Electronic Supplementary Material (ESI) for Analyst.

SUPPORTING INFORMATION

Metabolomics for Improved Treatment Monitoring of Phenylketonuria: Urinary Biomarkers for Non-invasive Assessment of Dietary Adherence and Nutritional Deficiencies

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Table S1; Figure S1-Figure S6

Table S1. Median concentration and their ranges for urinary Phe and three other catabolites from classic PKU patients (*n*=16) and their correlation with urinary Phe excretion.

Phe Catabolite	*m/z*:RMT:mode	Median (Range) Concentration[a]	Correlation (R^2)
o-Hydroxyphenylacetic acid	151.040:0.969(-)	324 (41-2835) µM	0.469, *n*=16
Phenylpyruvic acid	163.040:0.981(-)	650 (52-5818) µM	0.808, *n*=12
Phenylalanine	166.086:0.913(+)	300 (28-1280) µM	0.833, *n*=13*
N-Phenylacetylglutamine	263.104:0.801(-)	1264 (167-5194) µM	0.889, *n*=16

[a] *Median concentrations and their ranges for urinary Phe and related catabolites from single-spot urine samples of a sex-balanced cohort of PKU patients (median age of 14 y; 8 M, 8F), where Pearson correlation of urinary Phe concentrations with other urinary metabolites was performed with the exception of urinary Phe, which was correlated with plasma Phe concentrations from matching PKU patients (median = 410 µM; range of 38-1548 µM).*

