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RESEARCH PAPER

The impact of free or standardized lifestyle and urine sampling protocol on metabolome recognition accuracy

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Abstract Urine contains a clear individual metabolic signature, although embedded within a large daily variability. Given the potential of metabolomics to monitor disease onset from deviations from the "healthy" metabolic state, we have evaluated the effectiveness of a standardized lifestyle in reducing the "metabolic" noise. Urine was collected from 24 (5 men and 19 women) healthy volunteers over a period of 10 days: phase I, days 1–7 in a real-life situation; phase II, days 8–10 in a standardized diet and day 10 plus exercise program. Data on dietary intake and physical activity have been analyzed by a nation-specific software and monitored by published protocols. Urine samples have been analyzed by ^{1}H NMR followed by multivariate statistics. The individual fingerprint emerged and consolidated with increasing the number of samples and reaches ~100 % cross-validated accuracy for about 40 samples. Diet standardization reduced both the intra-individual and the interindividual variability; the effect was due to a reduction in the dispersion of the concentration values of several metabolites. Under standardized diet, however, the individual phenotype was still clearly visible, indicating that the individual's signature was a strong feature of the metabolome. Consequently, cohort studies designed to investigate the relation of individual metabolic traits and nutrition require multiple samples from each participant even under highly standardized lifestyle conditions in order to exploit the analytical potential of metabolomics. We have established criteria to facilitate design of urine metabolomic studies aimed at monitoring the effects of drugs, lifestyle, dietary supplements, and for accurate determination of signatures of diseases.

Keywords Diet · Exercise · Metabolomics · Short term · Urine · NMR

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Introduction

Diet is an important environmental exposure, and many dietary factors (nutrients and non-nutrients) are associated with disease prevention or causation, health promotion, and performance improvement (Bingham 2002; Gavaghan et al. 2000). Different nutritional habits are reflected in the small-molecule composition of urine. Metabolomic profiling, i.e., the measurement of the ensemble of compounds of molecular mass <1,500 Da in a biological sample, applied to urine analysis revealed dietary intake

patterns (Lenz et al. 2004; Lloyd et al. 2011; Solanky et al. 2005; Zuppi et al. 1998) and has been proposed as a powerful tool for a better understanding of the outcomes of dietary intervention (Andersen et al. 2014; ÓSullivan et al. 2011).

The presence of dietary intake patterns, on the other hand, introduces a major day-to-day intrapersonal variability as well as interpersonal differences in metabolomic profiles that can be considered as confounding factors in studies aimed at disease diagnosis, prognosis, and evaluation of the individual response to medical intervention. However, the presence of an invariant part of the urine metabolomic profile has been established via a multivariate statistical analysis of multiple collection of urine samples from each individual (Assfalg et al. 2008). This individual metabolic phenotype is stable over the timescale of years (Bernini et al. 2009) in healthy subjects, and deviations from the individual's profile can be used to monitor disease onset. Most metabolomics studies of clinical relevance rely on the comparison of the profiles from different subjects (patients vs. healthy donors, groups of patients undergoing different treatments or at various degrees of disease progression), and non-uniform lifestyle habits are expected to introduce a high level of metabolic noise. Examples of metabolomic studies aimed at evaluating the effect on urinary metabolic profiles induced by very short (24 h) (Lenz et al. 2003; Walsh et al. 2006; Winnike et al. 2009) or prolonged (2 weeks) (Winnike et al. 2009) standardized diet are available. In the latter case, it was established that any normalization that does occur in urine would do so within 24 h. A comparative study performed in humans and monkeys shows that dietary habits have little or none effects on the urinary metabolic phenotype (Saccent et al. 2014). Characteristic changes in metabolite levels have also been reported in response to physical exercise (Enea et al. 2010). Nevertheless, the extent to which the urine metabolome can be normalized by standardized lifestyle/dietary protocols is not yet described in detail, and the added discriminating value of normalization itself is not clear.

