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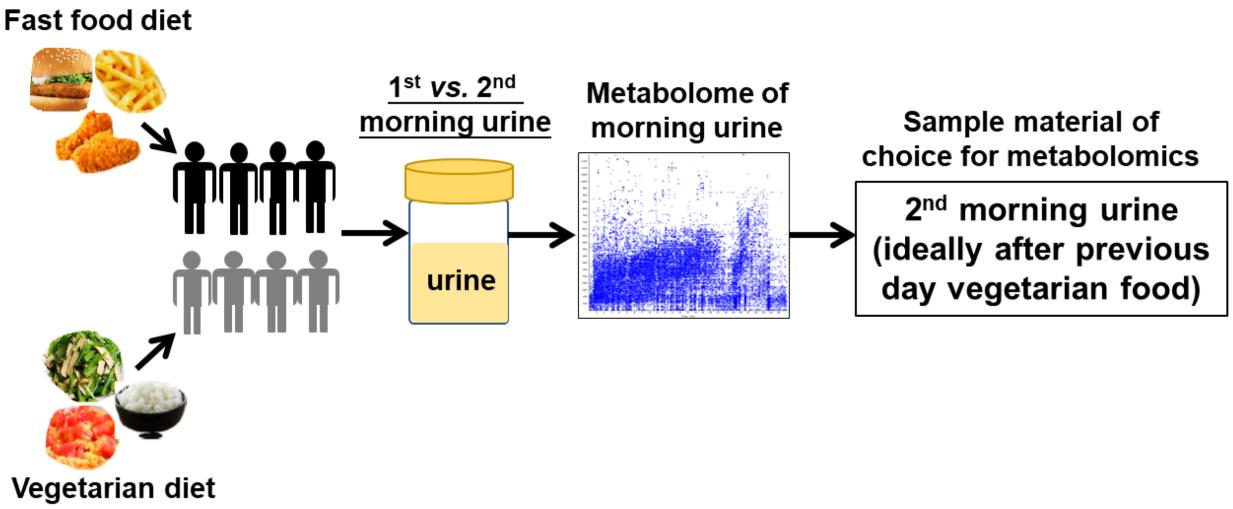

Which is the urine sample material of choice for metabolomics-driven biomarker studies?

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Abstract

Urine-based metabolomics-driven strategies for the discovery of biomarkers are increasingly developed and applied in analytical chemistry. But valid, data-based recommendations for a urine sample material of choice are lacking. We investigated first and second morning urine (MU), which are the most commonly used urine specimens. Potential major factors biasing metabolomics biomarker results in these sample materials were studied. First, 35 1st and 2nd MU samples were collected from healthy, young men after an overnight fast. Subsequently, two subgroups were built, one having fast food at lunch and dinner (n=17), the other vegetarian meals (n=18). Again 1st and 2nd MU were collected. Non-targeted liquid chromatography-mass spectrometry was applied for analyses. More than half of the >5400 urinary ion features showed a significant difference between 1st and 2nd MU. Just two fast food meals on previous day significantly affected around 30% of all metabolites in 1st and 2nd MU. In contrast, the effects of two vegetarian meals in 2nd MU were only minor. Additionally, we describe 47 metabolites in urine, possible hits in biomarker studies, which are susceptible to the diet the day before sample collection. They should be handled with caution until validation in diet-controlled studies. Based on our results we think the second MU, ideally collected after standardized vegetarian meals and drinking only water on the previous day, is most suitable for valid analysis of biomarkers in urine.

Keywords: first morning urine; second morning urine; metabolomics; biobank; diet; preanalytical

1. Introduction

Metabolomics strategies are frequently developed and applied by analytical chemists for the discovery of novel biomarkers. Up to now plasma or serum is the preferred sample material for these purposes, but the number of reports using urine is continuously growing [1-6]. Urine is collected in different ways, namely spot urine (collected at random), first morning urine (MU), second MU and 24h collection urine, which may all be used for metabolomics investigations. However, the specific characteristics of these sample materials are quite different, carrying pros and cons with respect to metabolomics analyses. First MU is collected in the bladder overnight and can therefore be seen as a kind of collection urine. Second MU is collected spontaneously after a 12h overnight fasting period, usually between 7 and 10 am after first MU is voided. 24h collection urine is preferred for quantitative measures in routine diagnostics, particularly for hospitalized patients.

