# Characterization of metabolic responses to healthy diets and association with blood pressure: application to the Optimal Macronutrient Intake Trial for Heart Health (OmniHeart), a randomized controlled study

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## ABSTRACT

**Background:** Interindividual variation in the response to diet is common, but the underlying mechanism for such variation is unclear.

**Objective:** The objective of this study was to use a metabolic profiling approach to identify a panel of urinary metabolites representing individuals demonstrating typical (homogeneous) metabolic responses to healthy diets, and subsequently to define the association of these metabolites with improvement of risk factors for cardiovascular diseases (CVDs).

**Design:** 24-h urine samples from 158 participants with prehypertension and stage 1 hypertension, collected at baseline and following the consumption of a carbohydrate-rich, a protein-rich, and a monounsaturated fat–rich healthy diet (6 wk/diet) in a randomized, crossover study, were analyzed by proton ($^1$H) nuclear magnetic resonance (NMR) spectroscopy. Urinary metabolite profiles were interrogated to identify typical and variable responses to each diet. We quantified the differences in absolute excretion of metabolites, distinguishing between dietary comparisons within the typical response groups, and established their associations with CVD risk factors using linear regression.

**Results:** Globally all 3 diets induced a similar pattern of change in the urinary metabolic profiles for the majority of participants (60.1%). Diet-dependent metabolic variation was not significantly associated with total cholesterol or low-density lipoprotein (LDL) cholesterol concentration. However, blood pressure (BP) was found to be significantly associated with 6 urinary metabolites reflecting dietary intake [proline-betaine (inverse), carnitine (direct)], gut microbial co-metabolites [hippurate (direct), 4-cresyl sulfate (inverse), phenylacetylglutamine (inverse)], and tryptophan metabolism [*N*-methyl-2-pyridone-5-carboxamide (inverse)]. A dampened clinical response was observed in some individuals with variable metabolic responses, which could be attributed to nonadherence to diet (≤25.3%), variation in gut microbiome activity (7.6%), or a combination of both (7.0%).

**Conclusions:** These data indicate interindividual variations in BP in response to dietary change and highlight the potential influence of the gut microbiome in mediating this relation. This approach provides a framework for stratification of individuals undergoing dietary management. The original OmniHeart intervention study and the metabolomics study were registered at www.clinicaltrials.gov as NCT00051350 and NCT03369535, respectively. *Am J Clin Nutr* 2018;107:323–334.

**Keywords:** diets, gut microbiome, hypertension, metabolic profiling, metabonomic, metabolomic, personalized health care

## INTRODUCTION

Of total global deaths, approximately half are attributed to cardiovascular diseases (CVDs), with elevated blood pressure (BP) being a key risk factor (1). Genome-wide association studies have identified common genetic variants associated with high BP (2) but these only account for a small proportion of the population variance in BP and do not take lifestyle factors such as physical inactivity or unhealthy diet into account. CVD remains the leading cause of mortality for noncommunicable diseases worldwide, even though the adoption of healthy dietary patterns such as those promoted by Dietary Approaches to Stop Hypertension (DASH) (3), Optimal Macronutrient Intake Trial for Heart Health (OmniHeart) (4), and Mediterranean diets (5) has unequivocally been shown to reduce CVD risk. Humans demonstrate substantial variation in response to dietary intervention, partially attributable to genetic heterogeneity (6, 7). For example, the apolipoprotein A-IV protein modulates cholesterol-lowering responses to high-fat diets (8, 9). However, supporting evidence for genetic influence on variable dietary responses remains conflicting (10) and modifiable factors such as changes in body weight (11, 12), or variation in the composition of the gut microbiome (13) and virome (14), have been implicated in variation in dietary responses.

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Metabolic phenotyping technologies provide a framework for investigating the influences of environmental and lifestyle factors on disease risk and have been successfully applied to investigate chronic diseases, e.g., diabetes (15). Systematic modulation of metabolism in response to food intake (16) has been reported and the impact of diet on a range of pathological conditions, including gastrointestinal cancer risk, has been assessed (17). Building on methodological approaches developed for characterizing interindividual variation in response to drug toxicity or therapies (18), we propose to demonstrate the feasibility of identifying interindividual variation in clinical response to 3 different healthy diets, using a $^{1}$H nuclear magnetic resonance (NMR)-based metabolic phenotyping approach, and establish the impact of this variation on CVD risk. We hypothesized that dietary change from a typical American diet to a healthy diet or between different healthy diets would result in typical changes in the urinary metabolic phenotypes for the majority of individuals, herein considered as the homogeneous dietary response (HDR) group. We ascertained that a minority of individuals demonstrated atypical dietary responses, herein referred to as the variable (heterogeneous or nonuniform) dietary response (VDR) group. We further hypothesized that these specific urinary dietary response phenotypes would be associated with BP. Variation in diet-specific biomarkers will further enhance our understanding of the link between variation in dietary response and the aetiopathogenesis of hypertension.

## METHODS

### OmniHeart study design

The OmniHeart study (N = 163) was a randomized, controlled, 3-period cross-over feeding study aiming to assess the effects of 3 healthy diets on BP and lipid profiles (19). The key findings and study design of the OmniHeart study have been previously published (4, 19). Briefly, all 3 OmniHeart diets had a similar nutrient composition to the established healthy DASH diet but varied in macronutrient composition. The Omniheart carbohydrate-rich diet (OmniCarb diet) provided 58% kcal from carbohydrate, 15% from protein, and 27% from fat; the remaining 2 diets replaced 10% of calories from carbohydrate with either protein, predominantly obtained from vegetable sources (OmniProt diet), or unsaturated fats, predominantly derived from monounsaturated fat (OmniMFA diet). Participants were randomly assigned to 1 of 6 possible orders of administration of the 3 diets, each intervention period lasting for 6 wk. During each intervention period, the participants were requested to only consume food prepared in the diet kitchen and were allowed to consume ≤2 alcoholic beverages/d and 3 noncaloric caffeinated beverages/d as part of the trial. Their main meal was consumed on-site on weekdays and all other meals were eaten at home. Participants completed a diary in which they indicated whether they had complied with the study food protocol during the feeding periods. During the screening visits and washout periods (≥2 wk), participants consumed their own food. The Willett food frequency questionnaire (20), administered by certified staff as a means to describe the usual food intake of participants during screening visits, indicated participants consumed a typical American diet at the outset of the study, corresponding to high intake of saturated fat, excessive refined sugar, and salt with low intake of fruit, vegetables, and omega-3-fat.

A total of 163 men and women aged 30–80 y from the Baltimore and Boston areas, with prehypertension (systolic BP of 120–139 mmHg and/or diastolic BP of 80–89 mmHg) or stage 1 hypertension (systolic BP of 140–159 mm Hg and/or diastolic BP of 90–99 mm Hg) and without diabetes or prior CVD, were recruited to the study. The minimum detectable, between-diet differences for primary (systolic BP) and secondary (diastolic BP, LDL cholesterol, HDL cholesterol, triglyceride, and total cholesterol) variables in the full cohort (n = 160) and in subgroups (n = 80 and 70) were at 80% and 90% power (2-sided alpha, $P = 0.05$).

