, Yu T, Strobel FH, Jones DP (2010) A practical approach to detect unique metabolic patterns for personalized medicine. Analyst 135:2864–2870

Johnson SC, Rabinovitch PS, Kaeberlein M (2013) mTOR is a key modulator of ageing and age-related disease. Nature 493: 338–345

Jones DP, Park Y, Ziegler TR (2012) Nutritional metabolomics: progress in addressing complexity in diet and health. Annu Rev Nutr 32:183–202

Kadowaki K, Fukino K, Negishi E, Ueno K (2007) Sex differences in PPARgamma expressions in rat adipose tissues. Biol Pharm Bull 30:818–820

Kaufman S (1999) A model of human phenylalanine metabolism in normal subjects and in phenylketonuric patients. Proc Natl Acad Sci USA 96:3160–3164

Krishna RV, Krishnaswamy PR, Rao DR (1971) Enzymic synthesis of *N*-acetyl-L-phenylalanine in *Escherichia coli* K12. Biochem J 124:905–913

Lee PJ et al (2009) Adults with late diagnosed PKU and severe challenging behaviour: a randomised placebo-controlled trial of a phenylalanine-restricted diet. J Neurol Neurosurg Psychiatry 80:631–635

Miller GW, Jones DP (2014) The nature of nurture: refining the definition of the exposome. Toxicol Sci 137:1–2

Miller RA, Buehner G, Chang Y, Harper JM, Sigler R, Smith-Wheelock M (2005) Methionine-deficient diet extends mouse lifespan, slows immune and lens aging, alters glucose, T4, IGF-I and insulin levels, and increases hepatocyte MIF levels and stress resistance. Aging Cell 4:119–125

Okano Y, Nagasaka H (2013) Optimal serum phenylalanine for adult patients with phenylketonuria. Mol Genet Metab 110:424–430

Phinney KW et al (2013) Development of a standard reference material for metabolomics research. Anal Chem 85:11732–11738

Rappaport SM, Smith MT (2010) Epidemiology. Environment and disease risks. Science 330:460–461

Roede JR, Uppal K, Liang Y, Promislow DE, Wachtman LM, Jones DP (2013) Characterization of plasma thiol redox potential in a common marmoset model of aging. Redox Biol 1:387–393

Schumacher U et al (2008) High concentrations of phenylalanine stimulate peroxisome proliferator-activated receptor gamma: implications for the pathophysiology of phenylketonuria. Neurobiol Dis 32:385–390

Simon-Manso Y et al (2013) Metabolite Profiling of a NIST Standard Reference Material for Human Plasma (SRM 1950): GC-MS, LC-MS, NMR, and Clinical Laboratory Analyses, Libraries, and Web-Based Resources. Anal Chem 85:11725–11731

Smith CA et al (2005) METLIN: a metabolite mass spectral database. Ther Drug Monit 27:747–751

Soltow QA, Strobel FH, Mansfield KG, Wachtman L, Park Y, Jones DP (2013) High-performance metabolic profiling with dual chromatography-Fourier-transform mass spectrometry (DC-FTMS) for study of the exposome. Metabolomics 9:132–143

Solverson P, Murali SG, Litscher SJ, Blank RD, Ney DM (2012) Low bone strength is a manifestation of phenylketonuria in mice and is attenuated by a glycomacropeptide diet. PLoS One 7:e45165

Stegink LD, Koch R, Blaskovics ME, Filer LJ Jr, Baker GL, McDonnell JE (1981) Plasma phenylalanine levels in phenylketonuric heterozygous and normal adults administered aspartame at 34 mg/kg body weight. Toxicology 20:81–90

Tardif SD, Smucny DA, Abbott DH, Mansfield K, Schultz-Darken N, Yamamoto ME (2003) Reproduction in captive common marmosets (*Callithrix jacchus*). Comp Med 53:364–368

Udenfriend S, Cooper JR (1953) Assay of L-phenylalanine as phenylethylamine after enzymatic decarboxylation; application to isotopic studies. J Biol Chem 203:953–960

Uppal K, Soltow QA, Strobel FH, Pittard WS, Gernert KM, Yu T, Jones DP (2013) xMSanalyzer: automated pipeline for improved feature detection and downstream analysis of large-scale, non-targeted metabolomics data. BMC Bioinform 14:15

Wachtman LM, Miller AD, Xia D, Curran EH, Mansfield KG (2011) Colonization with nontuberculous mycobacteria is associated with positive tuberculin skin test reactions in the common marmoset (Callithrix jacchus). Comp Med 61:278–284

Waisbren SE, Noel K, Fahrbach K, Cella C, Frame D, Dorenbaum A, Levy H (2007) Phenylalanine blood levels and clinical outcomes

in phenylketonuria: a systematic literature review and meta-analysis. Mol Genet Metab 92:63–70

Wanders D, Ghosh S, Stone KP, Van NT, Gettys TW (2014) Transcriptional impact of dietary methionine restriction on systemic inflammation: relevance to biomarkers of metabolic disease during aging. Biofactors 40:13–26

Wild CP (2005) Complementing the genome with an "exposome": the outstanding challenge of environmental exposure measurement in molecular epidemiology. Cancer Epidemiol Biomark Prev 14:1847–1850

Willett P, Barnard J, Downs G (1998) Chemical similarity searching. J Chem Inf Comput Sci 38:983–996

Yu T, Park Y, Johnson JM, Jones DP (2009) apLCMS–adaptive processing of high-resolution LC/MS data. Bioinformatics 25:1930–1936