Spot urine collected at random over the day should be avoided for metabolomics analyses, since the individual metabolite pattern in spot urine can be highly affected by various preanalytical factors like day time of collection, physical activity, fasting/feeding, and many others. The metabolome of 24h collection urine can be affected by the presence of living, metabolic active cells or released enzymes thereof and lack or variable cooling during the sampling period. Particularly at room temperature metabolically active cells like erythrocytes, leukocytes, bacteria, yeasts, or fungi can modify the urinary metabolome inside the sample collector and be a serious problem [7]. Stabilizers like thymol or borate may reduce the risk [8], but are not common in use. Moreover, the collection of 24h urine is error-prone, because it is usually collected by the patients without supervision by medical experts. To minimize such problems first MUs are an often used specimen in metabolomics studies [3, 9-15], which is also recommended by a recent white paper of the metabolomics society initiative [7]. The use of second MU in metabolomics studies is rarely reported. In general sample collection for

metabolomics should be tightly controlled, ideally applying a standard operating procedure (SOP) [7, 16, 17]. However, to the best of our knowledge, being faced with the decision which MU specimen is preferable for metabolomics-driven studies to discover novel biomarkers, up to now no data are available to facilitate the selection.

The goals of our study were twofold. First, we aimed to figure out differences between the metabolome of 1st and 2nd MU by non-targeted ultrahigh-performance liquid chromatography (UPLC) mass spectrometry (MS). Second, we investigated short-term effects of different diets consumed the day before sample collection on the metabolite biomarker pattern in 1st and 2nd MU. Finally, we draw conclusions from both investigations which urine could be the sample material of choice for metabolomics-driven biomarker studies.

2. Materials and Methods

2.1 Samples and study design

An overview showing the experimental design of the study is presented in Figure 1. 35 healthy, young, omnivore, male volunteers participated in the study, which was performed in accordance with the current revision of the Helsinki Declaration. All subjects gave written informed consent. The study protocol was approved by the local ethics committee. The participants were instructed to avoid nocturnal urinating, strenuous exercise as well as other unusual activities two days before and during the study. On experimental day 1, the participants started fasting at 7 p.m. (Figure 1). On subsequent experimental day 2, 1st and 2nd midstream morning urines (MU) were collected at 7 a.m. after the individuals awaked from sleep and at 8 a.m., respectively. Next, all participants had the same breakfast (steamed buns, rice porridge, pickled vegetables, eggs). Thereafter, participants were randomly assigned (stratified by BMI) to a fast food diet (FFD) group (n=17) or a vegetarian diet (VD) group (n=18) (Table S1). The FFD group consumed two hamburgers, two chicken wings and French fries at lunch and dinner on experimental day 2

(Figure 1). The VD group had a standardized vegetarian diet at lunch and dinner (fried celery with dry bean curd, rice, and scrambled egg with tomatoes). On subsequent experimental day 3 again 1st and 2nd MU were collected. All samples were immediately stored at 4°C after collection and aliquots were prepared and frozen at -80°C within 2h. All meals were pre-portioned, only drinking of water was allowed.

2.2 Sample preparation

Urine samples (100 µL) were thawed on ice, warmed to room temperature and strongly vortexed. Next, 400 µL of methanol containing nine stable isotope labelled internal standards (carnitine C2:0-d3, carnitine C6:0-d3, carnitine C10:0-d3, leucine-d3, phenylalanine-d5, tryptophan-d5, cholic acid-2,2,4,4-d4, and one unlabeled standard (leucine enkephalin) were added, vortexed, and centrifuged at 15,700 × *g* for 10 min at 4°C. 200 µL of the supernatant was dried in a speed-Vac. For non-targeted UPLC-MS analysis the samples were re-dissolved in 150 µL of 5% acetonitrile. Quality control (QC) samples were generated by pooling an identical aliquot from all urine samples. QC sample pretreatment was performed as described above. A QC sample was injected after every tenth sample.

2.3 Non-targeted metabolomics

Samples were analyzed by an ACQUITY™ UPLC coupled with a qTOF mass spectrometry (triple TOF5600 System; AB SCIEX, Framingham, USA). Chromatographic conditions (applied in positive and negative ionization mode): T3 HSS column (1.8 µm, 2.1×100 mm) (Waters, USA), column temperature 40°C; mobile phase A 0.1% formic acid in water; mobile phase B 0.1% formic acid in acetonitrile. The gradient elution initially started with 5% B, which was maintained for 1 min, then increased linearly to 50% B in 17 min, and finally increased linearly to 100% B in 0.5 min, maintained for 4 min and then returned to 5% B for 3 min equilibration

before next injection. Data acquisition was performed in full scan mode (scan range: m/z 50-1200) combined with information-dependent analysis mode. Mass spectrometric parameters were as follows: ion spray voltage: +5500 V (ESI+) and -4500 V (ESI-), curtain gas: 35 PSI, declustering potential: 100 V (ESI+) and -100 V (ESI-), collision energy: 10 V (ESI+) and -10 V (ESI-), and an interface heater temperature of 500 °C. For information-dependent analysis, the collision energy was 30 V in positive and -30 V in negative mode. Collision energy spread was equal to 10 in both ion modes.