Here, we have evaluated the effect of 3 days of standardized diet and physical activity in reducing the urinary "metabolic" noise by daily multiple collection of urine samples from 24 healthy volunteers over a period of 10 days, with days 1–7 of documented real life (phase I), followed by days 8–10 in a standardized diet and day 10 plus exercise program (phase II) (Fig. 1). Nuclear magnetic resonance (NMR) was used to acquire urinary metabolic profiles for all 1,335 collected samples. Multivariate statistical analysis of the profiles was used to assess the discriminatory power of samples collected during the different phases. Standardized diet resulted effectively in reducing the interindividual variability for several metabolites. The relevance of these results for the design of future metabolomics studies is discussed.

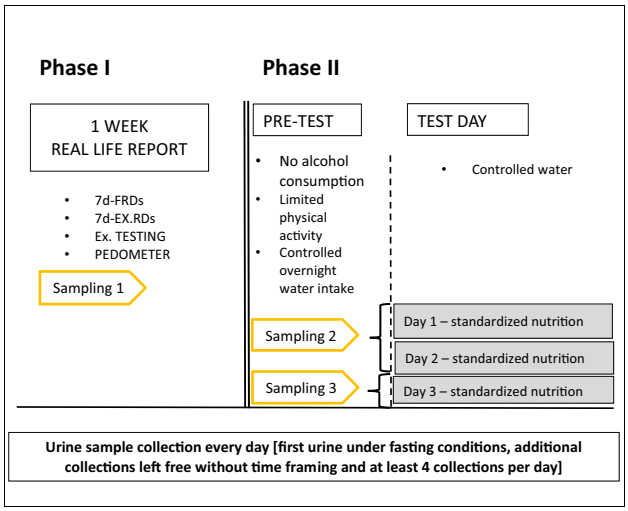

Fig. 1 Study design of data and biosample collection

Table 1 Demographic characteristics of the subjects participating in the study (mean ± SD)

N	24
Male/female	5/19
Age (year)	32.1 ± 10.6
Height (m)	169.8 ± 8.0
Body weight (kg)	64.4 ± 12.5
BMI (kg/m^2)	22.2 ± 3.4

Materials and methods

This study was conducted in accordance with the ethical principles of Good Clinical Practice and the Declaration of Helsinki. The local Research Ethics Committee of the Medical University of Graz approved the protocol before commencement of the study, and all subjects gave written informed consent.

Subject selection

Twenty-four healthy, 19 females and 5 males, subjects, over 21 years of age (22–57, mean 32.1 years) with a body mass index between 18 and 30, were recruited for this study, as summarized in Table 1. The volunteers are not genetically related. Individuals have been recruited by public announcement. Only one female has been screened, but not included in the study, because of time management problems. There were no dropouts. Exclusion criteria used in the selection of the subjects included current use of any regular medication or therapy, participation in another

study within 3 months before the start of the present study, and acute illness within the 2 weeks preceding the start of the study. In addition, subjects were excluded from the study if clinically significant abnormalities in clinical chemistry or hematology were present or there was evidence for a risk of transmitting, through blood or other body fluids, the agents responsible for acquired immune deficiency syndrome, hepatitis B or C. Excessive intake of alcohol, defined as a regular maximum weekly intake of greater than 28 units, was also used to exclude subjects from the study.

Study design

Data on dietary intake have been analyzed by a nation-specific software and nutrition database. Physical activity has been monitored by published protocols and pedometers (Denkwerkzeuge 2010; Hofmann and Tschakert 2011). Subjects were required to abstain from taking any medication (including over-the-counter remedies).

Phase I: Subjects completed a "1-week real-life report" on nutrition and physical activity (7d-Food Record, 7d-Exercise Report, ActiGraph©Pedometer-Report). Physical activity level has also been assessed by a standardized questionnaire (International Physical Activity Questionnaire, IPAQ). Urine was collected from all 24 volunteers for 7 days in a real-life situation and daily documentation of lifestyle factors.

Phase II: The same monitoring was done during 3 days in a standardized diet (Table 2), and an additional standardized exercise program on the third of these days (Fig. 1) was performed individually between 8:00 a.m. and 3:30 p.m.. The standardized diet criteria focused on minimizing the daily variations (low intake of phytochemicals, fiber, alcohol, and coffein) to obtain a more stable metabolic profile. We integrated the Fresubin®Energy drink (Fresenius Kabi), because this is a well-tolerated fiber-free, energy- and nutrient-defined (150 kcal/100 ml, 15 % of energy protein, 35 % Fat, 50 % carbohydrates) sip feed nutrition product.

