1 *Article*

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

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

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

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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]. These deleterious eating patterns increasingly impact cardiometabolic health across the lifespan [6]. In contrast, a Prudent diet and analogous health promoting diets (*e.g.,* DASH, Mediterranean, Nordic etc.) that include greater intake of fruits and vegetables, lean meats, and whole grains reduce blood lipids, improve blood sugar homeostasis, and lower blood pressure [7,8]. However, there is urgent need for more accurate dietary assessment tools for the validation of evidence-based nutritional policies that are effective for chronic disease prevention on a population level unlike the traditional diet-heart hypothesis [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/serum [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 plasma 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 after 2 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 may serve as responsive biomarkers reflecting short-term changes in habitual diet, which were measured in free-living individuals outside of a dedicated metabolic ward or hospital stay. These dietary biomarkers not only confirmed good adherence to assigned food provisions, but were also associated with healthy eating patterns indicative of a Prudent diet [26] as compared to a Western diet that increases overall risk for CVD [27].

94   **2. Experimental**

95   *2.1. Study Design, Participant Eligibility and Dietary Self-reporting.*

96   The Diet and Gene Intervention Study (DIGEST) was a 2-arm, parallel unblinded study to
97   compare the effects of 2 weeks of a Prudent diet as compared with a Western diet on CVD risk
98   factors and gene expression. Healthy participants were recruited using flyers, and self-referral
99   methods from McMaster University, and the surrounding areas. Exclusion criteria were an
100   unwillingness to eat an assigned diet, or serious disease or illness, including cardiometabolic and
101   neurodegenerative disorders, with the full cohort selection criteria described elsewhere [25]. A
102   subset of 42 participants from the DIGEST study with paired urine and serum samples were
103   selected for targeted and nontargeted metabolomics using three complementary instrumental
104   platforms, where participants had completed self-reported dietary records at baseline, and each
105   week for 2 weeks following food provisions. All participants from DIGEST picked up their food
106   allotment each week at the grocery store, during clinic visits, or food provisions were delivered to
107   their home by volunteers [25]. No detailed information was collected on food preparation methods
108   at home involving non-packaged foods, however all participants were requested to maintain their
109   normal lifestyle habits during the intervention period (*e.g.*, physical activity, cooking methods). A
110   Harris-Benedict equation plus an activity factor was used to estimate participant's energy intake,
111   compared this against their 7-day diet record, and assigned the appropriate diet plan at one of 9
112   energy levels (from 1600 through 3200 kcal) designed to maintain weight during the study period.
113   After 1 week of intervention, participants were re-weighed and the food for week 2 was maintained,
114   increased or decreased (using groups of 100-kcal snacks designed for each diet) as necessary to
115   maintain the baseline weight. In order to maximize treatment effects in this short-term dietary
116   intervention, participants were assigned into two parallel arms of contrasting diets, namely a
117   Western diet reflecting a typical Canadian macronutrient profile with higher intake of processed
118   foods (*e.g.*, burgers, fried chicken, cereals, processed cheeses), and a Prudent diet based around
119   minimally processed foods composed of lean protein (*e.g.*, poultry, fish, legumes), whole grains and
120   a high amount of fresh fruits and vegetables [25].

121   Briefly, one week prior to beginning the intervention diet, all DIGEST participants attended a
122   pre-study visit, where they were instructed by staff on how to complete a prospective 7-day food
123   diary. An instruction sheet and booklet was also provided. Participants were provided with a blank
124   diary, and asked to record each item consumed at each eating occasion (*e.g.*, food name, amount,
125   method of cooking, etc.). Common household measures were used for food and drink (*e.g.*, cups,
126   tablespoons, etc.). For composite foods that could not be readily split, such as a fruit pie or meat
127   casserole, participant were asked to provide recipe details. Foods eaten away from home were
128   recorded in as much detail as possible (*e.g.*, restaurant name, item, etc.). Study staff reviewed the
129   food diary with each participant on the day of the baseline visit. At the beginning of each week of
130   the intervention period, each participant was provided with a 7-day meal plan that listed the exact
131   food items and quantities they were to eat. As each food was eaten, participants were instructed to
132   check that item off the list. Participants also recorded any additional items consumed that were not
133   on the checklist. This checklist was returned at the end of each of the 2 weeks of intervention, and
134   study staff reviewed this with each participant. The pre-study diets were entered into, and analyzed
135   using The Food Processor (ESHA, Oregon, USA) for micro- and macronutrients, and servings of
136   fruits and vegetables. A diet quality index score was assigned for each participant at baseline and
137   an average over the 2 week intervention as follows; one "Prudent" point was assigned for each of
138   the following criteria: polysaturated:saturated fat ratio (Poly:Sat) > 1.0, saturated fat < 7%, total
139   fiber > 28 g, fruits+vegetables > 5 servings/day, and potassium > 3500/d. The maximum score was 5.
140   Similarly, one "Western" point was assigned for each of the following criteria: Poly:Sat < 0.5,
141   saturated fat > 16%, fiber < 9 g, fruits+vegetables < 5 servings/d, and potassium < 3500 mg/day. The
142   maximum score was 5. DIGEST participants were classified as "predominantly" Prudent if the
143   Prudent score minus the Western score was ≥ 2, or they were classified as "predominantly" Western
144   if the Western score minus the Prudent score was ≥ 2; however, based on these criteria, few
145   participants had Prudent diet characteristics (14%; 6 out of 42) with most having Western eating

146 habits at baseline (95%, 40 out of 42). Dietary adherence was a measure of the % of the foods
147 "prescribed" that they reported eating that was self-reported based on the foods they checked-off
148 from menu list that they consumed, which was > 95% for both treatment arms. A total of 20 micro-
149 and macronutrient categories (from over 120) from self-reporting dietary records were determined
150 to be different ($q < 0.05$; Bonferroni adjustment) between assigned Prudent and Western diets
151 among DIGEST participants, which were subsequently correlated with top-ranked plasma and
152 urinary metabolites when validating putative biomarkers of contrasting diets.

2.2 Chemicals and Reagents.

154 Ultra HPLC grade LC-MS solvents (water, methanol, acetonitrile) obtained from Caledon
155 Laboratories Ltd. (Georgetown, ON, Canada) were used to prepare all buffer and sheath liquid
156 solutions. Proline betaine was purchased from Toronto Research Chemicals (Toronto, Ontario,
157 Canada). All other chemicals were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA).