2.4 Multivariate and univariate analysis

MarkerView software (AB SCIEX, USA) was applied for peak matching and alignment. Areas of ion masses were calibrated by seven isotope-labeled and one non-labeled internal standards. In brief, in the QC samples all ion features were corrected by each internal standard. Subsequently, a suitable internal standard for each ion feature in the pooled QC samples was defined with respect to the lowest RSD %. Then each ion feature of the real, non-pooled urine sample was corrected according to the corresponding internal standard defined in the previous step in the pooled QC samples. In a second calibrating step the individual urine creatinine concentration was used to calibrate the metabolites in each sample individually. The creatinine calibration is very important for the analysis of urine samples to avoid a bias by signal differences between samples coming from variations of the drinking volume of the individuals, which lead to more or less diluted or concentrated urines. Further details were given in a previous publication [18]. Subsequently multivariate data analysis was performed.

SIMCA-P software (version 11.0; Umetrics) was used for principal component analysis (PCA) of the data and unit variance (UV) scaling was applied. For the comparison between 1st and 2nd MU, a paired nonparametric test with p-value < 0.05 and FDR limit ≤ 0.05 was applied.

3. Results and Discussion

In the first step, 1st morning urine (MU) and 2nd MU samples were compared by UPLC-MS driven non-targeted metabolomics. 1st MU was collected at 7 a.m. and 2nd MU at 8 a.m. at the same day, from 35 healthy young male volunteers. Figure 2A illustrates distinct differences between the metabolite profiles detected in 1st and 2nd MU in a principal component analysis (PCA). More than half of the >5400 detected urinary ion features were significantly different (paired nonparametric test; p-value < 0.05; FDR ≤ 0.05), and 59% of these were higher in 1st MU. Subsequently, in a further LC-MS driven analytical step, 165 out of the >5400 ion features was either identified by corresponding standard compounds, or the putative identity is based on the MS^2 fragmentation pattern and/or the exact mass (Table S2). A representative example of a LC-MS/MS metabolite identification is presented in Figure S1. Retention time, MS1 and MS2 information of the compound of interest in comparison to the corresponding standard compound was used to confirm the identity of the metabolites of interest. 40 of these 165 metabolites showed significant differences between 1st and 2nd MU (Table S3). For instance, in 1st MU higher relative levels of amino acid and derivatives, organic acids and derivatives thereof, caffeine and others, as well as lower relative levels of short- and medium-chain acylcarnitines, bile acids, etc. were detected. In 2nd MU we detected striking higher hydrocortisone relative levels although samples were collected only 60 min later than 1st MU (Figure 2B). Hydrocortisone is a hormone having one of the most distinct circadian rhythms in human physiology, peaking in the morning after getting up. Thus, effects of circadian rhythmicity on the urinary metabolome should be taken into account during decision making to collect 1st or 2nd MU, and standardization of the sampling time point in biomarker studies is needed.

Based on metabolomics data from long-term nutritional studies it can be concluded that dietary effects could be major factors biasing results of urinary biomarker studies [19]. This raises the question of whether short-term nutritional effects may also affect metabolite signatures

in MU. Consequently, we next aimed to figure out the impact of previous day meals on metabolite profiles in MU. Another goal was to detect in these short-term experiments distinct diet-associated urinary metabolites baring the risk for misleading interpretation of novel diagnostic biomarkers. To do so, the study group of 35 healthy young volunteers was divided at random in two groups which consumed two different kind of standardized meals the day before MU collection (Figure 1), namely a fast food (FFD, n=17) or a vegetarian diet (VD, n=18).