The test started with 3 min of passive rest sitting on the cycle ergometer and 3 min of warm up at either 20 W (female) or 40 W (male) subjects. Work load was increased by 10–15 W (female) and 20 W (male) dependent on exercise performance determined in an incremental exercise test before the start of the 10-day intervention period. Subjects completed the incremental increase of workload until reaching the target workload which was set midpoint between the first (LTP1) and the second (LTP2) lactate turn point to guarantee a metabolically balanced situation for the whole 45 min of constant load exercise. (Hofmann and Tschakert 2011) A 3-min active cool down at 20 W (female) or 40 W (male) and 3-min passive recovery finished the test.

Table 2 Standardized diet conditions

Day 7, after 18:00 p.m.	Days 8–10
No alcohol, tea, coffee, chocolate	No alcohol, tea, coffee, chocolate
Little physical activity	Little physical activity
Standardized evening meal:	Standardized meals:
1 × 200 ml Fresenius®Energy	5 (for women) and 7 (for men) × 200 ml Fresenius®Energy
Foods low in phytochemicals (for "bite pleasure")[a]	Foods low in phytochemicals (for "bite pleasure")[a]
At least 1.5 l water	At least 1.5 l water

[a] Allowed food in addition to Fresubin (if needed) was white bread, rolls, rice wafers, maize wafers, cornflakes, oat flakes, potatoes, white rice, noodles, milk, yogurt, curd, buttermilk, sour milk, gouda, emmentaler, cottage cheese, cream cheese. All diaries should be lactose-free

[b] Allowed beverages: water, mineral water, and herbal tea

Sample collection

For this analysis, we have collected a total of 1,335 urine samples from all 24 donors. Urine samples were collected from 24 healthy donors 4–6 times a day. Samples were immediately filtrated through a 0.2-µm Whatman GD/X filter (#6904-2502), kept at −20 °C for a minimum of 30 min to a maximum of 75 h before long-term storage in liquid nitrogen without additives. In consequence of our aim to reflect "normal lifestyle," we included 1 weekend in phase I in the probands testing, which caused herein the maximum of 75 h. Time points of collection, storage at −20 °C, and transfer into liquid nitrogen were recorded.

NMR sample preparation

According to a widely used protocol (Assfalg et al. 2008; Bernini et al. 2009; Weckwerth 2007), frozen urine samples were thawed at room temperature and shaken before use. A total of 540 µl of urine was added to 60 µl of sodium phosphate buffer (0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4 in 100 % $2H_2O$, pH 7.0), also containing 10 mM sodium trimethylsilyl [2,2,3,3–2H4] propionate (TSP) and 30 mM sodium azide. 450 µl of the mixture was pipetted into 4.25-mm NMR tubes (Bruker BioSpin srl).

NMR spectra

^{1}H-NMR spectra were acquired using a Bruker 600-MHz spectrometer operating at 600.13-MHz proton Larmor frequency and equipped with a 5-mm CPTCI ^{1}H-^{13}C-^{31}P-^{2}H cryo-probe including a *z*-axis gradient coil,

an automatic tuning-matching (ATM), and an automatic sample changer. A PT 100 thermocouple served for temperature stabilization at the level of approximately ±0.1 K at the sample. Before measurement, samples were kept for 3–5 min inside the NMR probehead, for temperature equilibration. For each urine sample, a one-dimensional (1D) NMR spectrum was acquired with water peak suppression using a standard pulse sequence (NOESYpresat; Bruker), 64 scans, 64 k data points, a spectral width of 12,019 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s, and a mixing time of 100 ms. ^{1}H-^{1}H J-resolved (J-res) spectra were also acquired in order to get more information about signal multiplicity and coupling patterns.

Spectral processing and analysis

Free induction decays were multiplied by an exponential function equivalent to a 1.0-Hz line-broadening factor before applying Fourier transform. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated using TopSpin 2.1 (Bruker Biospin). Spectra were aligned calibrating the TSP peak at 0.00 ppm. Each 1D spectrum in the range between 0.2 and 10.00 ppm was segmented into 0.02-ppm chemical shift bins, and the corresponding spectral areas were integrated using AMIX software (Bruker BioSpin). Regions between 6.0 and 4.5 ppm containing residual water and urea signals were removed. The total spectral area was calculated on the remaining bins, and prior to pattern recognition, normalization was carried out dividing each bin by the total spectral area value. Alternatively, probabilistic quotient normalization (PQN) was used as a further scaling method (Dieterle et al. 2006).

All resonances of interest were assigned on template one-dimensional NMR profiles by using matching routines of AMIX 3.8.4 (Bruker BioSpin) in combination with the BBIOREFCODE (version 2-0-0; Bruker BioSpin) reference database and published literature when available.

Statistical analysis

Unsupervised and supervised statistical analysis tools were applied to study the metabolomics profiles (Madsen et al. 2010). Principal component analysis (PCA) was used for first-step exploratory data analysis, to get a general overview of samples distribution. The supervised statistical procedure employed for data reduction and classification was a combination of PSA (Assfalg et al. 2008) or partial least squares (PLS) (Bertini et al. 2009) with canonical analysis that was used as the method for discriminating different groups, depending on the focus of the analysis performed (e.g., to discriminate individuals, to discriminate different days of the study, and to discriminate samples collected at different times of the day). The accuracy, sensitivity, and specificity for the classification were assessed by means of a double cross-validation scheme (Szymanska et al. 2012). The original dataset was split into a training set (80 % of the samples) and a test set (20 % of the samples) prior to any step of statistical analysis. To avoid overfitting, the number of PLS components was chosen on the basis of a 5-fold cross-validation performed on the training set only, and the best model was used to predict the samples in the test set, using a k-nearest neighbors learning method ($k = 5$) as the final classifier. The whole procedure was repeated inside a Monte Carlo cross-validation scheme, and the results were averaged.

To assess which metabolites (i.e., NMR peaks) were significantly different between different sets and to follow the metabolites' changes over different time points, univariate Wilcoxon test was used. A P value ≤ 0.05 (after Bonferroni correction for multiple tests) was considered statistically significant. The reason to use a univariate test to enlighten relevant metabolites is that univariate models are easy to interpret and provide a mean to assess the significance of the results, while multivariate models provide a full overview of the metabolic fingerprints, but often lack interpretability, especially in high-dimensional data (Hageman et al. 2008). All calculations were made using the R statistical environment (Ihaka and Gentleman 1996).

Results

The metabolic information contained in the NMR spectra of all samples was analyzed to derive information about phase I, phase II, and from the comparison of the two phases. In both phases, we based our analyses on the first urine in the morning and all urines of the day collected without regular times.

In phase I, each individual was under free lifestyle, but food intake was carefully recorded; a summary of the average per day energy and macronutrients intake is shown in the first column of Table 3.

When all the 928 samples (corresponding to an average of 38.7 samples/individual) collected during phase I were used to retrieve the individual phenotype, a good interindividual discrimination was obtained, with a mean accuracy of 98.7 % (Fig. 2a). The corresponding diagonal values of the confusion matrix, i.e., the recognition accuracy for each individual, are shown in Table 4. The mean value of accuracy drops to 88.7 % when only the first urine of the morning was used to build the dataset. These values were obtained using total area normalization. To exclude any possible bias deriving from the normalization method, a PQN approach was also used, which provided mean values of accuracy of 99.3 and 90.1 %, when using all

sample or the first urine of the morning only, respectively. This test demonstrates the reliability of the analysis irrespectively from the normalization method. The effect was reasonably attributable to the decreased number of samples per individual (7 vs. 38.7). Here, we verified the impact of the total number of samples on recognition accuracy, demonstrating that what counts the most for a high recognition accuracy is the total number of samples and that multiple collections within the same day are equally good as the same number of samples collected as first urine of the morning under fasting conditions in different days, despite the effect of food/beverage intake and the established modulation of the urine metabolome by the circadian clock (Slupsky et al. 2007). A circadian dependence of the NMR profile was also clearly visible in our samples. When a supervised analysis was done for all 24 individuals separately as a function of the collection time during the day (4–6 time points per day), we observed a good clustering of samples that belongs to one of 6 time points; in general, the cluster composed of the first samples in the morning is well separated from the other 5 clusters of the day.

A good separation existed between samples from male and female donors (5 male and 19 female donors); in a supervised analysis regarding gender of the individuals using samples from all 10 days, we obtained an accuracy of 98.7 % using all samples and of 94.06 % using the first urines only).

When a supervised PLS/CA analysis was performed on all samples of phases I and II providing the information about collection day (day 1–10), phase II samples are clearly separated from those of phase I (Fig. 3), indicating significant differences in the urinary metabolome between the free and standardized diet regimes. Within phase II, day 10, in which standardized exercise was introduced in

Table 3 Per-day/person average energy and macronutrients intake (mean ± SD) during the two phases of the study

	Phase I	Phase II
Energy (kcal/d/kg)	29.8 ± 7.3	25.6 ± 5.6
Protein (g/d)	71.1 ± 17.5	63.6 ± 18.6
Carbohydrate (g/d)	218.1 ± 57.1	207.2 ± 52.0
Fat (g/d)	69.6 ± 18.9	60.3 ± 18.7
Protein (% of energy)	15.8 ± 2.8	15.9 ± 0.1
Carbohydrate (% of energy)	47.7 ± 5.1	52.2 ± 4.3
Fat (% of energy)	30.3 ± 8.6	34.1 ± 3.8
Alcohol (g/d)	17.8 ± 4.1	–
Alcohol (% of energy)	10.2 ± 11.4	–
Fiber (g/d)	17.8 ± 4.0	2.9 ± 2.0

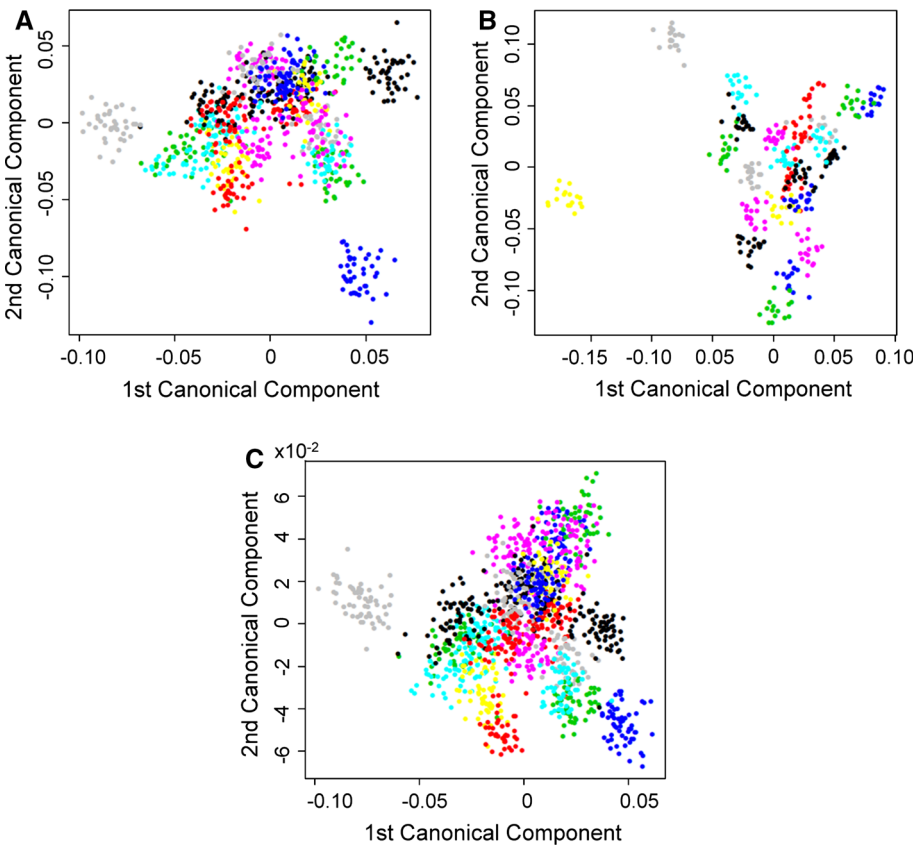

Fig. 2 PCA/CA score plots for the discrimination between individuals using: **a** Phase I samples. **b** Phase II samples. **c** all available samples. Samples are *colored* by individuals; some *colors* are repeated