The sample size of the trial (n = 160) was selected because it provided adequate power to detect between-diet differences in the primary outcome variables that have public health significance, both overall and in subgroups. Specifically, the minimum detectable effect size for systolic BP was <3 mmHg even in subgroups that comprised only 40% (n = 64) of participants. One individual completed just 1 dietary intervention period, and 4 individuals completed 2 intervention periods. The remaining 158 completed all 3 dietary interventions, provided four 24-h urine collections, and supplied anthropometric and sociodemographic metrics on CVD (**Supplemental Figure 1**). These four 24-h urine collections corresponded to the baseline screening visit and 1 at the end of each of the three 6-wk dietary interventions. NMR urine spectra for these 158 individuals were used for the analyses presented here. During the last 10 d of each dietary intervention period, a fasting blood specimen was obtained to measure lipid concentrations. BP was measured on 5 d by trained staff using the OMRON 907 device for those requiring a normal or large adult cuff, after participants had been seated for ≥5 min. The reported BP was based on the average of 9 BP measurements taken at screening visits and 15 measurements taken at the last 5 visits of each feeding period. Body weight for all participants was maintained within 2% of their baseline throughout the study period by adjusting caloric levels each weekday. Baseline sociodemographic and anthropometric characteristics were obtained for each participant. Institutional ethics committee approval was obtained for each site and all participants provided written informed consent.

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Supplemental Figures 1–4 and Supplemental Tables 1–5 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: BP, blood pressure; CVD, cardiovascular disease; DASH, Dietary Approaches to Stop Hypertension; HDR, homogeneous dietary response; mOPLSDA, multilevel orthogonal partial least squares discriminant analysis; NIHR, National Institute for Health Research; NMR, nuclear magnetic resonance; OmniCarb, OmniHeart carbohydrate-rich diet; OmniHeart, Optimal Macronutrient Intake Trial for Heart Health; OmniMFA, OmniHeart monounsaturated fat–rich diet; OmniProt, OmniHeart protein-rich diet; SHOCSY, statistical homogeneous cluster spectroscopy; VDR, variable dietary response.

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### NMR-based metabolic phenotyping and data processing

Urine specimens were analyzed by 600 MHz $^1$H NMR spectroscopy using a Bruker NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) according to a standard protocol (21) in our London metabolic phenotyping laboratory. Urine specimens were allowed to thaw at room temperature and centrifuged at 12,000 g for 5 min to remove particulates. For each specimen, 500 $\mu$L of urine was mixed with 250 $\mu$L of phosphate buffer solution at pH 7.4 $\pm$ 0.1. The resulting mixtures were left to stand for 10 min and then further centrifuged as before. A total volume of 500 $\mu$L of the supernatant was added to 50 $\mu$L of sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)-1-propionate (TSP) in deuterium oxide, giving a final concentration of 1 mM. This solution was transferred to a 5-mm NMR tube. The prepared urine specimens were placed in the auto-sampler and analyzed in a simple randomized order generated by computer. A 1-dimensional (1D) pulse sequence with a water saturation method (recycle delay–90°–$t_1$–90°–$t_m$–90°–acquisition) was used to acquire standard $^1$H NMR spectra of the urine. The spectra were acquired with 64K data points and 128 scans over a spectral width of 12 kHz. The recycle delay was set to 2 s with a mixing time ($t_m$) of 100 ms and a $t_1$ of 20 μs, providing an acquisition time of approximately 2.72 s. All $^1$H NMR spectra were phased, baseline corrected, and manually referenced to TSP at $\delta$ 0 with Topspin software (version 2.1, Bruker Biospin) prior to multiplication by an exponential weighting function corresponding to a line broadening of 0.3 Hz. The spectral regions containing the water ($\delta$ 4.5–5.05) and urea ($\delta$ 5.5–6.5) resonances, as well as the extreme ends ($<\delta$ 0.7 and $>\delta$ 9.5) of the spectra that contain minimal metabolic information, were removed. Initial analysis showed that the signal arising from the $–CH_2$ and $–CH_3$ group of the creatinine peaks dominated the analysis due to the high concentration of creatinine compared to other metabolites. Since there was no statistical difference in the clinical creatinine measurements at screening visit and at the end of each study period based on Jaffé reaction measurement ($P > 0.5$ for all comparisons between each diet and the baseline), we removed the creatinine regions containing the peaks at $\delta$ 3.035–3.062 and $\delta$ 4.052–4.075 from all subsequent analysis. A total of 23,998 NMR data variables, at a full resolution (0.0003 ppm), were then normalized by a probabilistic quotient method (22) using the median spectrum of the whole data-set as a reference and subsequently scaled to unit-variance.

### Data analysis

We applied Statistical HOmogeneous Cluster SpectroscopY (SHOCSY) (23) to the processed and normalized spectroscopic data. SHOCSY is a variant of statistical spectroscopic techniques such as Subset Optimization by Reference Matching (STORM) (24) and Statistical TOtal Correlation SpectroscopY (STOCSY) (25). SHOCSY involves clustering of the spectral data based on the similarity or dissimilarity of the spectral features followed by the association of clusters to different dietary groups using an enrichment test. The application of SHOCSY enables identification of the groups of spectra showing uniform or homogeneous urinary metabolic responses (HDR) and those showing variation from the coherent metabolic response (VDR) following the consumption of different OmniHeart diets. Due to the nature of the cross-over study design, we employed multilevel orthogonal partial least squares discriminant analysis (mOPLSDA) (26, 27), which incorporates the variation between and within participants in the data-set to optimize visualization of dietary response, in conjunction with SHOCSY. We performed this in a pairwise fashion, comparing the urinary spectral data from the screening visit (reflecting a basal dietary pattern) with those from the end of each dietary intervention, and modelled this separately for the urinary spectral data corresponding to an HDR (3 models, 1 model/diet) and those representing a VDR (3 models). Thus, each subgroup was compared to its own baseline. We also performed comparison between different OmniHeart diets and separately for the HDR (3 models) and VDR (3 models) groups, creating a total of 12 different mOPLSDA models (**Supplemental Table 1**). Each mOPLSDA comparison was validated using a 7-fold cross-validation procedure. The model statistics, $Q^2$Yhât (28), is defined as the proportion of variance in the data predicted by the mOPLSDA model and is therefore a measure of the robustness of the model. In addition, permutation testing was performed by randomly assigning classes to the samples and remodeling repeatedly 100 times. The $Q^2$Yhât statistic for the real model was then compared to the null hypothesis distribution obtained from the permuted $Q^2$Yhât *t* values and was considered significant when the *P* value of the real $Q^2$Yhât was $<0.05$ on those permuted values.

