Effect of acute dietary standardization on the urinary, plasma, and salivary metabolomic profiles of healthy humans\textsuperscript{1–3}

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ABSTRACT

Background: Metabolomics in human nutrition research is faced with the challenge that changes in metabolic profiles resulting from diet may be difficult to differentiate from normal physiologic variation.

Objective: We assessed the extent of intra- and interindividual variation in normal human metabolic profiles and investigated the effect of standardizing diet on reducing variation.

Design: Urine, plasma, and saliva were collected from 30 healthy volunteers (23 females, 7 males) on 4 separate mornings. For visits 1 and 2, free food choice was permitted on the day before biofluid collection. Food choice on the day before visit 3 was intended to mimic that for visit 2, and all foods were standardized on the day before visit 4. Samples were analyzed by using \textsuperscript{1}H nuclear magnetic resonance spectroscopy followed by multivariate data analysis.

Results: Intra- and interindividual variations were considerable for each biofluid. Visual inspection of the principal components analysis scores plots indicated a reduction in interindividual variation in urine, but not in plasma or saliva, after the standard diet. Partial least-squares discriminant analysis indicated time-dependent changes in urinary and salivary samples, mainly resulting from creatinine in urine and acetate in saliva. The predictive power of each model to classify the samples as either night or morning was 85\% for urine and 75\% for saliva.

Conclusions: Urine represented a sensitive metabolic profile that reflected acute dietary intake, whereas plasma and saliva did not. Future metabolomics studies should consider recent dietary intake and time of sample collection as a means of reducing normal physiologic variation. \textit{Am J Clin Nutr} 2006;84:531–9.

KEY WORDS Nutritional metabolomics, metabonomics, healthy human biofluids, metabolic profiles, multivariate data analysis

INTRODUCTION

Metabolomics, the global analysis of metabolites, provides nutrition research with an alternative to the traditional single-biomarker approaches used to assess health and disease. The integration of metabolomics with nutritional science will enhance current clinical and research practices by providing a deeper insight into the relations between various metabolite arrays and health status (1, 2). Metabolomics is achieved by maximum data capture from biofluid or tissue analysis through the use of technologies such as nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry, followed by pattern recognition statistics (multivariate data analysis) (3). Metabolomics has been successfully applied in the research fields of pharmacology, toxicology (4–6), and medical screening (7–10), but to date nutritional metabolomics is somewhat underdeveloped (2). Limited dietary metabolomics studies include an isoflavone intervention (11, 12), a chamomile tea intervention (13), and a comparison of healthy British and Swedish subjects (14).

To date, most metabolomics studies have been in animal models, which are particularly important in the area of drug discovery. The Consortium on Metabonomic Toxicology (COMET) is an international research body that has been set up to generate a comprehensive database of NMR spectra of rodent metabolic responses to \textasciitilde 150 toxins and treatments (15). The normal degree of physiologic variation in rodents is well understood and was recently reviewed (16). The factors that influence the metabolic composition of biological samples and contribute to variation in rodent metabolic profiles include species, strain, genetics, sex, age, hormone concentrations, diurnal cycles, diet, temperature, stress, and gut microflora. Humans are extremely diverse beings and it is not surprising that most human metabolomics studies have found that spectral outputs are strongly influenced by inter- and intra-individual variation (11–14, 17). Therefore, normal physiologic variation has the potential to be a strong confounder in human studies. With the exception of a few dietary studies previously mentioned, most of the human metabolomics studies that have been undertaken involved clinical cases and controls (7–10). It is not certain that the changes in metabolic profiles resulting from dietary intervention will be as easy to detect as the contrast between the metabolic profiles produced from individuals with a clinical disease compared with controls.

Therefore, in the study of human nutrition, it will be important to be able to control and understand the factors that contribute to

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normal physiologic variation so that normal metabolic fluctuations are not confused with biomarkers that represent a metabolic change due to nutritional intervention. In addition, investigating the effects of diet on biofluid metabolic profiles may improve our understanding of how certain dietary components influence metabolic pathways. The objective of this study was to determine the extent of variation in normal human metabolic profiles and to ascertain the influence of diet on these changes.