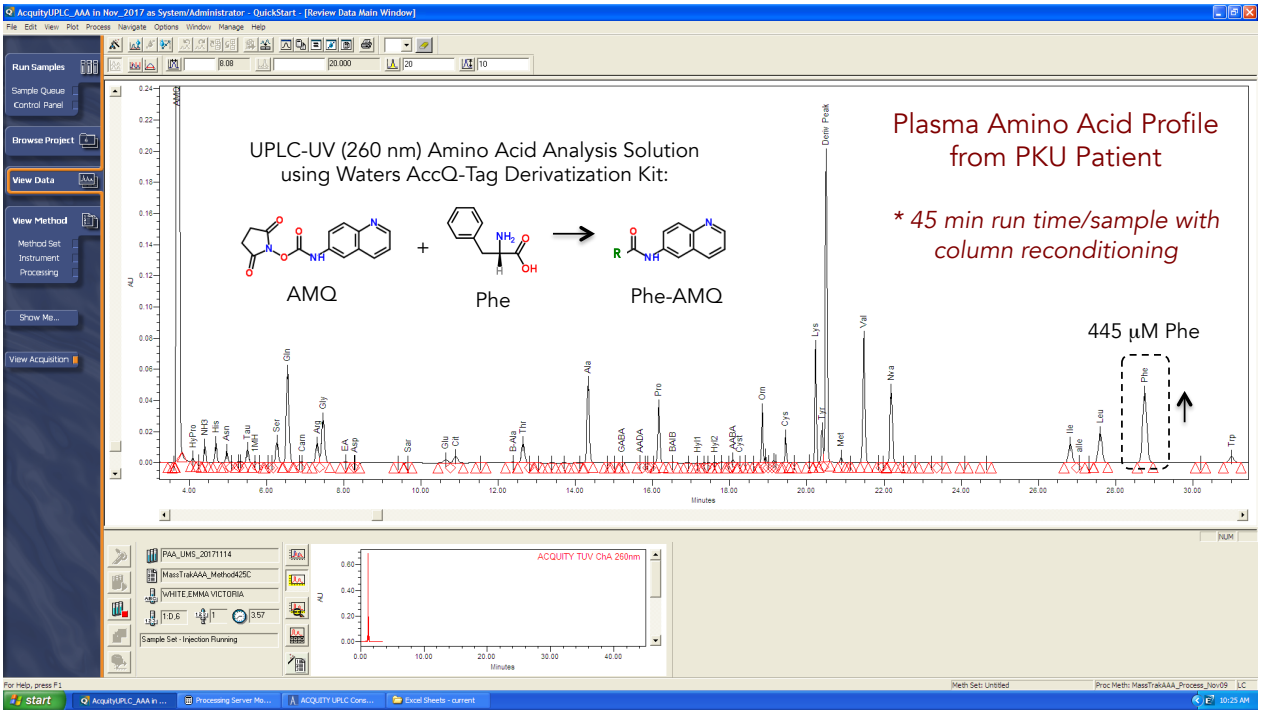

Figure S1. Representative UPLC-UV run that is used for amino acid analysis following pre-column chemical derivatization for plasma Phe (and Tyr) determination as required for confirmatory testing and treatment monitoring of PKU patients. All measurements were performed independently at McMaster Children's Hospital (Hamilton, ON, Canada) using standardized protocols/reagents supplied from Waters Inc. (Mass Trak Amino Acid Analysis).