The 3 criteria used to identify discriminatory metabolites were: (i) *P* values of the correlations between the spectral variable and the mOPLSDA scores vector should be $<1.85 \times 10^{-6}$ (corresponding to $P < 0.05$ after Sidák correction); (ii) a variable loading coefficient strength, $r^2 > 0.3$ as defined in Zou et al. (23); and (iii) the stability of the NMR variables, whereby a data point was considered significant when flanked by 2 NMR spectral variables conforming to criteria (i) and (ii). For peaks that were free from spectral overlap, the 24-h urinary excretion of each discriminatory metabolite was quantified by integration of the NMR signal intensities. Since we found no significant difference in the excretion of creatinine between different OmniHeart diets and the typical American diet ($P > 0.5$), the absolute excretion of each discriminatory metabolite was normalized to the corresponding 24-h urinary creatinine excretion (in mmol/L). The difference in absolute excretion of each discriminatory metabolite was determined for the comparison of each dietary intervention with baseline or between different OmniHeart dietary interventions. The association between the differences in absolute excretion of each discriminatory metabolite and changes in CVD risk factors (systolic and diastolic BP, LDL, total cholesterol) was established using linear regression for HDR groups. In addition, known covariates for hypertension, including urinary excretion of sodium, potassium, calcium, and phosphate, were also established for the HDR and VDR groups for the comparisons between baseline and each OmniHeart diet. The statistical significance of these covariates was adjusted by Bonferroni correction (0.05 divided by number of comparisons) to account for multiple testing. All analyses were performed using in-house software written in Matlab (version 2012a, MathWorks, Natick, MA).

### Identification of discriminatory metabolites

The discriminatory metabolites found to be significantly influenced by the healthy dietary interventions were confirmed by in-house and published database (29) references and

authenticated by spiking in standard compounds purchased from Sigma Aldrich. These compounds included: *N*-methyl-2-pyridone-5-carboxamide, 4-hydroxyphenylacetic acid, carnitine, creatine, dimethylglycine, *S*-methyl-L-cysteine-*S*-oxide, *N*-methyl nicotinic acid, *N*-methyl nicotinamide, proline-betaine, and hippurate. For the remaining urinary metabolites where they were not available commercially, identification was achieved using further analytical methods such as 2-dimensional NMR experiments, solid phase extraction chromatography experiments coupled with NMR, ultra-performance liquid chromatography coupled to mass spectroscopy, and statistical analysis such as STORM (24) as well as using published databases and/or literature.

## RESULTS

### Individuals show variation in urinary metabolic phenotypes on OmniHeart diets

Participants' demographics and changes in CVD risk factors following each OmniHeart diet are provided in **Table 1**. Each diet elicited a range of clinical responses over the 6-wk study in terms of reduction of CVD risk factors, which was reflected in the urinary metabolome. Interindividual differences in dietary response were observed; the majority of the participants showed a HDR to all of the OmniHeart diets when compared to the baseline profile: 71.5% ($n = 113$) for OmniProt, 80.4% ($n = 127$) for OmniMFA, and 86.7% ($n = 137$) for OmniCarb. The remaining individuals who did not demonstrate a "typical" response to a given diet were grouped into the VDR class: $n = 45$ for OmniProt, $n = 31$ for OmnMFA, and $n = 21$ for OmniCarb. A similar modeling strategy was applied to compare between pairs of OmniHeart diets. We found >70% participants showed consistent metabolic differences between diets (Supplemental Table 1).

### OmniHeart diets show distinctive urinary metabolic phenotypes

Each of the 3 OmniHeart diets was associated with a distinct metabolic phenotype in the majority of participants (the HDR group). For the OmniHeart–baseline comparisons, the discriminatory metabolites were predominantly related to: (i) dietary intake—increased excretion of proline-betaine, *N*-acetyl-S-methyl-L-cysteine sulfoxide, *S*-methyl-L-cysteine-*S*-oxide, creatine, and carnitine; (ii) tryptophan–NAD degradation—reduced excretion of *N*-methyl-2-pyridone-5-carboxamide and *N*-methyl nicotinamide, and increased excretion of *N*-methyl nicotinic acid; and (iii) gut microbial mammalian metabolism—increased excretion of hippurate and dimethylglycine, and reduced excretion of 4-hydroxyphenylacetic acid (Supplemental Table 2). Compared to the baseline profiles, proline-betaine was the only metabolite uniformly increased in the urinary phenotypes of HDR groups across all 3 diets, consistent with increased citrus fruit consumption (30). Increased excretion of carnitine and creatine in the OmniProt diet reflected the increase in protein intake (31).

Additional pairwise comparisons ($P < 10^{-5}$) between different OmniHeart diets further indicated that each diet was associated with a distinct metabolic phenotype. The HDR group of the OmniProt diet was generally characterized by higher excretion of urinary creatine, *N*-methyl-2-pyridone-5-carboxamide and 2 gut microbial mammalian co-metabolites, phenylacetylglutamine, and 4-cresyl sulfate compared to the other 2 OmniHeart diets; whilst the HDR group for the OmniCarb diet consistently showed higher excretion of hippurate and guanodinoacetate (**Supplemental Tables 3** and **4**). The differences in the markers for dietary intake of cruciferous vegetables (*S*-methyl-L-cysteine-*S*-oxide and *N*-acetyl-S-methyl-L-cysteine sulfoxide) (32) and markers for citrus fruit intake (proline-betaine) (30) observed when comparing urine of OmniHeart diets with the baseline profiles, were generally not observed for pairwise comparisons between the OmniHeart diets since all 3 diets included higher proportions of fruits or vegetables than the baseline.

### Urinary metabolites significantly associated with BP

We quantified 10 discriminatory metabolites altered in response to 1 or more OmniHeart diets and assessed their associations with BP and lipid profiles using the HDR groups only. Although no significant associations were found between dietary phenotypes and LDL or total cholesterol, we found significant associations between 2 of these food-related metabolites and BP. Proline-betaine was inversely associated with systolic and diastolic BP for OmniCarb and OmniMFA diets when compared to baseline ($P < 0.05$, **Table 2**). A similar trend was observed for the OmniProt diet although it was not statistically significant. A direct association was found between systolic BP and carnitine for the OmniProt diet when compared to baseline ($P < 0.05$). We found 3 metabolites related to host-gut microbial pathways that were significantly associated with BP (hippurate, phenylacetylglutamine, and 4-cresyl sulfate). Hippurate showed a direct association with systolic BP ($P < 0.001$) and diastolic BP ($P < 0.01$) levels for the OmniCarb diet compared to baseline, whereas 4-cresyl sulfate and phenylacetylglutamine (distal colonic microbial metabolites of tyrosine and phenylalanine, respectively) were inversely associated with BP for the comparison between OmniMFA and OmniProt diets. *N*-methyl-2-pyridone-5-carboxamide (tryptophan–NAD metabolite) was also found to be inversely associated with systolic and diastolic BP levels for the OmniCarb–baseline comparison ($P < 0.05$). These data demonstrate healthy diets can elicit coherent changes in the urinary metabolic phenotypes for the majority of individuals and that some of these metabolites are either directly or inversely associated with BP.

