Characterization of metabolic responses to healthy diets and the association with blood pressure: application to the Optimal Macronutrient Intake Trial for Heart Health (OmniHeart), a Randomized Control Study

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Short running title: Metabolic response to diets and the link with BP

Abbreviation: 1D, one-dimensional; CVDs, cardiovascular diseases; BP, blood pressure; CI, confidence interval; DASH, Dietary Approaches to Stop Hypertension; HDR, homogenous dietary response; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; mOPLS-DA, multilevel orthogonal partial least squares discriminant analysis; NAD, nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; OmniHeart, Optimal Macronutrient Intake Trial for Heart Health; OmniCarb, OmniHeart carbohydrate rich diet; OmniMFA, OmniHeart monounsaturated fat rich diet; OmniProt, OmniHeart protein rich diet; SD, standard deviation; SHOCSY, statistical homogeneous cluster spectroscopy; TSP, sodium 3-trimethylsilyl-(2,2,3,3-$^{2}H_4$)-1-propionate; VDR, variable dietary response.

Clinical Trial Registration: The original OmniHeart intervention study is registered at www.clinicaltrials.gov as NCT00051350 and metabolomics study is registered at www.clinicaltrials.gov as

ABSTRACT

Background: Inter-individual 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 (CVD).

Design: 24-h urine samples from 158 participants, with pre-hypertension 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-weeks per 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 three 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 cholesterol levels. However, blood pressure (BP) was found to be significantly associated with six 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 non-adherence to diet (up to 25.3%), variation in gut microbiome activity (7.6%) or a combination of both (7.0%).

Conclusion: These data indicate inter-individual variations in BP in response to dietary change and highlight the potential influence of the gut microbiome in mediating this relationship. This approach provides a framework for stratification of individuals undergoing dietary management.

Keywords: diets; gut microbiome; hypertension; metabolic profiling, metabonomic, metabolomic; and personalized health care.

INTRODUCTION

Of the total global deaths, approximately half are attributed to cardiovascular diseases (CVDs), with elevated 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 non-communicable 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) have 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.

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 in a range of pathological conditions, including gastrointestinal cancer risk, has been assessed (17). Building on methodological approaches developed for characterizing inter-individual variation in response to drug toxicity/therapies (18), we propose to demonstrate the feasibility of identifying inter-individual variation in clinical response to three different

healthy diets, using a ^{1}H 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 homogenous dietary response (HDR) group. We ascertained that a minority of individuals demonstrated atypical dietary responses, herein referred to as variable (heterogenous/non-uniform) dietary responders (VDR). 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, three period cross-over feeding study aiming to assess the effects of three healthy diets on BP and lipid profiles (19). The key findings and the study design of OmniHeart Study have been previously published (4, 19). Briefly, all three 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% kcals from carbohydrate, 15% from protein and 27% from fat; the remaining two 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 one of six possible orders of administration of the three diets, each intervention period lasting for 6-weeks. During each intervention period, the participants were requested to only consume food prepared in the

diet kitchen and were allowed to consume up to 2 alcoholic beverages and 3 non-caloric caffeinated beverages per day 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 (at least 2 weeks), 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 consume 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 between 30 to 80 years from the Baltimore and Boston areas, with pre-hypertension (systolic BP of 120 to 139 mmHg and/or diastolic BP of 80 to 89 mmHg) or stage 1 hypertension (systolic BP of 140 to 159 mm Hg and/or diastolic BP of 90 to 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, low density lipoprotein cholesterol [LDL], high density lipoprotein cholesterol [HDL], 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 one dietary intervention period, and four individuals completed two intervention periods. The remaining 158 completed all three 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 one at the end of each of the three 6-week dietary interventions. NMR urine spectra for these 158 individuals were used for the analyses presented here. During the last 10 days of each dietary intervention period, a fasting blood specimen was obtained to measure lipid levels. BP was measured on 5 days by trained staff using the OMRON 907 device for those requiring a normal or large adult cuff, after participants had been seated for at least 5 mins. The reported BP was based on the average of nine BP measurements taken at screening visits and 15 measurements taken at the last five 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 week-day. Baseline socio-demographic and anthropometric characteristics were obtained for each participant. Institutional ethics committee approval was obtained for each site and all participants provided written informed consent.