SUBJECTS AND METHODS

Study design

The project was approved by the Faculty of Health Sciences Ethics Committee, Trinity College, Dublin, and all participants provided written informed consent. Thirty healthy, free-living subjects were recruited at Trinity College, Dublin. All volunteers attended a screening session at which heights and weights were measured and a screening blood sample was taken. Exclusion criteria were a body mass index (in kg/m²) <18.5 or >30.0, vegetarianism, iron deficiency anemia (hemoglobin < 11.5 g/dL for females and < 13.5 g/dL for males), receiving prescribed medication (contraceptive pills were permitted), or having an oral or urinary tract infection within 1 mo of commencing the study.

Biological samples were collected at 4 laboratory visits to the Trinity Centre for Health Sciences at St James’s Hospital, Dublin. Volunteers were asked to record their dietary intake and physical activity on the day before each laboratory visit and to fast from midnight until the biofluids were collected the following morning. For the first and second visit, the volunteers were instructed to carry out their normal daily routine while recording their diet and physical activity on the records provided. On the third visit, they were asked to repeat exactly what they recorded on the second visit, this was intended to assess intraindividual variation in metabolic profiles. Finally, on the forth visit, the volunteers were provided with a full range of foods to form a standard diet and were asked to avoid any vigorous activity. This was intended to assess interindivdual variation. In addition, diurnal variation was assessed by asking the volunteers to collect a urine and a saliva sample on the evening before as well as on the morning of the final laboratory visit.

Diet and physical activity

Diet and physical activity data were not collected with the intention of analysis of nutrient intake or energy expenditure. The intention was to use these as a reference for the interpretation of unusual profiles and for identification of foods that could have particularly influenced biofluid metabolic profiles. For example, large trimethylamine N-oxide peaks have been observed in profiles from individuals who have consumed fish (15, 16).

Biofluid collection

Volunteers collected their first void urine at home in a chilled graduated container before each laboratory visit. The volume of the spot urine sample was recorded and then a 10-mL portion was transferred to a plastic tube. A sample of unstimulated whole saliva was also collected at home, before teeth-brushing, by using a chilled plain salivette (Sarstedt, Aktiengesellschaft & Co, Germany). The salivette and urine sample were transported to the Trinity Centre on ice in an insulated pack. During the laboratory visit, a plasma sample was collected in a 7-mL lithium heparin tube and the subjects’ diet and physical activity records were checked for clarity. All samples were centrifuged at 2500 × g for 10 min at 4 °C to separate the plasma from the blood samples and to remove any solid debris from the urine and saliva samples. Portions (500 µL) of the supernatant fluid were then stored at −20 °C until 1H NMR analysis.

NMR spectroscopy

Urine samples were prepared by the addition of a phosphate buffer (0.2 mol KH₂PO₄/L, 0.8 mol KH₂PO₄/L) to the samples in a 2:1 ratio. Sodium trimethylsilyl propionate and 10% D₂O were added to each sample to serve as a chemical shift reference and a field frequency lock, respectively. Spectra were acquired with 32,000 data points and 128 scans over a spectral width of 8 kHz on a 500 MHz DRX NMR spectrometer (Bruker Biospin, Karlsruhe, Germany) with the use of a Noesypresat pulse sequence. Water suppression was achieved during the relaxation delay (2.5 s) and the mixing time (100 ms).

Plasma and saliva samples were prepared by the addition of sodium trimethylsilyl propionate and 10% D₂O. Spectra of plasma samples were acquired by using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with 32 k data points and 64 scans. Spectra of saliva samples were acquired by using a Noesypresat pulse sequence with 32 k data points and 256 scans over 8 kHz. Water suppression was achieved during the relaxation delay (2.5 s) and mixing time (100 ms).

All 1H NMR spectra were processed with Bruker software by using suitable weighting functions and were corrected for baseline. The spectra were then reduced by integrating into bins across spectral regions of 0.04 ppm by using AMIX (Bruker Biospin, Karlsruhe, Germany). The water region (4–6 ppm) was excluded and the data were normalized to the sum of the spectral integral.

Statistical analysis of NMR data

The data were imported to SIMCA-P+ (version 10.0; Umetrics, Umeå, Sweden), mean centered, and Pareto scaled (1/√SD). Principal components analysis (PCA) is an unsupervised multivariate statistical tool that is used to analyze data sets consisting of a large number of variables. It reduces this multidimensional data into principal components that can be viewed by using low-dimensional plots. PCA was applied to each data set (plasma, urine, and saliva), and then the score plots were visually inspected for clustering trends. The corresponding loadings plot provided information on the contribution of each variable to the pattern on the scores plot. To further investigate the variation between samples from week to week and to support the visual interpretation of the PCA score plots, the SD of the score values for each laboratory visit was determined (each score represented a sample). The 3 biofluids were assessed separately. In addition, we calculated the CV for each bin region (NMR variable) and reported the average CV at each of the 4 laboratory visits. The CVs were calculated for the information-rich region of the spectra (0.5–4.0 ppm) to reduce the influence of noise.