### Urinary metabolic phenotypes can identify nonadherence to diets

The urinary spectral data for the VDR groups for each of the OmniHeart diets typically produced fewer dietary-specific discriminatory metabolites than the HDR groups (Supplemental Tables 2 and 3). The VDR groups also showed discordance in the concentrations of proline-betaine and hippurate when compared to the HDR groups. Since increased consumption of citrus fruits was a feature of all dietary interventions, we therefore classified individuals with a lower concentration of proline-betaine (a direct marker of citrus fruit intake) (33, 34) as nonadherent to these diets on the assumption that this was generally indicative of dietary behavior. We found the majority of participants in the VDR groups excreted lower 24-h urinary

**TABLE 1**
Characteristics of participants who completed all 3 OmniHeart diets ($n = 158$)[1]

| Characteristics | | *P* values |
|---|---|---|
| Age, mean ± SD | 53.1 ± 10.8 | |
| Ethnicity, *n* (%) | | |
| African American | 86 (54.4) | |
| Non-African American | 72 (45.6) | |
| Gender, *n* (%) | | |
| Male | 88 (55.7) | |
| Female | 70 (44.3) | |
| Hypertension, *n* (%) | | |
| Prehypertension | 127 (80.4) | |
| Hypertension | 31 (19.6) | |
| Obesity status, *n* (%) | | |
| Normal range | 32 (20.3) | |
| Overweight | 53 (33.5) | |
| Obese | 73 (46.2) | |
| Smoking, *n* (%) | | |
| Current | 18 (11.4) | |
| Former | 42 (26.6) | |
| Never | 98 (62) | |
| Alcohol intake | | |
| No alcohol, *n* (%) | 88 (56) | |
| Serving per week among drinkers, mean ± SD | 4.17 ± 3.5 | |
| Education, *n* (%) | | |
| ≤ High school | 32 (20.3) | |
| Some college | 53 (33.5) | |
| College graduate | 73 (46.2) | |
| Mean changes in SBP from baseline (95% CI), mm Hg | | |
| OmniCarb diet | –8.0 (–9.4, –6.6) | ‡ |
| OmniMFA diet | –9.4 (–10.7, –8.1) | ‡ |
| OmniProt diet | –9.4 (–10.8, –8.1) | ‡ |
| Mean changes in DBP from baseline (95% CI), mm Hg | | |
| OmniCarb diet | –4.1 (–4.9, –3.3) | ‡ |
| OmniMFA diet | –4.9 (–5.7, –4.1) | ‡ |
| OmniProt diet | –5.3 (–6.1, –4.4) | ‡ |
| Mean changes in LDL cholesterol from baseline (95% CI), mg/dL | | |
| OmniCarb diet | –11.6 (–14.6, –8.6) | ‡ |
| OmniMFA diet | –13.2 (–16.5, –9.9) | ‡ |
| OmniProt diet | –14.4 (–17.7, –11.1) | ‡ |
| Mean changes in HDL cholesterol from baseline (95% CI), mg/dL | | |
| OmniCarb diet | –1.5 (–2.6, –0.3) | * |
| OmniMFA diet | –0.4 (–1.4, 0.6) | |
| OmniProt diet | –2.7 (–3.7, –1.7) | † |
| Mean changes in triglyceride from baseline (95% CI), mg/dL | | |
| OmniCarb diet | –0.2 (–9.1, 8.7) | |
| OmniMFA diet | –9.7 (–17.9, –1.5) | * |
| OmniProt diet | –16.5 (–25.8, –7.3) | * |
| Mean changes in total cholesterol from baseline (95% CI), mg/dL | | |
| OmniCarb diet | –12.5 (–15.8, –9.1) | ‡ |
| OmniMFA diet | –15.6 (–19.2, –11.9) | ‡ |
| OmniProt diet | –20.2 (–23.7, –16.7) | ‡ |

[1] *T* test comparison between baseline clinical data and after each dietary intervention: $^{*}P < 0.05$; $^{\dagger}P < 10^{-5}$; $^{\ddagger}P < 10^{-10}$. DBP, diastolic blood pressure; OmniCarb, OmniHeart carbohydrate-rich diet; OmniHeart, Optimal Macronutrient Intake Trial for Heart Health; OmniMFA, OmniHeart monounsaturated fat-rich diet; OmniProt, OmniHeart protein-rich diet; SBP, systolic blood pressure.

concentrations of proline-betaine when compared to the HDR groups. Fifteen of the 21 individuals (71.4%) from the OmniCarb-VDR group showed a 24-h urinary excretion of less than the lower 95% CI obtained for proline-betaine excretion of the OmniCarb-HDR group. A similar trend was observed for the OmniMFA-VDR (21/31, 67.7%) and OmniProt-VDR (35/45, 77.8%) groups. The overall estimation of nonadherence to each diet was: 9.5% (n = 15) for the OmniCarb, 13.3% (n = 21) for the OmniMFA, and 22.2% (n = 35) for the OmniProt diet. Despite sub-classification of VDR groups as adherent or nonadherent, contrasting patterns remained in the VDR and HDR groups, as exemplified for hippurate (a gut microbial co-metabolite of dietary phenols), where increased excretion of hippurate was characteristic for the HDR but not either of the VDR (diet adherent or