FFD and VD led to marked differences between the urinary metabolome in 1st MU (Figure 2C), which persist also in 2nd MU (Figure 2D). To figure out specific effects of particular diets on urinary biomarkers, we next compared the metabolite profiles in each group before vs after dietary intervention. In 1st MU of the fast food group 30% of the >5400 urinary ion features per individual were significantly changed, and in the VD group 21% were altered (paired nonparametric test; p-value < 0.05; FDR limit ≤ 0.05). In contrast, in 2nd MU only 6% of all ion features were significantly altered by vegetarian diet, while in the FFD group still 34% were changed. Noteworthy, the dietary effects are not based on long-term dietary habits, but only by the meals the day before sample collection. To gain deeper insights in distinct metabolites behind these diet-associated changes, we investigated the relative levels of the 165 metabolites shown in Table S2 before and after the dietary intervention. The findings presented in Table 1 and 2 are in line with the results from the non-targeted investigations described above, i.e. again FFD-associated changes are more pronounced and persist also in 2nd MU. We detected in total 47 metabolites (Table 1 and 2 together), which could be possible hits in diagnostic or pathomechanistic biomarker studies, but are susceptible to the diet the day before sample collection. We want to remark, that some limitations of our study should be considered for the interpretation of the presented data. One is, that defined by the chromatographic selectivity of reversed-phase chromatography on the HSS T3 column, only a portion of the whole metabolome present in urine is covered. Consequently, applying a different analytical approach, for example,

the use of hydrophilic interaction or normal phase chromatography, may lead to additional, complementary findings. The other one limitation is the small number of investigated subjects (only 35 in total, i.e. 18 and 17 per group).

Almost half of the metabolites affected by FFD in 1st and 2nd MU are acylcarnitines (Table 1). Acylcarnitines, as well as free carnitine, have been reported to originate from diets rich in meat [20-22]. Interestingly C11:1 carnitine and 2,6 dimethylheptanoyl carnitine, two major products of peroxisomal oxidation of pristanic acid and phytanic acid [23], are amongst the metabolites showing the most striking diet-related increases in the FFD group (Table 1). In the VD group the relative levels of these metabolites are decreased (Table 2). Acetylcarnitine, a major product of the mitochondrial beta-oxidation, is also >5-fold increase in 1st and 2nd MU after FFD and reduced to 0.5 after VD (Tables 1 vs 2). This could be a hint for enhanced oxidation of fatty acids in the FFD group. In general, many metabolites showing increased relative levels after FFD on the previous day display reverse effects in the VD group, which is particularly true for acylcarnitines (Table 1 vs 2). We detected in 1st MU in both groups a pronounced decrease in caffeine or related metabolites (methylxantine, dimethyluric acid) after the dietary intervention, possibly caused by the cessation from tea and coffee during the dietary experiments (Tables 1 and 2, Figure S2). This finding is of interest, since beverages can also affect the composition of the urinary metabolome [24, 25], and the drop in the caffeine metabolites in the urines of our study participants clearly indicates that the participants followed the instruction to drink only water.

A closer look on the detected metabolites in 2nd MU reinforced the impression that the effect of VD on the urinary metabolome is less pronounced (Table 2), because only 9 out of these 165 metabolites showed significant differences between the urines collected before and after VD. Overall, the metabolome of 2nd MU after the vegetarian diet used in our study is much less affected.

In 1st MU dietary effects on metabolite profiles have already been studied in clinical and population settings by several groups [9-14], but, to the best of our knowledge, none has investigated also 2nd MU. The diets in these studies were applied either for several weeks [12, 14] or at least for 48h [10], but not solely on the day before sample collection. All these studies reported food effects on the urinary metabolome of 1st MU [10-14]. It has also been demonstrated that standardization of the diet reduced the intra-individual variability of the metabolome in 1st MU [14, 26]. Recently country-dependent effects on the urinary metabolome caused by typical local food were demonstrated [11, 13]. These findings may be of consequence for international biomarker studies using urine, but also for national multi-center studies in countries like China with great regional variations in food preferences. Noteworthy, all these studies were NMR-driven [10-14]. Our data go beyond these reports, because the sensitivity of NMR is much lower than LC-MS (mmol/L vs pmol/L) [27], therefore covering solely the most abundant metabolites [28]. Concerning the detected urine metabolites reported in our study we want to make a general critical remark with respect to the selective coverage of LC-MS approaches in metabolomics. The coverage of all metabolites present in a body fluid can hardly be achieved therefore every chosen chromatographic material favors the detection of distinct portions of the metabolome based on chemical characteristics of the corresponding metabolites. We consciously decided to use a reversed-phase column, because this stationary phase is commonly used in metabolomics investigations of urine, serum and plasma[29, 30]. This should allow the transfer of our findings to the projects of the majority of analytical chemists dealing with the investigation of human urine samples.