Diurnal variation was initially assessed by using PCA to visualize the data and to check for clustering patterns. The difference between night and morning samples was then further explored by using partial least-squares discriminant analysis (PLS-DA), a supervised data analysis technique. To test the
PLS-DA model describing diurnal variation, a cross-validation step was carried out by using a training set constructed from 50% of the samples. This training set was used to determine class membership (night or morning) of the remaining 50% of the samples (test set). Paired-sample t tests were used to compare the independently determined creatinine concentration of the night and morning urine samples.

RESULTS

Thirty healthy volunteers (23 women and 7 men) aged 19–31 y completed the study. Their demographic characteristics (x ± SD) are shown in Table 1. Typical 1H NMR spectra obtained for urine, plasma, and saliva are shown in Figure 1.

<table>
<thead>
<tr>
<th></th>
<th>Women (n = 23)</th>
<th>Men (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height (m)</td>
<td>1.68 ± 0.05</td>
<td>1.80 ± 0.09</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>64.0 ± 7.4</td>
<td>74.1 ± 12.6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.7 ± 2.8</td>
<td>22.8 ± 2.1</td>
</tr>
<tr>
<td>Age (y)</td>
<td>24 ± 3</td>
<td>27 ± 4</td>
</tr>
</tbody>
</table>

1 All values are x ± SD.
2 Significantly different from women, P < 0.05 (independent t tests).

Intra- and interindividual variation in urinary, plasma, and salivary metabolites

Urine

The PCA scores plot of the 1H NMR urine data are shown in Figure 2. This plot displays the first 2 principal components and accounts for 26% of the variation in the samples. Seven observations fell outside the Hotelling’s T² 95% confidence ellipse. Consultation of the original 1H NMR spectra revealed that the spectra corresponding to visit 1 for subjects 31 and 36 were very weak in comparison with the other urine samples. Examination of the spectra for subjects 36 (visit 2), 38 (visit 1 and 2), and 45 (visit 3) revealed large peaks due to ethanol. The dietary records for these individuals were checked, and it was confirmed that these subjects consumed alcohol on the evening before sample collection.

Visual inspection of the PCA scores plot in Figure 2 shows that both intra- and interindividual variation were relatively high among the samples. However, intraindividual variation appeared to be lower than interindividual variation, with the samples for each visit remaining within the same quadrant of the plot for most subjects (subjects 3 and 35 are labeled as an example). Laboratory visit 3 was intended to be a replica of visit 2 with regard to diet and physical activity, but the observations from visits 2 and 3 did not cluster more closely to each other, which indicates that repeating the visit 2 protocol did not reduce intraindividual variation in the urinary metabolic profiles. After the standard diet on

![FIGURE 1. Typical 1H nuclear magnetic resonance spectra of biofluids obtained from a healthy volunteer: (A) urine, (B) saliva, and (C) plasma. TMAO, trimethylamine-N-oxide; DMA, dimethylamine. The spectra were acquired as described in Subjects and Methods.](image-url)
visit 4, a reduction in interindividual variation was observed. When the 7 outlying samples were removed, the reconstructed PCA scores plot showed the same trend with the visit 4 samples forming a tighter cluster.

SDs (excluding outliers) of the scores for each visit and the CVs for the variables from the information-rich region of the $^1$H NMR spectra provided quantitative data regarding the variation among samples from each visit (Table 2). After the standard diet, the SD of each principal component was lower and the average CV of the $^1$H NMR spectral intensities was reduced.

**Table 2**

<table>
<thead>
<tr>
<th></th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
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<tbody>
<tr>
<td><strong>Urine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC 1</td>
<td>8.6</td>
<td>5.5</td>
<td>6.6</td>
<td>4.7</td>
</tr>
<tr>
<td>PC 2</td>
<td>4.8</td>
<td>6.7</td>
<td>7.0</td>
<td>4.0</td>
</tr>
<tr>
<td>CV</td>
<td>30</td>
<td>29</td>
<td>26</td>
<td>18</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC 1</td>
<td>8.6</td>
<td>8.0</td>
<td>10.4</td>
<td>12.3</td>
</tr>
<tr>
<td>PC 2</td>
<td>4.5</td>
<td>5.3</td>
<td>4.9</td>
<td>6.2</td>
</tr>
<tr>
<td>CV</td>
<td>36</td>
<td>31</td>
<td>29</td>
<td>36</td>
</tr>
<tr>
<td><strong>Saliva</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC 1</td>
<td>12.2</td>
<td>12.0</td>
<td>12.5</td>
<td>13.4</td>
</tr>
<tr>
<td>PC 2</td>
<td>7.7</td>
<td>6.2</td>
<td>7.3</td>
<td>6.2</td>
</tr>
<tr>
<td>CV</td>
<td>35</td>
<td>36</td>
<td>41</td>
<td>33</td>
</tr>
</tbody>
</table>

1 PC 1, SD of principal component 1; PC 2, SD of principal component 2. SDs were calculated from the scores at each laboratory visit; the value of each score represents a single sample and is derived from the original $^1$H NMR data. CVs were calculated for each bin region ($^1$H NMR spectral intensity variables), and the average CV for each week is reported.

**Plasma**

The PCA scores plot of the $^1$H NMR plasma data are shown in Figure 3. This plot displays the first 2 principal components and accounts for 74% of the variation in the samples. The 4 observations representing samples from each laboratory visit for subject 33 fell outside the Hotelling's T^2 95% confidence ellipse. Consultation of the corresponding loadings plots and inspection of the $^1$H NMR spectra revealed that the spectra for subject 33 had very large lipid peaks in comparison with the spectra from other subjects. After removal of subject 33, the two-component model accounted for 66% of the variation in the samples. Visual inspection of the PCA scores plot in Figure 3 showed that both intra- and interindividual variation were considerable. Overall, intraindividual variation appeared to be lower than interindividual variation, with the samples for most subjects congregating in the same region of the plot (subjects 15, 34, and 41 are labeled as examples).

The observations from visits 2 and 3 in Figure 3 did not appear to cluster closer than the other observations, which indicates that repeating the visit 2 protocol on visit 3 did not reduce intradi
dividual variation in the plasma metabolic profiles. The standard diet on visit 4 did not appear to reduce interindividual variation, because the visit 4 data did not cluster more closely together in the center (Figure 3). After removal of the outlying samples, the reconstructed PCA scores plot did not reveal any extra relevant information. SD calculations (excluding outliers) of the scores for each visit and the average CV of the variables from the information-rich region of the $^1$H NMR spectra also suggested that the variation in plasma was not reduced after the standard diet (Table 2).

**Saliva**

The PCA scores plot of the $^1$H NMR saliva data are shown in Figure 4. This plot displays the first 2 principal components and
accounts for 59% of the variation in the samples. Adding another component to the model increased the variation accounted for by 9%. The 2-component model (Figure 4), identified 2 outliers, corresponding to samples from subjects 8 (visit 1) and 38 (visit 4), and the 3-component model identified subject 34 (visit 1) as an outlier. Inspection of the corresponding 1H NMR spectra revealed a large peak representing acetate for subject 38 (visit 4), but no unusual peaks were found for the other outliers. Visual inspection of the PCA scores plot in Figure 4 showed that both intra- and interindividual variation were relatively high among the samples. Overall, intrapersonal variation was lower than interindividual variation, but subject 38 showed particularly high intrapersonal variation and subjects 4, 8, and 36 each had one sample that deviated more from the others. The scores plot did not indicate that there was more consistency between the samples for visits 2 and 3 compared with the other visits, which indicates

FIGURE 3. Principal components analysis scores plot derived from plasma 1H nuclear magnetic resonance spectra for 30 healthy subjects. Symbols are coded according to the laboratory visit in which the samples were collected as follows: □, visit 1; △, visit 2; ○, visit 3; and ■, visit 4. There were no dietary restrictions for visit 1 or 2, visit 3 was intended to replicate visit 2, and on visit 4 the diet was standardized.

FIGURE 4. Principal components analysis scores plot derived from salivary 1H nuclear magnetic resonance spectra for 30 healthy subjects. Symbols are coded according to the laboratory visit in which the samples were collected as follows: □, visit 1; △, visit 2; ○, visit 3; and ■, visit 4. There were no dietary restrictions for visit 1 or 2, visit 3 was intended to replicate visit 2, and on visit 4 the diet was standardized.
that repeating on visit 3 what the subjects recorded for visit 2 did not reduce intra-individual variation in salivary metabolic profiles. After the standard diet on visit 4, no change in inter-individual variation was observed. After the removal of the outlying samples, the reconstructed PCA scores plots did not reveal any extra relevant information. SD calculations (excluding outliers) of the scores for each visit and the average CV of the variables from the information-rich region of $^1$H NMR also suggested that the variation in saliva was not reduced after the standard diet (Table 2).

**Diurnal variation**

**Urine**

A 2-component model was computed that accounted for 36% of the variation in the data. Inspection of the PCA scores plot showed that 3 samples were positioned outside the Hotelling’s $T^2$ 95% confidence ellipse. These corresponded to the nighttime samples from subjects 15, 36, and 45. Inspection of the $^1$H NMR spectra indicated that the sample belonging to subject 45 had peaks corresponding to ethanol. This sample was subsequently removed from the analysis. No unusual peaks were found in the spectra from the other outlying subjects; hence, they were not removed from further analysis. A PLS-DA model was constructed, and the plot is depicted in Figure 5 ($R^2 = 0.368$, $Q^2 = 0.342$), which shows separation of the night and morning samples by the first principal component ($t[1]$). The principal metabolite contributing to this separation was acetate, and the predictive ability of the model to differentiate between night and morning samples was found to be 75%.

**Saliva**

Analysis of the $^1$H NMR saliva data produced a 4-component model with an $R^2$ of 0.837 and a $Q^2$ of 0.683. The nighttime saliva samples from subjects 4, 13, 30, 38, and 41 were removed from the analysis because their corresponding $^1$H NMR spectra had intense peaks in the 3–4 ppm region, which may be contamination from the salivette. The morning sample from subject 38 appeared as an outlier. Evidence of separation between the night and morning samples was observed along the third principal component ($t[3]$). A PLS-DA model was constructed, and the plot is depicted in Figure 6 ($R^2 = 0.368$, $Q^2 = 0.342$), which shows separation of the night and morning samples by the first principal component ($t[1]$). The principal metabolite contributing to this separation was acetate, and the predictive ability of the model to differentiate between night and morning samples was found to be 75%.

**DISCUSSION**

The principal objective of the present study was to assess the effects of standardizing acute dietary intake on the extent of variation in urinary, plasma, and salivary metabolic profiles. Our findings show that consumption of a standard diet on the day before sample collection can reduce inter-subject variation in the urinary metabolic profiles of healthy individuals but does not reduce variation in plasma or salivary metabolic profiles. Lenz et al (17) conducted a similar investigation in which they assessed the variability in both plasma and urine in 12 healthy males on 2 separate days. However, there are notable differences in the design of the 2 studies. The current study included both males and females, the standard diet was provided on the day.
before rather than the day of sample collection, samples were collected in the morning on 4 separate occasions rather than on 2, and plasma samples were collected after the subjects had fasted overnight. Lenz et al (17) collected plasma samples 1.5 h after a standard breakfast and concluded that there was a relatively low level of inter- and intraindividual variation among the samples. In the current study, we have shown that a standard diet followed for 24 h before plasma collection does not appear to reduce variation in fasting plasma samples. The extent to which acute nutrient ingestion can influence the plasma metabolome depends on a balance between the rate of entry of the ingested or exogenous form and the rate of entry of the endogenous form into the plasma metabolome. The actual enduring effect of diet on plasma metabolomic profiles occurs when variation in nutrient intake alters the balance of many metabolic pathways to create a new homeostasis. In this study, any metabolic effects would have been acute and unlikely to influence endogenous metabolism.

Regarding urinary metabolic profiles, Lenz et al (17) observed a reduction in variation in the 0–12-h and 12–24-h samples during the standard diet, whereas first-void urine, collected before the start of the diet, varied the most. Similarly, the current study observed a high level of variation in first-void urine samples, but we have shown that this variation can be reduced by standardizing dietary intake on the day before sample collection. When the diet was not standardized, the metabolites that contributed most to variation in the urinary profiles were hippurate, creatinine, and an unknown. Variation along the first principal component (Figure 1) was dominated by hippurate. Hippurate is produced by the metabolism of benzoate, which may be derived from sodium benzoate, a common food additive. It is also derived from 3-phenylpropionic acid, a product of the gut microflora (18). In addition, urinary hippuric acid excretion has been positively associated with consumption of dietary polyphenols, particularly those from tea (19–22). Wang et al (13) observed an increase in urinary hippurate and glycine excretion and a decrease in creatinine excretion after a chamomile tea intervention. Variation along the second principal component in Figure 1 was dominated by creatinine and an unknown (3.70–3.74 ppm). It is uncertain why creatinine excretion appeared less variable when nutrient intakes were standardized. Urine receives the end products of many metabolic processes and its composition is not meticulously controlled. Therefore, it is more likely to accumulate residues from the metabolized diet than is plasma or saliva. This may explain why the first void urinary metabolic profiles showed an acute response to diet.

Currently, no published metabolomics studies have used saliva as a medium for investigation, and only a few NMR investigations have used saliva (23–25). Saliva is considered to be useful for the diagnosis of various diseases and endocrine disorders and for drug use assessment (26). The current study assessed the extent of variation in salivary metabolic profiles and found a relatively high level of both inter- and intraindividual variation. The extent of this variation did not appear to be reduced by standardizing dietary intake on the day before sample collection. Future studies are needed to assess the utility of saliva as a medium for metabolomics research.

Several factors must be considered to elucidate the components of the diet responsible for initiating an effect on urinary profiles. Changes in trace element intakes are unlikely, because they would not have been identified by 1H NMR. There was no evidence to suggest variation in the excretion of water-soluble vitamins or changes in acid-base balance. The gut microflora, also known as the microbiome (27), has an integrative relation with their host’s own metabolic processes, and animal studies have shown their influence on metabolic profiles (28–30). Although the gut microflora may have been involved in the metabolic breakdown of the standard diet, it is unlikely that dietary intake over one day caused any alterations in the microflora.
population. Therefore, the reduced variation observed in urinary metabolic profiles after the standard diet may not have been associated with the gut microflora. The contribution of nonnutrient food components to metabolic profiles has been discussed previously (2). These nonnutrient food components include dietary phytochemicals, and previous studies have shown that these phytochemicals exert an effect on urinary composition (31, 32). Considering that urinary hippurate has been associated with polyphenol consumption (19–22), it is possible that the reduced variation observed after the standard diet resulted mainly from the standardization of nonnutrients. A possible explanation for the decrease in variation in urinary hippurate is that the standard diet provided all subjects with 2 servings of tea on the day before the 4th visit, whereas on the other study days, tea consumption varied (0–6, 0–7, and 0–9 servings/d before visits 1, 2, and 3, respectively). Further studies will be needed to investigate the influence of nonnutrients, particularly phytochemicals, on biofluid metabolic profiles.

This study detected diurnal variation in both the urinary and salivary metabolic profiles. Creatinine was identified as the principal metabolite that changed between night and morning urine samples, with increased creatinine excretion in the first-void samples for most subjects. Urinary creatinine was 162.1 ± 88.9 mg/L in the morning samples compared with 125.6 ± 65.5 mg/L in the nighttime samples (P < 0.05). Increased creatinine excretion has been observed in male rats after energy restriction (33); therefore, it is possible that the increased creatinine in the first-void urine was attributed to the overnight fast. This observation warrants further consideration given that normalization of clinical measurements to creatinine is commonly practiced. Bollard et al (16, 34) reported that rats also show diurnal variation in creatinine excretion, with lower concentrations found in daytime urine samples. Acetate was identified as the main metabolite responsible for diurnal variation in the saliva samples. The higher amounts of acetate in the samples collected at night may be attributed to carbohydrate fermentation in the mouth after earlier eating occasions or may be exogenous, because no mouth irrigation was performed before sample collection. The morning samples were taken before any oral activity.

The current study is an important step toward a full understanding of the influence of diet on metabolic variation in humans. Our results highlight the need for careful protocol planning in metabolomics studies where factors such as recent dietary intake or time of sample collection could confound the findings of an investigation. Despite developments in data filtering, such as orthogonal signal correction (35), it remains important that we develop a greater understanding of the dietary determinants of human metabolic profiles, and to that end, further foundation studies are needed.

We offer our sincere thanks to the volunteers for their commitment and patience during the study and to Valerie Trimble and Brendan Fitzpatrick for their invaluable technical assistance.

MCW contributed to the study design and was responsible for conducting the experiment, data interpretation, and writing the manuscript. LB was responsible for conducting the experiment, data interpretation, and manuscript editing. JPGM contributed to the experimental design and manuscript editing. HMR contributed to experimental design and manuscript editing. MJG was responsible for the conception and design of the experiment, data interpretation, and manuscript editing. None of the authors had a conflict of interest.

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