**TABLE 2**
Estimated mean differences in CVD risk factors[1]

| Urinary metabolites | 2 SD excretion (mmol/L) | SBP (mm Hg) | DBP (mm Hg) | LDL-C (mg/dL) | Total cholesterol (mg/dL) |
|---|---|---|---|---|---|
| Homogeneous dietary responders for OmniCarb diet vs. baseline ($n = 137$) | | | | | |
| Proline-betaine | 1.25 | –4.10 (–2.90) † | –1.77 (–2.15)* | –3.90 (–1.26) | –3.94 (–1.17) |
| Hippurate | 3.47 | 6.14 (4.64) ‡ | 2.27 (2.79) † | –1.47 (–0.48) | 2.70 (0.80) |
| *N*-methyl-2-pyridone-5-carboxamide | 0.21 | –3.03 (–2.24)* | –1.77 (–2.19)* | 2.22 (0.75) | 3.49 (1.11) |
| *N*-methyl nicotinic acid | 0.27 | –0.20 (–0.14) | 0.59 (0.71) | –1.94 (0.64) | –0.25 (–0.07) |
| *N*-methyl nicotinamide | 0.03 | –0.86 (–0.60) | –0.75 (–0.97) | 0.60 (0.20) | –0.55 (0.17) |
| Homogeneous dietary responders for OmniMFA vs. baseline ($n = 127$) | | | | | |
| Proline-betaine | 0.87 | –3.53 (–2.76) † | –1.73 (–2.20)* | 2.41 (0.73) | 1.87 (0.48) |
| Homogeneous dietary responders for OmniProt vs. baseline ($n = 113$) | | | | | |
| Proline-betaine | 0.74 | –1.16 (0.74) | 0.14 (0.16) | 1.31 (0.33) | –1.42 (–0.33) |
| Carnitine | 0.29 | 3.11 (1.99)* | 1.13 (1.38) | 0.45 (0.11) | 1.03 (0.24) |
| Creatine | 1.62 | 1.54 (1.05) | –1.19 (–0.24) | –4.34 (–1.11) | –6.87 (–1.65) |
| Homogeneous dietary responders for OmniCarb vs. OmniMFA ($n = 113$) | | | | | |
| Guanodinoacetate | 0.95 | 0.29 (0.35) | 0.06 (0.09) | –0.51 (–0.21) | 2.70 (0.94) |
| Homogeneous dietary responders for OmniCarb vs. OmniProt ($n = 134$) | | | | | |
| Phenylacetylglutamine | 0.70 | –0.89 (–0.96) | –0.93 (–1.42) | –2.82 (–1.18) | –0.44 (–0.15) |
| 4-cresyl sulfate | 0.27 | 0.06 (0.07) | 0.73 (1.11) | –0.91 (–0.79) | 1.02 (0.35) |
| Homogeneous dietary responders for OmniMFA vs. OmniProt ($n = 118$) | | | | | |
| Phenylacetylglutamine | 0.68 | –1.7 (–1.93) | –1.89 (–3.32) † | 0.31 (0.13) | –0.33 (–0.14) |
| 4-cresyl sulfate | 0.30 | –2.68 (–3.05) † | –2.15 (–3.74) † | 0.73 (0.31) | –0.27 (–0.11) |

[1]The SBP, DBP, LDL-C, and total cholesterol mean differences per 2-SD increase in absolute excretion for the comparison between baseline and post-OmniHeart diets and between different OmniHeart diets for the HDR groups. The correlations between changes in metabolites and CVD factors were evaluated by linear regression. 2 SD excretion of each urinary metabolite was calculated by the absolute differences between dietary comparisons. Numbers in parenthesis are $z$ scores, i.e., regression coefficient divided by standard error ($z$ score $\geq$ 1.96, $P < 0.05$; $\geq$2.58, $P < 0.01$; $\geq$3.89, $P < 0.001$). NMR chemical shifts (multiplicity) used for quantification: proline-betaine, $\delta$3.11 (singlet); hippurate, $\delta$7.64 (triplet); *N*-methyl-2-pyridone-5-carboxamide, $\delta$6.67 (doublet); *N*-methyl nicotinic acid, $\delta$4.44 (singlet); *N*-methyl nicotinamide, $\delta$8.89 (triplet); carnitine, $\delta$3.23 (singlet); creatine, $\delta$3.93 (singlet); guanodinoacetate, $\delta$3.80 (singlet); phenylacetylglutamine, $\delta$7.43 (triplet); 4-cresyl sulfate, $\delta$2.35 (singlet). CVD, cardiovascular disease; DBP, diastolic blood pressure; LDL-C, LDL cholesterol; NMR, nuclear magnetic resonance; OmniCarb, OmniHeart carbohydrate-rich diet; OmniHeart, Optimal Macronutrient Intake Trial for Heart Health; OmniMFA, OmniHeart monounsaturated fat–rich diet; OmniProt, OmniHeart protein-rich diet; SBP, systolic blood pressure.

*$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

nonadherent) subgroups for OmniCarb. Differential metabolite patterns were also observed for different subgroups within the OmniMFA (**Figure 1**).

### Urinary metabolic variation reflects interindividual differences in clinical responses

Discarding the nonadherent VDR group, we assessed the effect of each diet, stratified by HDR group compared to adherent-VDR group, on urinary electrolyte concentrations. We found significant overall changes in mean urinary sodium (decrease) and mean urinary potassium (increase) in the HDR groups for all OmniHeart diets when compared to baseline values (**Supplemental Table 5**). The mean changes in urinary electrolytes were of slightly greater magnitude when considering the subset of prehypertensive individuals within the HDR groups for sodium: –31.3 mmol/d (OmniCarb), –44.9 mmol/d (OmniMFA), and –35.9 mmol/d (OmniProt); and potassium 26.4 mmol/d (OmniCarb), 28.4 mmol/d (OmniMFA), and 24.7 mmol/d (OmniProt), $P < 0.001$ (data not shown). This general trend in mean urinary sodium and potassium levels was apparent for the adherent-VDR groups but the changes from baseline level were insignificant. With regard to the intercomparison between OmnniHeart diets, no systematic differences were observed in the electrolyte levels with the exception of higher urinary sodium and phosphate levels being characteristic of the OmniProt-HDR when compared to the OmniMFA-HDR group ($P < 0.01$, data not shown). No systematic differences in electrolytes were expected as micronutrients such as potassium, sodium, calcium, and magnesium were indexed to the energy level from the diet for each participant (19).

We also investigated the changes in CVD risk factors post-diet and found a significant ($P < 10^{-10}$) reduction in all HDR diet groups when compared to the baseline for systolic and diastolic BP, LDL cholesterol, and total cholesterol. Additionally, the reduction in serum triglyceride concentrations was significant for the OmniProt-HDR group; and HDL cholesterol for the OmniCarb-HDR and OmniProt-HDR groups, $P < 0.05$ (**Figure 2**). High-risk individuals such as those who were hypertensive or those with nonoptimal lipid profiles in the HDR groups showed greater reduction in these CVD risk factors than low-risk individuals (**Supplemental Figure 2**). For all the VDR groups, a dampened reduction in CVD risk factors was generally observed when compared to the corresponding HDR comparator groups (Figure 2). A significant ($P < 0.05$) reduction in systolic and diastolic BP was observed in both the adherent- and nonadherent-OmniMFA-VDR and the non-adherent-OmniProt-VDR groups; whilst the adherent- and