4. Conclusions

Standardization of the diet before sample collection seems to be as important as the decision of sample specimen and collection time point in metabolomics-driven biomarker studies

investigating morning urine. The dietary effects in 1st MU are strong. Fast food meals affect the metabolome in 1st and 2nd MU in a similar marked way. Only minor alterations were detected in 2nd MU after vegetarian diet. We recommend 2nd MU as the preferential sample material for biomarker studies. However, our study revealed that a perfect urine specimen for metabolomics-driven biomarker studies is not feasible. If 2nd MU is intended to be used, ideally a carefully standardized specimen collection should be combined with a vegetarian diet starting 20h before. Since standardization of the diet one day before sample collection is difficult to achieve, we suggest as a compromise to harmonize at the least the composition of the meals and request to drink only water. Finally, novel urinary biomarkers detected in samples of non-diet-controlled studies should be handled with caution until further confirmation.

List of abbreviations

ESI, electrospray ionization; FDR, false discovery rate; FFD, fast food diet; MS, mass spectrometry; MU, morning urine; QC, quality control; UPLC, ultrahigh-performance liquid chromatography; PCA, principle component analysis; VD, vegetarian diet.

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Table 1 Effect of previous day fast food meals (FFD) on relative levels of metabolites in morning urine. Relative levels of metabolites in 1st MU after FFD were compared to relative levels in 1st MU before the dietary intervention. The same comparison was performed for 2nd MU. Conspicuous metabolites with significant ≥1.2-fold higher or ≤ 0.8-fold lower levels after FFD are given. 1st MU was collected at 7 a.m. after an overnight fasting period of 12h and 2nd MU was collected 60 min thereafter at 8 a.m. on the same day (for experimental details see Figure 1). The metabolites were grouped by their species and sorted by fold-changes in descending order. Based on literature search possible sources of the metabolites are listed. The comparison was performed by paired nonparametric test with FDR limit ≤0.05 (n=17).

	1st morning urine		2nd morning urine	
Metabolite	**Fold change after FFD**	***p value***	**Fold change after FFD**	***p value***
carnitine C11:1[a,c,k]	8.77	*<0.001*	5.92	*<0.001*
carnitine C2:0[a,c,d]	6.66	*<0.001*	5.18	*<0.001*
2,6 dimethylheptanoyl carnitine[a,i]	5.29	*<0.001*	3.99	*<0.001*
carnitine C3:0[a,c,d]	4.87	*<0.001*	4.38	*<0.001*
carnitine[b,g]	4.38	*<0.001*	3.72	*<0.001*
carnitine C5:0[a,c,e]	2.86	*<0.001*	2.25	*<0.001*
carnitine C8:0+OH[a,c,k]	2.78	*<0.001*	2.30	*<0.001*
carnitine C10:1[a,c,e]	2.5	*<0.001*	2.12	*<0.001*
carnitine C6:0[a,c,g]	1.9	*<0.01*	1.57	*<0.01*
carnitine C8:4[a,c,f]	1.76	*<0.01*	n.s.	
carnitine C4:0[a,c,f]	1.71	*<0.001*	1.48	*<0.001*
carnitine C12:2[a,c,i]	1.67	*<0.01*	1.69	*<0.01*
carnitine C12:1[a,c,e]	1.42	*<0.05*	1.54	*<0.01*
carnitine C9:1[a,c,g]	1.31	*<0.01*	n.s.	
carnitine C8:0[a,c,e]	2.84	*<0.01*	2.70	*<0.01*
carnitine C10:0[a,c,e]	1.2	*<0.01*	n.s.	
carnitine C10:4[a,c,e]	-	-	1.34	*<0.01*

carnitine C10:3[a,c,e]	-	-		1.25	*<0.05*
acetyl-L-leucine[b,k]	1.98	*<0.001*		n.s.	
homocysteine[b,g]	1.61	*<0.01*		1.51	*<0.01*
citrulline[b,g]	1.34	*<0.01*		n.s.	
lysine[b,d]	1.33	*<0.01*		n.s.	
valine[b,i]	1.27	*<0.01*		1.24	*<0.001*
proline[b,i]	-	-		2.14	*<0.01*
2- or 3-hydroxy phenylacetic acid[a,c,g]	2.47	*<0.001*		1.91	*<0.001*
2-methylglutaric acid[b,k]	1.82	*<0.001*		1.44	*<0.001*
pimelic acid[b,k]	1.58	*<0.001*		n.s.	
methylimidazoleacetic acid[a,k]	1.41	*<0.01*		1.29	*<0.01*
methylsuccinic acid[b,k]	1.31	*<0.01*		n.s.	
co-enzyme Q1[a,i]	1.66	*<0.01*		n.s.	
prolyl leucine[a,f]	1.45	*<0.01*		n.s.	
caffeine[b,i]	0.11	*<0.001*		0.13	*<0.01*
1,3-dimethyluric acid[b,d]	0.19	*<0.01*		0.19	*<0.01*
3-methylxanthine/7-methylxanthine[a,k]	0.29	*<0.001*		0.28	*<0.001*
2-methylhippuric acid[b,k]	0.34	*<0.01*		0.23	*<0.001*
DL-(-)-3-phenyllactic acid[b,k]	0.62	*<0.01*		n.s.	
6-methyladenosine[b,k]	0.64	*<0.001*		0.61	*<0.001*
N-acetyl-L-tryptophan[a,k]	0.70	*<0.01*		0.69	*<0.01*
α-D-glucoheptonic acid[a,i]	0.75	*<0.01*		0.69	*<0.01*
1-ribosyl-N-ω-caproyllhistamine[a,e]	-	-		0.76	*<0.05*