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 12,000g for 5 mins to remove particulates. For each specimen, 500uL of urine was mixed with 250μL of phosphate buffer solution at pH 7.4±0.1. The resulting mixtures were left to stand for 10 mins and then further centrifuged as before. A total volume of 500uL of the supernatant was added to 50uL of sodium 3-trimethylsilyl-(2,2,3,3-$^{2}H_4$)-1-propionate (TSP) in Deuterium Oxide, giving a final concentration of 1mM. This solution was transferred to a 5mm NMR tube. The prepared urine specimens were placed in the auto-sampler, analyzed in a simple randomized order generated by computer. A

one-dimensional (1D) pulse sequence with a water saturation method (recycle delay – 90º –t_1-90º-t_m-90º-acquisition) was used to acquire standard ^{1}H NMR spectra of urine. The spectra were acquired with 64K data points and 128 scans over a spectral width of 12kHz. The recycle delay was set to 2s with a mixing time (t_m) of 100ms and a t_1 of 20µs, providing an acquisition time of approximately 2.72s. All ^{1}H NMR spectra were phased, baseline corrected, and manually referenced to sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)-1-propionate (TSP) at δ 0 with Topspin software (version 2.1, Bruker Biospin) prior to multiplication by an exponential weighting function corresponding to a line broadening of 0.3Hz. The spectral regions containing the water (δ 4.5 to 5.05) and urea (δ 5.5 to 6.5) resonances, as well as the extreme ends (<δ 0.7 and > δ 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 comparison between each diet and the baseline), we removed the creatinine regions containing the peaks at δ 3.035-3.062 and δ 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 dataset 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 the 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/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/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 cross-over study design, we employed multilevel orthogonal partial least square-discriminant analysis (mOPLSDA) (26, 27), which incorporates the variation between and within participants in the dataset 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 a HDR (3 models, 1 per 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 seven-fold cross-validation procedure. The model statistics, Q^2Yhât (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 for 100 times. The Q^2Yhât statistic for the real model was then compared to the null hypothesis distribution obtained from the permuted Q^2Yhât t values and was considered significant when the p-value of the real Q^2Yhât was <0.05 on those permuted values.

The three criteria used to identify discriminatory metabolites were: i) P-values of the correlations between the spectral variable and the mOPLS-DA scores vector should be <

185 1.85×10^{-6} (corresponding to $p < 0.05$ after Sidák correction); ii) a variable loading coefficient
186 strength, $r^2 > 0.3$ as defined in Zou et al (23); and iii) the stability of the NMR variables,
187 whereby a data point was considered significant when flanked by two NMR spectral variables
188 conforming to criteria i) and ii). For peaks that were free from spectral overlap, the 24-h
189 urinary excretion of each discriminatory metabolite was quantified by integration of the NMR
190 signal intensities. Since we found no significant difference in the excretion of creatinine
191 between different OmniHeart diets and the typical American diet ($P > 0.5$), the absolute
192 excretion of each discriminatory metabolite was normalized to the corresponding 24-h urinary
193 creatinine excretion (in mmol/L). The difference in absolute excretion of each discriminatory
194 metabolite was determined for the comparison of each dietary intervention with baseline or
195 between different OmniHeart dietary interventions. The association between the differences
196 in absolute excretion of each discriminatory metabolite and changes in CVD risk factors
197 (systolic and diastolic BP, LDL, total cholesterol) was established using linear regression for
198 HDR groups. In addition, known covariates for hypertension including urinary excretion of
199 sodium, potassium, calcium and phosphate, were also established for HDR and VDR groups
200 for the comparison between baseline and each OmniHeart diet. The statistical significance of
201 these covariates was adjusted by Bonferroni correction (0.05 divided by number of
202 comparisons) to account for multiple testing. All analyses were performed using in-house
203 software written in Matlab (version 2012a, MathWorks, Natick, MA).
204
205 **Identification of discriminatory metabolites**
206 The discriminatory metabolites found to be significantly influenced by the healthy dietary
207 interventions were confirmed by in-house and published database (29) references and
208 authenticated by spiking in standard compounds purchased from Sigma Aldrich. These
209 compounds included: N-methyl-2-pyridone-5-carboxamide, 4-hydroxyphenylacetic acid,