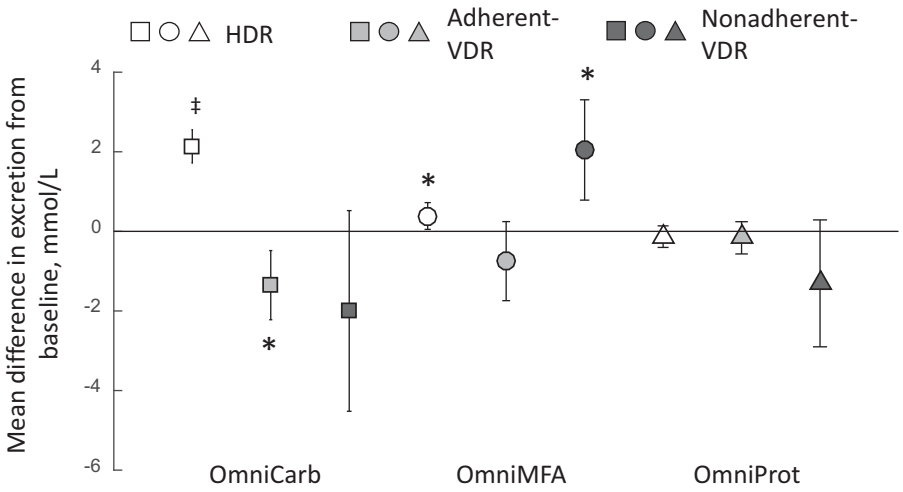


**FIGURE 1** The observed mean differences in excretion for hippurate between HDR and adherent- and nonadherent-VDR groups when OmniHeart diets and their corresponding baseline spectra were compared. Open square, OmniCarb-HDR ($n = 137$); light-grey closed square, adherent-OmniCarb-VDR ($n = 6$); dark-grey closed square, nonadherent-OmniCarb-VDR ($n = 15$); open circle, OmniMFA-HDR ($n = 127$); light-grey closed circle, adherent-OmniMFA-VDR ($n = 10$); dark-grey closed circle, nonadherent-OmniMFA-VDR ($n = 21$); open triangle, OmniProt-HDR ($n = 113$); light-grey closed triangle, adherent-OmniProt-VDR ($n = 10$); dark-grey closed triangle, nonadherent-OmniProt-VDR ($n = 35$). Error bars indicate 95% CIs. Significant *t* test comparison between baseline and post OmniHeart diets: $^{*}P < 0.05$; $^{\ddagger}P < 10^{-10}$. HDR, homogeneous dietary response; OmniCarb, OmniHeart carbohydrate-rich diet; OmniHeart, Optimal Macronutrient Intake Trial for Heart Health; OmniMFA, OmniHeart monounsaturated fat–rich diet; OmniProt, OmniHeart protein-rich diet; VDR, variable dietary response.

non-adherent-OmniProt-VDR groups also generally showed significant reductions for LDL, HDL, and total cholesterol although the magnitude of the change in CVD risk factors was generally more variable than that observed for the corresponding HDR groups. The observed lack of dietary-induced clinical benefit in the adherent-VDR groups may be partially due to the reduced sample size ($n < 10$) following stratification of the cohort. In addition to the observation that HDR groups of all 3 OmniHeart diets generally elicited a reduction in CVD risk factors when compared to typical American diets, we also found the HDR-OmniProt group generally showed a larger overall reduction in CVD risk factors when compared to the HDR-OmniMFA and HDR-OmniCarb groups (**Supplemental Figure 3**).

### Stratification of individual response based on urinary metabolic phenotypes

From a cohort of 158 individuals, who partook in all 3 dietary interventions, we were able to stratify individuals according to diet-response specific urinary phenotypes: corresponding to those who demonstrated: HDR to all 3 diets ($n = 95$, 60.1%; Group 1); HDR to 2 diets but VDR to 1 diet ($n = 35$, 22.2%; Group 2); HDR to only 1 diet but VDR to 2 diets ($n = 22$, 13.9%; Group 3); non-adherent-VDR to all 3 diets ($n = 4$, 2.5%; Group 4); and mix of nonadherent- and adherent-VDR to all 3 diets ($n = 2$, 1.3%; Group 5). Moreover, we were able to further sub-stratify individuals in the VDR groups that demonstrated a dampened clinical response into those participants that were: (a) adherent to diets but showed differences in metabolic phenotypes from the majority of participants (including gut-microbial co-metabolites; $n = 12$, 7.6%); (b) nonadherent to ≥1 diet ($n = 40$, 25.3%); or (c) a combination of the 2 ($n = 11$, 7.0%); see **Table 3**. We found that individuals consistently classified as HDR for all 3 OmniHeart diets generally manifested a greater reduction in CVD risk factors than those that were classified as HDR for just 1 or 2 of the OmniHeart diets (**Supplemental Figure 4**).

## DISCUSSION

We show that the majority, but not all, of the participants responded similarly in terms of their expressed metabolic phenotype to a particular diet and that each of the 3 diets had a distinct effect on the metabolism. However, regardless of the macronutrient differences between the 3 OmniHeart diets and the diet-specific impact on the metabolic profile, the majority of participants (60.1%), demonstrated post-diet improvement in clinical risk factors for CVD. We applied an agnostic multivariate statistical tool to identify participants who showed a coherent biochemical response (HDR) to each of the diets and subdivided the data-set into high- and low-risk individuals based on their BP status or lipid profiles. Although both groups demonstrated a coherent biochemical response irrespective of the CVD risk status, the high-risk groups generally demonstrated a larger reduction in CVD risk factors than low-risk individuals. Our results thus demonstrate that manipulation of dietary macronutrient content, without alteration of caloric intake and body weight, can elicit coherent changes in metabolic profiles and contribute to beneficial effects on both BP levels and lipid profiles.

Notably, we identified 2 gut microbial-host co-metabolites associated with BP: phenylacetylglutamine and 4-cresyl sulfate, deriving from phenylalanine and tyrosine, respectively, resulting from bacterial putrefaction of protein in the distal colon. The gut microbiota, in particular Firmicutes and Bacteroidetes, can adapt to dietary changes and induce changes in host metabolism (35): an increase of Firmicutes-to-Bacteroidetes ratio has been demonstrated in spontaneous hypertensive rats (36). Other researchers

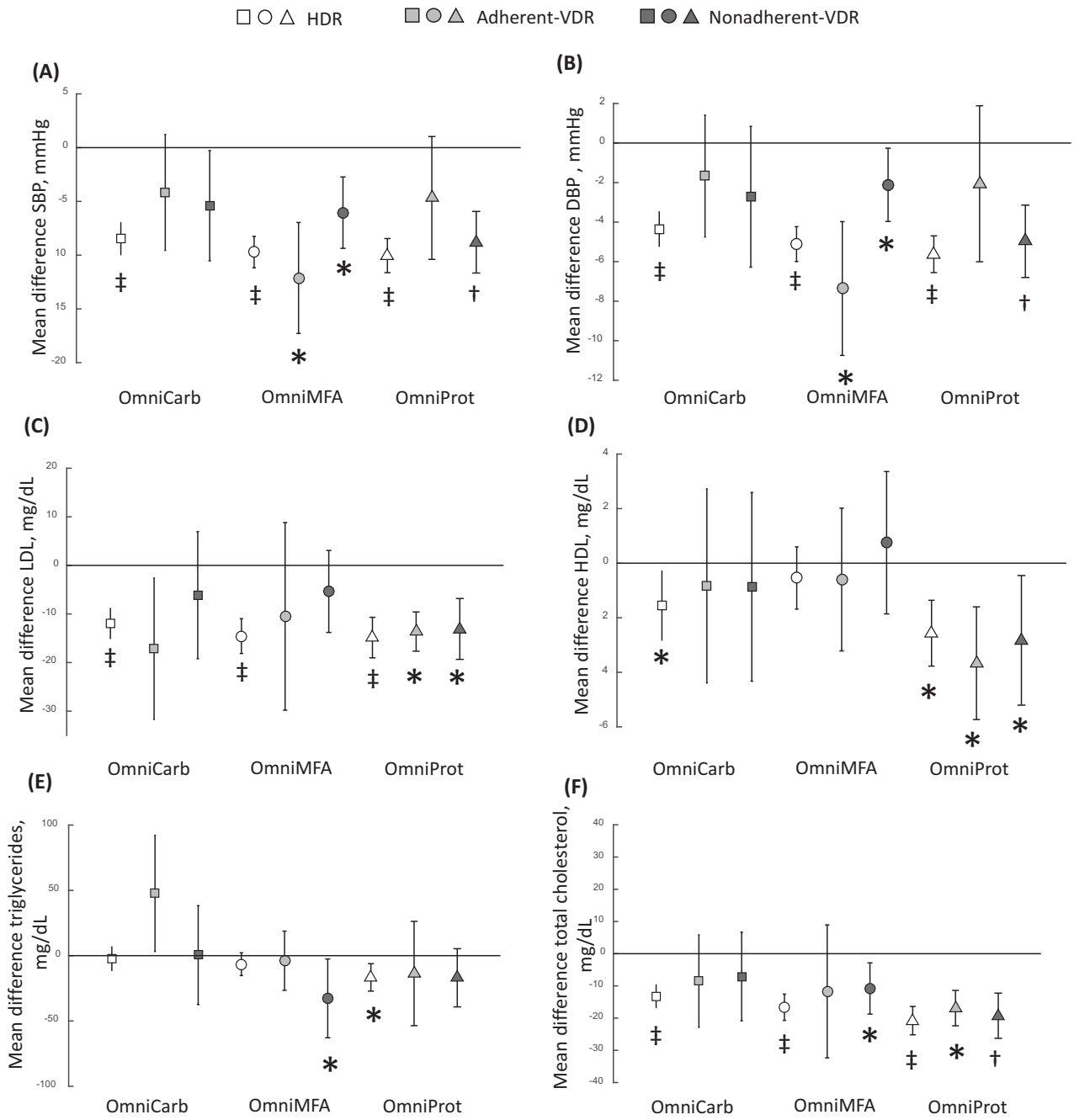


**FIGURE 2** Key observations for changes in the cardiovascular disease risk factors showing differences in HDR and VDR groups for the comparisons between each OmniHeart diet and baseline corresponding to the changes in systolic BP (A), diastolic BP (B), LDL cholesterol (C), HDL cholesterol (D), triglycerides (E), and total cholesterol (F). Open square, OmniCarb-HDR ($n = 137$); light-grey closed square, adherent-OmniCarb-VDR ($n = 6$); dark-grey closed square, nonadherent-OmniCarb-VDR ($n = 15$); open circle, OmniMFA-HDR ($n = 127$); light-grey closed circle, adherent-OmniMFA-VDR ($n = 10$); dark-grey closed circle, nonadherent-OmniMFA-VDR ($n = 21$); open triangle, OmniProt-HDR ($n = 113$); light-grey closed triangle, adherent-OmniProt-VDR ($n = 10$); dark-grey closed triangle, nonadherent-OmniProt-VDR ($n = 35$). Error bars indicate 95% CIs. Missing data include LDL cholesterol ($n = 2$ for OmniMFA-VDR, OmniProt-VDR, OmniMFA-HDR, OmniProt-HDR and $n = 3$ for OmniCarb-HDR), HDL cholesterol ($n = 1$ for OmniMFA-VDR and OmniProt-VDR), triglycerides ($n = 1$ for OmniMFA-VDR and OmniProt-VDR), and total cholesterol ($n = 1$ for OmniMFA-VDR and OmniProt-VDR). Significant $t$ test comparison between baseline and post-OmniHeart diets: $^{*}P < 0.05$; $^{\dagger}P < 10^{-5}$; $^{\ddagger}P < 10^{-10}$. DBP, diastolic blood pressure; HDR, homogeneous dietary response; OmniCarb, OmniHeart carbohydrate-rich diet; OmniHeart, Optimal Macronutrient Intake Trial for Heart Health; OmniMFA, OmniHeart monounsaturated fat–rich diet; OmniProt, OmniHeart protein-rich diet; VDR, variable dietary response.

**TABLE 3**
Stratification by urinary phenotypes[1]

| Summary of sub-phenotypes | N | % |
|---|---|---|
| Group 1: HDR to all 3 diets | 95 | 60.1 |
| Group 2: HDR to 2 diets but VDR to 1 diet | 35 | 22.2 |
| *(a) Nonadherent-VDR to the other diet* | *25* | *15.8* |
| *(b) Adherent-VDR to the other diet* | *10* | *6.3* |
| Group 3: HDR to 1 diet but VDR to 2 diets | 22 | 13.9 |
| *(a) Nonadherent-VDR to the other 2 diets* | *11* | *7.0* |
| *(b) Adherent-VDR to the other 2 diets* | *2* | *1.2* |
| *(c) Mixed response—nonadherence-VDR to 1 diet and adherent-VDR to the other diet* | *9* | *5.7* |
| Group 4: Nonadherent-VDR to all 3 diets | 4 | 2.5 |
| Group 5: Mix of nonadherent and adherent to all 3 diets | 2 | 1.3 |

[1]Individuals were stratified based on diet-specific urinary phenotypes. HDR, homogeneous dietary response; VDR, variable dietary response.

have manipulated gut microbiota balance via probiotic administration with consequent beneficial effects on BP levels (37). More recently, blood concentrations of phenylacetylglutamine were found to be strongly anti-correlated with BP, consistent with our results, and with carotid-femoral pulse-wave velocity, a measure of aortic stiffness (38). Although 4-cresyl sulfate has never been formally linked to BP, its dietary excretion has been shown to be highly correlated with that of phenylacetylglutamine (16).

The association between gut-microbial co-metabolites and BP is further evidenced in the direct association we found between BP and hippurate, originating from the conversion of benzoic acid by gut microflora via the shikimate pathway (39). In contrast to our results, hypertensive rats showed an anti-correlation between hippurate and BP (40) but interpolation from animal data to human must be performed with care due to the differences in gut microbiomes between species. An inverse association between excretion of hippurate and BP has been reported in humans but this association was not significant after adjusting for BMI, alcohol intake, and urinary excretion of sodium and potassium (41). A controlled feeding study by Wu et al. (35) showed that changes in the gut microbiome occurred within 24 h of initiating a change in diet and that BMI and weight loss can also influence the gut microbiome composition. However, in our dietary intervention study, all participants consumed a consistent healthy dietary pattern for 6 wk and maintained their body weight, with micronutrients being indexed to the energy level of their diets. Our data, therefore, suggest modulation of diets can affect gut microbiome activity and that this may lead to a direct effect on BP regulation.

We observed an inverse association of *N*-methyl-2-pyridone-5-carboxamide (tryptophan–NAD metabolite) and BP. Bartus et al. (42) showed that ingestion of 1-methylnicotinamide in hypertriglyceridemic rats resulted in an increase of 1-methylnicotinamide and its metabolites such as *N*-methyl-2-pyridone-5-carboxamide and found that ingestion of 1-methylnicotinamide in both the diabetic and hypertriglyceridemic rats can ameliorate the nitric oxide-dependent vasodilation, a surrogate marker for atherosclerosis. Others have found that 1-methylnicotinamide demonstrates anti-thrombotic activity (43). Our findings further support the beneficial impact of *N*-methyl-2-pyridone-5-carboxamide on CVD health. We suggest the tryptophan–NAD pathway may offer a new target for pharmacological treatment of hypertension.

We also confirmed the association of dietary markers with BP including: a direct association between BP and carnitine (a marker for protein ingestion); and an inverse association with proline-betaine (citrus fruit ingestion). Our results are consistent with previous studies linking hypertension with blood concentration of carnitine (44) and variations in BP following carnitine treatment in rats (45). Similarly our results support the previously postulated benefit of citrus fruit intake in reduction of BP (34). Specifically for the OmniProt diet, despite the increased excretion of carnitine, a marker which was linked to higher BP, overall beneficial reductions in CVD risk factors (both BP levels and lipid profiles) were elicited and these benefits persisted for those who were considered as typical (HDR) as well as variable (VDR) responders. The specific mechanisms for this remain unclear although it may be hypothesized that the altered large-bowel microbiome following protein-rich dietary intervention may play a significant role.

We investigated our data stratified by responders (HDR groups) and nonresponders (VDR groups) to ascertain whether the lack of demonstrated response was purely due to poor adherence to diet. We used a marker of citrus fruits, proline-betaine, as a proxy for dietary adherence to OmniHeart diets, as participants were given citrus fruits as part of their diets. Using the level of proline-betaine excretion at <95% CI of the HDR groups as a cutoff, we estimated that nonadherence contributed to the dampened clinical responses for 9.5–22.2% of the participants, depending on the type of OmniHeart diet. These nonadherence values are considerably higher than the <5% nonadherence estimated from the self-reported data from this study (4) and provided an additional objective measure to the mean urine urea nitrogen measurements, reflecting protein intake, which was highest on the protein-rich diet. Our modeling strategy thus provided an objective method for classification of individuals in the VDR groups as nonadherent to each of the OmniHeart diets. The remaining discrepancy in metabolic response in individuals showing good dietary adherence was mainly attributable to variation in the excretion of gut microbial metabolites (7.6%). These results are consistent with findings from a recent study by Zeevi et al. (46) who showed interindividual differences in glycemic response to foods and that these were correlated with differences in the composition of the microbiome.

As a feeding study, this study has several strengths including: the provision of all meals to participants where their body weights

were held constant throughout the feeding periods, thereby removing the confounding effect of weight loss; the inclusion of 24-h urine collection; and the randomized cross-over design, which all add rigor to the study. Further, we have included individuals from high CVD risk groups such as African American (~50%) and prehypertensive patients (~80%), which strengthens the general applicability of our stratification pipeline, although we recognize a large proportion of our participants was either overweight or obese and therefore not reflective of the general population. However, this reflects the higher incidence of obesity among the African American population. Since, by design, participants' weight remained the same throughout the study, our models were not adjusted for BMI. We also did not adjust for socioeconomic status based on previous findings in a large-scale cross-sectional study, which demonstrated that the inverse association with BP was explained mostly by dietary differences (47).

Our study represents one of the largest dietary interventions of its kind where many prior nutritional metabolic phenotyping studies have typically involved a small number of participants ($N < 25$) (48, 49). In this study, we used food-frequency questionnaires to describe participants' food intake during the screening visit (baseline) and this information was used to estimate the average intended food intake to maintain the participants' body weight throughout the isocaloric feeding periods. However, one limitation is that we were unable to perform more detailed analysis on individual dietary components and the dose–response relation with BP. An additional limitation of the current study was the use of NMR spectroscopy as the sole method of metabolic profiling. Although the robustness of the technique is advantageous for generating high-quality data, mass spectrometry would offer better sensitivity and selectivity and may have identified further candidate biomarkers relating to BP. Nonetheless, we were able to uncover a number of biomarkers related to BP and these biomarkers were structurally authenticated.

In this global profiling study, we opted to use urine as our choice of biofluid as urine constitutes a rich source of information encompassing the influence of dietary and gut microbiota. We and others (41, 50) have successfully identified urinary discriminatory metabolites related to BP. However, future studies should validate our findings by the use of urine specimens collected from independent epidemiological studies. Further to validating the candidate biomarkers related to dietary modulation of BP, a series of in vivo studies to establish causality would be necessary. For example, Menni et al. (51) have shown a possible causal relation between hexadecanedioate and BP using rodent models.

Our strategy illustrates the feasibility of adopting a rational stratification approach for diabetologists, cardiologists, or dietitians to identify individuals' nonadherence to diets and to optimize clinical responses to therapy. Extending this concept, we can envisage that further characterization of interindividual responses to healthy diets as determined by an individual's phenotypic patterns and further determining an individual's longitudinal phenotypic stability prior to a healthy dietary intervention would need to be developed for the identification of latent sub-phenotypes. This may confer a public health benefit with potential to provide a personalized approach to dietary recommendations aimed at optimizing prevention of CVD and related disorders.

In conclusion, variation in metabolic phenotypes in response to specific healthy diets may hold clues as to the mechanisms underlying interindividual variations in response to dietary modulation and points to the potential importance of the gut microbiome in accounting for differences in dietary response and the subsequent impact on BP. The workflow presented here provides a clinically actionable framework to develop tailored dietary interventions designed to reduce BP and other CVD risk factors.

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The authors' responsibilities were as follows—RLL: designed metabolic profiling research; LJA: designed OmniHeart research; RLL and XZ: conducted the research and analyzed data; RLL, EH, and XZ: wrote the manuscript; RLL: had primary responsibility for final content; EH and JKN: facilitated access to MRC-NIHR National Phenome Centre and related work; RLL, EH, and JKN: conducted metabolite identification; and all authors: reviewed and approved the final manuscript. None of the authors reported any conflicts of interest.

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