n.s. = not significant

[a)] based on exact mass

[b)] confirmed by a standard compound

[c)] based on fragmentation pattern

[d]) corrected by internal standard carnitine C2:0-d3

[e]) corrected by internal standard carnitine C6:0-d3

[f]) corrected by internal standard carnitine C10:0-d3

[g]) corrected by internal standard leucine-d3

[h]) corrected by internal standard tryptophan-d5

[i]) corrected by internal standard phenylalanine-d5

[j]) corrected by internal standard cholic acid-2,2,4,4-d4

[k]) corrected by internal standard leucine enkephalin

Table 2 Effect of previous day vegetarian meals (VD) on relative levels of metabolites in morning urine. Relative levels of metabolites in 1st MU after VD were compared to relative levels in 1st MU before the dietary intervention. The same comparison was performed for 2nd MU. Conspicuous metabolites with significant ≥1.2-fold higher or ≤ 0.8-fold lower levels after VD are given. 1st MU was collected at 7 a.m. after an overnight fasting period of 12h and 2nd MU was collected 60 min later at 8 a.m. on the same day (for experimental details see Figure 1). The comparison was performed by paired nonparametric test with FDR limit ≤0.05 (n=18).

	1st morning urine		2nd morning urine	
Metabolites	**Fold-changes after VD**	***p value***	**Fold change after VD**	***p value***
glycodeoxycholic acid sulfate (GDCS)[a,c,k]	1.45	*<0.01*	n.s.	
glycochenodeoxycholate sulfate (GCDCS)[a,k]	1.38	*<0.01*	n.s.	
indoleacetic acid[b,g]	1.22	*<0.01*	1.34	*<0.001*
L-threonine[b,g]	1.28	*<0.01*	1.32	*<0.01*
carnitine C17:2+OH[a,c,i]	0.23	*<0.01*	n.s.	
carnitine C2:0[b,d]	0.51	*<0.01*	0.55	*<0.001*
carnitine C3:0[b,d]	0.63	*<0.01*	n.s.	
carnitine C12:2[a,c,i]	0.65	*<0.01*	n.s.	
carnitine C6:0[a,c,g]	0.65	*<0.01*	0.78	*<0.01*
carnitine C11:1[a,c,k]	0.66	*<0.01*	0.74	*<0.01*
carnitine C10:1[a,c,e]	0.67	*<0.01*	n.s.	
carnitine C11:0[b,e]	0.70	*<0.01*	n.s.	
carnitine C8:0+OH[a,c,k]	0.70	*<0.05*	n.s.	
carnitine C10:2[a,c,e]	0.71	*<0.01*	n.s.	
2,6-dimethylheptanoyl carnitine[a,i]	0.77	*<0.01*	n.s.	
carnitine C10:0[a,c,e]	0.78	*<0.01*	n.s.	
carnitine C10:3[a,c,e]	0.79	*<0.01*	n.s.	
1,3-dimethyluric acid[b,d]	0.44	*<0.01*	0.55	n.s.[l)]

2-furoylglycine[b,g]	0.44	*<0.001*		n.s.	
N-acetyl-L-tryptophan[a,k]	0.53	*<0.001*	0.57	*<0.001*	
methylimidazoleacetic acid[a,k]	0.63	*<0.01*	0.65	*<0.01*	
proline betaine[b,d]	0.65	*<0.001*	0.71	*<0.001*	
coenzyme Q1[a,i]	0.70	*<0.01*		n.s.	
3-(1-pyrazolyl)-L-alanine[b,d]	0.78	*<0.01*		n.s.	