carnitine, creatine, dimethylglycine, S-methyl-L-cystiene-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 two dimensional NMR experiments, solid phase extraction chromatography experiments coupled with NMR, ultra-performance liquid chromatography coupled to mass spectroscopy, and statistical analysis such as Subset Optimization by Reference Matching (STORM) (24) as well as using published databases and/or literature.

RESULTS

Individuals show variation in urinary metabolic phenotypes to 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 six-week study, in terms of reduction of CVD risk factors, which was reflected in the urinary metabolome. Inter-individual differences in dietary response were observed; the majority of the participants showed a HDR to all of the OmniHeart diets when compared with 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 modelling 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 three 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-nicotinamide-adenine dinucleotide (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 three 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 two gut microbial mammalian co-metabolites, phenylacetylglutamine and 4-cresyl sulfate compared to the other two OmniHeart diets; whilst the HDR group for the OmniCarb diet consistently showed higher excretion of hippurate and guanodinoacetate (**Supplemental Table 3 and 4**). The differences in the markers for dietary intake of cruciferous vegetables (S-methyl-L-cystiene-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 three diets included higher proportions of fruit/vegetables than the baseline.

Urinary metabolites significantly associated with BP

We quantified ten discriminatory metabolites altered in response to one 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 two of these food related metabolites with 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 three 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 non-adherence 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 levels 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 level of proline-betaine (a direct marker of citrus fruit intake) (33, 34), as non-adherent 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 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 95% confidence interval (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 non-adherence 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 non-adherent, 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 non-adherent) subgroups for OmniCarb. Differential metabolite patterns were also observed for different subgroups within the OmniMFA (**Figure 1**).

Urinary metabolic variation reflects inter-individual differences in clinical responses

Discarding the non-adherent VDR group, we assessed the effect of each diet, stratified by the HDR versus adherent-VDR, 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 pre-hypertensive individuals within the HDR

groups for sodium: -31.3mmol/day (OmniCarb), -44.9mmol/day (OmniMFA), and -35.9mmol/day (OmniProt); and potassium 26.4 mmol/day (OmniCarb), 28.4 mmol/day (OmniMFA) and 24.7 mmol/day (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 inter-comparison 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 and total cholesterol. Additionally, the reduction in serum triglyceride concentrations was significant for the OmniProt-HDR group; and HDL 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 non-optimal 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 non-adherent-OmniMFA-VDR and the non-adherent-OmniProt-VDR groups; whilst the adherent- and 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 three 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 the 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 three dietary interventions, we were able to stratify individuals according to diet-response specific urinary phenotypes; corresponding to those who demonstrated: HDR to all three diets (N=95, 60.1%; Group 1); HDR to two diets but VDR to one diet (N=35, 22.2%; Group 2); HDR to only one diet but VDR to two diets (N=22, 13.9%; Group 3); non-adherent-VDR to all three diets (N=4, 2.5%; Group 4); and mix of non-adherent- and adherent-VDR to all three 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) non-adherent to one or more diet (N=40, 25.3%); or c) a combination of the two (N=11, 7.0%), **Table 3**. We found that individuals consistently classified as HDR for all three OmniHeart diets generally manifested a greater reduction in CVD risk factors than those that were classified as HDR for just one or two of the OmniHeart (**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 three diets had a distinct effect on the metabolism. However, regardless of the macronutrient differences between the three 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 diet and sub-divided the dataset 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 two 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 have manipulated gut microbiota balance via probiotic administration with consequent beneficial effects on BP levels (37). More recently, blood levels 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 the gut microbiome 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 body-mass-index, alcohol intake, and urinary excretion of sodium and potassium (41). A controlled feeding study by Wu et al showed that changes in the gut microbiome occurred within 24-h of initiating a change in diet (35) and that body-mass-index 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 weeks 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 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 hypertriglyceridemia rats can ameliorate the nitric oxide dependent vasodilation, a surrogate marker for atherosclerosis (42). 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) was 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 non-responders (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 non-adherence contributed to the dampened clinical responses for 9.5% to 22.2% of the participants, depending on the type of OmniHeart diet. These non-adherence values are considerably higher than the <5% non-adherence 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 non-adherent 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 inter-individual differences in glycaemic response to foods and that this was 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 all add rigor to the study. Further, we have included individuals from high CVD risk groups such as African American (~50%) and pre-hypertensive patients (~80%), which strengthens the general applicability of our stratification pipeline, although we recognize large proportion of our participants were either overweight or obese and therefore not reflective of the general population. However, this reflects the higher incidence of obesity among the African American. Since, by design, participants' weight remained the same throughout the study, our models were not adjusted for body-mass-index. 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 was that we were unable to perform more detailed analysis on individual dietary components and the dose-response relationship 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 contains 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 have shown a possible causal relationship between hexadecanedioate with BP using rodent models (51).

Our strategy illustrates the feasibility of adopting a rational stratification approach for diabetologists/cardiologists/dieticians to identify individuals' non-adherence to diets and to optimize clinical responses to therapy. Extending this concept, we can envisage that further characterization of inter-individual 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 inter-individual variations in response to dietary modulation and points 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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Conflict of Interest: None

Authors' contributions:

RLL: designed metabolic profiling research;

LJA designed OmniHeart research;

RLL and XZ: conducted the research;

RLL and XZ 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.

All authors reviewed and approved the manuscript.

Data and materials availability: The OmniHeart study description together with the study protocol and associated metadata are available from the Biologic Specimen and Data Repository Information Coordinating Center (BioLINCC) at https://biolincc.nhlbi.nih.gov/static/studies/omniheart/MOP.pdf?link_time=2017-07-02_01:45:33.646682.

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Table 1: Characteristics of participants completed all three OmniHeart diets (N = 158).

Characteristics		P values
Age, mean (SD)	53.1 (10.8)	
Ethics, N (%)		
African American	86 (54.4%)	
Non-African American	72 (45.6%)	
Gender, N (%)		
Male	88 (55.7%)	
Female	70 (44.3%)	
Hypertension, N (%)		
Pre-hypertension	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 drinker, 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 of SBP from baseline (95% CI), mmHg		
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 of DBP from baseline (95% CI), mmHg		
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 of LDL 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 of HDL 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 of 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 of total cholesterol from baseline (95% CI), mg/dL		
OmniCarb diet	-12.5 (-15.8, -9.1)	‡
OmniMFAdiet	-15.6 (-19.2, -11.9)	‡
OmniProt diet	-20.2 (-23.7, -16.69)	‡

Abbreviations: N, number of individuals; SD, standard deviation; CI, confidence interval; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol.

T-test comparison between baseline clinical data and after each dietary intervention: * $p<0.05$; † $p<10^{-5}$; ‡ $p<10^{-10}$.

Table 2: Estimated mean differences in CVD risk factors. The systolic and diastolic BP, LDL and total cholesterol mean differences per 2 standard deviation (SD) increase in absolute excretion for the comparison between baseline and post OmniHeart diets; and between different OmniHeart diets for the HDR groups.

Urinary metabolites	2SD excretion (mmol/L)	SBP (mmHg)	DBP (mmHg)	LDL (mg/dL)	Total Cholesterol (mg/dL)
	Homogeneous dietary responder 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)

Abbreviations: N, number of individuals; SD, standard deviation; CI, confidence interval; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein cholesterol. Key: * $p<0.05$; † $p<0.01$; ‡ $p<0.001$

The correlation between changes of metabolites and CVD factors were evaluated by linear regression. 2SD 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 ($|Z\text{-score}|\geq 1.96$, $p<0.05$; ≥ 2.58, $p<0.01$; ≥ 3.89, $p<0.001$). NMR chemical shifts (multiplicity) used for quantification: proline-betaine, δ3.11 (singlet); hippurate, δ7.64 (triplet); N-methyl-2-pyridone-5-carboxamide, δ6.67 (doublet); N-methyl nicotinic acid, δ4.44 (singlet); N-methyl nicotinamide, δ8.89 (triplet); carnitine, δ3.23 (singlet); creatine, δ3.93 (singlet); guanodinoacetate, δ3.80 (singlet); phenylacetylglutamine, δ7.43 (triplet); 4-cresyl sulfate, δ2.35 (singlet).

Table 3: Stratification by urinary phenotypes. Individuals were stratified based on diet-specific urinary phenotypes.

Summary of sub-phenotypes	**N**	**%**
Group 1: HDR to all three diets	95	60.1%
Group 2: HDR to two diets but VDR to one diet	35	22.2%
a) Non-adherent-VDR to the other diet	25	15.8%
b) Adherent-VDR to the other diet	10	6.3%
Group 3: HDR to one diet but VDR to two diets	22	13.9%
a) Non-adherent-VDR to the other two diets	11	7.0%
b) Adherent-VDR to the other two diets	2	1.2%
c) Mixed response – non-adherence-VDR to one diet and adherence-VDR to the other diet	9	5.7%
Group 4: Non-adherence-VDR to all three diets	4	2.5%
Group 5: Mix of non-adherence and adherence to all three diets	2	1.3%

Figure 1: The observed mean differences in excretion for hippurate between homogeneous (HDR), adherent- and non-adherent variable dietary response (VDR) groups when OmniHeart diets and their corresponding baseline spectra were compared. Open squares, OmniCarb-HDR (N=137); light-grey closed squares, adherent- OmniCarb-VDR (N=6); dark-grey closed square, non-adherent-OmniCarb-VDR (N=15); open circle, OmniMFA-HDR (N=127); light-grey closed circle, adherent- OmniMFA-VDR (N=10); dark-grey closed circle, non-adherent-OmniMFA-VDR (N=21); open triangle, OmniProt-HDR (N=113); light-grey closed triangle, adherent- OmniProt-VDR (N=10); dark-grey closed triangle, non-adherent-OmnProt-VDR (N=35). Error bars indicate 95% confidence interval. Significant t-test comparison between baseline and post OmniHeart diets: * $p<0.05$; † $p<10^{-5}$; ‡ $p<10^{-10.}$

Figure 2: Key observations for changes in the cardiovascular disease risk factors showing differences in homogeneous and variable dietary response groups for the comparisons between each OmniHeart diet and baseline corresponding to the changes in: (A) systolic BP; (B) diastolic BP; (C) LDL; (D) HDL; (E) triglycerides; (F) total cholesterol. Open squares, OmniCarb-HDR (N=137); light-grey closed squares, adherent- OmniCarb-VDR (N=6); dark-grey closed square, non-adherent-OmniCarb-VDR (N=15); open circle, OmniMFA-HDR (N=127); light-grey closed circle, adherent- OmniMFA-VDR (N=10); dark-grey closed circle, non-adherent-OmniMFA-VDR (N=21); open triangle, OmniProt-HDR (N=113); light-grey closed triangle, adherent- OmniProt-VDR (N=10); dark-grey closed triangle, non-adherent-OmnProt-VDR (N=35). Error bars indicate 95% confidence interval. Missing data include: LDL (N=2 for OmniMFA-VDR, OmniProt-VDR, OmniMFA-HDR, OmniProt-HDR and N=3 for OmniCarb-HDR); HDL (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: * $p<0.05$; † $p<10^{-5}$; ‡ $p<10^{-10}$.