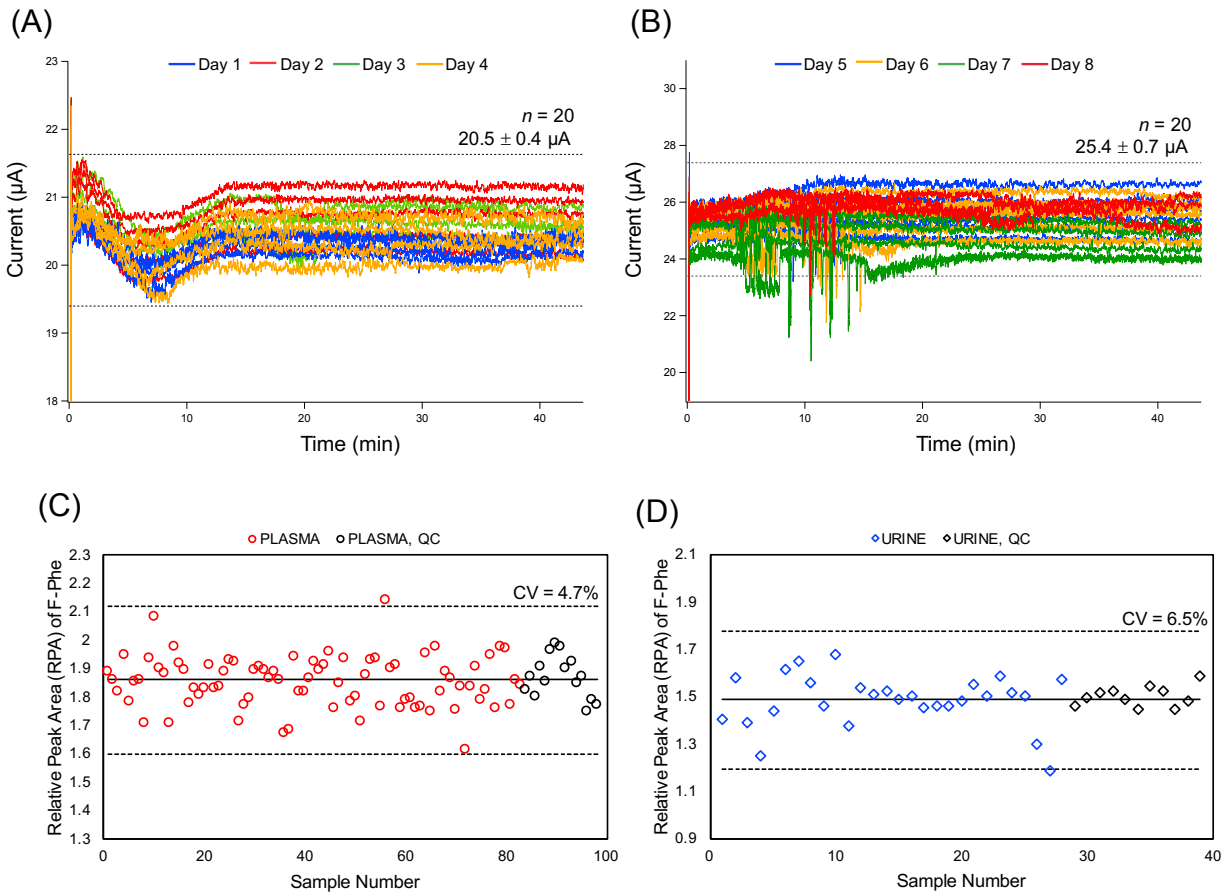

Figure S2. Overview of the long-term technical precision of MSI-CE-MS for 40 runs performed over 8 days when using MSI-CE-MS (*n*=136), which included plasma (*n*=83), urine (*n*=28) and repeated analysis of pooled samples as QC (*n*=25). Overlay of CE current traces for 40 runs over 8 days by for metabolomic analyses of plasma and urine samples with full-scan data acquisition in (A) positive and (B) negative ion mode detection. There was excellent inter-day precision in CE current traces in positive and negative ion mode with average CV of 1.8% and 2.6%, where upper and lower action limits are indicated by dashed line (± 3s). Also, excellent robustness and long-term precision is also demonstrated by control charts for the quantification of the recovery standard, F-Phe that was added to all samples analyzed (n=136), where the solid line represents the average relative peak area, and the dotted lines represent represent the upper and lower action limits (± 3s).

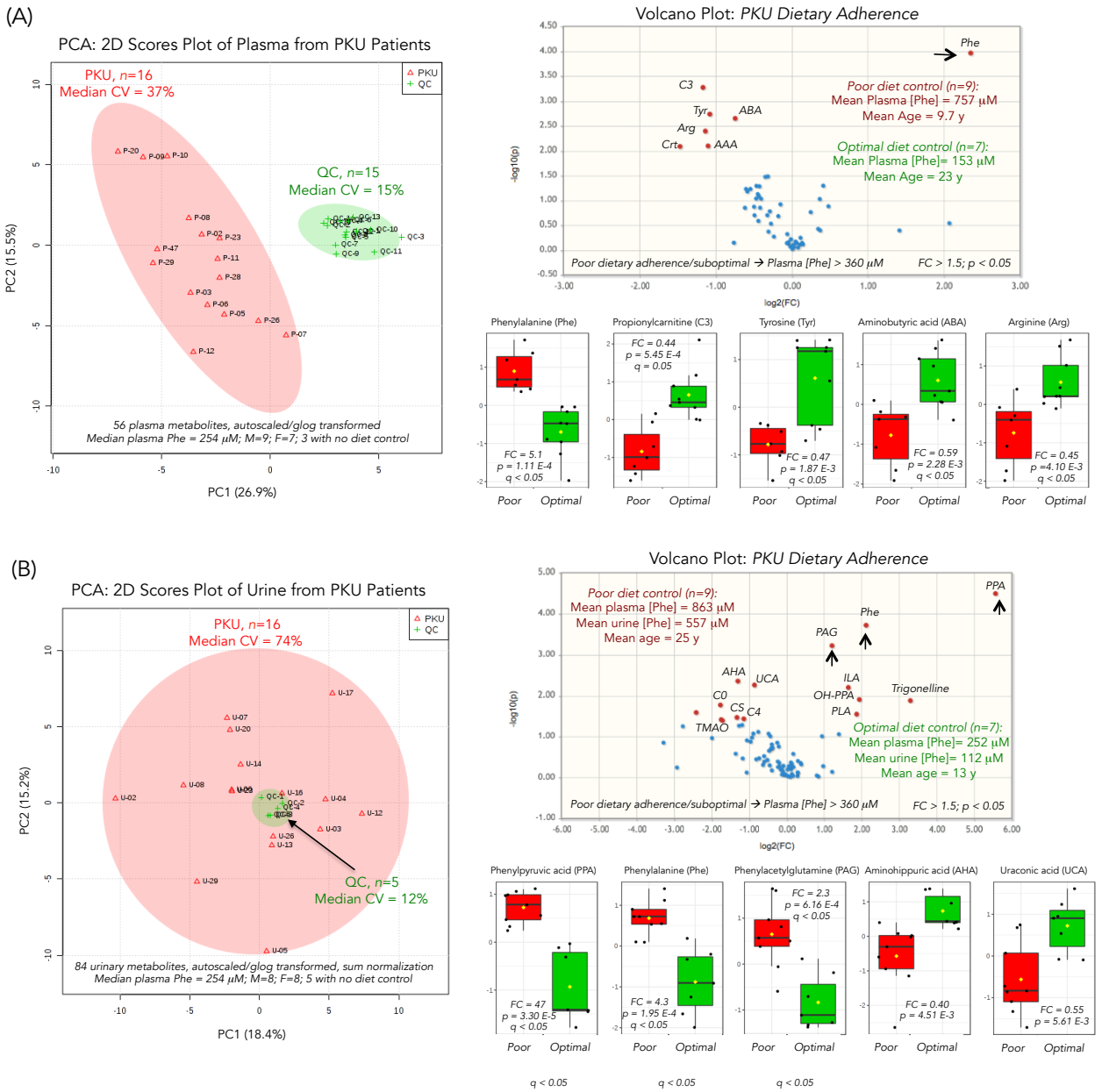

Figure S3. Data overview when using principal component analysis (PCA) and top-ranked metabolites in **(A)** plasma, as well as **(B)** urine from PKU patients reflecting poor adherence to dietary Phe-restriction that exceed recommended therapeutic range (> 360 µM plasma Phe) based on volcano plots (*FC* > 1.5; *p* < 0.05). Overall, elevated plasma Phe in PKU patients with poor dietary maintenance was inversely correlated to circulating levels of Tyr, propionylcarnitine (C3), arginine (Arg) and α-aminobutyric acid (ABA), whereas urine reflected increased excretion of Phe, and several Phe catabolites, including phenylpyruvic acid (PPA), phenylacetylglutamine (PAG), as well as hydroxyphenylpyruvic acid (OH-PPA) and phenyllactic acid (PLA), together with lower levels of aminohippuric acid (AHA), as well as a histidine catabolite, uraconic acid (UCA). Overall, poor dietary adherence with corresponding elevated plasma Phe concentrations were associated with adult PKU patients not following specialized diets for Phe restriction (*e.g.,* amino acid supplemental formula).

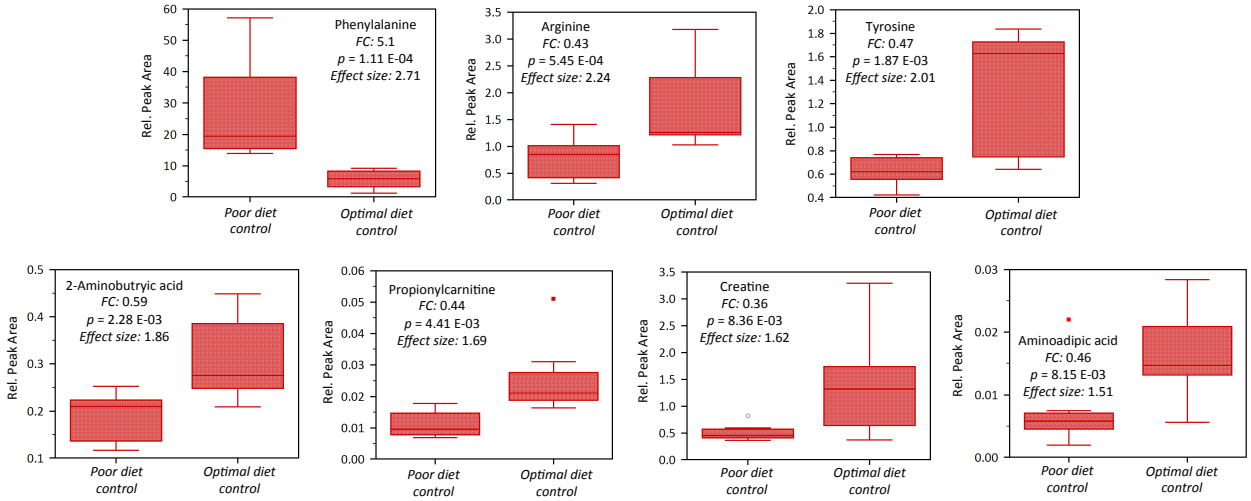

Figure S4. Box-whisker plots for the 7 top-ranked plasma metabolites associated with PKU phenotype that was associated with poor diet control (plasma Phe > 360 μM, *n*=9) as compared to optimal diet control (plasma Phe < 360 μM, *n*=7) when using nontargeted metabolite profiling of plasma by MSI-CE-MS. As expected, plasma Phe was independently confirmed to be significantly elevated among poor diet control PKU sub-group with the largest fold-change (*FC*) increase in response, and strong effect size. Additionally, 6 other plasma metabolites were inversely correlated to Phe and found to be significantly lower/deficient in poor diet control PKU sub-group as compared to treated PKU patients following a Phe-restricted diet and amino acid supplementation as summarized in **Table 2**.

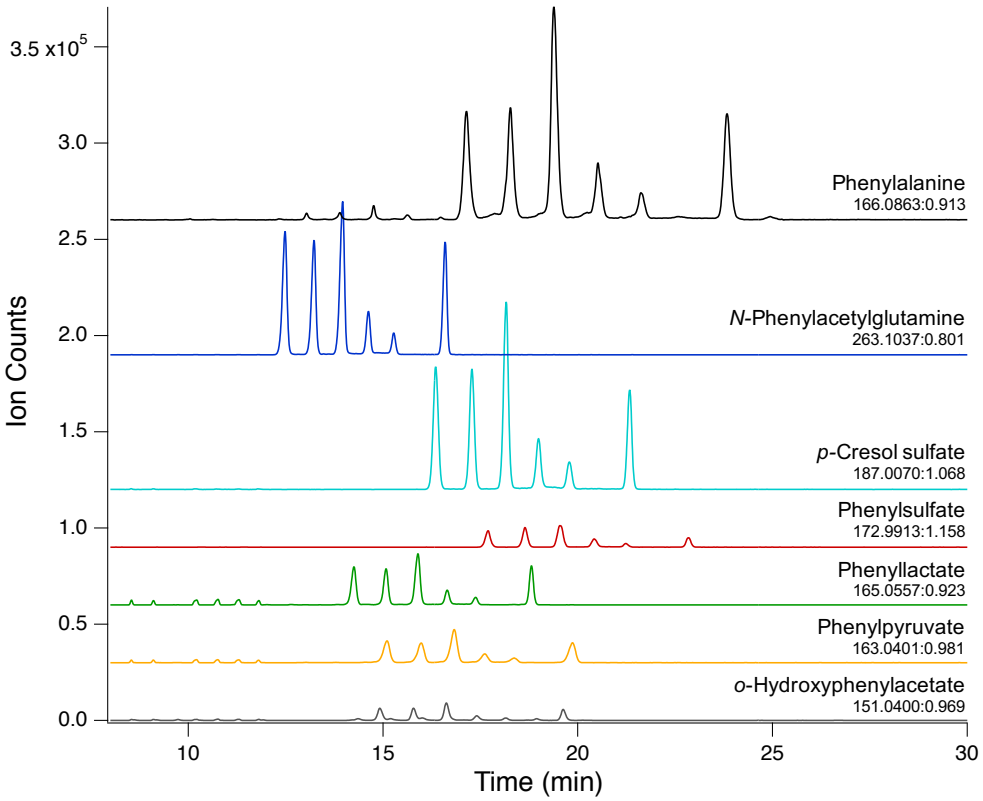

Figure S5. Extracted ion electropherogram overlay for Phe, *N*-phenylacetylglutamine, *p*-cresol sulfate, phenylsulfate, phenyllactate, phenylpyruvate, and *o*-hydroxyphenylacetate for a representative pooled urine QC sample using MSI-CE-MS with a serial dilution trend filter. Phe was detected in positive ion mode with ion responses scaled down by a factor of 2, whereas all other anionic metabolites were detected in negative ion mode.

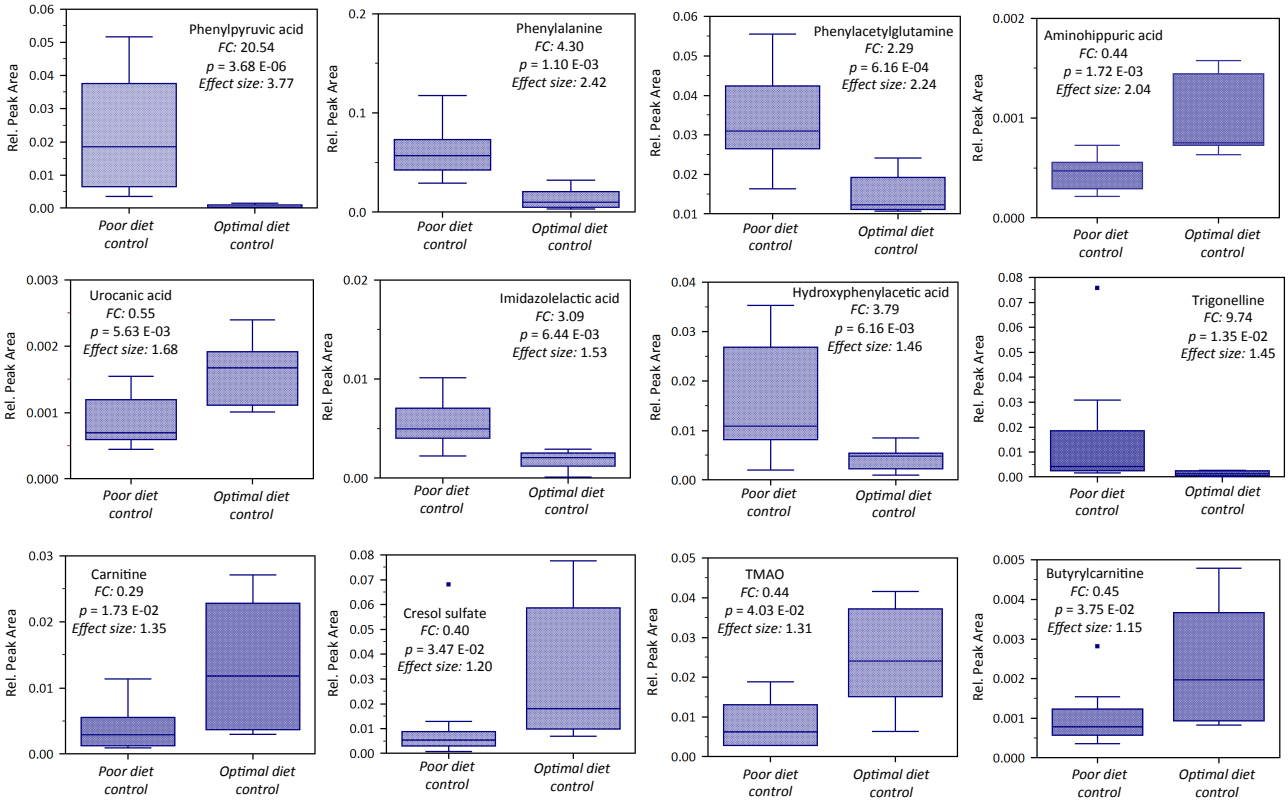

Figure S6. Box-whisker plots for the 12 top-ranked urinary metabolites associated with PKU phenotype that was associated with poor diet control (plasma Phe > 360 μM, *n*=9) as compared to optimal diet management (plasma Phe < 360 μM, *n*=7) when using nontargeted metabolite profiling of single-spot urine samples by MSI-CE-MS. As expected, urinary Phe was independently confirmed to be elevated among poor diet control PKU sub-group; however, the major urinary catabolite of Phe excreted among PKU patients was phenylpyruvic acid, which had with the largest fold-change (*FC*) increase in response with a strong effect size. Additionally, a series of 10 other urinary metabolites were positively (PAG, OH-PAA, ILA, TGL) or inversely (AHA, UCA, C0, CS, TMAO, C4) correlated to PKU phenotype and found to be significantly higher or lower/deficient in poor diet control PKU sub-group, respectively, as compared to treated PKU patients following a Phe-restricted diet as summarized in **Table 2**.