n.s. = not significant

[a)] based on exact mass

[b)] confirmed by a standard compound

[c)] based on the fragmentation pattern

[d]) corrected by internal standard carnitine C2:0-d3

[e]) corrected by internal standard carnitine C6:0-d3

[f]) corrected by internal standard carnitine C10:0-d3

[g]) corrected by internal standard leucine-d3

[h]) corrected by internal standard tryptophan-d5

[i]) corrected by internal standard phenylalanine-d5

[j]) corrected by internal standard cholic acid-2,2,4,4-d4

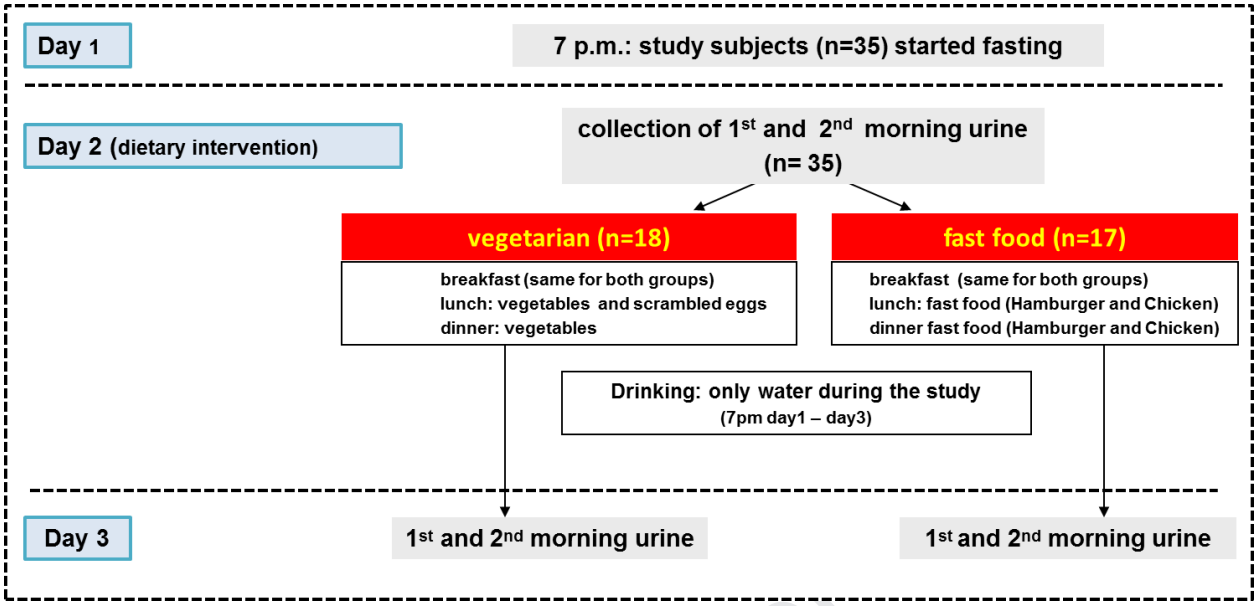
[k]) corrected by internal standard leucine enkephalin