addition to standardized diet, was only slightly different from days 8 and 9. Although standardization started at 6:00 p.m. of day 7 (see Fig. 1), the first urine of day 8 was still closer to the samples of phase I than those from phase II. Subdividing samples into three groups, a better recognition accuracy was obtained maintaining the first urine of day 8 together with samples of phase I: an accuracy of 92.7 % was obtained when considering group I composed by all samples of phase I plus the first urine of day 8, group 2 composed by all samples of day 8 but the first urine and all samples of day 9, and group 3 composed by all samples of day 10; the accuracy is 91.3 % when the first urine of day 8 was included in group 2.

The observed differences between samples in phases I and II originated from significant changes in the levels of several metabolites. During phase I, the metabolite levels measured for different subjects varied without following any specific trend; instead, clear differences were observed between phase I and phase II, with an overall reduction in the spreading of the concentration of most metabolites among the different individuals as a result of the diet standardization (Fig. 4). This analysis was performed using only the first urine of the morning, and urine of day 8 was associated with the samples of phase I. More homogenous metabolite levels were observed already on day 9. Additionally, hippuric acid ($p = 9.643 \times 10^{-7}$), m-hydroxy phenyl-proprionic acid (m-HPPA; $p = 0.000919$), trigonelline ($p = 1.381 \times 10^{-6}$), and trimethylamine N-oxide (TMAO; $p = 0.002159$) displayed significantly lower concentrations in phase II than in phase I (Fig. 4) reflecting the different contents of many food compounds in the free and standardized diets (Table 3). Under the standardized diet regime, the nutrients contents were extremely similar at each meal. Still, differences among samples collected at different time points during the day reproduced the same pattern observed in phase I, thus demonstrating how daily changes in urinary profiles are dominated by circadian rhythms.

The observed homogenization of the metabolome composition under standardized diet in phase II contributes to the reduction in the intrapersonal variability, as visible from the comparison of panels A and B of Fig. 2, without any significant loss in the interindividual discrimination; the mentioned recognition accuracy of 98.7 % obtained with samples of phase I compares well with the accuracy of 98.9 and 99.2 % obtained, respectively, using the 407 samples of phase II only, or all 1,335 samples of both phases together. It should be noted that these calculations were conducted on different numbers of samples. To evaluate the real advantage of collecting samples under a standardized regime in clinical studies, we have also compared the accuracies obtained for groups of samples coming from 3 days of phase I with all sample from phase

Table 4 Individual recognition accuracies using all samples of phase I (PCA/CA/KNN analysis, supervised by individual)

1 M	97.7 %	13 F	98.7 %
2 F	95.5 %	14 F	97.8 %
3 F	97.6 %	15 F	98.6 %
4 F	96.6 %	16 F	99.3 %
5 F	98.7 %	17 F	99.1 %
6 F	96.5 %	18 F	100 %
7 F	99.5 %	19 M	100 %
8 M	99.2 %	20 F	98.6 %
9 F	97.4 %	21 M	99.2 %
10 M	99.4 %	22 F	100 %
11 F	96.6 %	24 F	100 %
12 M	100 %	25 F	96.6 %

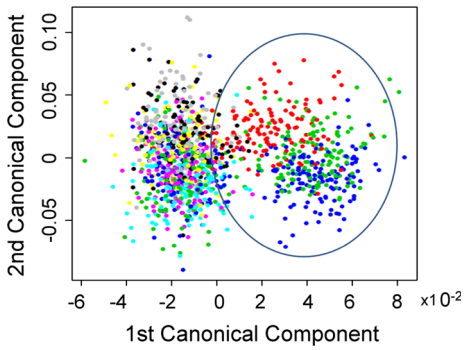

Fig. 3 PLS/CA score plots for the discrimination between days of study. Phase II days are *encircled*. Samples are *colored* by the day of study

II (in such a way to have a similar number of samples in each group). Under these conditions, the PCA score plot shows more compact clusters for each individual in phase II than in phase I (Fig. 2c). Additionally, to explain the 99.9 % of total variance, a lower number (89) of PCA components was needed for phase II than for phase I (94 components).

Discussion

In this metabolomics study, we present the impact of the total number of samples on the recognition accuracy and report on the extent to which the urine metabolome can be normalized by our standardized lifestyle/dietary protocols.

The daily multiple collection of urine samples during phase I was used to retrieve the individual phenotype. In phase II, we evaluated the effect of 3 days of standardized diet and exercise in reducing the urinary "metabolic" noise. The use of only the first urine of the morning to build the dataset dropped the mean value of accuracy from 98.7 to 88.7 % (or from 99.2 to 90.1 using the PQN approach),

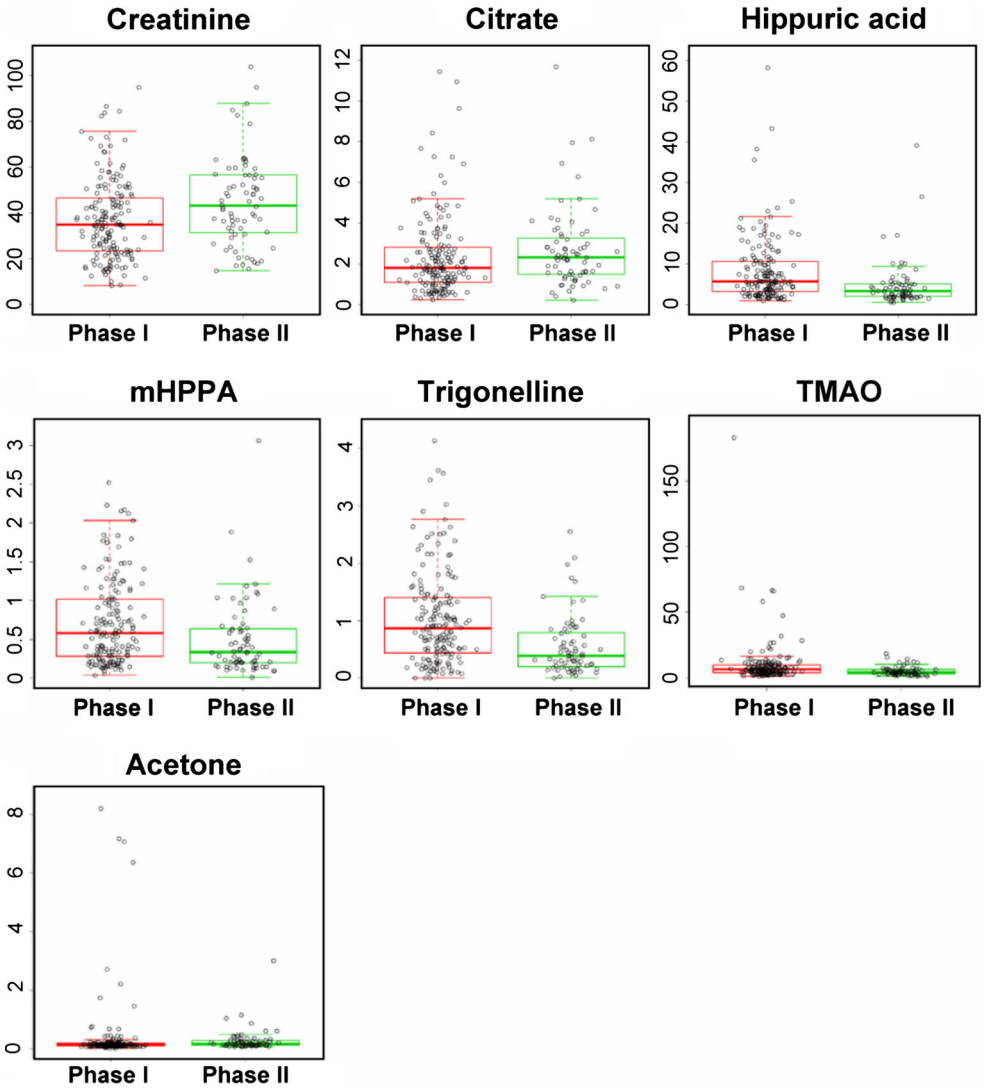

Fig. 4 *Box* whiskers plots showing the trends of significant metabolites in the first urine of the morning in phases I and II. Relative intensities are reported in arbitrary units

an effect that is reasonably attributable to the decreased number of samples per individual. An optimal minimum number of samples per individual of the order of 40 has been indeed already identified in previous works (Assfalg et al. 2008). Compared with previous studies, where the 40 samples had been collected as first urine of the morning under fasting conditions in different days, we showed here that what has the greatest impact on a high recognition accuracy is the total number of samples and that multiple collections within the same day are equally good, despite the effect of food/beverage intake and a possible modulation of the urine metabolome by the circadian clock (Slupsky et al. 2007). The last effect was also clearly visible in our samples. Our data support that small variations due to meal composition represent a random background that do not alters the individual metabolome. In a recent paper, the authors reported only minor effects due to the meal on the urinary metabolome. The authors reported small intra-individuals variations during the day, with major variations only for the first void urines (Lenz et al. 2003). Dallmann and colleagues demonstrated that there is a strong direct effect of the endogenous circadian clock on multiple human metabolic pathways that is independent of sleep or meals (Dallmann et al. 2012).

Urinary metabolomics is well established as a tool to monitor dietary intake and to evaluate the outcome of dietary intervention (Andersen et al. 2014; Holmes et al. 2008; Lenz et al. 2004; Lloyd et al. 2011; ÓSullivan et al. 2011; Solanky et al. 2005; Zuppi et al. 1998). The metabolite levels measured for different subjects varied without following any specific trend in phase I, but were different between phase I and phase II. More homogenous metabolite levels were observed already on day 9, confirming earlier observations that the effects of diet on the urinary metabolome are already visible within the first 24 h (Lenz et al. 2003; Walsh et al. 2006). Accordingly, under

the standardized diet of phase II, we could observe a clear overall homogenization in metabolites levels and significant decreases in the concentration of four molecules, namely hippuric acid, m-HPPA, trigonelline, and TMAO, which are reconducible to exclusion of a number of food and beverages in this phase, as detailed below. Elevated urinary excretion of hippuric acid is associated with the intake of tea, coffee, wine, fruits, and vegetables (Cathcart-Rake et al. 1975; DuPont et al. 2002; Gonthier et al. 2003; Mulder et al. 2005; Olthof et al. 2003; Rechner et al. 2002; Toromanovic et al. 2008), which were all excluded in phase II. m-HPPA is one of the major metabolites of ingested caffeic acid and of the phenolic degradation products of proanthocyanidins, the most abundant polyphenol present in chocolate (Konishi and Kobayashi 2004; Rios et al. 2003), and coffee and chocolate consumption was forbidden in phase II. Trigonelline is a product of the metabolism of niacin (vitamin B3), which is excreted in the urine; it is also found in coffee and several plants such as oats and potatoes (Wishart et al. 2013). TMAO is biosynthesized endogenously from trimethylamine, which is derived from choline, which can be derived from dietary lecithin (phosphatidylcholines) or dietary carnitine; carnitine is found in red meat, and lecithin is found in eggs and is commonly used as an ingredient in processed food; high levels of TMAO are found in many seafoods (Stella et al. 2006; Wang et al. 2005; Wishart et al. 2013).

The reduction in the differences among the NMR profiles induced by standardized diet/lifestyle gave rise to a reduction in the distance between the points associated with each individual in the metabolic space of Fig. 2. Furthermore, a small reduction in interindividual distances was also observed. However, each donor was still well discriminated from the others. This is a very important result that provides a clear evidence of the fact that the individual lifestyle is not the key determinant of the invariant part of its metabolome and negligibly contributes to the individual phenotype. Diet standardization may help identifying the invariant part of the urinary metabolome removing some of the day-to-day intrapersonal and interpersonal variability, thus possibly reducing the minimum set of required samples and facilitating the statistical analysis, but the interpersonal differences associated with the metabolic phenotype are still well "visible." This finding is further proof of concept of the relevance of metabolomics in biomedical research; the presence of a strong signature of the personal phenotype, which goes beyond the individual lifestyle, will permit to assign deviations from individual-specific metabolic traits to biochemical alterations of his/her "healthy" status for an early disease diagnosis. To further explore individual-specific metabolic traits and its relation to nutrition, future cohort studies should collect multiple samples for each study participant even when lifestyle conditions are highly standardized.

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Conflict of interest None.

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