2.3 Nontargeted Metabolite Profiling of Plasma and Urine by MSI-CE-MS.

159 Fasting plasma (EDTA) samples together with matching single-spot urine samples were
160 collected from all DIGEST participants during clinic visits on day 1 and day 14, which were then
161 stored at -80 °C [25]. Multisegment injection-capillary electrophoresis-mass spectrometry (MSI-CE-
162 MS) was the major platform used for nontargeted profiling of polar/ionic metabolites from both
163 plasma and urine samples [28], which was performed on an Agilent G7100A CE (Agilent
164 Technologies Inc., Mississauga, ON, Canada) equipped with a coaxial sheath liquid (Dual AJS)
165 Jetstream electrospray ion source coupled to an Agilent 6230 TOF-MS system. All separations used
166 uncoated fused-silica capillaries (Polymicro Technologies, AZ, USA) with a total length of 120 cm
167 and inner diameter of 50 μm. About 7 mm of polyimide coating was removed from both distal ends
168 to avoid sample carry-over and prevent polyimide swelling from contact with organic solvent [29].
169 The background electrolyte (BGE) consisted of 1 M formic acid with 15% *vol* acetonitrile (pH 1.8)
170 under positive ion mode, and 50 mM ammonium bicarbonate (pH 8.5) under negative ion mode for
171 analysis of the ionic metabolome, including cationic and anionic metabolites from matching plasma
172 and urine specimens, respectively. All CE separations were performed under normal polarity with
173 an applied voltage of 30 kV at 25 °C. However, a pressure gradient of 2 mbar/min from 0 to 40 min
174 was used for MSI-CE-MS analyses under negative ion mode conditions to shorten analysis times for
175 highly charged anionic metabolites (*e.g.*, citrate). The TOF-MS system was operated with full-scan
176 data acquisition over a mass range of *m/z* 50-1700 and an acquisition rate of 500 ms/spectrum. The
177 sheath liquid was comprised of 60% *vol* MeOH with 0.1% *vol* formic acid for positive ion mode, and
178 50% *vol* MeOH for negative ion mode. The ESI conditions were Vcap =2000 V, nozzle voltage = 2000
179 V, nebulizer gas =10 psi, sheath gas = 3.5 L/min at 195 °C, drying gas 8 L/min at 300 °C. whereas, the
180 MS voltage settings were fragmentor = 120 V, skimmer = 65V and Oct1 RF= 750 V.

181 A 7-sample serial injection format was used in MSI-CE-MS [31-33] consisting of a serial
182 injection of 6 individual urine or plasma samples together with a pooled quality control (QC)
183 within each experimental run; the latter sample was used to assess technical variance while also
184 allowing for robust batch correction due to long-term signal drift in ESI-MS [31, 33]. Multiplexed
185 separations in MSI-CE-MS was performed by programming a hydrodynamic injection sequence
186 with each sample (5 s at 100 mbar) interspaced with a BGE spacer (40 s at 100 mbar) prior to voltage
187 application as described elsewhere [31-33]. Non-targeted metabolite profiling of plasma or urine
188 specimens by MSI-CE-MS was conducted by pairing together matching baseline and post-treatment
189 samples from 3 DIGEST participants together with a QC in a randomized injection position for each
190 run. Different serial sample injection configurations in MSI-CE-MS were also applied for rigorous
191 authentication of metabolites using a dilution trend filter, as well as acquisition of calibration curves
192 for metabolite quantification [31-33]. Briefly, authentic metabolites were defined by their unique
193 accurate mass and relative migration time (*m/z*:RMT) under positive (+) or negative (-) ion mode
194 detection after filtering out spurious signals, background ions, as well as redundant adducts, in-
195 source fragments and isotopic features that comprise the majority of signals in MS-based
196 metabolomics [34]. Additionally, only frequently detected plasma or urinary metabolites measured

197 in the majority of samples from DIGEST participants (> 75%) with acceptable technical precision
198 based on repeated analysis of QC samples (mean *CV* < 30%) were included in the final data matrix
199 as a way to reduce false discoveries and data over fitting [35]. Missing (*i.e.,* zero) data below
200 method detection limits were replaced with a minimum value corresponding to half of the lowest
201 responses measured for a given metabolite in all samples analyzed. Overall, 70 and 50 authentic
202 polar/ionic metabolites were measured consistently in the majority of urine and plasma samples by
203 MSI-CE-MS, respectively including unknown ions and recovery standards that fully satisfied our
204 multi-level metabolomics selection criteria, and rigorous filtering procedure. Also, creatinine
205 concentrations were measured by MSI-CE-MS, which were used to reduce biological variance and
206 correct for differences in hydration status for single-spot urine samples analyzed in this study.

207 *2.4 Unknown Metabolite Identification by MS/MS.*

208 High resolution tandem mass spectrometry (MS/MS) was employed for structural elucidation
209 of unknown metabolites of biological significance from pooled samples in this study. Collisional–
210 induced dissociation (CID) experiments were performed on an Agilent G7100A CE system with a
211 coaxial sheath liquid Jetstream electrospray ion source connected to an Agilent 6500 iFunnel QTOF
212 instrument. Metabolite identification in this work adopted reporting standards recommended from
213 the Metabolomics Standards Initiative [36], including unambiguous identification (level 1) that was
214 confirmed with matching MS/MS spectra and co-migration with an authentic standard acquired on
215 the same instrument, tentative/probable identification (level 2) by comparison of MS/MS spectra
216 from public databases or published literature, partial annotation of MS/MS spectra guided by *in*
217 *silico* software tools with metabolite class (level 3), and compounds with unknown chemical
218 structure that were characterized by their most likely molecular formula (level 4). The latter case
219 typically occurred for low abundance metabolites that had inadequate responses for their precursor
220 ion when acquiring MS/MS spectra. Also, the electromigration behavior of polar/ionic metabolites
221 (*i.e.,* RMT) provided useful qualitative information that complemented MS/MS when selecting
222 among potential isobaric or isomeric candidate ions. MS/MS spectra were acquired from pooled
223 plasma or urine samples that were injected hydrodynamically using a conventional single sample
224 injection plug at 50 mbar for 10 s followed by 5 s with BGE. Precursor ions were selected for CID
225 experiments at different collision energies (*e.g.,* 10, 15, 20 or 40 V). Mirror plots comparing MS/MS
226 spectra of unknown metabolites under an optimal collision energy were then compared to their
227 respective authentic reference standard (if available), which were generating using the
228 "InterpretMSSpectrum" R Package. Otherwise, MS/MS spectra were annotated based on their
229 characteristic product ions or neutral losses for *de novo* structural elucidation (level 2 or 3), which
230 was guided by *in silico* MS/MS spectra generated by CFM-ID [37] or comparison to MS/MS spectra
231 deposited in open-access public repositories (HMDB, http://www.hmdb.ca) or published in
232 literature. The exact stereochemistry was uncertain for tentatively identified metabolites in plasma
233 or urine if authentic standards were lacking. MS/MS spectra for 5 urinary metabolites associated
234 with a Prudent diet that were identified in this work were deposited onto HMDB.

235 *2.5 Total Plasma Fatty Acid Determination by GC-MS.*

236 A GC-MS method was used for targeted analysis of 26 total (hydrolyzed) fatty acids (FA) and
237 their isomers from plasma extracts on an Agilent 6890 GC coupled to an Agilent 5973 single
238 quadrupole mass spectrometer with electron impact ionization (EI) with minor modifications [38].
239 Total hydrolyzed plasma FA were analyzed by GC-MS as their methyl ester derivatives (FAME)
240 using *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) (≥ 98.5%, GC from Sigma-Aldrich).
241 Isotopically-labelled myristic acid-d27 (98%), stearic acid-d35 (98%) and pyrene-d10 (98%) were
242 obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). HPLC grade chloroform (≥
243 99.5%, GC), methanol (99.8%, GC), hexanes (≥ 99.5%, GC) and Ultra LC-MS grade water were
244 purchased from Caledon Laboratories Ltd. (Georgetown, ON, Canada). The antioxidant, butylated
245 hydroxytoluene (BHT, 490 μL) was added to all FA calibrant solutions prepared in methanol to
246 prevent autooxidation. Briefly, a 10 μL aliquot of thawed plasma was mixed with 5 μL of a 1.0
247 mg/mL C18:0-d35 recovery standard. Concentrated sulfuric acid (10 μL) was added as a
248 transesterification catalyst and vortexed for 2 min before incubation for 4 h at 80 °C to produce

249 FAME for improved volatility. Cooled samples were then mixed with 500 μL of 9 g/L NaCl and 200
250 μL hexanes and vortexed for 2 min prior to centrifugation at 14,000 *g* at 4 ºC for 5 min. GC inserts
251 were prepared with 45 μL of hexane supernatant with 5 μL of internal standard pyrene-d10 and
252 vortexed for 2 min before injection. Total plasma FAME were resolved on a Supleco SP-2380
253 column (30 m x 0.25 mm x 0.20 μm) using an optimal temperature program within 30 min. Samples
254 were injected in 1.0 μL volumes using a splitless injector held at 250 °C, the carrier gas was helium
255 at 1.0 mL/min and the transfer line was held at 270 °C. Pooled QC and blank extracts were analyzed
256 together with each batch of 8-10 randomized plasma samples when using GC-MS in order to assess
257 technical precision and monitor for background contamination. A temperature program used for
258 resolution of major FAME from plasma hydrolysates comprised of a temperature gradient of
259 20 °C/min starting from 2 min at 80 °C until 20 min at 160 °C, which was further increased to 190 °C
260 for 3 min prior to elution at 300 °C for 5 min with a total run time of 28 min. In most cases, FAME
261 were quantified in GC-MS based on integration of the relative response ratio of their [M-15]+
262 fragment ion relative to pyrene-d10 as internal standard when using single ion monitoring (SIM)
263 mode detection. Calibrations curves from FAME standards were used for FA quantification, as well
264 as their identification when comparing their characteristic EI-MS spectra (70 eV) and elution times.

265 *2.6 Targeted Urinary Electrolyte Analysis.*

266 Targeted analysis of 8 inorganic/involatile electrolytes in urine was performed using two
267 complementary CE-UV methods adapted from Nori de Macedo *et al.* [39] and Saoi *et al.* [32] for
268 anionic (*e.g.,* nitrate) and cationic (*e.g.,* sodium) electrolytes, respectively. First, analysis of major
269 cationic electrolytes was performed on frozen urine samples that were thawed, vortexed for 30 s
270 and centrifuged at 14,000 *g* for 5 min. An aliquot of the urine supernatant was diluted with
271 deionized water and spiked with 0.5 mM lithium as an internal standard. Samples were analyzed
272 on an Agilent G7100A CE system with UV photodiode array detection with indirect absorbance
273 detection at 214 nm. All samples were injected hydrodynamically for 10 s (at 35 mbar) and
274 separation was performed under normal polarity at 30 kV at 25 °C using a 50 μm inner diameter
275 capillary with 60 cm total length. The background electrolyte (BGE) was 5 mM formic acid
276 containing 12.5 mM creatinine (Crn) and 4 mM 18-crown-6 ether at pH 4.0 (adjusted with 1 M
277 sulfuric acid. In this case, ammonium (NH_4^+), sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and
278 magnesium (Mg^{2+}) were analyzed in urine samples [32]. Additionally, all urine samples were
279 analyzed using a complementary CE assay for UV-absorbing inorganic anions, including nitrate
280 (NO_3^-), iodide (I^-) and thiocyanate (SCN^-) [39]. In this case, the BGE was comprised of 180 mM
281 lithium hydroxide, 180 mM phosphoric acid, and 10 mM α-cyclodextrin (α-CD) at pH 3 (adjusted
282 with 1 M phosphoric acid), where 1,5-naphthalene disulfonate (NDS) was used as an internal
283 standard. Samples were injected hydrodynamically for 80 s at 0.5 psi and analyzed at 25 °C under a
284 reversed polarity at -18 kV with UV absorbance detection at 226 nm (288 nm for NDS). In both CE-
285 UV assays, a pooled QC was measured intermittently after every batch of 6 urine samples.

286 *2.7 Data Preprocessing and Statistical Analysis.*

287 All MSI-CE-MS data were integrated and analyzed using Agilent MassHunter Qualitative
288 Analysis B.07.00 and Microsoft Excel and Igor (Wavemetrics Inc., OR, USA). In all cases, the
289 integrated ion response (*i.e.,* peak area) for each metabolite was normalized to an internal standard,
290 4-chlorotyrosine (Cl-Tyr) migrating from the same sample by MSI-CE-MS. Also, a QC-based batch
291 correction algorithm was applied to creatinine-normalized urine metabolomic data to adjust for
292 long-term signal drift in ESI-MS during data acquisition as outlined in a recent work [31]. This
293 algorithm is based on an empirical Bayesian frameworks, [33] which takes advantage of QC
294 samples included in each serial injection run when using MSI-CE-MS, as well as batch and injection
295 sequence information. However, batch correction did not provide any significant improvement in
296 the overall technical precision of plasma metabolome data, and thus was not applied in this case.
297 All non-batch (plasma) and batch-corrected (creatinine normalized urine) metabolomic data was
298 pre-processed using generalized *log*-transformation and autoscaling prior to multivariate statistical
299 analysis using MetaboAnalyst 4.0 (www.metaboanalyst.ca) [40], including volcano plots, principal
300 component analysis (PCA), hierarchical cluster analysis (HCA)/2D heat maps, receiver operating

301 characteristic (ROC) curves, orthogonal partial least squares-discriminant analysis (OPLS-DA), as
302 well as multivariate empirical Bayes analysis (MEBA) of variance; the latter method is optimal for
303 analyzing time-series data [41] relevant to identifying metabolic trajectories reflective of contrasting
304 diets. To validate each OPLS-DA model, cross-validation and permutation testing (n=1000) on
305 paired metabolome data sets (*i.e.,* ratio of metabolite response based on assigned diet/baseline diet
306 for each subject) following *glog* transformation and autoscaling, whereas Hotelling's *T*-squared
307 distribution using MEBA was performed on *glog*-transformed metabolomic time series data for
308 DIGEST participants at baseline and following 2 weeks of food provisions. These complementary
309 statistical approaches were used for unsupervised data exploration to identify overall trends, as
310 well as supervised data analysis for ranking metabolites modulated by contrasting diets without
311 adjustments for covariates. Additionally, normality tests, partial Pearson correlation analysis, and
312 mixed model ANOVA were performed on top-ranked dietary biomarker candidates using the
313 Statistical Package for the Social Sciences (SPSS, version 18.0). In this case, a partial listwise Pearson
314 correlation analysis of lead plasma and creatinine-normalized metabolite responses to 20 major
315 nutrient categories from self-reported food records from DIGEST participants (n=42) were adjusted
316 for age, sex, and post-intervention *BMI*. Only metabolites that had a correlation coefficient of $r > \pm$
317 0.300 and $p < 0.05$ for more than 2 nutrient categories were considered as significant in this work. A
318 repeat measures general linear mixed ANOVA model was also performed with the number of
319 levels set at 2 for the repeat sampling (*i.e.,* time; baseline diet, and 2 weeks after assigned diet) while
320 setting the intervention diet (*i.e.,* treatment arm; P-W, and W-P) as the between-subject factor with
321 age, sex and post-intervention *BMI* as potential covariates. Overall, plasma and urine metabolites
322 that satisfied MEBA and/or mixed ANOVA models, as well as partial correlation analysis to > 2
323 nutrient categories from diet records were defined as robust dietary biomarkers in our study.

3. RESULTS

325 *3.1. Study Design, Baseline Habitual Diet and Metabolomics Workflow.*

326   The DIGEST study was a two-arm parallel dietary intervention involving healthy/non-
327 smoking participants recruited from the local community as described elsewhere [25]. A CONCERT
328 diagram summarizes eligibility criteria (Figure S1), where all participants completed a 7-day
329 prospectively collected diet record and then were randomly allocated to eat a weight-maintaining
330 Prudent or Western diet over 2 weeks. Participants (n=42) with contrasting habitual diets were
331 selected in this unblinded metabolomics study based on availability of matching plasma and urine
332 samples with complete diet records as depicted in Figure 1(A). There were more women (64%)
333 recruited than men, however there were no differences in age (mean age of 47 years ranging from
334 20 to 69 years), body composition (mean *BMI* of 27 kg/m^2 with 26% defined as obese) and average
335 caloric intake (mean of 1940 kcal/day) between assigned diet groups, and most participants were
336 Caucasian (78%) with no self-identified tobacco smokers (Table S1). Also, no differences in baseline
337 blood lipids, fasting glucose, inflammatory biomarkers and blood pressure were evident between
338 the two treatment arms. Since Prudent diet scores (< 2) were low for this cohort overall (Table S1),
339 18 participants were classified as having a "Prudent-like" diet at baseline, but this was due to their
340 lower average Western diet score (2.61 ± 0.69); this group was randomized to an assigned Western
341 diet (referred to as P-W) in this study. In contrast, 24 participants having a predominate Western
342 diet at baseline due to their higher average Western diet score (3.42 ± 0.93) were randomized to a
343 Prudent diet (referred to as W-P). Importantly, there were no significant changes in plasma or urine
344 metabolome between these two sub-groups at baseline reflecting similar habitual dietary patterns.

345   Figure 1(B) and (C) depict 2D heat maps for matching plasma and urine metabolomes from
346 DIGEST participants (n=42) collected at baseline and following 2 weeks of assigned food provisions.
347 A total of 80 serum metabolites and 84 urinary metabolites were reliably measured ($CV < 30\%$) in
348 the majority of participants (> 75%) when using a validated data workflow for nontargeted
349 metabolite profiling with stringent QC [30-32]. A rigorous approach to metabolite authentication
350 was implemented to reject spurious, redundant and background ions that comprise the majority of

351
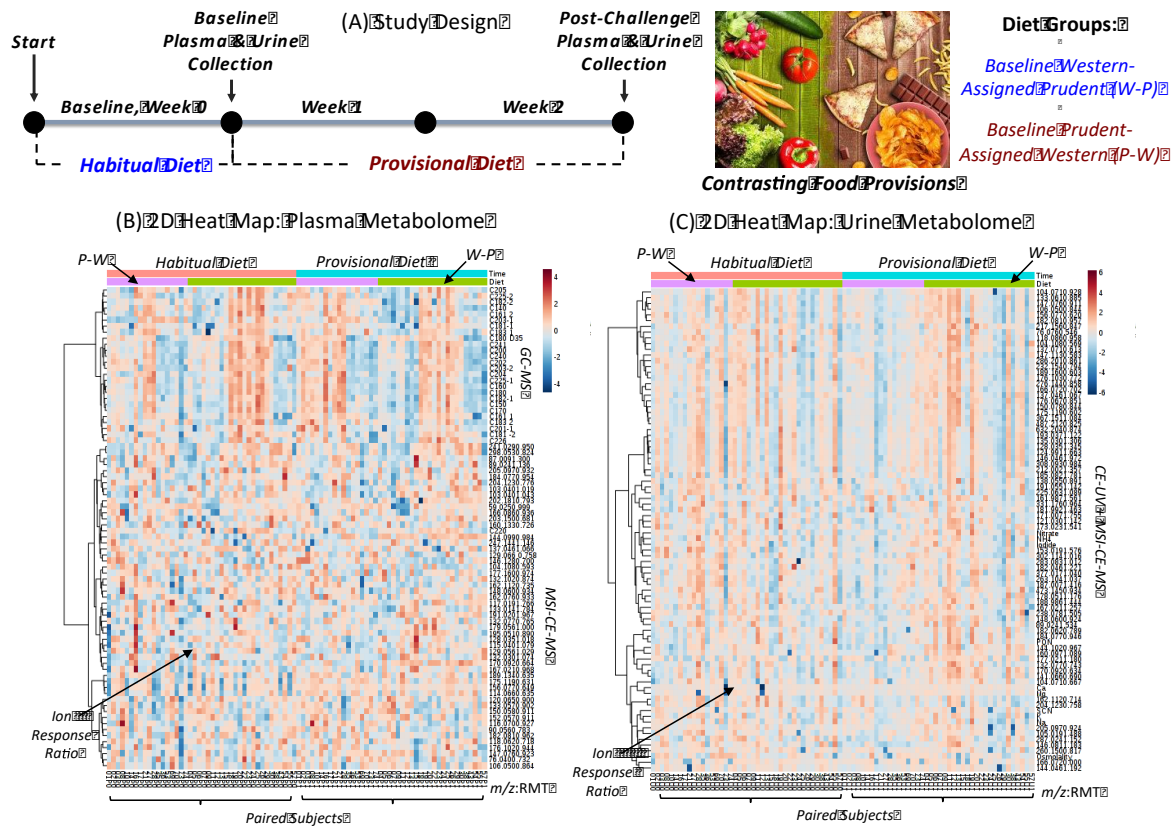
Figure 1. (A) Overview of study design in this parallel two-arm dietary intervention study involving participants from DIGEST (*n*=42) who were assigned a contrasting Prudent or Western diet over a 2-week period with matching urine and plasma samples collected at baseline and post-intervention. (B) A 2D heat map with hierarchical cluster analysis (HCA) of the plasma metabolome consistently measured in the majority of participants, including non-targeted analysis of polar/ionic metabolites by MSI-CE-MS, and total hydrolyzed fatty acids by GC-MS. (C) A 2D heat map with HCA of the urine metabolome consistently measured in majority of participants, including non-targeted analysis of polar/ionic metabolites, and targeted electrolytes by CE with UV detection. A generalized *log* transformation and autocaling was performed on metabolomic datasets together with batch correction and 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 who had lower Western diet score at baseline, but were assigned a Western diet during the intervention period.

365 molecular features detected in ESI-MS [34] in order to reduce false discoveries in metabolomics [35]. Overall, 3 orthogonal instrumental platforms were used to characterize polar/ionic metabolites from plasma and urine using MSI-CE-MS, as well as total (hydrolyzed) plasma fatty acids (FA) by GC-MS, and inorganic urinary electrolytes by CE with UV detection (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 from 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 6 individual samples together with a QC. Also, control charts for recovery standards provided further evidence of acceptable long-term precision (mean CV < 9%) with few outliers exceeding confidence intervals (Figure S3).

377 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, mass error, level of
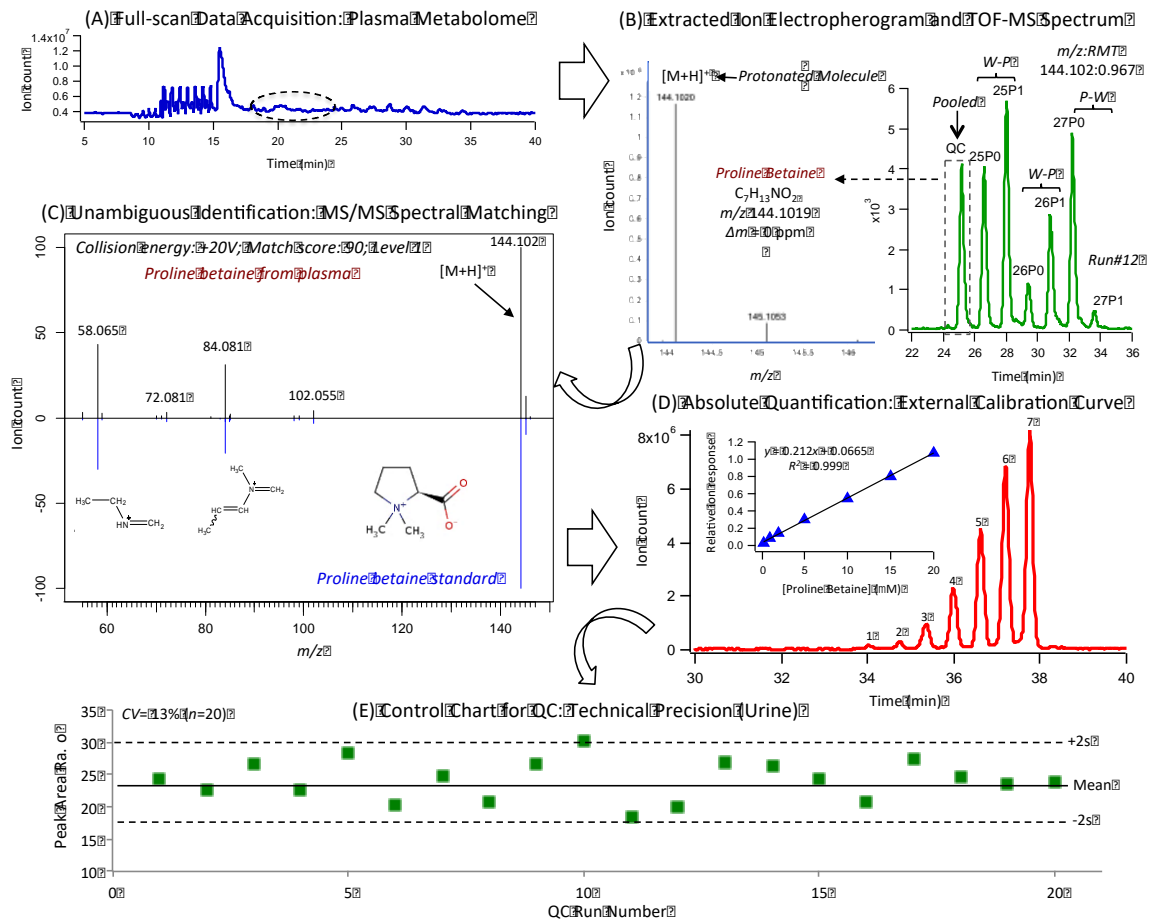
Figure 2. Summary of the metabolomics data workflow when using MSI-CE-MS for the identification and quantification of biomarkers associated with a Prudent diet, including (A) a total ion electropherogram when using full-scan data acquisition under positive ion mode. (B) Extracted ion electropherogram for an authentic unknown cation (*i.e.*, protonated molecular ion) annotated by its *m*/z:RMT based on serial injection of 7 plasma (or urine) samples within a single run. Each run comprised 3 pairs of samples from DIGEST participants (*i.e.*, baseline diet, and 2 weeks after dietary intervention) together with a pooled sample as QC for assessing technical precision, and correcting long-term signal drift. High resolution MS spectra allows for determination of its most likely molecular formula, and (C) MS/MS spectra allows for structural elucidation when compared to an authentic standard with a mirror plot that is consistent with ProBet. (D) Quantification of ProBet is 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 repeated measurements of QC samples in every MSI-CE-MS run demonstrates acceptable technical precision (*CV* = 13%, *n*=20) over 3 days.

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 deposited in public data bases (HMDB, Metlin); otherwise, spectral annotation was guided by *in silico* fragmentation [37] using recommended reporting standards for metabolite identification [36,42]. An overview of this metabolomics workflow is outlined in Figure 2, which shows the detection of an unknown protonated molecule [MH+] in plasma, followed by its annotation using 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. Quantification of ProBet using an external calibration curve is also demonstrated, and good technical precision (*CV* < 15%, *n*=20) was achieved as shown in a control chart based on repeated analysis of a QC in every run throughout study.

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 + Vegetable intake (servings/2000 kcal/day)	(3.6 ± 1.4)	(-1.8 ± 1.3)	*p* = 7.3 E-15; Greater fruit + vegetable intake 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 vegetable 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 (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 2-tailed student's t-test with equal variance.

3.2. Changes in Dietary Intake and Biomarker Classification.

Major changes in self-reported dietary patterns among DIGEST participants were evident after 2 weeks of assigned diets 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^{2+}), fruit and/or vegetable, vitamins (A, C and E), 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.

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). Overall,

426 contrasting diets generated pronounced changes in a wide range of plasma and urinary metabolites
427 that was largely absent for the same participants at baseline given modest differences in their
428 habitual diets prior to the start of food provisions (Table S1). For instance, 10 plasma and 16 urinary
429 metabolites were differentially expressed in W-P as compared to P-W diet groups, including 4
430 metabolites satisfying a Benjamini-Hochberg/FDR adjustment ($q < 0.05$), including ProBet, 3-
431 methylhistidine (Me-His) and two unknown urinary metabolites subsequently identified (level 2) as
432 hydroxypipecolic acid (OH-PCA) and imidazole propionic acid (ImPA). The identification and
433 quantification of Me-His was confirmed in both plasma and urine (Figure S6), whereas several
434 unknown urinary metabolites were putatively identified (level 1 or 2) based on their characteristic
435 MS/MS spectra, such as OH-PCA (Figure S7) and acesulfame K (ASK; Figure S8). Similarly, targeted
436 analysis of FAME from hydrolyzed plasma extracts using GC-MS (Figure S9) allows for resolution
437 of low abundance *trans* isomers (linoelaidic acid, C18:2n-6*trans*) and saturated fatty acids (myristic
438 acid, C14:0) from abundant dietary fatty acids (linoleic acid, C18:2n-6*cis*). As expected, several
439 circulating fatty acids (Figure S5) were consistently elevated in circulation following a Western diet
440 due to higher average consumption of total fats as compared to a Prudent diet.

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

442   Complementary statistical methods that take advantage of the repeated-measures study design
443 were used to classify metabolites responses to contrasting dietary patterns. A paired orthogonal
444 partial least-squares discriminant analysis (OPLS-DA) model (Figure 3) was used to rank
445 metabolites in plasma and urine that were modulated by contrasting diets relative to each
446 participant's baseline habitual diet (*i.e.*, ion response ratio). Both OPLS-DA models demonstrated
447 good accuracy ($R^2 > 0.840$) with adequate robustness ($Q^2 > 0.200$) after permutation testing ($p < 0.05$,
448 n=1000). S-plots confirmed that ProBet and Me-His were consistently elevated following a Prudent
449 diet (W-P) in both plasma and urine samples, whereas total plasma C14:0 and C18:2n-6*cis* had the
450 most significant increase following an assigned Western diet (P-W). Additionally, top-ranked
451 creatinine-normalized urinary metabolites excreted at higher levels following a Prudent diet
452 included ImPA, OH-PCA, dihydroxybenzoic acid (DHBA), enterolactone glucuronide (ETL-G),
453 nitrate and an unknown cation (*m/z* 217.156, MH+) tentatively identified as a dipeptide, valinyl-
454 valine (Val-Val). In contrast, urinary excretion of ASK was only modestly increased ($p = 0.0686$)
455 following a Western diet. Additionally, excellent discrimination among DIGEST participants
456 following a Prudent or Western diet was achieved when using top-ranked single or ratiometric
457 biomarkers from a receiver operating characteristic (ROC) curve (*AUC* > 0.820; $p < 1.0$ E-5) for
458 plasma and creatinine-normalized urine samples (Figure S10). For instance, plasma ProBet and the
459 ratio of Me-His/C18:3 n-6*trans* demonstrated good sensitivity and specificity (≈ 80-90%) for
460 classifying DIGEST participants based on their assigned diets similar to urinary OH-PCA and the
461 ratio of OH-PCA/Na+. A multivariate empirical Bayes analysis of variance (MEBA) [41] was also
462 used to characterize time-dependent metabolite profiles related to contrasting diets after two weeks
463 of food provisions. In this case, metabolic trajectories with distinctive time-course profiles following
464 a Prudent or Western diet were ranked based on their Hotelling's T^2 distribution as shown for
465 plasma (Figure S11) and urine (Figure S12), which were consistent with metabolites identified as
466 dietary biomarkers from volcano plots, ROC curves and OPLS-DA models.

467   A mixed ANOVA model, and a partial Pearson correlation analysis to self-reported diet
468 records after adjustment for sex, age and *BMI* were next applied to further validate the relevance of
469 dietary biomarkers identified from multivariate statistical models. Table 2 highlights that ProBet
470 and Me-His were the most robust plasma metabolites associated with a Prudent diet that satisfied
471 several statistical parameters (T^2, F-value, r, adjusted p-value). For instance, ProBet was positively
472 associated ($r \approx 0.520$, $p = 0.001$) with self-reported intake of fruit (cup eq./2000 kcal), vitamin C
473 (mg/2000 kcal) and fruit+vegetable servings (servings/2000 kcal), as well as negatively associated
474 with fat intake ($r > -0.530$, $p < 0.001$), including *trans* and saturated fat (%energy). Me-His had
475 strong positive correlations ($r = 0.530-0.570$, $p < 0.001$) with protein (%energy), insoluble fiber
476 (g/2000 kcal), electrolytes (Mg, K; mg/2000 kcal), as well as fruit, and fruit+vegetable intake
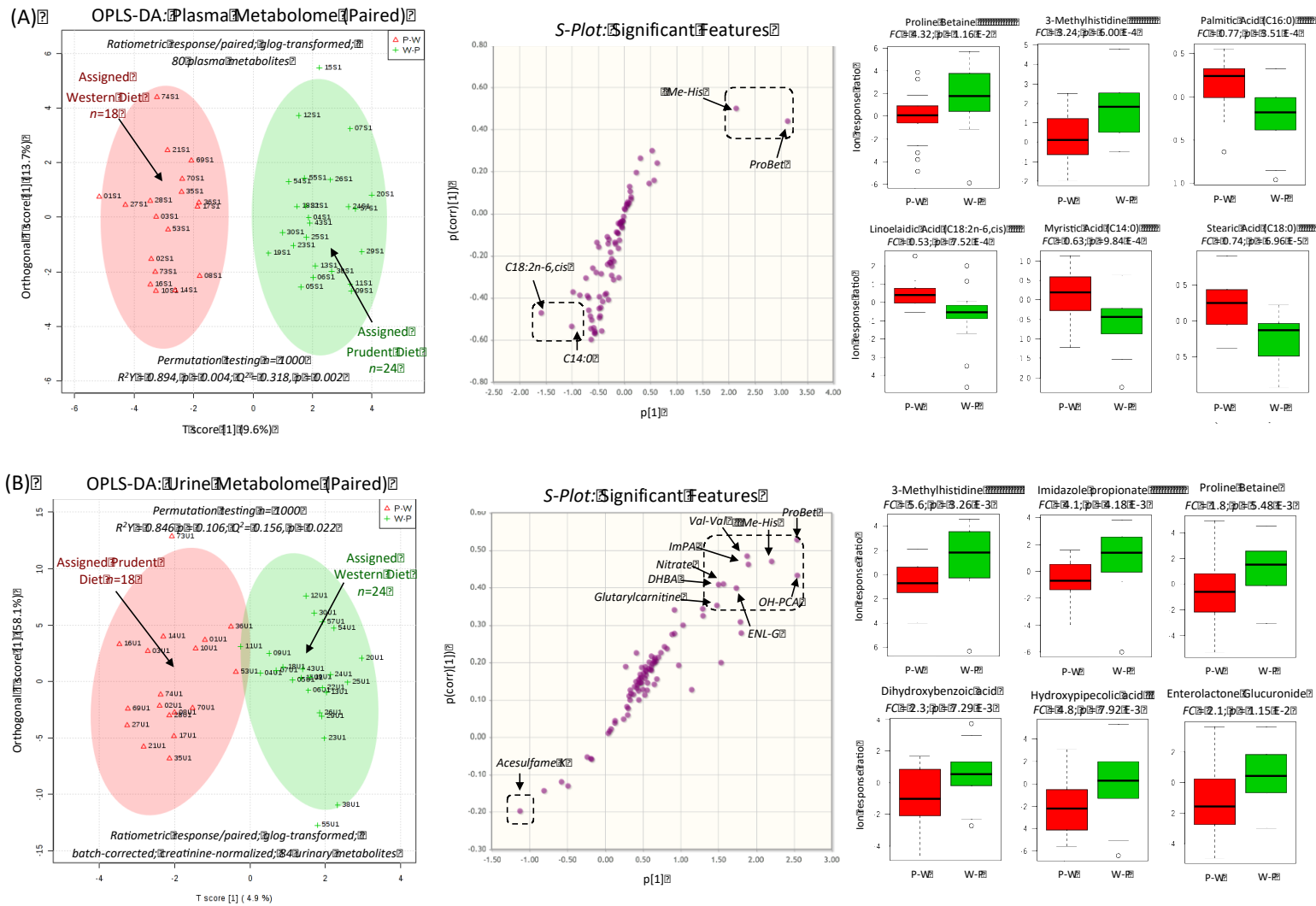
Figure 3. Paired supervised multivariate data analysis of (A) plasma and (B) creatinine-normalized urine metabolomic data based on orthogonal partial least-squares-discriminant analysis (OPLS-DA) using the ratio of ion responses or concentrations for metabolites following 2 weeks of food provisions to their baseline habitual diet values. 2D scores plot highlight differences in the metabolic phenotype from matching biofluids collected from DIGEST participants assigned a Prudent (W-P) or 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 differentially expressed between treatment arms ($p < 0.05$).

Table 2. Top-ranked plasma metabolites associated with contrasting diets by DIGEST 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+Vegetable
3-Methylhistidine (Me-His) 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+Vegetable
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 -0.362 -0.359	0.002 0.010 0.019 0.026 0.027	*trans* fat %energy Fruit+Vegetable Vegetable Fruit Protein %energy
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+Vegetable Vitamin E Vitamin C
Deoxycarnitine or γ-Butyrobetaine (dC0) HMDB01161	146.128:0.700 (+) MSI-CE-MS $C_7H_{16}NO_2$ Level 1	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 1	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+Vegetable 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 1	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+Vegetable 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 1	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+Vegetable 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 *trans* fat %energy Poly:Sat Vit A Fruit+Vegetable
α-Linoleic acid (C18:3n-6 *cis*)	292/79.1:15.096 GC-MS	7.0	11.6	0.002	-0.441 -0.397 -0.391	0.006 0.013 0.015	Poly:Sat Vitamin A Fruit+Vegetable

Metabolite					r	p	Nutrient category
HMDB001388	$C_{18}H_{30}O_2$ Level 1				0.391 -0.387	0.015 0.016	*trans* fat %energy Vitamin E
Ketovaline or α-Isovaleric acid (kVal) HMDB00019	115.040:1.079 (-) MSI-CE-MS $C_5H_8O_3$ Level 1	6.3	2.4	0.125	0.489 0.472 0.466 0.458 0.451	0.002 0.003 0.003 0.004 0.004	Protein %energy Fiber (kcal) Fruit+Vegetable 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+Vegetable 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 1	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+Vegetable 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 > ± 0.30 and p < 0.05.* [d] *Top-five categories from food records significantly correlated to urinary metabolites following provisional diets.*

reflecting a Prudent diet. Other plasma metabolites classified as dietary biomarkers of contrasting diets in this study included, 2 carnitines (*e.g.*, carnitine, C0; deoxycarnitine, dC0), 2 amino acids (*e.g.*, proline, Pro; alanine, Ala), 3 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) plasma 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+vegetable, Poly:Sat, and vitamins. Table 2 summarizes 14 plasma metabolites that function as robust biomarkers of contrasting diets since they satisfied at least 2 of the 3 statistical models ($p < 0.05$) following adjustment for covariates between groups while also having a significant correlation ($r > ± 0.3$, $p < 0.05$) with at least 2 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, 2 plant-derived phenolic metabolites in urine, namely ETL-G and DHBA were also correlated to healthy eating patterns, such as greater intake of fruits, vegetables and micronutrients. However, creatinine-normalized Val-Val and DMG in urine were weakly correlated with only 2 nutrient categories ($p ≈ 0.05$) 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 from self-reported diet records despite showing treatment responses to contrasting diets.

Table 3. Top-ranked creatinine-normalized metabolites associated with contrasting diets by DIGEST 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 (Me-His) 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+Vegetable 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+Vegetable 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 + Vegetable 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+Vegetable 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) HMDB0240377	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+Vegetable Vegetable (cup eq.)
Dihydroxybenzoic acid (DHBA)* HMDB0001856	153.019:1.576 (-) MSI-CE-MS $C_7H_6O_4$ Level 2	8.0	7.3	0.010	-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 Vegetable (cup eq.) Fruit+Vegetable
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+Vegetable 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.*

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 (Figure S13) specimens from DIGEST participants. 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, 2 amino acids (Pro, Ala) and 2 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 among certain individuals due to potential dietary non-adherence and/or inaccurate self-reporting. Figure 4 illustrates metabolic trajectory plots for ProBet and Me-His as they were among the most sensitive biomarkers responsive to contrasting diets, and they were also measured consistently in both plasma and urine samples. 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), 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 the 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), and indicative on non-adherence to assigned diet. In contrast, a second participant (P-W, #28) had higher than average Me-His concentrations in both plasma and urine despite their low self-reported protein intake from diet records that was suggestive of reporting bias.
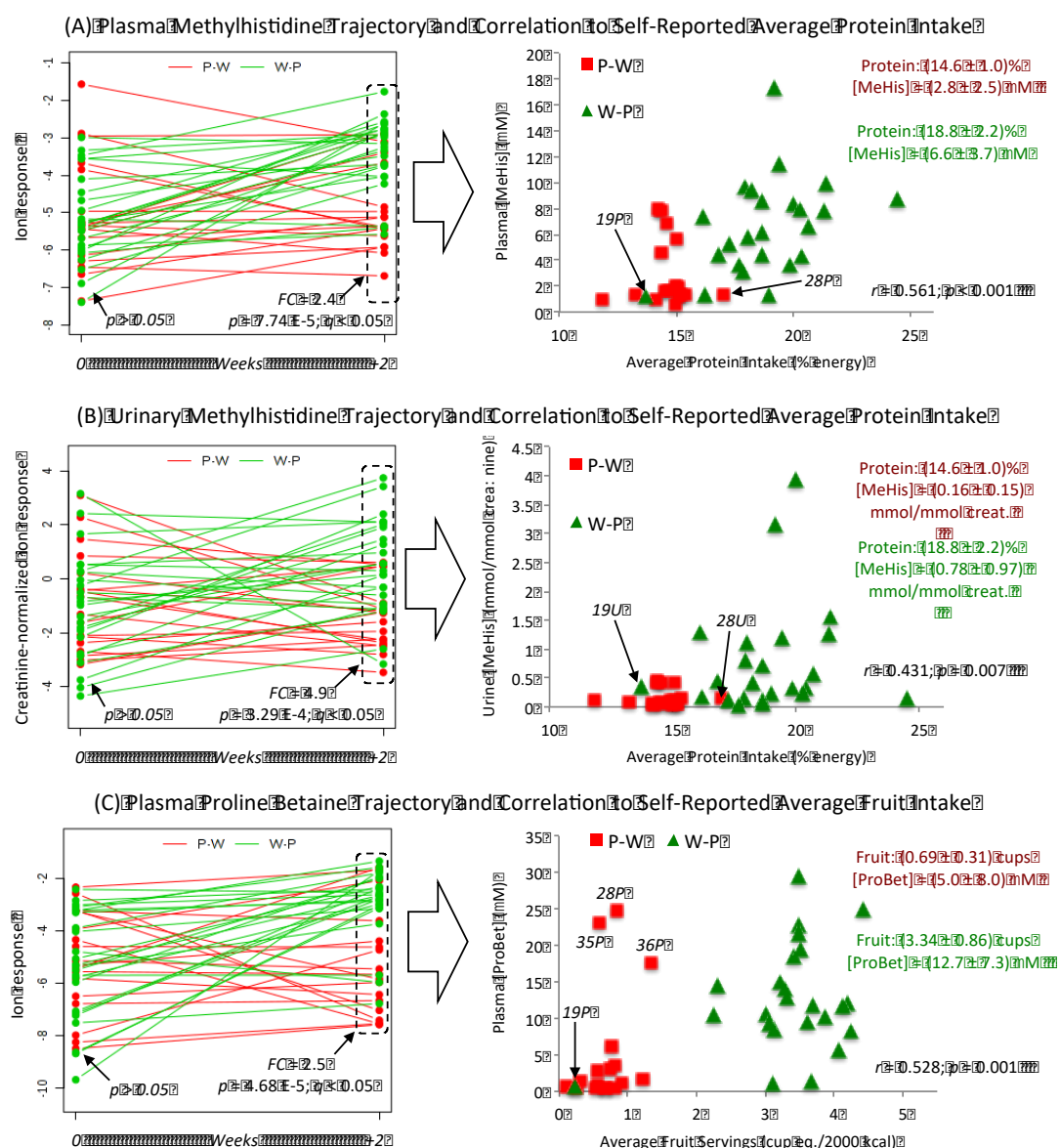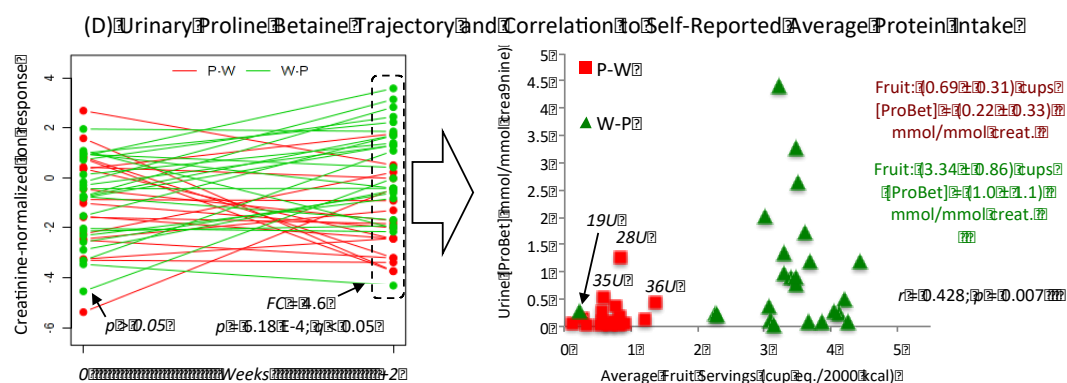
Figure 4. Metabolic trajectories for two prominent dietary biomarkers measured in both plasma and urine specimens that increased significantly following an assigned Prudent diet (W-P) as compared to Western diet (P-W), namely Me-His and ProBet. Both metabolites were not different at baseline, but undergo significant changes after 2 weeks of food provisions ($q < 0.05$, FDR) with concentrations moderately correlated ($r > 0.400$) to self-reported diet records, such as health-promoting foods reflective of a Prudent diet, such as higher intake of fruit (ProBet), and protein (Me-His). Good dietary adherence was demonstrated for DIGEST participants with few exceptions (labeled on plots) who had metabolic phenotypes inconsistent with their contrasting assigned diets.

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 window when analyzing 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 biotransformed plant-derived phenol metabolites in urine, DHBA and ETL-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).

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 [43]. However, few validated biomarkers exist for routine monitoring of habitual diet [44], such as omega-3 fatty acids [45], and water insoluble fiber [46]. 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) [30-32].

Since DIGEST participants had poor Prudent diet eating habits with modest differences associated with their aggregate Western diet scores (p = 0.0037) at baseline (Table S1), 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 1; 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 [47,48]. 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* ≈ 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 ≈ 0.40-0.60, p < 0.001) with eating patterns reflecting a Prudent diet, including a higher intake of fiber, fruit, fruit+vegetable, protein, vitamins, and 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 [49] since it is not prevalent in most other foods [50]. In fact, ProBet has been replicated in large-scale observational studies as a robust dietary biomarker (r ≈ 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 [51], 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 [52]. 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 [37] 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 [54], which has recently been identified as a product of gut microbiota activity that also regulates insulin sensitivity [55]. 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 [47]. 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 [56] and/or citrus fruits [57] when following a Prudent diet even though it also represents an endogenous lysine metabolite [58] also produced by gut microbiota [59]. Indeed, urinary OH-PCA or its ratio to sodium (OH-PCA/Na$^+$) discriminated between DIGEST participants from the 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 ETL-G and DHBA. In the case for ETL-G, a MS/MS spectral match based on 3 characteristic product ions, including a neutral loss of a glucuronide is in close agreement with published data [60]. 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). ETL-G is a major phytoestrogen from dietary plant lignins, and is excreted in urine as its monoglucuronide conjugate following biotransformation by human intestinal bacteria [61]. 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 [62], which has been reported to possess putative anticancer, antioxidant and/or estrogenic activity [63]. DHBA is a major phenolic acid constituent from most cereals (*e.g.,* wheat, rye) [64], which can serve as a biomarker of dietary fiber intake allowing for differentiation of contrasting low and high (> 48 g/day) fiber diets [65]. In fact, urinary DHBA was the only biomarker differentially excreted at baseline that likely reflected modest differences in fiber intake between DIGEST participant sub-groups (Figure S5). Urinary Val-Val and DMG were also biomarkers related to a Prudent diet, but had weak correlations with only 2 nutrients (Table 3), whereas the artificial/low calorie sweetener ASK, and inorganic nitrate were not associated with any nutrients 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 [66]. In contrast, nitrate exposure has been reported to be mainly from vegetable consumption due to agricultural fertilizer usage [67] that is consistent with its increase in urine following a Prudent diet.

The major circulating ketone body, OH-BA and 2 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 [32], 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 having 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 [47,48]. 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 [68]. Plasma kVal and kLeu were also positively correlated ($r \approx 0.45$-0.50, $p < 0.004$) with key nutrients 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 [69]. The metabolism of branched-chain amino acids plays other critical roles in human health, including ammonia detoxification, protein biosynthesis and insulin sensitivity [70] while serving as predictive biomarkers of type 2 diabetes [71]. 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, 2 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, vegetables 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 [72]. 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* [73]. 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 2 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, and 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) [74]; however, plasma or urinary TMAO were not found to be 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 [75]. 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 [76]. 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, and a lower consumption of poly:sat, vitamins, and fruits and/or vegetables (Table 2). For example, 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* were among the most significant plasma FA that increased following 2 weeks of a Western diet. Indeed, high intake of omega-6 [77] and saturated [78] 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 [79]. 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 human health may likely be beneficial [80]. In contrast, greater consumption of processed foods containing vegetable oils rich in C18:2n-6*cis*, and other omega-6 fatty acids is likely a major dietary culprit for CVD prevalence in developed countries [81]. 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 [82]. These trends are consistent with data measured in our work, as fasting plasma concentrations of C18:2n-6*trans* were about 0.34% of its stereoisomer and most abundant FA 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 2 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, ETL-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 vitamins/electrolytes, 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 included ASK, nitrate, DMG and Val-Val, but they 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 center without strict dietary adherence monitoring, or recording of food preparation methods. 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 between participants. Also, certain dietary biomarkers tentatively identified in this study require further structural elucidation to confirm their exact stereoisomer configuration. The advent of high throughput and low cost metabolomics technologies allows for more accurate assessment of diet exposures applicable to large-scale epidemiological and clinical intervention studies. Overall, our work provides strong corroborating evidence of the utility of food exposure biomarkers to accurately differentiate complex dietary patterns in a free-living healthy population outside a laboratory setting. Urine offers a less invasive and more convenient biofluid than blood with a wider detection window for tracking dietary biomarkers in observational studies, which are often further biotransformed by host metabolism and gut microbiota. This is urgently needed for new

advances in nutritional epidemiology and chronic disease prevention relevant to cardiometabolic and neurodegenerative disorders that increasingly impact an aging population. Future work will also focus on assessing the impact of maternal nutrition on fetal development early in life, and its impact on obesity and metabolic syndrome risk in childhood.

Supplemental Material: The following are available online. Description of 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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