[l)] p-value: 0.008, but FDR > 0.05

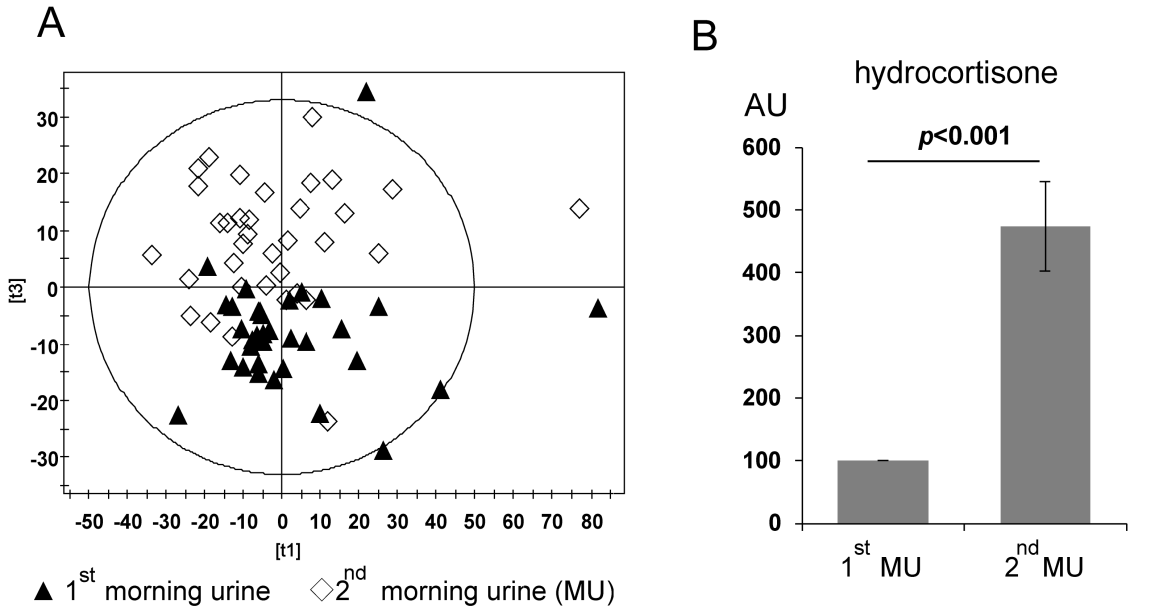
Figure Legends

Figure 1 Scheme of the experimental design of the study.

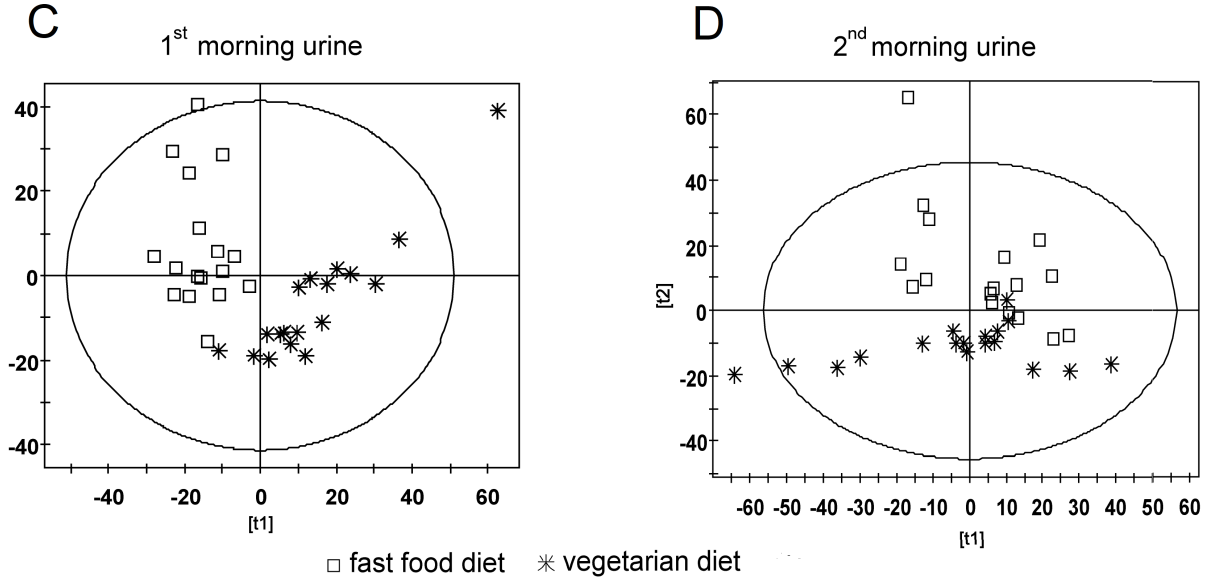
Figure 2 Comparison of the urinary metabolite profiles of first and second morning urine (MU) and effects of previous day meals on the individual metabolomes in first and second MU. (A) A principal component analysis (PCA) scores plot showing individual metabolite fingerprints of 1st and 2nd MU collected at 7 a.m. and 8 a.m. at the same day from the same 35 healthy individuals (for experimental details see Figure 1). Exemplarily the PCA scores plot analyzed in negative electrospray ionization mode (ESI-) is given. Black triangles = first MU; open diamonds = second MU; **(B)** relative levels of hydrocortisone detected in 1st and 2nd MU (n=35). Hydrocortisone data are presented as means ± SEM; **(C)** A comparison of urinary metabolite pattern in 1st MU after previous day standardized meals, either fast food (n = 17) or vegetarian style (n = 18); **(D)** same comparison in 2nd MU after previous day standardized meals. Exemplarily the PCA scores plots analyzed in negative electrospray ionization mode (ESI-) are given. Squares: fast food; stars: vegetarian.



1st *vs.* 2nd morning urine



Effects of previous day meals



Highlights

- 1st morning urine samples are greatly affected by the previous day food
- Previous day vegetarian meals affect the metabolome in urine only little
- Mix-up of 1st and 2nd morning urine is not recommended, since the metabolome is rather different
- 2nd morning urine collected after standardized vegetarian meals and drinking only water is the preferential sample material for metabolomics
- 47 metabolites in urine, possible hits in biomarker studies but susceptible to the diet the day before sample collection, are reported

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: