Article

Metabolic Trajectories Following Contrasting Prudent and Western Diets from Food Provisions: Robust Biomarkers of Short-term Changes in Habitual Diet

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Abstract: A large body of evidence has linked unhealthy eating with an alarming increase in obesity and chronic disease worldwide. However, existing methods of assessing dietary intake rely on food frequency questionnaires or dietary records that are prone to bias and selective reporting. Herein, metabolic phenotyping was performed on 42 healthy participants from the Diet and Gene Intervention (DIGEST) pilot study, a parallel two-arm randomized clinical trial that provided complete diets to all participants. Matching urine and plasma specimens were collected at baseline and following 2 weeks of provision of either a Prudent or Western diet with a weight-maintaining menu plan designed by a dietician. Targeted and nontargeted metabolite profiling was conducted using three complementary analytical platforms, where 80 serum metabolites and 84 creatinine-normalized urinary metabolites were reliably measured (CV < 30%) in the majority of participants (> 75%) after implementing a rigorous data workflow for metabolite authentication with stringent quality control. We classified a panel of metabolites with distinctive trajectories following 2 weeks of food provisions when using complementary univariate and multivariate statistical models. Unknown metabolites associated with contrasting dietary patterns were identified with high resolution MS/MS and/or co-elution after spiking with authentic standards. Overall, 3-methylhistidine and proline betaine concentrations increased consistently when participants were assigned a Prudent diet ($q < 0.05$) in both plasma and urine samples with a decrease in the Western diet group. Similarly, creatinine-normalized urinary imidazole propionate, hydroxypipecolic acid, dihydroxybenzoic acid, and enterolactone glucuronide, as well as plasma ketoleucine and ketovaline increased with a Prudent diet ($p < 0.05$) after adjustments for age, sex and BMI. In contrast, plasma myristic acid, linoelaidic acid, linoleic acid, α-linoleic acid, pentadecanoic acid, alanine, proline, carnitine and deoxycarnitine, as well as urinary acesulfame K increased among participants following a Western diet. Most metabolites were also correlated ($r > \pm 0.30$, $p < 0.05$) to changes in average intake of specific nutrients from self-reported diet records reflecting good adherence to food provisions. This study revealed robust biomarkers sensitive to short-term changes in habitual diet that can be used to reliably monitor healthy eating patterns for new advances in nutritional epidemiology, as well as the design of evidence-based public health policies for chronic disease prevention.

Keywords: Metabolomics; Metabolite profiling; Prudent diet; Western diet; Food provisions; Diet records; Nutritional epidemiology; Mass spectrometry

1. Introduction

A global epidemic of obesity and chronic non-communicable diseases threaten to reduce life expectancy and impose a severe burden on public health [1,2]. Diet and lifestyle are two key modifiable determinants of human health of particular importance for risk of cardiovascular disease (CVD), type 2 diabetes and some cancers [3]. CVD remains the leading cause of death globally [4] which has been associated with a Western diet. Contemporary Western diets rich in *trans* fats, processed foods and red meat, including regular consumption of sweetened beverages and high glycemic index foods lacking adequate fiber, have been strongly linked to chronic inflammation and metabolic syndrome [5] that increasingly impacts the metabolic health across the lifespan. [6] In contrast, a Prudent eating pattern (*e.g.*, DASH, Mediterranean, Nordic diets etc.) that includes greater intake of fruits & vegetables, lean meats and whole grains reduces blood lipids, improves blood sugar homeostasis and lowers blood pressure [7,8]. However, there is urgent need for more accurate dietary assessment tools for the design of evidence-based nutritional policies that are effective for chronic disease prevention on a population level [9].

Nutritional epidemiologists face unique challenges in light of the highly complex chemical composition of foods, whose physiological effects are often confounded by interactions of diet with genes, lifestyle, microbiome and other environmental exposures [10]. To date, observational studies in nutrition mainly rely on self-reported measures of dietary intake, including methods of recall (*e.g.*, food frequency questionnaires, 24 h dietary recall) or real-time recording (*e.g.*, food diaries) that are prone to bias and selective reporting [11]. Alternatively, targeted assays exist for measuring energy expenditure (*e.g.*, doubly-labeled water), as well as specific macronutrients (*e.g.*, protein), electrolytes (*e.g.*, sodium) and micronutrients (*e.g.*, vitamin D) with established reference ranges associated with nutritional status and/or chronic disease risk. However, these methods are not routinely applied in large-scale human studies due to cost barriers while representing only a small fraction of total food exposures [12,13]. In this context, new advances in high throughput metabolomics offer a holistic approach to measure complex dietary patterns in lieu of specific nutrients in human biofluids, such as urine and plasma [14]. Recent metabolomic studies have identified dietary biomarkers [15,16] to monitor for dietary adherence, as well as validate or correct standard dietary assessment tools used in nutritional epidemiology [17-21]. However, few dietary biomarkers are unique to specific foods nor adequately validated as quantitative measures of recent or habitual food intake in well-controlled randomized clinical trials [22-24].

Herein, metabolic phenotyping of matching blood and urine specimens were analyzed from healthy participants from the Diet and Gene Intervention (DIGEST) pilot study, which was a randomized controlled trial to explore the short-term effects of a Prudent diet on CVD risk factors where individuals were provided all foods to prepare at home [25]. A modest reduction in systolic and diastolic blood pressure and total cholesterol was reported for participants following a Prudent diet for two weeks as compared to a Western diet; however, dietary adherence relied on participant self-reporting and food preparation methods were not standardized likely contributing to variability in treatment responses [25]. In this work, we sought to identify specific metabolic trajectories in plasma and urine that function as responsive biomarkers reflecting short-term changes in habitual diet, that were measured in free-living individuals outside of a dedicated metabolic ward or hospital stay. These dietary biomarkers not only confirmed good adherence to food provisions, but were also associated with healthy eating patterns indicative of a Prudent diet [26] unlike a Western diet that increases overall risk for CVD [27].

2. Experimental

2.1. Study Design, Participant Eligibility and Dietary Self-reporting.

The Diet and Gene Intervention Study (DIGEST) was a 2-arm, parallel unblinded study to compare the effects of two weeks of a Prudent diet compared with a Western diet on cardiovascular risk factors and gene expression in apparently healthy adults. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the

Declaration of Helsinki, and the protocol was approved by the Hamilton Integrated Research Ethics Board (#12-260), and it was also registered with clinicaltrials.gov (NCT01658137). Healthy participants were recruited using flyers, and self-referral methods from McMaster University and the surrounding areas. Exclusion criteria were an unwillingness to eat an assigned diet, or serious disease or illness. The full inclusion and exclusion criteria are described elsewhere [25]. A subset of 42 participants from the DIGEST study with paired urine and serum samples were selected for targeted and nontargeted metabolomics analysis using three complementary instrumental platforms (Experimental of Supplemental Material), where participants had completed self-reported dietary records at baseline and two weeks following food provisions. All participants from DIGEST picked up their food allotment each week at the grocery store, during clinic visits, or food provisions were delivered to their home by volunteers [25]. Additionally, cooking suggestions with meal plans were provided by a dietician that still allowed for flexibility in food preparations while participants were requested to maintain their normal lifestyle habits (*e.g.,* physical activity). In order to maximize treatment effects in this short-term dietary intervention pilot study, subjects were assigned into two parallel arms of contrasting diets, namely a Western diet reflecting a typical Canadian macronutrient profile with higher intake of processed foods (*e.g.,* burgers, fried chicken, cereals, processed cheeses), and a Prudent diet based around minimally processed foods composed of lean protein (*e.g.,* poultry, fish, legumes), whole grains and a high amount of fresh fruits and vegetables.

An aggregate diet quality index score was used to classify participants as having a predominately Prudent or Western habitual diet at baseline based on five categories assessed from self-reporting questionnaires, including polyunsaturated/saturated fatty acid ratio (poly/sat > 1.0), relative intake of saturated fatty acids (< 7%), total fibre (> 28 g/day), daily servings fruits and vegetables (> 5), and daily potassium intake (> 3500 mg/day). Briefly, habitual dietary patterns at baseline was evaluated by having participants attend a screening visit 1 week prior to beginning DIGEST where they were also provided a diary to record all their foods. Both diet groups were balanced with respect to age and adiposity (*BMI*), however most participants in this study were female (64%), and a majority were Caucasian (78%) with no self-identified tobacco smokers. Each participant was assigned a 'Prudent score' and 'Western score' from 1 (low) to 4 (high) based on their quartile rankings [25]. Habitual diet was classified as predominately Western if the difference of [Western score - Prudent score] ≥ 2. All participants from DIGEST were informed to maintain their usual lifestyle and physical activity routine during the study period and they were provided a 7-day menu plan for each of the 2 weeks, which listed all of the foods they were to eat at specific meals. Servings of Prudent-type (*e.g.,* fruit & vegetables, lean meats, high fiber) and Western-type (*e.g.,* red meat, salty food, high saturated fats) diet was scored and ranked in quartiles [25]. Dietary adherence was a measure of the % of the foods "prescribed" that they reported eating that was self-reported based on the foods they checked-off from menu list that they consumed, which was > 95% for both treatment arms. A total of 20 micro- and macronutrient categories (from over 120) from self-reporting dietary records were determined to be significantly ($q < 0.05$; Bonferroni adjustment) different between assigned Prudent and Western diets for DIGEST participants in this pilot study, which were subsequently correlated with top-ranked plasma and urinary metabolites when validating putative dietary biomarkers of contrasting diets. Further details on chemicals/reagents, instrumental platforms for targeted and nontargeted metabolite profiling of plasma and urine samples, unknown metabolite identification by MS/MS, as well as data processing and statistical analysis are described in the Experimental of the Supplemental Material.

3. RESULTS

3.1. Study Design, Baseline Habitual Diet and Metabolomics Workflow.

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The DIGEST study was a two-arm parallel dietary intervention involving healthy/non-smoking participants recruited from the local community as described elsewhere [25]. A CONCERT diagram summarizes eligibility criteria (Figure S1), where all participants completed a 7-day

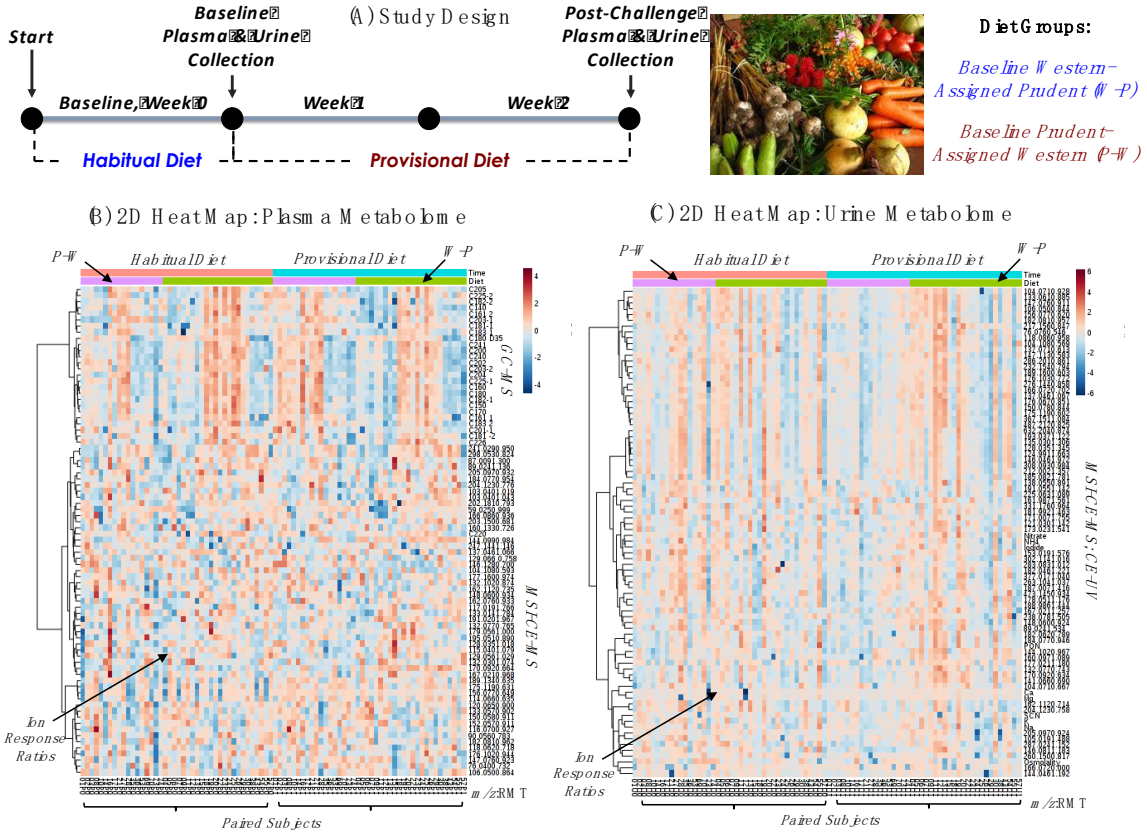

Figure 1. (A) Overview of study design in this parallel two-arm dietary intervention study involving participants from DIGEST (n=42) who were assigned a Prudent or Western diet over a 2-week period with matching urine and plasma samples collected at baseline and post-intervention. (B) A 2D heat map with hierarchical cluster analysis (HCA) of the plasma metabolome that were consistently measured in the majority of participants, including non-targeted analysis of polar/ionic metabolites by MSI-CE-MS and total fatty acids by GC-MS. (C) A 2D heat map with hierarchical cluster analysis of the urine metabolome that were consistently measured in majority of participants, including non-targeted analysis of polar/ionic metabolites and targeted electrolytes by CE with indirect UV absorbance. A generalized *log* transformation and autocaling was performed on metabolomic datasets together with creatinine normalization for single-spot urine specimens. Participants classified as having a predominate Western diet at baseline who were then assigned a Prudent diet are designated as "W-P" (n=24), whereas "P-W" (n=18) refers to participants had lower Western diet score at baseline, but were assigned a Western diet.

prospectively collected diet record and then were randomly allocated to eat a weight-maintaining Prudent or Western diet over 2 weeks. Participants (n=42) with contrasting habitual diets were selected in this unblinded metabolomics study based on availability of matching plasma and urine samples with complete diet records as depicted in Figure 1(A). There were more women (64%) recruited than men, however there were no differences in age (mean age of 47 years ranging from 20 to 69 years), body composition (mean *BMI* of 27 kg/m^2 with 26% defined as obese) and average caloric intake (mean of 1940 kcal/day) between assigned diet groups (Table S1). Also, no differences in baseline blood lipids, fasting glucose, inflammatory biomarkers and blood pressure were measured between the two treatment arms. In this study, 18 participants were classified as having a Prudent-like diet at baseline (*i.e.*, low Western diet score) who were randomized to a Western diet (referred to as P-W), and 24 participants with a predominate Western diet at baseline were

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randomized to a Prudent diet (referred to as W-P). Overall, participants reported excellent adherence to all provided food items [25].

Figure 1(B) and (C) depict 2D heat maps for matching plasma and urine metabolomes from DIGEST participants (n=42) collected at baseline and following 2 weeks of assigned food provisions. A total of 80 serum metabolites and 84 urinary metabolites were reliably measured (CV < 30%) in the majority of participants (> 75%) when using a validated data workflow for nontargeted metabolite profiling with stringent quality control (QC) [28-30]. A rigorous approach to metabolite authentication was implemented to reject spurious, redundant and background ions that comprise the majority of molecular features detected in ESI-MS [31] in order to reduce false discoveries in metabolomics [32]. Overall, three orthogonal platforms were used to characterize polar/ionic metabolites in plasma and urine samples using multisegment injection-capillary electrophoresis-mass spectrometry (MSI-CE-MS), as well as total (hydrolyzed) plasma fatty acids as their methylester derivatives (FAMEs) by GC-MS, and inorganic urinary electrolytes by CE with indirect UV detection (Supporting Experimental; Figure S2). Also, 2D scores plots from principal component analysis (PCA) of plasma and creatinine-normalized urine metabolome demonstrated good technical precision from pooled samples used as QCs (median CV = 4-12%) as compared to the biological variance measured in random/single-spot urine (median CV = 65-78%) and fasting plasma (median CV = 32-53%) metabolomes (Figure S3). A batch-correction algorithm was also applied to urine metabolome data to minimize signal drift when using MSI-CE-MS [29], where each run comprised a serial injection of six randomized samples together with a pooled QC. Also, control charts for recovery standards provide further evidence of acceptable intermediate precision (mean CV < 9%) with few outliers (Figure S3).

A complete list of authenticated metabolites reliably measured in this study (Table S2) is annotated by their accurate mass and relative migration time (m/z:RMT) under positive (+) or negative (-) ion detection mode, as well as their most likely molecular formula and mass error, level of identification, and compound name. Unambiguous identification of metabolites associated with contrasting diets was performed by spiking with authentic standards (if available) in conjunction with high resolution MS/MS, which were compared to reference spectra available in public databases (HMDB, Metlin); otherwise, spectral annotation was guided by *in silico* fragmentation [33] using recommended reporting standards for metabolite identification [34]. An overview of this metabolomics workflow is outlined in Figure 2, which shows the detection of an unknown protonated molecule (MH^+) in plasma by MSI-CE-MS, followed by its annotation by high resolution MS and subsequent identification (level 1) as proline betaine (ProBet) using MS/MS after comparison to an authentic standard at an optimal collision energy. Reliable quantification of ProBet using an external calibration curve is also demonstrated with good technical precision (CV < 15%, n=20) as shown in a control chart based on repeated analysis of a QC in every run over the duration of the study.

3.2. Changes in Dietary Intake and Biomarker Classification.

Major changes in self-reported dietary patterns among DIGEST participants were evident after 2 weeks as summarized in Table 1. Although there were no significant changes in BMI or average caloric intake between the two treatment arms, greater palatability and satiety was previously reported for participants assigned to a Prudent diet [25]. As expected, the Prudent diet group (W-P) had higher intake of dietary fiber (total, insoluble, soluble), major electrolytes (K, Mg) fruit and/or vegetable, vitamins, poly:sat, protein, and sugar or total carbohydrates, whereas the P-W group had higher intake of fat (total, saturated, and *trans*), sodium and cholesterol. Figure S4 illustrates the relationship among 20 of the most significant nutrient categories reflecting contrasting diets when using PCA along with a hierarchical cluster analysis (HCA) and 2D heat map. There was strong co-linearity (r > ± 0.70) among most nutrient categories with two distinctive clusters reflecting opposing Prudent and Western eating patterns assigned to DIGEST participants.

Volcano plots (Figure S5) were initially used to evaluate changes in the metabolic phenotype of participants using minimum cut-off thresholds (*i.e.*, mean fold-change or FC > 1.3; p < 0.05).

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Overall, contrasting diets generated pronounced changes in a wide range of plasma and urinary metabolites that was largely absent for the same participants at baseline given modest differences in their habitual diets prior to the start of food provisions (Table S1). For instance, 10 plasma and 16

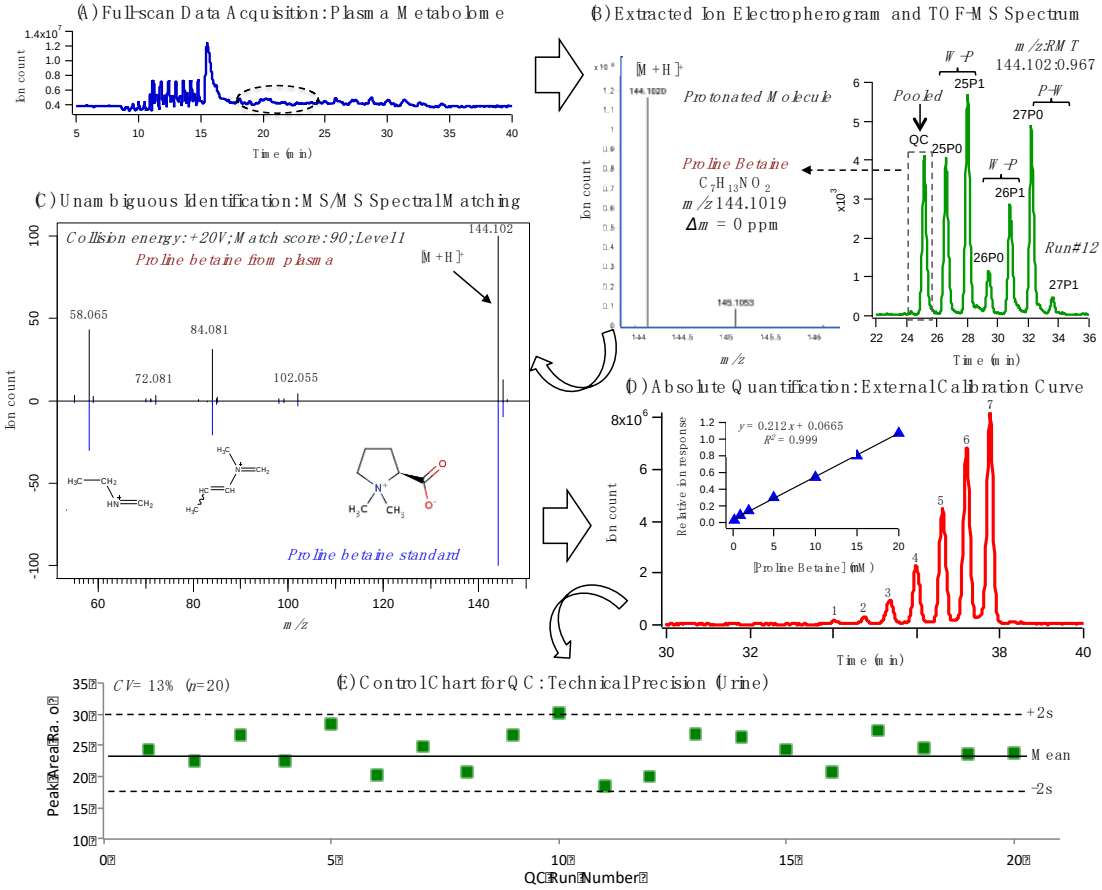

Figure 2. (A) Metabolomics data workflow for the identification and quantification of biomarkers of a provisional Prudent diet (*e.g.*, proline betaine annotated based on its *m*/z:RMT) when using full-scan data acquisition. (B) Multiplexed separations by MSI-CE-MS based on serial injection of seven plasma filtrate (or diluted urine) samples within a single run, including paired samples from each DIGEST participant (*i.e.*, baseline/post-treatment) together with a pooled sample as QC for assessing technical precision and long-term signal drift. High resolution MS under positive ion mode detection allows for determination of most likely molecular formula for unknown cation (*i.e.*, protonated molecular ion), whereas (C) MS/MS spectra is used for its structural elucidation when compared with an authentic standard. (D) Quantification for metabolites is then performed by external calibration when using an internal standard (Cl-Tyr) for data normalization by MSI-CE-MS. (E) A control chart for ProBet from pooled urine samples as QC analyzed in random positions in every run demonstrates acceptable technical precision over 3 days.

urinary metabolites were differentially expressed in W-P as compared to P-W diet groups, including four metabolites satisfying a Benjamini-Hochberg/FDR adjustment ($q < 0.05$), including ProBet, 3-methylhistidine (Me-His) and two unknown urinary metabolites subsequently identified (level 2) as hydroxypipecolic acid (OH-PCA) and imidazole propionic acid (ImPA). The identification and quantification of Me-His was confirmed in both plasma and urine (Figure S6), whereas several unknown urinary metabolites were putatively identified (level 1 or 2) based on their characteristic MS/MS spectra, such as OH-PCA (Figure S7) and acesulfame K (ASK; Figure S8). Similarly, targeted analysis of FAMEs from hydrolyzed plasma extracts using GC-MS (Figure S9) allows for resolution of low abundance *trans* isomers (linoelaidic acid, C18:2n-6*trans*) and saturated fatty acids (myristic acid, C14:0) from abundant dietary fatty acids (linoleic acid, C18:2n-6*cis*). As

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expected, several circulating fatty acids (Figure S5) were consistently elevated following a Western diet due to higher average consumption of total fats as compared to a Prudent diet.

Table 1. Major changes in dietary patterns after a 2-week assigned Prudent and Western diet relative to baseline habitual diet of DIGEST participants (*n*=42) based on self-reported diet records.

Diet Category[a]	W-P, *n*=24	P-W, *n*=18	*P* for comparison/outcome
Δ Insoluble fiber intake (g/2000 kcal/day)	(14.0 ± 5.3)	(-5.0 ± 3.5)	*p* = 1.4 E-15; Greater insol. fiber intake in Prudent arm
Δ Mg intake (mg/2000 kcal/day)	(189 ± 89)	(-134 ± 70)	*p* = 3.5 E-15; Greater Mg intake in Prudent arm
Δ Fruit & veggie intake (servings/2000 kcal/day)	(3.6 ± 1.4)	(-1.8 ± 1.3)	*p* = 7.3 E-15; Greater intake in fruit/veggie in Prudent arm
Δ Total fiber intake (g/2000 kcal/day)	(16.6 ± 8.4)	(-13.4 ± 8.1)	*p* = 5.2 E-14; Greater total fiber intake in Prudent arm
Δ Energy from sat. fat (%)	-(5.4 ± 3.2)	(4.6 ± 2.4)	*p* = 1.8. E-13; Greater intake of sat. fat in Western arm
Δ Vegetable intake (cup eq./2000 kcal/day)	(1.8 ± 0.80)	(-0.91 ± 0.92)	*p* = 2.4 E-12; Greater veggie intake in Prudent arm
Δ K intake (mg/2000 kcal/day)	(1338 ± 617)	(-854 ± 667)	*p* = 2.5 E-13; Greater K intake in Prudent arm
Δ Vitamin E (mg/2000 kcal/day)	(7.7 ± 5.3)	(-7.0 ± 4.0)	*p* = 5.1 E-12; Higher intake of vit. E in Prudent arm
Δ Poly:sat fatty acid (ratio)	(0.47 ± 0.21)	(-0.14 ± 0.18)	*p* = 8.2 E-12; Greater intake of poly:sat in Prudent arm
Δ Vitamin C (mg/2000 kcal/day)	(149 ± 69)	(-40 ± 54)	*p* = 1.2 E-11; Higher intake of vit. C in Prudent arm
Δ Soluble fiber intake (g/2000 kcal/day)	(3.9 ± 2.1)	(-1.5 ± 1.5)	*p* = 2.3 E-11; Greater total fiber intake in Prudent arm
Δ Fruit intake (cup eq./2000 kcal/day)	(1.79 ± 0.93)	(-0.92 ± 0.99)	*p* = 5.9 E-11; Greater fruit intake in Prudent arm
Δ Energy from fat (%)	-(7.5 ± 5.6)	(5.6 ± 5.6)	*p* = 9.0 E-10; Greater intake of total fat in western arm
Δ Na intake (mg/2000 kcal/day)	-(694 ± 590)	(754 ± 658)	*p* = 6.4 E-9; Greater Na intake in western arm
Δ Vitamin A (µg/2000 kcal/day)	(12973 ± 56344)	(-7847 ± 14060)	*p* = 1.4 E-7; Higher intake of vit. E in Prudent arm
Δ Energy from sugar (%)	(8.9 ± 5.4)	(-1.5 ± 5.8)	*p* = 7.3 E-7; Higher sugar intake in Prudent arm
Δ Energy from protein (%)	(1.9 ± 3.6)	(-3.2 ± 2.7)	*p* = 1.5 E-5; Greater intake of protein in Prudent arm
Δ Energy from carbohydrates (%)	(8.5 ± 7.8)	(-0.35 ± 5.7)	*p* = 2.9 E-4; Greater intake of total carbs in Prudent arm
Δ Cholesterol[b] (mg/2000 kcal/day)	-(101 ± 140)	(54 ± 110)	*p* = 4.8 E-4; Greater intake of cholesterol in Western arm
Δ Energy from trans fat (%)	-(0.26 ± 0.55)	(0.27 ± 0.23)	*p* = 6.4 E-4; Greater intake of trans fats in Western arm

[a] Mean differences (Δ) in self-reported dietary patterns were evaluated from food records collected twice over a 2-week period at clinical visits as compared to the baseline habitual diet of each participant. [b] There were no significant changes in measured total, LDL and HDL cholesterol based on standard clinical blood measurements when using a two-tailed student's t-test with equal variance.

3.3. Biomarkers of Contrasting Diets and Correlation with Diet Records.

Complementary statistical methods that take advantage of the repeated-measures study design were used to classify metabolites responses to contrasting dietary patterns. A paired orthogonal partial least-squares–discriminant analysis (OPLS-DA) model (Figure 3) was used to rank metabolites in plasma and urine that were modulated by assigned diets relative to each participant's baseline habitual diet (*i.e.,* ion response ratio). Both OPLS-DA models demonstrated good accuracy ($R^2 > 0.840$) with adequate robustness ($Q^2 > 0.200$) after permutation testing ($p < 0.05$, n=1000). S-plots confirmed that ProBet and Me-His were consistently elevated following a Prudent diet (W-P) in both plasma and urine samples, whereas total plasma C14:0 and C18:2n-6*cis* had the

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most significant increase following an assigned Western diet (P-W). Additionally, top-ranked creatinine-normalized urinary metabolites excreted at higher levels following a Prudent diet included ImPA, OH-PCA, dihydroxybenzoic acid (DHBA), enterolactone glucuronide (Ent-G), nitrate and an unknown cation (*m*/*z* 217.156, MH^+) tentatively identified as a dipeptide, valinyl-valine (Val-Val), whereas ASK was only modestly increased ($p = 0.0686$) following a Western diet. Additionally, excellent discrimination among DIGEST participants following a Prudent or Western

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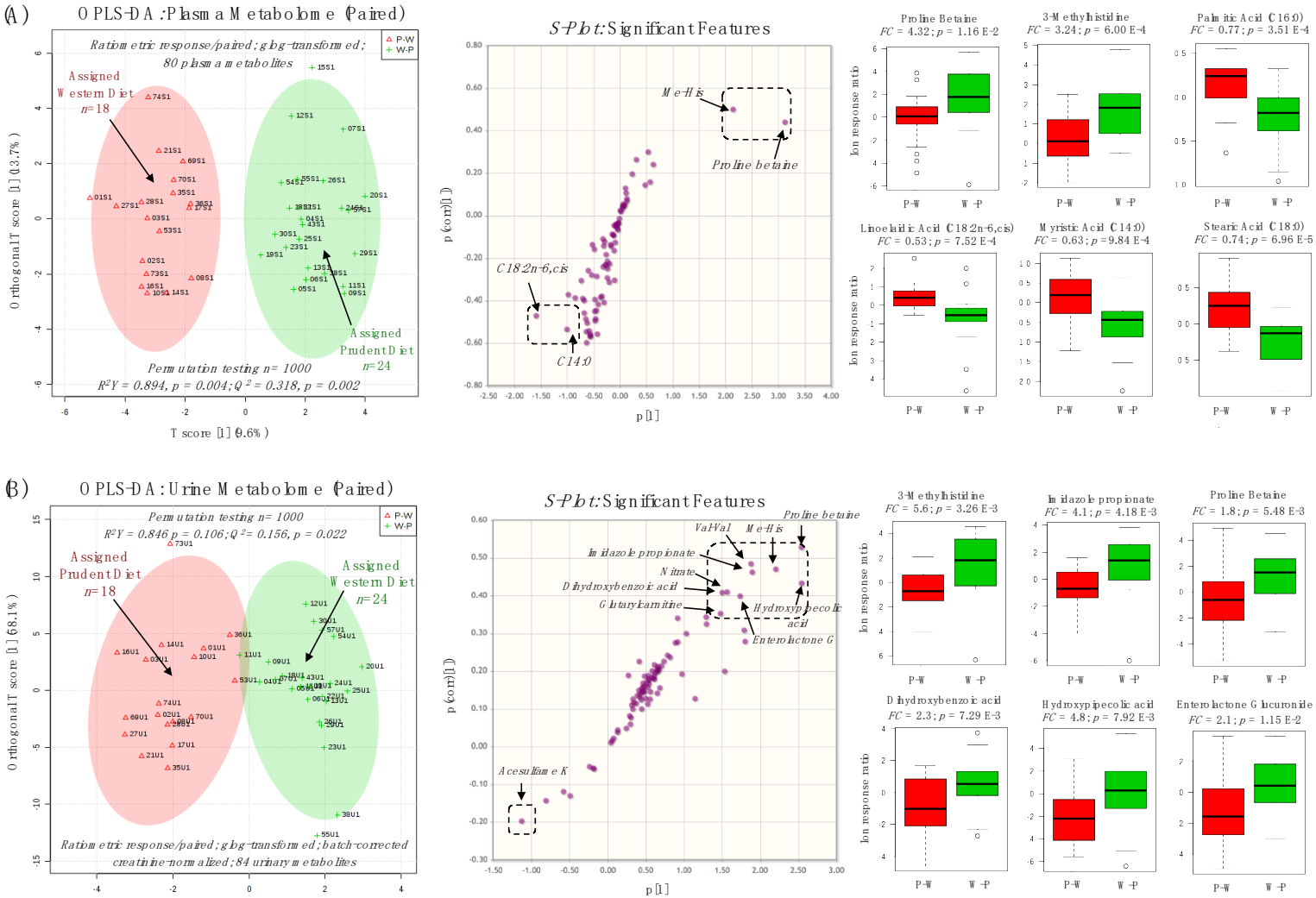

Figure 3. Paired supervised multivariate data analysis of (A) plasma and (B) creatinine-normalized urine metabolomic data based on orthogonal partial least-squares-discriminant analysis (OPLS-DA) using the ratio of ion responses or concentrations for metabolites following 2 weeks of food provisions to their baseline habitual diet values. 2D scores plot highlight differences in the overall metabolic phenotype from matching biofluids collected from DIGEST participants assigned to a Prudent (W-P) as compared to a Western (P-W) diet based on a sub-set of metabolites identified from S-plots, as well as univariate statistical analysis as shown in box-whisker plots for top-ranked metabolites significantly different between the treatment arms ($p < 0.05$).

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diet was achieved when using top-ranked single or ratiometric biomarkers from a receiver operating characteristic (ROC) curve (*AUC* > 0.820; p < 1.0 E-5) for plasma and creatinine-normalized urine samples (Figure S10). For instance, plasma ProBet and the ratio of Me-His/C18:3n-6*trans* demonstrated good sensitivity and specificity (≈ 80-90%) for differentiating DIGEST participants based on their assigned diets similar to urinary OH-PCA and the ratio of OH-PCA/Na. A multivariate empirical Bayes analysis of variance (MEBA) [35] was also used to characterize time-dependent metabolite profiles related to contrasting diets after two weeks of food provisions. In this case, metabolic trajectories with distinctive time-course profiles following a Prudent or Western diet were ranked based on their Hotelling's T^2 distribution as shown for plasma (Figure S11) and urine (Figure S12), which were consistent with metabolites identified as dietary biomarkers from volcano plots, ROC curves and OPLS-DA models.

A mixed ANOVA model, and a partial Pearson correlation analysis to self-reported diet records after adjustment for sex, age and *BMI* were next applied to further validate the relevance of dietary biomarkers identified from multivariate statistical models. Table 2 highlights that ProBet and Me-His were the most robust plasma metabolites associated with a Prudent diet that satisfied several statistical parameters (T^2, F-value, r, adjusted p-value). For instance, ProBet was positively associated ($r \approx 0.520$, $p = 0.001$) with self-reported intake of fruit (cup eq./2000 kcal), vitamin C (mg/2000 kcal) and fruit & vegetable servings (servings/2000 kcal), as well as negatively associated with total fat intake ($r > -0.530$, $p < 0.001$), including *trans* and saturated fat (% energy). Me-His had strong positive correlations (r = 0.530-0.570, $p < 0.001$) with protein (%energy), insoluble fiber (g/2000 kcal), electrolytes (Mg, K; mg/2000 kcal), as well as fruit, and fruit & vegetable intake reflecting a Prudent diet. Other plasma metabolites classified as dietary biomarkers of contrasting diets in this study included two carnitines (*e.g.*, carnitine, C0; deoxycarnitine, dC0), two amino acids (*e.g.*, proline, Pro; alanine, Ala), three ketone bodies/intermediates (*e.g.*, ketoleucine; kLeu; ketovaline, kVal; 3-hydroxybutyric acid, OH-BA), and several long-chain fatty acids (*e.g.*, C14:0, C15:0, C18:2n-6*trans*, C18:3n-6*cis*, C18:2n-6*cis*).

Overall, all total hydrolyzed fatty acids were positively correlated to a Western diet with a higher average intake of fats (*trans* fats, saturated fats) and a corresponding lower intake of fruit & vegetable, poly:sat and micronutrients (vitamins A, C and E). Similar outcomes were also measured for plasma carnitines and amino acids, which were positively correlated to a Western diet. In contrast, metabolic intermediates of branched-chain amino acids and energy metabolism, namely plasma kLeu, kVal and OH-BA, were positively associated with a Prudent diet, including higher average intake of protein, fiber, fruit & veggie, poly:sat, and vitamins. Table 2 summarizes 14 plasma metabolites that function as robust biomarkers of contrasting diets since they satisfied at least two of the three statistical models ($p < 0.05$) following adjustment for covariates between groups while also having a significant correlation ($r > \pm 0.3$, $p < 0.05$) with at least two nutrient categories from self-reported diet records. An analogous strategy was also used to identify 8 creatinine-normalized urinary metabolites significantly associated with contrasting diets (Table 3). Urinary Me-His and ProBet were among the top-ranked metabolites sensitive to short-term changes in habitual diet with strong positive associations with healthful eating patterns indicative of a Prudent diet. Additionally, several other urinary metabolites were also associated with a Prudent diet, including OH-PCA and ImPA. Furthermore, two plant-derived phenolic metabolites in urine, namely Ent-G and DHBA were also correlated to healthy eating patterns with a greater intake of fruit and/or vegetable and micronutrients, and a lower intake of total fat. However, creatinine-normalized Val-Val and DMG in urine were weakly correlated with only 2 nutrient categories ($p \approx 0.05$) from self-reported diet records. Interestingly, urinary ASK, nitrate and an unidentified cation (m/z:RMT, 276.144:0.858, MH^+) were not correlated to any major nutrient category despite showing treatment responses to contrasting diets.

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Table 2. Top-ranked plasma metabolites associated with a 2 week Prudent or Western provisional diet on healthy participants (n=42) when using time series MEBA, mixed ANOVA and a partial correlation analysis.

Metabolite/ID	Identifier/MSI	T^{2a}	*F*-value[b]	*p*-value[b]	*r*[c]	*p*-value[c]	Food record[d]
Proline betaine (ProBet) HMDB04827	144.102:0.984 (+) MSI-CE-MS $C_7H_{13}NO_2$ Level 1	24.6	8.7	0.007	-0.601 -0.544 -0.528 0.528 0.518	< 0.001 < 0.001 0.001 0.001 0.001	Change %fat *trans* fat %energy Sat fat %energy Fruit/Vitamin C Fruit & Veggie
3-Methylhistidine (MeHis) HMDB00479	170.092:0.664 (+) MSI-CE-MS $C_7H_{11}N_2O_3$ Level 1	24.9	14.0	0.001	0.573 0.561 0.553 0.546 0.534	< 0.001 < 0.001 < 0.001 < 0.001 0.001	Magnesium Protein %energy Insoluble Fiber Potassium Fiber/Fruit & Veggie
Proline (Pro) HMDB00162	116.070:0.927 (+) MSI-CE-MS $C_5H_9NO_2$ Level 1	14.6	5.9	0.020	0.495 -0.412 -0.378 -0.373 -0.362	0.002 0.010 0.019 0.021 0.026	*trans* fat %energy Fruit & Veggie (serv) Veggie Fruit & Veggie Fruit
Carnitine (C0) HMDB00062	162.112:0.735 (+) MSI-CE-MS $C_7H_{15}NO_3$ Level 1	12.2	8.9	0.005	-0.464 0.426 -0.404 -0.386 -0.368	0.003 0.008 0.012 0.017 0.023	Poly:Sat *trans* fat %energy Fruit & Veggie Vitamin E Vitamin C
Deoxycarnitine or γ-Butyrobetaine (dC0) HMDB01161	146.128:0.700 (+) MSI-CE-MS $C_7H_{16}NO_2$ Level 2	11.9	7.9	0.008	0.367 0.366 -0.352 0.340 -0.336	0.024 0.024 0.030 0.037 0.039	Change %fat Cholesterol Magnesium Sodium Poly:Sat
Linoelaidic acid (C18:2n-6 *trans*) HMDB06270	294/67.1:15.289 GC-MS $C_{18}H_{32}O_2$ Level 2	10.3	21.5	< 0.001	-0.579 -0.555 -0.486 0.485 0.464	< 0.001 < 0.001 0.002 0.002 0.003	Poly:Sat Fruit & Veggie/Vit. E Vitamin C Sat fat %energy *trans* fat %energy
Pentadecanoic acid (C15:0) HMDB000673	294/67.1:14.171 GC-MS $C_{18}H_{32}O_2$ Level 2	9.9	16.8	< 0.001	-0.471 0.408 -0.403 -0.379 0.379	0.003 0.011 0.012 0.019 0.019	Poly:Sat Change %fat Fruit & Veggie Vitamin A Change %sat fat
Alanine (Ala) HMDB00161	90.056:0.783 (+) MSI-CE-MS $C_3H_7NO_2$ Level 1	9.6	6.2	0.018	0.452 0.439 0.428 -0.395 0.386	0.004 0.006 0.007 0.014 0.017	Change %sat fat Change %fat *trans* fat %energy Protein %energy Sat fat %energy
Ketoleucine or 4-Methyl-2-oxopentanoic acid (kLeu) HMDB00695	129.056:1.209 (-) MSI-CE-MS $C_6H_{10}O_3$ Level 2	7.7	4.4	0.043	0.493 -0.459 0.456 0.453 0.452	0.002 0.004 0.004 0.004 0.004	Fruit & Veggie Sat fat %energy Fruit Poly:Sat Protein %energy/ Vitamin C/E
3-Hydroxybutyric acid (OH-BA) HMDB00357	103.040:1.043 (-) MSI-CE-MS $C_4H_8O_3$ Level 1	7.6	2.9	0.097	0.437 -0.429 0.425 0.419 0.415	0.006 0.007 0.008 0.009 0.01	Fruit Sat/trans fat %energy Poly:Sat Vit A Fruit & Veggie
α-Linoleic acid (C18:3n-6 *cis*) HMDB001388	292/79.1:15.096 GC-MS $C_{18}H_{30}O_2$ Level 2	7.0	11.6	0.002	-0.441 -0.397 -0.391 0.391 -0.387	0.006 0.013 0.015 0.015 0.016	Poly:Sat Vitamin A Fruit & Veggie *trans* fat %energy Vitamin E
Ketovaline or α-Isovaleric acid (kVal)	115.040:1.079 (-) MSI-CE-MS	6.3	2.4	0.125	0.489 0.472	0.002 0.003	Protein %energy Fiber (kcal)

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HMDB00019	$C_5H_8O_3$ Level 2				0.466 0.458 0.451	0.003 0.004 0.004	Fruit & Veggie Vitamin E Poly:Sat
Myristic acid (14:0) HMDB00826	242/74.1:10.336 GC-MS $C_{15}H_{30}O_2$ Level 1	5.0	15.2	< 0.001	-0.535 -0.512 0.503 0.465 -0.463	0.001 0.001 0.001 0.003 0.009	Poly:Sat Fruit & Veggie Change %fat Change %sat. fat Vitamin A
Linoleic acid (C18:2n-6 *cis*) HMDB000673	294/67.1:14.171 GC-MS $C_{18}H_{32}O_2$ Level 2	2.6	16.4	< 0.001	-0.438 0.420 0.412 -0.382 -0.370	0.006 0.009 0.005 0.018 0.022	Poly:Sat Change %fat Change %sat. fat Fruit & Veggie Vitamin A

[a] Hotelling's T-squared distribution using MEBA on glog-transformed metabolomic time series data. [b] Mixed ANOVA model derived from within-subject (diet x time interaction, $p < 0.05$) contrasts when adjusted for sex, age and BMI. [c] Partial Pearson correlation of urinary metabolites to food records with listwise deletion when adjusted for sex, age and BMI, where $r > \pm 0.30$ and $p < 0.05$. [d] Top-five categories from food records significantly correlated to urinary metabolites following provisional diets.

3.4. Metabolic Trajectories and Metabolite Correlation Analysis.

Representative metabolic trajectories are depicted for top-ranked biomarkers of contrasting diets that were measured in plasma (Figure S12) and urine specimens (Figure S13). In all cases, metabolic phenotype changes were evident following 2 weeks of food provisions with the exception of urinary DHBA, which was the only compound different between assigned diet groups at baseline (p = 8.03 E-3). The majority of dietary biomarkers underwent an increase in response for participants following a Prudent diet except for circulating fatty acids, two amino acids (Pro, Ala) and two carnitines (C0, dC0) in plasma, which increased following a Western diet. Metabolic trajectory plots also highlight considerable between-subject variances to assigned diets while also identifying outliers due to potential dietary non-adherence and/or inaccurate self-reporting. Figure 4 illustrates four metabolic trajectory plots for ProBet and Me-His as they were among the most sensitive biomarkers responsive to contrasting diets measured consistently in both plasma and urine samples. Also, scatter plots show the quantitative relationship between Me-His and ProBet concentrations in plasma as compared to their excreted concentrations in urine with self-reported average intake of protein (% energy) and fruit servings (servings/2000 kcal) over 2 weeks, respectively. For example, there was a 2.4-fold increase in mean plasma Me-His concentration following 2 weeks of food provisions that corresponded to a 28% greater intake of dietary protein when comparing Prudent (W-P, n=24) and Western (P-W, n=18) diet groups. Similar results were also evident when comparing creatinine-normalized concentrations of Me-His in urine, which generated a 4.8-fold higher mean concentration in Prudent relative to Western diet treatment arm. Overall, there was a strong correlation between Me-His concentrations and self-reported dietary protein intake (r = 0.430 to 0.560) with few exceptions, such as one participant (W-P, #19) who had consistently low Me-His concentrations in both biofluids consistent with self-reported protein intake that was characteristic of the Western diet group (P-W) indicative on dietary non-adherence. In contrast, a second participant (P-W, #28) had higher than average Me-His concentrations in both plasma and urine samples despite their low self-reported protein intake from diet records suggestive of diet record bias.

Figure 4 also depicts metabolic trajectories for plasma and urinary ProBet concentrations after 2 weeks of food provisions as compared to their baseline habitual diet along with scatter plots depicting their correlation (r = 0.430 to 0.530) to daily fruit servings. Similar to Me-His, the same participant (W-P, #19) had lower ProBet concentrations in both plasma and urine with diet records reflecting a Western diet low in fresh fruit intake despite being assigned a Prudent diet. Additionally, 3 participants had higher than expected ProBet concentrations in circulation (P-W, #28, 35, 36) inconsistent with self-reported diet records; interestingly, ProBet concentrations for these same participants were far less elevated in urine likely due to differences in the detection time

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Table 3. Top-ranked creatinine-normalized metabolites associated with a 2 week Prudent or Western provisional diet on healthy participants (n=42) when using time series MEBA, mixed ANOVA and a partial correlation analysis.

Metabolite/ID	Identifier/MSI	T^{2a}	*F*-test[b]	*p*-value[b]	*r*[c]	*p*-value[c]	Food record[c]
3-Methylhistidine (MeHis) HMDB00479	170.092:0.664 (+) MSI-CE-MS $C_7H_{11}N_2O_3$ Level 1	17.9	7.8	0.008	0.524 0.517 0.457 -0.432 0.431	0.001 0.001 0.004 0.007 0.007	Fiber (kcal) Fruit & Veggie Vitamin E trans fat %energy Protein %energy
5-Hydroxypipecolic acid (OH-PCA)* HMDB0029246	146.081:1.180 (+) MSI-CE-MS $C_6H_{11}NO_3$ Level 2	16.3	1.1	0.293	-0.468 0.397 0.390 0.381 0.374	0.003 0.013 0.016 0.018 0.021	Change fat Fiber (kcal) Fruit &Veggie Vitamin E Poly:Sat
Imidazole propionic acid (ImPA) HMDB02271	141.066:0.690 (+) MSI-CE-MS $C_6H_8N_2O_2$ Level 2	16.1	10.8	0.002	0.515 0.511 0.471 0.463 0.444	0.001 0.001 0.003 0.003 0.005	Fiber (kcal) Fruit &Veggie Protein %energy Vitamin E Poly:Sat
Proline betaine (ProBet) HMDB04827	144.099:0.984 (+) MSI-CE-MS $C_7H_{13}NO_2$ Level 1	15.5	10.8	0.002	0.487 -0.487 0.482 0.480 0.469	0.002 0.002 0.002 0.002 0.003	Poly:Sat trans fat %energy Fiber (kcal) Fruit &Veggie/Vit. E Fiber (insoluble)
Valinyl-valine (Val-Val) HMDB0029140	217.156:0.847 (+) MSI-CE-MS $C_{10}H_{20}N_2O_3$ Level 3	10.9	3.8	0.060	0.320 0.320	0.050 0.050	Poly:Sat Vitamin E
Enterolactone glucuronide (ETL-G) HMDB ---	473.145:0.934 (-) MSI-CE-MS $C_{24}H_{25}O_{10}$ Level 2	8.0	7.3	0.010	-0.434 0.387 0.340 0.332 0.316	0.006 0.016 0.037 0.042 0.054	Fat (kcal) Vitamin C Fruit (cup eq.) Fruit &Veggie Veggie (cup eq.)
Dihydroxybenzoic acid (DHBA) or protocatachuic acid* HMDB0001856	153.019:1.576 (-) MSI-CE-MS $C_7H_6O_4$ Level 2	7.9	10.3	0.003	-0.403 0.383 0.355 0.324 0.310	0.012 0.018 0.029 0.047 0.058	Fat (kcal) Sugar %energy Vitamin C Veggie (cup eq.) Fruit &Veggie
Dimethylglycine (DMG) HMDB0000092	104.108:0.569 (+) MSI-CE-MS $C_4H_9NO_2$ Level 1	2.9	3.6	0.065	0.356 0.322	0.028 0.049	Fruit &Veggie (serv.) Fiber (kcal)

[a] Hotelling's T-squared distribution using MEBA on glog-transformed metabolomic time series data. [b] Mixed ANOVA model derived from within-subject (diet x time interaction, $p < 0.05$) contrasts when adjusted for sex, age and BMI. [c] Partial Pearson correlation of urinary metabolites to food records with listwise deletion when adjusted for sex, age and BMI, where $r > \pm 0.30$ and $p < 0.05$. [d] Top-five categories from food records significantly correlated to urinary metabolites following provisional diet.

window when analyzing these complementary biofluids for exogenous dietary biomarkers of recent food intake, such as ProBet. Overall, there was a strong positive correlation between Me-His (r = 0.638) and ProBet (r = 0.547) concentrations measured from matching plasma and urine samples (Figure S13) collected at baseline and following assigned diets (n=84). Additionally, 2D heat maps and correlation matrices for top-ranked plasma (14) and urinary (11) metabolites provide insights into their underlying biochemical relationships (Figure S14). As expected, urinary imidazole metabolites derived from histidine, Me-His and ImPA (r = 0.956), plasma saturated fatty acids, C14:0 and C15:0 (r = 0.873), plasma branched-chain amino acid intermediates, kLeu and kVal (r = 0.705), as well as plant-derived phenol metabolites in urine, DHBA and Ent-G (r = 0.662) were among a group of highly co-linear metabolites correlated to similar nutrient categories from diet records (Table 2; Table 3).

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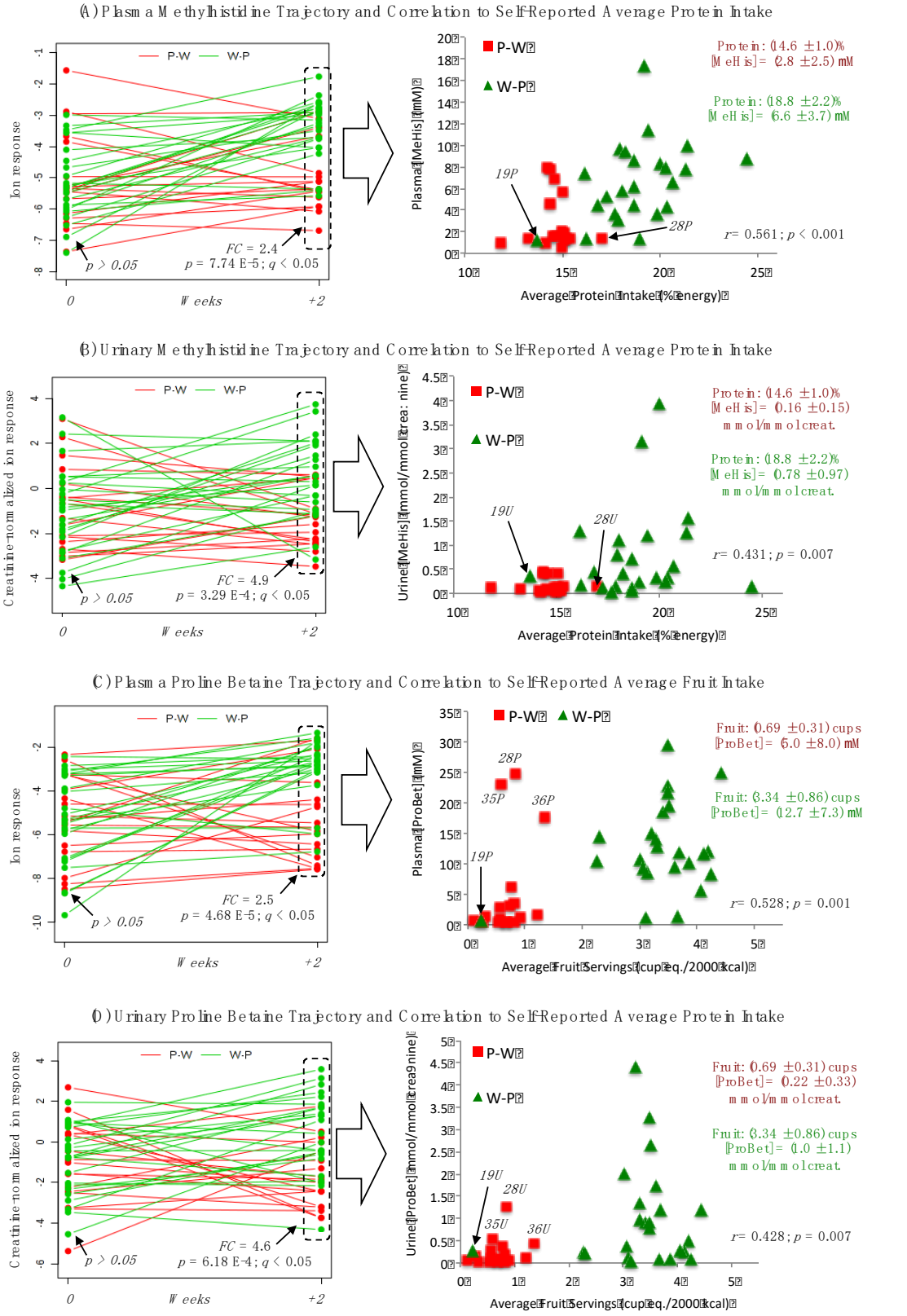

Figure 4. Metabolic trajectories for two distinctive biomarkers measured consistently in both plasma and urine specimens that increase significantly following a provisional Prudent diet (W-P) as compared to an assigned Western diet (P-W), namely Me-His and ProBet. Both metabolites were not different at baseline, but undergo notably changes after two weeks of food provisions ($q < 0.05$, FDR) with concentrations moderately correlated ($r > 0.400$) to self-reported diet involving health-promoting foods from a Prudent diet. Overall, good dietary adherence was demonstrated for the majority of DIGEST participants with few exceptions (labeled on plots) who had metabolite phenotypes inconsistent with their assigned diet group.

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4. DISCUSSION

4.1. Contrasting Diets from Food Provisions.

Accurate assessment tools of complex dietary patterns are needed to promote human health since sub-optimal diet is responsible for about 20% of preventable deaths from non-communicable diseases worldwide [36]. However, few validated biomarkers exist for routine monitoring of habitual diet [37], such as omega-3 fatty acids [38] and water insoluble fiber [39]. In this work, a panel of metabolites from plasma and urine was demonstrated to respond to short-term dietary changes when applying a cross-platform metabolomics approach with stringent QC (Figure 1; Figure 2) and a rigorous data workflow for metabolite authentication (Figure S2; Figure S3) [28-30]. Since all DIGEST participants had poor Prudent diet eating habits at baseline (Table 1), we hypothesized that assigning a Prudent diet (W-P) from food provisions would likely induce a more pronounced metabolic phenotype change than a Western diet (P-W); indeed, several top-ranked metabolites ($q < 0.05$, FDR) measured in plasma and urine were largely positively associated with a Prudent diet as shown in volcano plots (Figure S5). Unlike controlled feeding studies within a laboratory setting, DIGEST participants were provided cooking suggestions with meal plans by a dietician that still allowed for flexibility in food preparations [25]. In this study, short-term dietary changes were found to impact the intake of 20 specific nutrient categories from self-reported diet records (Table 2; Table S1; Figure S4). For instance, a Prudent diet was consistent with a higher consumption of dietary fiber, fruit and/or vegetables, electrolytes and vitamins, but with lower intake of dietary fat, sodium and cholesterol in contrast to a Western diet. To the best of our knowledge, this is the first metabolomics study to investigate the impact of contrasting diets using food provisions. As dietary adherence, potential misreporting and variations in food preparations represent uncontrolled variables in this study, we aimed to identify metabolites from plasma and urine that can serve as robust biomarkers of habitual diet applicable to a free-living population.

4.2. Robust Biomarkers of a Prudent Diet Measured in Both Plasma and Urine.

ProBet (Figure 2) and Me-His (Figure S6) were among the most significant metabolites ($q < 0.05$, FDR) associated with a Prudent diet, an eating pattern that promotes good health while contributing to chronic disease prevention [40,41]. In this case, ProBet and Me-His displayed opposing metabolic trajectories in both plasma and urine after 2 weeks of an assigned Prudent or Western diet with no differences measured at baseline (Figure 3). This was a consistent outcome from univariate and multivariate (Figure S5; Figure S10) statistical methods after adjustments for covariates (sex, age, *BMI*), including mixed ANOVA and correlation models (Table 2; Table 3). Indeed, plasma ProBet or the ratio of Me-His/C18:3n-6*cis* provided good discrimination ($AUC \approx 0.82$ to 0.87, $p < 3.0$ E-5) of contrasting diets (Figure S10). Additionally, ProBet and Me-His concentrations in plasma and urine were positively associated ($r \approx 0.40$-0.60, $p < 0.001$) with eating patterns reflecting a Prudent diet, including a higher intake of fiber, fruit, fruit & vegetable, protein and vitamins/electrolytes, with a lower consumption of *trans* or saturated fats as compared to a Western diet. In fact, ProBet is an exogenous biomarker specific to citrus fruit that has been validated in well-controlled feeding studies [42] since it is not prevalent in most other foods [43]. In fact, ProBet has been replicated in large-scale observational studies as a robust dietary biomarker ($r \approx 0.40$) of recent citrus fruit/juice intake when compared to standardized FFQs, which can be measured in either blood or urine specimens [18].

Me-His has long been reported as an index of myofibrillar muscle protein turn-over under fasting conditions [44], whereas it also can serve as a biomarker of recent meat consumption (*e.g.*, chicken) with lower plasma concentrations measured in vegetarians as compared to omnivores [45]. Consequently, fasting plasma and creatinine-normalized urinary concentrations of ProBet and Me-His were associated with average fruit (servings/2000 kcal) and protein (%energy) as the most likely primary food sources (Figure 4), which also confirmed excellent dietary adherence with few exceptions. For instance, one participant following a Prudent diet (#19, W-P) had consistently lower than expected concentrations of ProBet and Me-His in both plasma and urine samples, which

correctly corresponded to their self-reported diet records. In contrast, three participants following a Western diet (#25, 36, 38, P-W) were found to have higher than expected plasma ProBet when compared to their diet record, but this trend was less apparent in their matching urine samples. These observations are likely due to incidental intake of fruit juice or citrus beverages not included with food provisions that also highlights the different detection windows for dietary biomarkers when relying on "single-spot" plasma or random urine samples [16]. For instance, ingestion of ProBet or orange juice results in a peak concentration in circulation (< 1-2 h) that reflects more recent intake as compared to its later excretion in urine (< 2-24 h) [46]. Nevertheless, there was a strong linear correlation between circulating and excretory concentrations of ProBet (r = 0.638) and Me-His (r = 0.547) measured in matching plasma and urine samples collected in this study (Figure S13).

4.3. Novel Biomarkers Identified Following a Prudent Diet.

Two urinary metabolites were also identified by MS/MS (level 2, Figure S7) as sensitive dietary biomarkers (q < 0.05, FDR) reflecting a Prudent diet, namely OH-PCA and ImPA (Table 3). Other potential isomeric/isobaric candidates for these metabolites were ruled out by comparing their MS/MS spectra with those predicted *in silico* using CFM-ID [33] in the absence of authentic standards for more confident identification (level 1). Their metabolic trajectories (Figure S12) displayed a notable increase (FC ≈ 4 to 6, p < 0.001) in excretion following a Prudent diet with no differences measured at baseline similar to trends observed for urinary ProBet and Me-His excretion. ImPA is a normal constituent of human urine derived from the metabolism of histidine [47], which has recently been identified as a product of gut microbiota activity that also regulates insulin sensitivity [48]. This highlights the fact that many dietary biomarkers are not only dependent on habitual dietary intake and host (liver) metabolism, but are also co-metabolized by gut microbiota with poorly understood effects on human health. Urinary excretion of ImPA was significantly correlated with fiber, fruit & vegetable and protein intake (r ≈ 0.50, p ≈ 0.001), which comprise eating patterns consistent with a Prudent diet [40]. Similarly, urinary OH-PCA was found to have a moderate correlation with fiber and fruit & vegetable intake, and inversely related to total fat. This data indicates that higher excretion of OH-PCA in urine is likely derived from intake of leguminous plants [49] and citrus fruits [50] when following a Prudent diet, but represents an endogenous lysine metabolite [51] also produced by gut microbiota [52]. Indeed, urinary OH-PCA or its ratio to sodium (OH-PCA/Na) discriminated between DIGEST participants from two diet treatment arms (Figure S10) with good accuracy (AUC ≈ 0.83 to 0.88, p < 3.0 E-4), as well as sensitivity and specificity (≈ 90%).

Two other metabolites derived from edible plant sources were also identified by MS/MS (Figure S8) since they were elevated in urine (FC ≈ 2.5 to 3.8) following a Prudent diet as shown by their urinary metabolic trajectory plots (Figure S12), namely Ent-G and DHBA. In the case for Ent-G, a MS/MS spectral match based on three characteristic product ions, including a neutral loss of a glucuronide is in close agreement with published data [53]. These urinary metabolites were consistently associated (r ≈ 0.30-0.40, p < 0.05) with fruit, vegetable, vitamin C, and/or total sugar intake, and inversely correlated to total fat (Table 3). Ent-G is a major phytoestrogen from dietary plant lignins, and is excreted in urine as its monoglucuronide conjugate following biotransformation by human intestinal bacteria [54]. Even in controlled feeding studies, there is considerable between-subject variation in urinary excretion of enterolignin metabolites due to complex interactions with liver and colonic environments [55], which has been reported to possess putative anticancer, antioxidant and/or estrogenic activity [56]. Also, DHBA is a major phenolic acid constituent from most cereals (*e.g.*, wheat, rye) [57], which can serve as a biomarker of dietary fiber intake allowing for differentiation of contrasting low and high (> 48 g/day) fiber diets [58]. In fact, urinary DHBA was the only biomarker differentially excreted at baseline that reflected modest differences in fiber intake between assigned DIGEST participant groups (Figure S5). Urinary Val-Val and DMG were also biomarkers related to a Prudent diet, but had weak correlations with only two nutrients (Table 3), whereas the artificial/low calorie sweetener ASK, and inorganic nitrate were

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not associated with any nutrient categories from self-reported diet records (Figure 3). ASK was elevated following a Western diet, but was rather sporadic with frequent missing data (*i.e.,* below detection limit) since it reflects recent intake of certain sugar-sweetened beverages [59]. In contrast, nitrate exposure has been reported to be mainly from vegetable consumption due to agricultural fertilizer usage [60] that is consistent with its increase in urine following a Prudent diet.

The major circulating ketone body, OH-BA and two branched-chain amino acid intermediates, kVal and kLeu also increased in plasma following a Prudent diet as compared to a Western diet (Table 2) as shown by their metabolic trajectories (Figure S11). Increases in OH-BA from the liver during ketosis occurs during prolonged fasting or following strenuous exercise [30], as well as abrupt changes in habitual diet, such as adopting a low glycemic index or very low carbohydrate diet [19]. In our work, plasma OH-BA was moderately correlated ($r \approx 0.42$, $p < 0.01$) to increases in fruit, fruit & vegetable and poly:sat consumption and inversely associated with saturated and *trans* fat intake. Since a Prudent diet is characterized by greater consumption of fiber-rich foods with a lower glycemic index, this may contribute to a mild ketogenic physiological state unlike a Western diet that include regular consumption of processed foods high in salt and added refined sugar yet low in dietary fiber [40,41]. Indeed, a Prudent diet composed of whole foods elicits fewer adverse health effects with better adherence than highly restrictive ketogenic diets, which is effective in regulating insulin sensitivity in type 2 diabetes and pre-diabetic patients [61]. Plasma kVal and kLeu were also positively correlated ($r \approx 0.45$-0.50, $p < 0.004$) with key nutrient categories associated with a Prudent diet, including higher intake of protein, fruit and/or vegetable, poly:sat and vitamins. Both plasma metabolites are generated by extra-hepatic branched-chain amino acid transferases prior to oxidative decarboxylation and subsequent utilization as energy substrates within muscle tissue [62]. The metabolism of branched-chain amino acids plays other critical roles in human health, including ammonia detoxification, protein biosynthesis and insulin sensitivity [63] while serving as predictive biomarkers of type 2 diabetes [64]. A correlation matrix/heat map (Figure S14) confirms that plasma kLeu and kVal were strongly co-linear ($r \approx 0.70$, $p = 6.8$ E-14) while also being closely associated with OH-BA ($r \approx 0.48$, $p = 3.7$ E-6) reflecting common dietary patterns that influence their circulating concentrations. Also, urinary Me-His and ImPA ($r \approx 0.96$, $p <$ 1.0 E-15), as well as plasma C14:0 and C15:0 ($r \approx 0.87$, $p = 8.4$ E-15) were among the most strongly correlated metabolites that originate from consumption of foods rich in dietary histidine and saturated fats, respectively.

4.4. Novel Biomarkers Identified Following a Western Diet.

Unlike branched-chain amino acid intermediates, two circulating amino acids, Ala and Pro were associated with greater intake of dietary fats (saturated, *trans*, total), and inversely correlated to a Prudent diet due to lower intake of fruit, vegetable or protein (Table 2). As a result, their plasma metabolic trajectories increased when DIGEST participants were assigned a Western diet for 2 weeks (Figure S11). Fasting amino acid concentrations reflect long-term habitual diet rather than recent dietary intake, where Ala has been reported to be inversely associated to plant-based protein diets [65]. This is consistent with outcomes in our study, since plasma Ala was negatively correlated to average protein intake ($r \approx -0.40$, $p = 0.014$). Similar to Ala, plasma Pro was reported to be inversely associated with a Prudent diet as measured in a cross-sectional observational study that was adjusted for age, sex and *BMI* [66]. This was consistent with our findings since plasma Pro was inversely related to healthy eating patterns, such as lower intake of fruits and/or vegetables and higher consumption of processed foods with *trans* fats (Table 2). As expected, plasma Pro was correlated ($r \approx 0.46$-0.49, $p <$ 1.0 E-5) with circulating levels of Ala, as well as C0 indicative of a Western diet (Figure S14). Similar outcomes were also measured for two carnitine metabolites (C0 and dC0) since they had metabolic trajectories that increased for DIGEST participants following a Western diet, which were correlated with greater intake of dietary fat, sodium or cholesterol (Table 2). Although *de novo* synthesis of C0 is derived from dC0 via lysine metabolism, red meat represents a major dietary source of C0 that is also metabolized by gut microbiota with subsequent host hepatic conversion to generate the thrombosis-promoting metabolite, *N*-trimethylamine oxide

(TMAO) [67]; however, plasma or urinary TMAO were not modulated by a short-term, contrasting diets in our study. In fact, recent studies have shown that anaerobic gut microbiota species can also generate TMAO via its atherogenic intermediate, dC0 due to chronic C0 exposure from the diet [68]. Nevertheless, C0 is still widely promoted as a nutritional supplement and ergogenic aide to improve fatty acid energy metabolism, as well as alleviate muscle injury from strenuous exercise [69]. Lastly, a series of plasma total (hydrolyzed) fatty acids had metabolic trajectories that increased when following a Western diet, which were directly associated with greater intake of total, saturated and *trans* fats, but lower consumption of poly:sat, vitamins and fruits & vegetables (Table 2); these included a low abundance circulating *trans* fatty acid, C18:2n-6*trans*, as well as saturated fats (C14:0, C15:0), and omega-6 fatty acids, namely C18:3n-6*cis* and C18:2n-6*cis*. Indeed, high intake of omega-6 [70] and saturated [71] fatty acids has long been associated with a Western diet that increases systemic inflammation and chronic disease risk. Nevertheless, there remains on-going controversy regarding the optimal dietary fat composition needed to promote cardiometabolic health [72]. Recent clinical trials and observational studies have demonstrated that circulating C14:0, C17:0 and notably C15:0 represent dietary biomarkers of dairy fat intake whose impact on cardiometabolic health may likely be beneficial [73]. In contrast, greater consumption of processed foods containing vegetable oils rich in C18:2n-6*cis* and other omega-6 fatty acids is hypothesized to be a major dietary culprit for cardiovascular disease prevalence in developed countries [74]. Public health policies have been far more effective in the past decade to reduce dietary *trans* fat intake to less than 1% energy based on WHO recommendations with animal meats/dairy now being more significant than industrial sources from partial hydrogenation of vegetable oils [75]. These trends are consistent with data measured in this study, as fasting plasma concentrations of C18:2n-6*trans* were about 0.34% of its stereoisomer and most abundant fatty acid in circulation, C18:2n-6*cis* (Figure S9).

5. Conclusions

In summary, a panel of dietary biomarkers that reflect contrasting Prudent and Western diets were identified based on their distinctive metabolic trajectories measured in matching plasma and urine samples using a cross-platform metabolomics strategy. All DIGEST participants were provided whole foods for consumption over a two week period while maintaining normal lifestyle habits with no significant changes in their caloric intake, BMI, blood pressure, as well as standard lipid or inflammatory biomarkers as compared to baseline. Me-His and ProBet were the most significant dietary biomarkers associated with a Prudent diet consistently measured in both plasma and urine. Also, urinary ImPA, OH-PCA, Ent-G and DHBA, as well as fasting plasma OH-BA, kVal and kLeu were also positively associated with a Prudent diet. These dietary biomarkers reflect greater consumption of health-promoting foods containing insoluble fiber, protein, essential nutrients and bioactive phytochemicals with a low glycemic index as compared to highly processed foods in contemporary Western diets. Also, a series of circulating saturated and polyunsaturated fatty acids, as well as plasma Ala, Pro, C0 and dC0 were classified as dietary biomarkers of a Western diet reflecting greater intake of fats, cholesterol and salt, but having lower overall nutrient and fiber quality. Other urinary biomarkers of contrasting diets including ASK, nitrate, DMG and Val-Val, did not have strong associations with any specific nutrient categories from self-reported food records. Strengths of this study include the use of complementary statistical methods with appropriate adjustments, access to matching biospecimens and food records from participants, and use a validated metabolomics data workflow for biomarker discovery and authentication with stringent QC. However, there were several study limitations, including the short duration of the dietary intervention, as well as modest sample size involving participants recruited from a single centre without strict dietary adherence monitoring. Future studies that include multiple time points for biomonitoring of long-term changes in habitual diet with greater study power are recommended. Also, the integration of metabolomics with fecal microbiome data is needed given the important roles of commensal microbiota in nutrient generation and metabolite biotransformation that varies considerably between participants. Also, certain dietary biomarkers

tentatively identified in this study still require further structural elucidation to confirm their exact stereoisomer configuration. Overall, our work provides strong corroborating evidence of the utility of food exposure biomarkers to accurately differentiate complex dietary patterns that is generalizable to a free-living, healthy population. This is urgently needed for new advances in nutritional epidemiology and chronic disease prevention, including assessing the impact of maternal nutrition on fetal development early in life and metabolic syndrome risk in childhood.

Supplemental Material: The following are available online. Description of experimental and participant metadata (Experimental), baseline characteristics of DIGEST cohort (Table S1), summary of authenticated plasma and urinary metabolites (Table S2), a CONCERT flow diagram illustrating participant selection criteria (Figure S1), overview of three instrumental platforms used for nontargeted and targeted metabolite profiling (Figure S2), 2D scores plots from PCA summarizing metabolomic data quality with control charts (Figure S3), 2D scores plots from PCA of contrasting Prudent and Western diets including key discriminating nutrients from self-reporting dietary records (Figure S4), volcano plots depicting differentiating plasma and urine metabolites at baseline and following assigned Prudent and Western dietary treatment arms (Figure S5), data workflow for identification and quantification of Me-His by MSI-CE-MS with QC (Figure S6), identification of cationic metabolites, OH-PCA, ImPA, and Val-Val by high resolution MS/MS (Figure S7), identification of anionic metabolites, ASK, Ent-G, and DHBA by high resolution MS/MS (Figure S8), identification of plasma fatty acids, C14:0 and C18:2n-6*cis* by GC-MS (Figure S9), ROC curves for top-ranked single and ratiometric plasma and urinary biomarkers of contrasting Prudent and Western diets (Figure S10), trajectory plots for plasma metabolites as biomarkers of contrasting diets for individual DIGEST participants (Figure S11), trajectory plots for creatinine-normalized urinary metabolites as biomarkers of contrasting diets for individual DIGEST participants (Figure S12), linear correlation plots for plasma and creatinine-normalized concentrations of Me-His and ProBet in plasma and urine for DIGEST participants (Figure S13), and 2D heat maps and correlation matrices for top-ranked plasma and urinary metabolites associated with contrasting diets from food provisions (Figure S14).

Author Contributions: S.S.A., M.Z., R.J.S. and D.D. conceived and supervised the study, including participant recruitment, collection of dietary records, and coordination of biospecimen collection. N.W., M.S. and S.A. were involved in non-targeted metabolite profiling of urine and plasma samples by MSI-CE-MS, whereas S.A., J.B., A.M. and R.L. contributed to targeted analysis of total plasma fatty acids and urinary electrolytes, including sample pretreatment and data acquisition. N.W. and P.B.M. were involved in data analysis and wrote the manuscript, including statistical analysis, metabolite identification and data interpretation with S.S.A. and R.J.S. contributing to the final version of the manuscript.

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