The effect of sampling procedures and day-to-day variations in metabolomics studies of biofluids

Guro F. Giskeødegård[1], Trygve Andreassen[2], Helena Bertilsson[3,4], May-Britt Tessem[1], Tone F. Bathen[1]

1. Department of Circulation and Medical Imaging, Norwegian University of Science and Technology (NTNU), Trondheim, Norway

2. MR Core Facility, Department of Circulation and Medical Imaging, NTNU, Trondheim, Norway

3. Department of Clinical and Molecular Medicine, NTNU, Trondheim, Norway

4. Department of Urology, St. Olav University Hospital, Trondheim, Norway

Corresponding authors:

Guro F. Giskeødegård, guro.giskeodegard@ntnu.no

Tone F. Bathen, tone.f.bathen@ntnu.no

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Abbreviations: CPMG, Carr–Purcell–Meiboom–Gill pulse sequence; HSQC, heteronuclear single quantum coherence spectroscopy; ICC, intraclass correlation coefficient; NOESY, Nuclear Overhauser effect spectroscopy; PCA, principal component analysis; PLSDA, partial least squares discriminant analysis.

Abstract

Metabolomics analysis of biofluids is a feasible tool for disease characterization and monitoring due to its minimally invasive nature. To reduce unwanted variation in biobanks and clinical studies, it is important to determine the effect of external factors on metabolic profiles of biofluids. In this study we examined the effect of sample collection and sample processing procedures on NMR measured serum lipoproteins and small-molecule metabolites in serum and urine, using a cohort of men diagnosed with either prostate cancer or benign prostatic hyperplasia. We determined day-to-day reliability of metabolites by systematic sample collection at two different days, in both fasting and non-fasting conditions. Study participants received prostate massage the first day to assess the differences between urine with and without prostate secretions. Further, metabolic differences between first-void and mid-stream urine samples, and the effect of centrifugation of urine samples before storage were assessed. Our results show that day-to-day reliability is highly variable between metabolites in both serum and urine, while lipoprotein subfractions possess high reliability. Further, fasting status clearly influenced the metabolite concentrations, demonstrating the importance of keeping this condition constant within a study cohort. Day-to-day reliabilities were however comparable in fasting and non-fasting samples. Urine sampling procedures such as sampling of first-void or mid-stream urine, and centrifugation or not before sample storage, were shown to only have minimal effect on the overall metabolic profile, and is thus unlikely to constitute a confounder in clinical studies utilizing NMR derived metabolomics.

1. Introduction

Metabolomics, the study of small-molecular intermediates and end-products of metabolism, provides a snapshot of the active cellular processes. The most commonly used platforms for metabolic profiling is nuclear magnetic resonance (NMR) spectroscopy and chromatography-based mass spectrometry (MS). While MS has the advantage of high sensitivity (picomolar for MS versus micromolar for NMR), NMR has high reproducibility and a simpler sample-preparation which is more suitable for large-scale reproducibility and quality studies [1]. Metabolomics analysis of biofluids are gaining increased interest due to its minimally invasive nature, and several studies have shown that different biofluids contain important information related to cancer diagnosis, prognosis and treatment response [2]. The most commonly used biofluids for metabolomics analyses are blood (serum or plasma) and urine, however other biofluids such as saliva [3] and cerebral spinal fluid [4] has also shown promising results for disease characterization. Sampling of blood and urine, often termed liquid biopsies, is less invasive than e.g. cerebrospinal fluid or tissue biopsy and therefore suitable for screening, surveillance and patient monitoring. However, in comparison to metabolomics analysis of tissue samples, the biofluid metabolome will reflect the metabolic state of the entire organism being studied. Serum and urine metabolism are therefore influenced by several endogenous and exogenous factors, such as health and disease [2], diet [5, 6], and activity level [7-10]. Such factors may change over time, causing variation in the measured metabolome that can hinder the identification of clinically relevant biomarkers. Additionally, previous studies have demonstrated that urine and serum metabolomes are affected by circadian rhythms [11, 12]. Characterizing how the biofluid metabolome varies between days will facilitate identification of robust biomarkers for clinical use.

To minimize the effect of irrelevant factors, collecting samples from fasting participants and standardizing the sampling protocols are desired in biobanks and clinical studies. The effect of time from sample

collection to freezing on NMR measured metabolic profiles in serum and plasma has been assessed [13, 14], in addition to different centrifugation methods for urine samples [15], demonstrating that sample handling will affect the measured metabolic profile. However, different biobanks may follow different protocols for sample collection and handling. Collecting urine and serum samples from fasting condition will reduce the influence of immediate diet effects on the measured metabolic profile. For urine samples, collecting mid-stream samples is usually recommended to avoid contaminations such as bacteria and epithelial cells [16]. However, it is not always feasible to collect fasting samples, for instance in large-scale population biobanks or in cases where fasting will be inconvenient for the patients in clinical trials. Additionally, study participants may not always follow the given instructions. Thus, it is important to characterize how deviations from standard protocols affects the metabolic profile.

Prostate cancer is the most common cancer type in men, and there is an urgent need of defining biomarkers for accurate diagnosis of prostate cancer, and further to separate aggressive from indolent cancers. Urine is an interesting biofluid in the search for prostate cancer biomarkers, due to its close proximity to the prostate. The main function of the prostate is to produce and secrete prostatic fluid which mixes with sperm upon ejaculation. By applying prostate massage (stroking the prostate through the rectum), prostatic fluid will be released into the urethra, and the first urine collected after prostate massage will be a mixture of urine and prostate secretion. The difference in metabolic profile between urine with and without prostate secretions has not been assessed.

The purpose of this study was to examine how sample collection and processing affects NMR measured serum lipoproteins and small-molecule metabolites of serum and urine, using a cohort of men diagnosed with either prostate cancer or benign prostatic hyperplasia. To achieve this, we examined day-to-day variations in the NMR measured serum and urine metabolome by systematic sample collection at two

different days, and we characterized how non-fasting serum and urine samples differ from fasting samples in a setting where samples were collected before and after a meal. Further, we describe metabolic differences between urine samples acquired with and without prior prostate massage, the effect of centrifugation of urine samples before storage, and metabolic differences between first- void and mid-stream urine samples.

2. Materials and methods

2.1 Study participants

Serum and urine samples were collected as part of a pilot study including prostate cancer patients (n=29) and controls diagnosed with lower urinary tract symptoms and benign prostatic hyperplasia (n=21)[17]. Samples were collected at St. Olavs University Hospital, Trondheim, Norway, at two different days within a week (Monday (Day 1) and Thursday (Day 2)). At Day 1, the patients received prostate massage by three finger strokes over the prostate prior to urination in order to collect urine containing prostate secretion. Fasting morning serum and urine samples were collected, and non-fasting samples were then collected after participants ate a meal of their own choice. Median time since last meal were 11 hours (range: 9-15 hours) for fasting samples and 2 hours (range: 0.5-2 hours) for non-fasting samples.

First-void and mid-stream urine was collected from both fasting and non-fasting. The first 20-30 mL of urine was defined as first-void urine (or urine containing prostate secretion at the morning of day 1) and collected in separate tubes. One aliquot of urine was immediately frozen after collection, while another was centrifuged (10 000g, 10 minutes) before collection and freezing of the supernatant. This resulted in 16 urine samples per study participant (Figure S1), giving a total of 800 urine samples.

Serum samples were collected in 10 mL Vacuette tubes without gel, turned five times and left to coagulate for at least 30 minutes (max 120 minutes), before all samples were centrifuged (1800g, 10 minutes) and aliquoted. Serum samples (four samples per study participants, Figure S1) and urine samples were stored in Biobank1, St.Olavs University Hospital, at -80 °C for approximately one year before NMR analysis.

This study was approved by the Regional Committee for Medical and Health Research Ethics (Norwegian Health Region III) (REK 2011/540) and informed written consent was obtained from all study participants.

2.2 NMR analyses

Thawed urine samples (540 µL) were mixed with a bacteriostatic buffer (60 µL) (pH 7.4, 1.5 mM KH_2PO_4 in D_2O, 0.1% sodium 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionate (d4-TSP), 2 mM NaN_3) and transferred to 5 mm NMR tubes. Urine samples containing prostate secretion (1 mL) were centrifuged at 13000 g for 5 min before NMR analysis, and 540 µL supernatant was used for analysis. Thawed serum samples (100 µL) were mixed with 100 µL buffer (pH 7.4, 0.075 mM Na_2HPO_4, 5 mM NaN_3, 5 mM d4-TSP) and transferred to 3 mm NMR tubes.

All NMR experiments were recorded on a Bruker Avance III 600 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a 5 mm QCI Cryoprobe with integrated, cooled preamplifiers for ^{1}H, ^{2}H and ^{13}C. Experiments were fully automated using the SampleJet™ in combination with Icon-NMR on TopSpin 3.1 software (Bruker Biospin). Samples were stored at 6 °C in the autosampler prior to analysis. Urine samples were analysed at 27 °C, using a 1D ^{1}H Nuclear Overhauser effect spectroscopy (NOESY) pulse sequence (noesygppr1d) with 32 scans (NS), 64k data points (TD), 20 ppm spectral width, 4 s relaxation delay and mixing time of 10 ms. Serum samples were recorded at 37 °C using a Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence (cpmgpr1d) with 64 scans (NS), 64k data points (TD), 20 ppm

spectral width and 4 s relaxation delay. Both pulse sequences utilized water presaturation (25 Hz) during the relaxation delay. The spectra were Fourier transformed to 64K real data points after 0.3 Hz exponential line broadening. 2D heteronuclear single quantum coherence spectroscopy (HSQC) were acquired for metabolite identification. Results from analysis of serum samples showing metabolic differences between prostate cancer and benign prostatic hyperplasia from day 1 have been published previously [17].

2.3 Data preprocessing

Data were transferred to Matlab R2017a for further processing. Urine NOESY NMR spectra were referenced to the creatinine peak at 3.05 ppm. The region of interest between ppm 0.73-9.42 was peak aligned using icoshift [18] using the spectrum with the highest correlation to the remaining spectra as reference. Asymmetric least squares baseline correction [19] was used to correct the baseline of two spectra with uneven baseline due to poor water-suppression. Spectra were normalized by probabilistic quotient normalization [20] after removal of the water peak between ppm 4.63-5.00, using the median spectrum as a normalization reference. Four spectra were removed due to poor spectral quality, and four spectra were removed due to insufficient water suppression. Urine samples from one patient at day 2 were excluded due to large contaminating peaks from paracetamol intake.

Serum spectra were referenced to the left peak of the alanine doublet at 1.47 ppm. One spectrum was removed due to contaminations from unknown origin resulting in massive peaks in the spectrum. The spectra were baseline corrected by setting the lowest point to zero. The region of interest between 0.29-8.52 ppm, excluding the water peak between 4.31-5.15 ppm, was normalized to equal total area.

Metabolites were assigned using HSQC data, Chenomx NMR suite 7.7 (Chenomx Inc., Alberta, Canada) and the Human Metabolome Database [21]. Metabolite peaks were integrated from normalized spectra

using Matlab, and for metabolites with more than one resonance, either the mean of resonances or the resonance in a non-overlapping region of the spectrum was chosen. For serum samples, the metabolite levels were normalized after quantification to remedy the influence of large lipid peaks in the spectra.

Lipoprotein subfractions were predicted from the fasting serum NMR spectra using commercially available procedures from Bruker Biospin (Bruker IVDr Lipoprotein Subclass Analysis (B.I.LISA)), as described previously for this dataset [17]. This method measures the concentration of cholesterol, phospholipids, triglycerides and/or apolipoproteins A1, A2 and B in the serum sample (total values) and in each of the lipoprotein subfractions (total very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL), in addition to the subclasses VLDL-1–6, LDL-1–6 and HDL-1–4) [22].

2.4 Statistical analysis

Intraclass correlation coefficients (ICC) for each metabolite and lipoprotein subfraction were calculated between measurements from the two days of sample collection to assess day-to-day reliability. ICCs were calculated from random intercept models with no covariates in R (version 3.5.2, R Foundation for Statistical Computing) using the nlme package. Random intercept models are linear mixed models and allows for modelling of random effects in clustered data, such as repeated measures collected from the same individuals. The ICC is an estimate of the correlation between clustered observations, and is calculated as the variance of the random effect divided by the total variation of the model.

To examine metabolic differences between days (day 1 vs day 2, in fasting and non-fasting condition), principal component analysis (PCA) trajectory plots were calculated from autoscaled metabolite levels. Systematic differences between samples with different sampling procedures (fasting vs non-fasting serum

and urine samples, centrifuged vs non-centrifuged urine samples, urine with and without prostate secretion, first-void vs mid-stream urine samples) were examined by multilevel partial least squares discriminant analysis (multilevel PLSDA) [23] of autoscaled metabolite levels. Multilevel PLSDA puts focus on the individual changes in metabolic profile due to sampling procedures, by separating the within- and between-subject variation. Models were orthogonalized for easier interpretation [24]. Loadings were colored according to variable importance in the projection (VIP) score, reflecting the variable's influence on the classification [25].

For urine samples, centrifuged, mid-stream samples were used for assessment of day-to-day variation, fasting/non-fasting samples and to compare urine with and without prostate secretion. For examining differences between first-void and mid-stream urine samples, fasting, centrifuged samples from day 2 (without prostate massage) were used. For examining differences between centrifuged and non-centrifuged urine samples, fasting mid-stream samples from day 2 were used. When comparing fasting and non-fasting condition, samples from both days were used. Multilevel PLSDA models were validated by leave-n-patients-out cross-validation (where n=10% of patients) with 20 iterations. Permutation testing using 1000 permutations was performed for testing the significance of the resulting models, and models with permutation p-values (p_{perm}) ≤ 0.05 were considered significant. Multivariate analyses were performed in Matlab R2017a using PLS_toolbox 8.2.1. Boxplots showing changes in metabolite concentrations related to urine sampling procedures were made in R version 3.5.2 using the package ggplot2, and shows the median, first and third quartiles (in box) and up to the smallest/largest observation within 1.5 * interquartile range from the box (whiskers). For visual simplicity, outliers are not shown.

3. Results

3.1 Day-to-day variations in the biofluid metabolome

Relative concentrations of 25 serum metabolites and 34 urine metabolites were quantified (Table S1 and S2 for serum and urine metabolites, respectively), while 105 lipoprotein subfractions were quantified from fasting serum samples as previously described (Table S3) [17]. There are clear day-to-day variations in fasting serum metabolites, urine metabolites and serum lipoproteins, as demonstrated by PCA trajectory score plots (Figure 1). The amounts of variation explained by the first two principal components are 39.9%, 24.0% and 64.4% for serum metabolites, urine metabolites and lipoprotein subfractions, respectively. For some individuals, metabolite measurements from two different days cluster together in the PCA score plot, while in several cases the variation between two measurements from one individual appears comparable to the between-subject variation. The serum lipoproteins have smaller day-to-day variations compared to the small-molecule serum metabolites, as two samples from the same study participant are plotted closer together in the PCA score plot. Similar results were evident also in further PCs. PCA trajectory score plots for non-fasting serum and urine samples are shown in Figure S2, showing comparable day-to-day variations as for fasting samples.

ICC values for quantified serum (Figure 2A) and urine metabolites (Figure 3A) show comparable day-to-day reliability in fasting and non-fasting samples. Nine serum metabolites (36%) had ICC ≥ 0.5 for both fasting and non-fasting samples. Methionine levels had low ICC values (≤ 0.2) for both fasting and non-fasting samples. Histidine and tyrosine had ICC<0.2 in fasting samples, while ICC > 0.5 for non-fasting samples. Mean ICC values were 0.47 and 0.52 for fasting and non-fasting serum samples, respectively.

Urine samples had ICC ≥ 0.5 for both fasting and non-fasting samples for 16 metabolites (47%) (Figure 3A). Dimethylamine and tartaric acid had ICC<0.2 for both fasting and non-fasting samples, while 3-

aminoisobutyrate, citrate, and ascorbate had ICC values ≥ 0.8 in fasting and non-fasting condition. Mean ICC were 0.51 and 0.52 for fasting and non-fasting urine samples, respectively.

ICC values for fasting lipoprotein subfractions are shown in Table S3. 103 subfractions (98%) had ICC values > 0.5 and 85 subfraction (81%) had ICC values ≥ 0.8. ICC values ranged from 0.42-0.95, with a mean of 0.84.

3.2 The effect of fasting and non-fasting condition on the biofluid metabolome

There were significant differences in serum metabolite levels between fasting and non-fasting serum samples, with a multilevel PLSDA classification accuracy of 93.2% ($p_{perm} < 0.001$). Orthogonalized PLSDA score and loading plots (Figure 2B-C) show that non-fasting samples had higher levels of leucine and icoleucine compared to fasting samples, while fasting serum samples were characterized by higher relative levels of glutamine, dimethyl sulfone and glycine.

The difference between fasting and non-fasting urine samples was assessed on centrifuged, mid-stream urine samples. Multilevel PLSDA showed highly significant differences due to fasting status with a classification accuracy of 97.3% ($p_{perm}<0.001$) (Figure 3B-C). Non-fasting samples had higher levels of alanine, glycine, allantoin, and 2-furoylglycine, and lower levels of phenylacetylglutamine, p-cresol sulfate, dimethylamine, creatinine, and hippurate compared to fasting samples.

PCA trajectory analysis of fasting and non-fasting samples shows that the within-subject variation due to fasting status is of comparable influence as the between-subject variation for both serum and urine samples (Figure S3).

3.3 Metabolic differences between urine with and without prostate secretion

PCA trajectory plot (Figure 4A) shows that metabolite levels from first-void urine samples collected immediately after prostate massage, containing prostate secretion, show small, but systematic differences compared to mid-steam urine samples. For most individuals, the between-subject variation is larger than the variation between measurements from the same individual. These systematic differences between prostate secretion and urine resulted in perfect separation in a multilevel PLSDA model with a classification accuracy of 100.0% (p_{perm}<0.001), showing that there are clear metabolic differences between the two sample types (Figure S4). Differences in metabolite levels between urine samples with and without prostate secretion are shown in Figure 5A. As evident from both percentage differences and OPLSDA loadings, it is clear that urine samples had higher levels of all quantified metabolites except for citrate and the NMR signal at 3.11 ppm, consisting of signals from spermine, proline betaine, cis-aconitic acid and 3-aminoisobutyrate.

3.4 Metabolic differences between first-void and mid-stream urine samples

First-void and mid-stream urine samples from the same individuals could be well separated by multilevel PLSDA with a classification accuracy of 95.0% (p_{perm}<0.001), demonstrating clear differences in metabolite levels (Figure S4). However, PCA trajectory analysis (Figure 4B) show that although systematic, the metabolic differences between first-void and mid-stream urine are minor compared to between-subject variations, with overlapping samples in the PCA score plot for most of the individuals. This is also evident by the very small percentage differences, fluctuating around zero, between first-void and mid-stream urine samples shown in Figure 5B.

3.5 Metabolic difference between centrifuged and non-centrifuged data

Separation of centrifuged and non-centrifuged urine samples by multilevel PLSDA was highly significant (classification accuracy 91.0%, p_{perm}<0.001, Figure S4). PCA trajectory analysis (Figure 4C) however shows that the within-subject variation resulting from differences in first-void and-mid stream samples are minor compared to the between-subject variation, and the samples from the same individual are overlapping. Median percentage change in metabolite concentrations fluctuate around zero, as shown in Figure 5C. (

4. Discussion

In this study we demonstrate clear day-to-day variations in the NMR measured metabolome of serum and urine samples. However, most metabolites show correlated levels in measurements from two different days, with mean ICC values of approximately 0.5 for both serum and urine samples. Serum lipoprotein subfractions had smaller day-to-day variations and higher ICC values compared to small-molecule serum metabolites measured simultaneously. We further describe metabolic differences in samples acquired with and without prior fasting, and demonstrate a change in the metabolic profile of urine after prostate massage releasing prostate secretion. Additionally, we show that urine sampling procedures such as sampling of first-void or mid-stream urine, and performing or not performing centrifugation before sample storage introduces systematic, but very small, differences in the metabolite levels.

The high ICC values observed for NMR derived lipoprotein subfractions, with a mean value of 0.84, are in agreement with a previous study showing low coefficients of variation for serum total and HDL cholesterol in samples acquired days apart [26]. The observation of higher day-to-day reliability of serum lipoprotein subfractions compared to small-molecule serum metabolites is expected. The small-molecule metabolites will be more influenced by immediate factors, such as last meal, recent activity level, and circadian rhythm, while lipoproteins, commonly measured to assess an individual's cardiovascular health, represent a measure of long-term habitual diet and lifestyle.

Interestingly, mean ICC values over all quantified metabolites were comparable in fasting and non-fasting samples for both serum and urine samples. In agreement with our results, a previous study by Carayol et al. examining serum samples collected from individuals at two different days two years apart found comparable reliability according to fasting status for most amino acids [27]. However, the estimated ICC values in our studies are not in agreement for all amino acids. While our data show low ICC values for methionine for both fasting and non-fasting samples, Carayol et al. find ICCs of 0.6 for methionine in fasting samples. Similarly, we find ICC values above 0.6 for fasting and non-fasting leucine and isoleucine, while Carayol et al. describe ICCs below 0.3 for non-fasting samples. This discrepancy may result from differences in study design, as they acquired samples two years apart, and their non-fasting samples were collected from women only. Further, in the study by Carayol et al, the first batch of samples was stored for two years longer than the second batch, and it is possible that metabolite degradation during sample storage affects the reliability measure of certain metabolites. Thus, ICC values for different metabolites might be affected by sample handling. However, previous studies have described how serum metabolic concentrations fluctuate within the first hours after a meal [28, 29], and in our study postprandial samples were acquired approximately after two hours. Sampling at varying time points after food intake, which would represent a more realistic setting for a study cohort including non-fasting samples, might have provided lower day-to-day reliability for non-fasting samples.

Serum dimethyl sulfone had high day-to-day reliability with ICC values above 0.9 for fasting and non-fasting samples. We previously described serum dimethyl sulfone as a possible biomarker for separating prostate cancer and benign prostatic hyperplasia in the same cohort [17]. A good biomarker should have reliable measurements between days, thus this study further supports dimethyl sulfone as a feasible biomarker for prostate cancer. Histidine levels were also in our panel of possible biomarkers for prostate

cancer, however this amino acid shows low reliability in fasting samples, and may therefore be a less valuable biomarker candidate.

Urinary levels of 3-aminoisobutyrate and ascorbate had ICCs above 0.8 in both fasting and non-fasting samples, thus showing high day-to-day reliability. 3-aminoisobutyrate is an end-product of pyrimidine degradation, while ascorbate, or vitamin C, is an important antioxidant and contributes to immune defence [30]. On the contrary, urine dimethylamine, trimethylamine N-oxide (TMAO) and tartaric acid all had ICC values below 0.25 in both fasting and non-fasting conditions. Both TMAO and dimethylamine are abundantly present in urine, and can result from metabolism of trimethylamine by gut microbiota [31], while dietary intake is the major contributor to urinary tartaric acid [32]. Thus, our results show that metabolites typically resulting from diet may have low reliability also in fasting samples. Creatinine is a breakdown product of creatine phosphate in muscles, and is claimed to be produced at a relatively constant rate in the body. This is in accordance with our findings of ICC values above 0.5 for creatinine levels measured both in fasting and non-fasting serum and urine samples.

The effect of habitual diet has been investigated in several large epidemiological studies [33-35], showing that habitual dietary patterns will be reflected in serum metabolic data. Further, a previous study showed more diet-specific metabolites being present in urine than in serum samples [36]. Fasting samples are usually recommended to reduce the direct effect of dietary intake, however this is not always achievable, and not all subjects will be fasting even if required. Despite comparable ICC values between fasting and non-fasting samples, we detected large differences in metabolic profiles of fasting and non-fasting samples both in urine and serum. Participants in our study had a freely chosen meal prior to the non-fasting sample collection, and our cross-over study design allows analysis of fasting effects within individuals. The very high classification results for separating fasting and non-fasting samples indicate that

the metabolic patterns we observe are characteristic of fasting status, independent of which type of food has been consumed.

For serum, the main effect of non-fasting condition was increased leucine and isoleucine levels, and decreased dimethyl sulfone and glycine levels. Both leucine and isoleucine are essential amino acids, and food intake will increase their serum concentrations. Similarly, a study of postprandial serum metabolomics three hours after intake of three different breakfast meals found increased leucine levels after intake of dairy and meat containing meals, while isoleucine was not quantified in their study [37]. In urine, non-fasting samples were characterized by higher levels of alanine, glycine, allantoin and 2-furoylglycine levels, and lower levels of p-cresol sulfate and creatinine. Increased alanine levels in urine were also an important discriminator in a study comparing fasting samples to one and two hours postprandial samples following meals containing wheat bran and aleurone [38], while 2-furoylglycine is a suggested biomarker for coffee consumption [39]. Overall, our data show that differences in fasting status between study participants may introduce noise and possibly be a confounder in clinical studies using NMR measured metabolic profiling of biofluids.

We showed clear separation between urine samples with and without centrifugation prior to storage, and between first-void and mid-stream urine. However, it should be noticed that the metabolic changes related to these sampling procedures are very small, as evidenced by the PCA trajectory plots showing nearly complete overlap between samples, and median percentage changes in metabolite concentrations fluctuating around zero. By using multilevel PLSDA analyses, we separate the within and between patient variation, in that way putting emphasis on the individual metabolic differences resulting from the sample processing. Despite high classification accuracies, the observed between-patient variation is much higher

than the systematic variation resulting from the protocol, thus centrifugation and urine type will not be a confounder of high influence in clinical studies where these factors are varying between patients.

Larger within-patient variations were evident between urine samples acquired with and without prior prostate massage. Urine samples had higher relative concentrations of most metabolites compared to urine with prostate secretion, possibly due to larger protein content after prostate massage, with the exception of levels of citrate and the singlet peak at 3.11 ppm. In urine spectra, this peak is frequently referred to as proline betaine, which can be related to intake of citrus fruits [40]. However 2D HSQC NMR experiments showed that this peak also contained signals from spermine, in addition to signals from cis-aconitic acid, 3-aminoisobutyrate and an unknown compound. Both citrate and spermine are produced in large amounts by the healthy prostate. Although the overall metabolic content in urine seems reduced after prostate massage compared to regular urine, the high levels of citrate and spermine released from the prostate upon massage seems to compensate for this and increase relative citrate and spermine levels. Our results thus show that prostate massage affects the metabolic content of urine by secretion of prostate-related metabolites into the urine.

In this study we examined a cohort of men with either prostate cancer or benign prostatic hyperplasia. It is possible that the presence of a non-healthy prostate affects the biofluid metabolites, and that results would differ in cohorts including men without prostatic conditions, or in cohorts including women. We and others have previously described metabolic changes as a results of circadian rhythms in both urine and blood samples [11, 12]. Our results may be influenced by diurnal variations, as the fasting samples were acquired in the morning while non-fasting samples were acquired mid-day. However, this study design allows comparison of fasting status within individuals, which is a strength of the study.

5. Conclusion

We conclude that day-to-day reliability vary between metabolites in both serum and urine, which should be taken into account when searching for clinical biomarkers, as a good biomarker should possess low day-to-day variation. Further, fasting or non-fasting condition highly influenced the metabolite concentrations, thus it is important to keep this condition constant within a study cohort to avoid confounding results. Day-to-day reliability were however comparable in fasting and non-fasting samples in this setting where non-fasting samples were acquired two hours postprandial. Urine sampling procedures such as sampling of first-void or mid-stream urine, and centrifugation or not before sample storage, only had minimal effect on the overall metabolic profile, and is unlikely to constitute a confounder in clinical studies.

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Competing interests

The authors declare no competing interests.

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Figures:

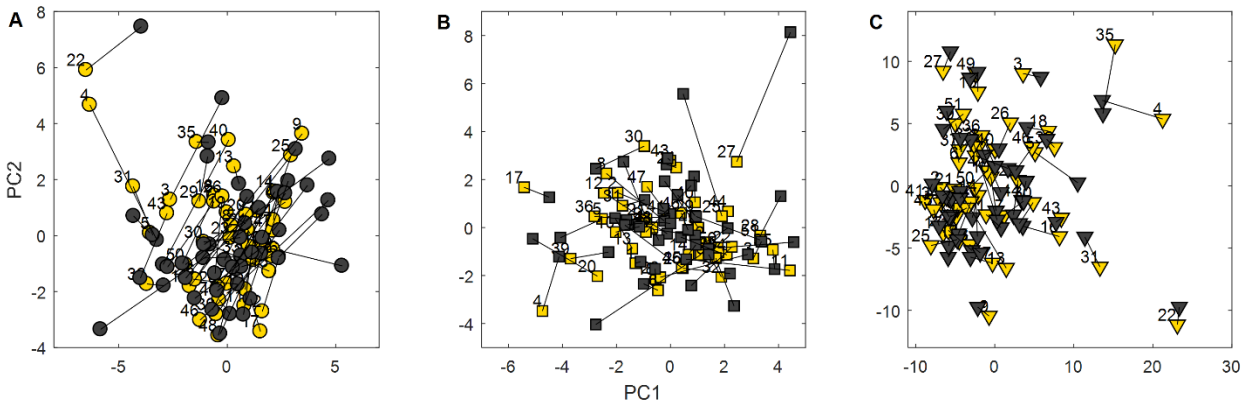

Figure 1: Day-to-day variations in the biofluid metabolome. PCA score plots showing day-to-day variations in A) fasting serum metabolites, B) fasting urine metabolites and C) fasting lipoprotein subfractions between day 1 (yellow) and day 2 (black). Measurements from the same individual are connected with black lines. Variations explained by PC1/PC2 were 22.2/17.6%, 14.3/9.7% and 42.9/21.6% for A, B, and C, respectively.

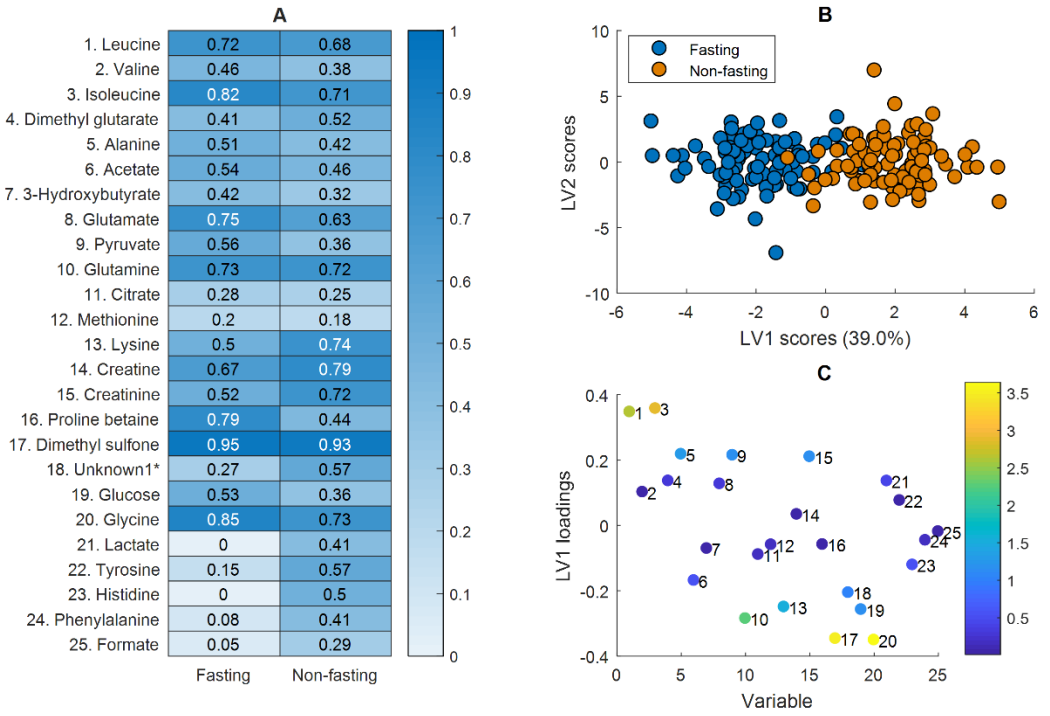

Figure 2: Day-to-day reliability and effect of fasting condition in serum metabolites. A) ICC values for fasting and non-fasting serum metabolites measured from the same individuals at two different days. B,C) Orthogonalized multilevel PLSDA results separating fasting from non-fasting condition. The score plot (B) shows clear separation between fasting and non-fasting samples, while the corresponding loading plot (C) shows each metabolite's influence on the model, colored by the metabolites' VIP scores. Metabolites in C) are numbered according to A). *Unknown1: singlet at 3.36 ppm.

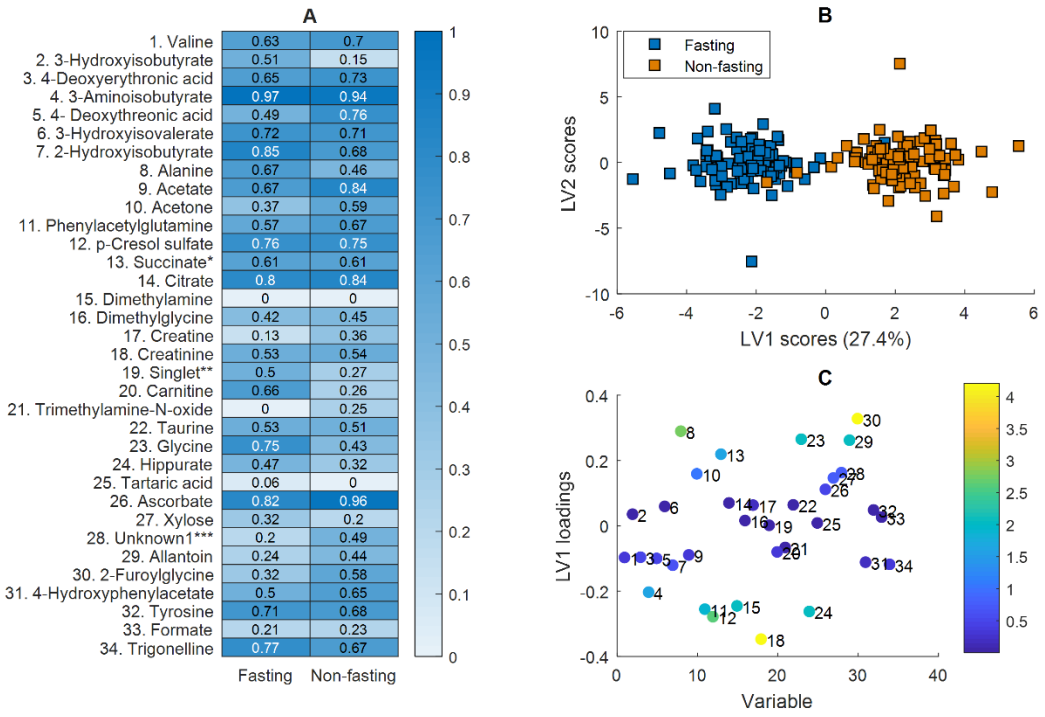

Figure 3: Day-to-day reliability and effect of fasting condition in urine metabolites. A) ICC values for fasting and non-fasting urine metabolites measured from the same individuals at two different days. B,C) Orthogonalized multilevel PLSDA results separating fasting from non-fasting condition. The score plot (B) shows clear separation between fasting and non-fasting samples, while the corresponding loading plot (C) shows each metabolite's influence on the model, colored by the metabolites' VIP scores. Metabolites in C) are numbered according to A).*in some spectra, the succinate peak is overlapping with an unknown peak at 2.41/32.5 in HSQC. **Singlet at 3.11 ppm consisting of signals from spermine, proline betaine, cis-aconitic acid, 3-aminoisobutyrat and an unknown compound. ***Unknown1: multiplet at 5.25 ppm.

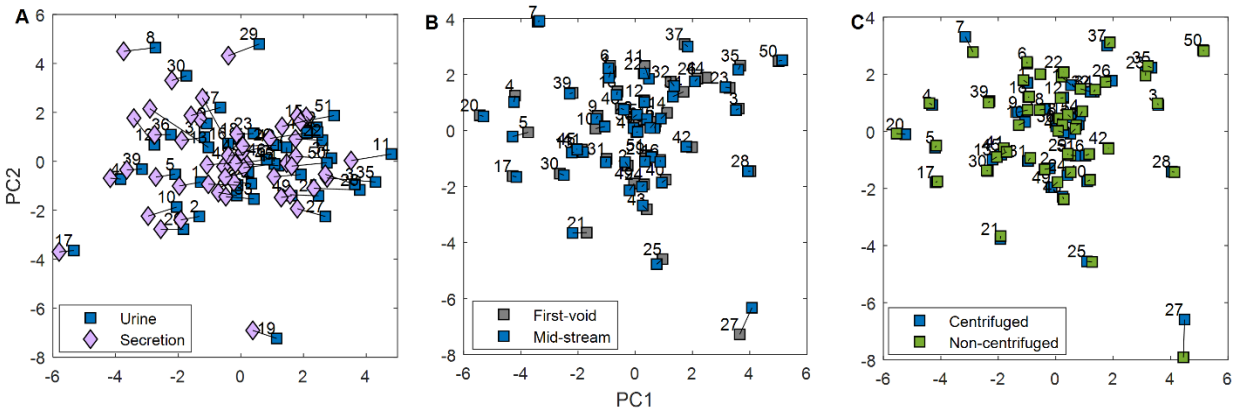

Figure 4: Variation in urine metabolite levels related to sampling and sample processing procedures

PCA trajectory score plots comparing A) prostate secrete and mid-stream urine samples, B) first-void and mid-stream urine samples, and C) centrifuged and non-centrifuged urine samples. Variations explained by PC1/PC2 were 13.9/11.0%, 14.9/11.6%, and 14.8/11.4% for A, B, and C, respectively.

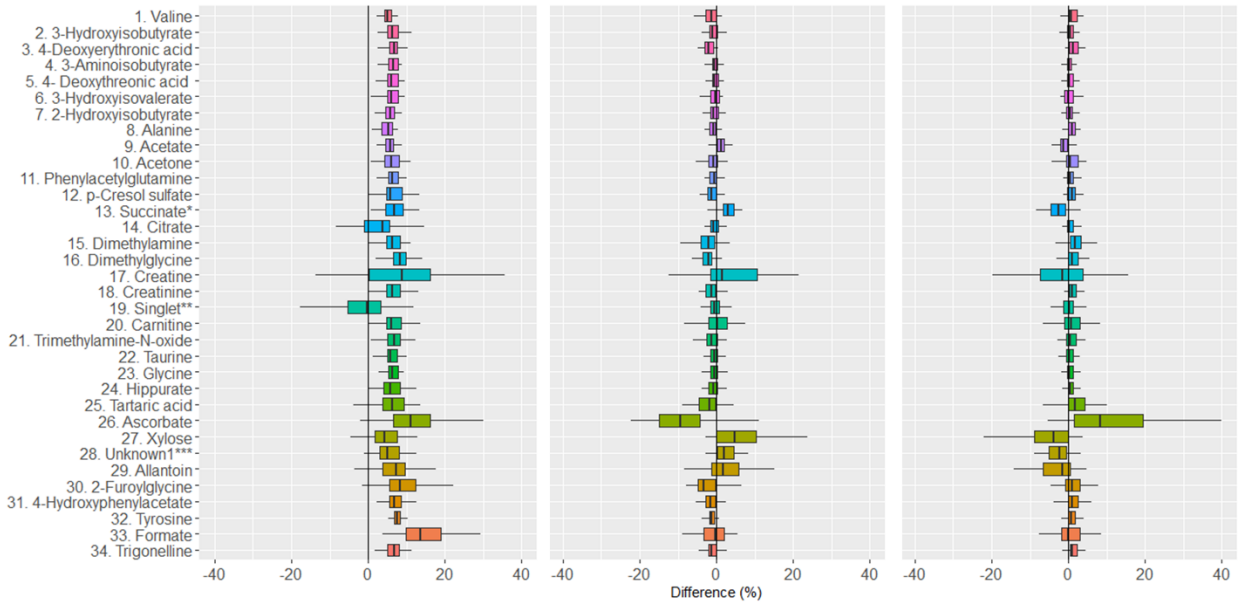

Figure 5: Differences in urine metabolite concentrations related to sampling and sample processing procedures. The figure shows boxplots of percentage differences in relative metabolite concentrations between A) prostate secrete and mid-stream urine samples, B) first-void and mid-stream urine samples, and C) centrifuged and non-centrifuged urine samples. A positive differences represents a higher level in mid-stream urine, mid-stream urine, and non-centrifuged samples for A, B, and C, respectively. *in some spectra, the succinate peak is overlapping with an unknown peak at 2.41/32.5 in HSQC. **Singlet at 3.11 ppm consisting of signals from spermine, proline betaine, cis-aconitic acid, 3-aminoisobutyrat and an unknown compound. ***Unknown1: multiplet at 5.25 ppm.

Supplementary figures:

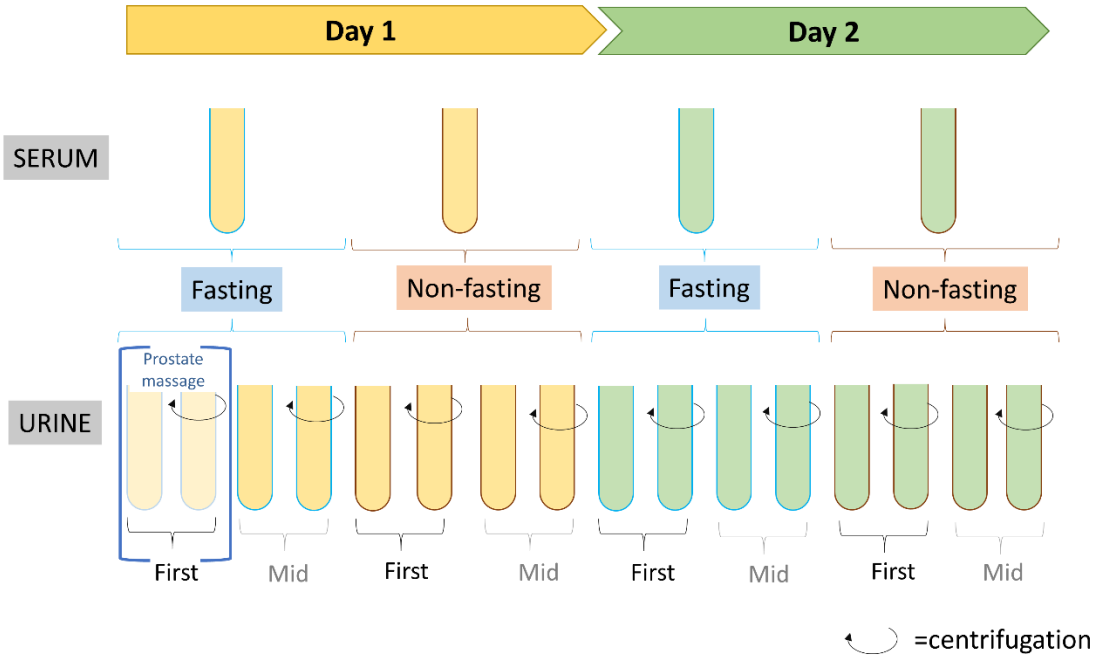

Figure S1: Serum and urine samples collected from each study participant. A total of 16 urine samples were collected from each study participant; first-void and mid-stream samples from fasting and non-fasting condition at two different days. One aliquot of each urine sample was immediately frozen after collection, while another was centrifuged before collection and freezing of the supernatant. Four serum samples were collected from each study participant, fasting and non-fasting samples at two different days.

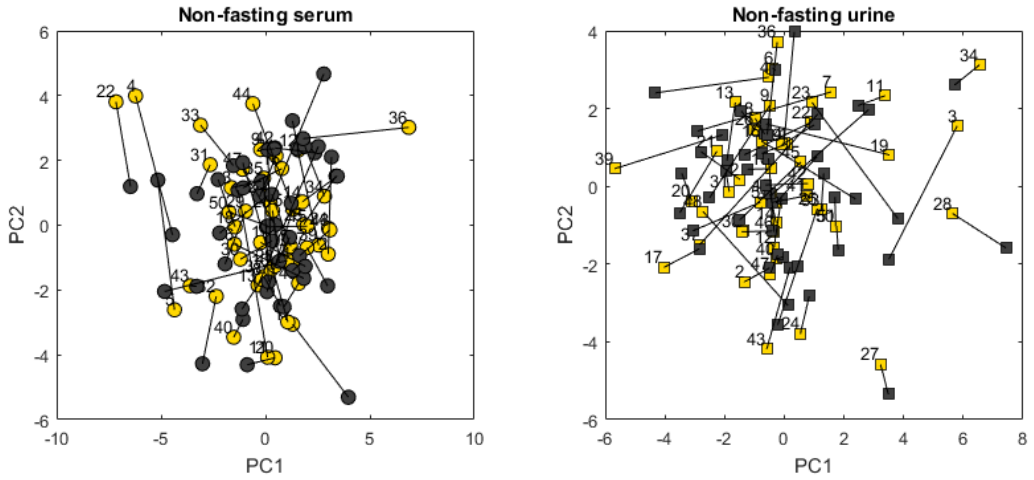

Figure S2: Day-to-day variations in the non-fasting biofluid metabolome. PCA score plots showing day-to-day variations in non-fasting serum and urine metabolites between day 1 (yellow) and day 2 (black). Measurements from the same individual are connected with black lines. The amounts of variation explained by the first two principal components are 39.8% for serum metabolites and 25.9% for urine metabolites.

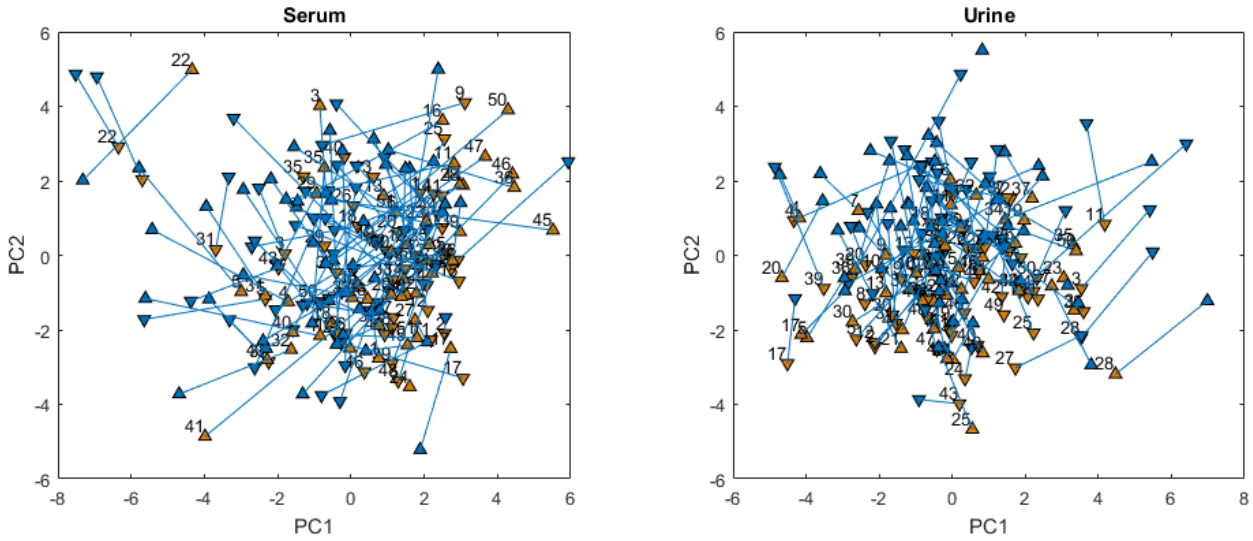

Figure S3: Variations due to fasting status in the biofluid metabolome. PCA score plots of fasting (blue) and non-fasting (orange) samples of serum and urine metabolite levels. Samples collected at day 1 are labelled by down-pointing triangles while day 2 samples are labelled by up-pointing triangles. PCA with two principal components explained 67.0% and 22.5% of the total variation for serum and urine metabolites, respectively.

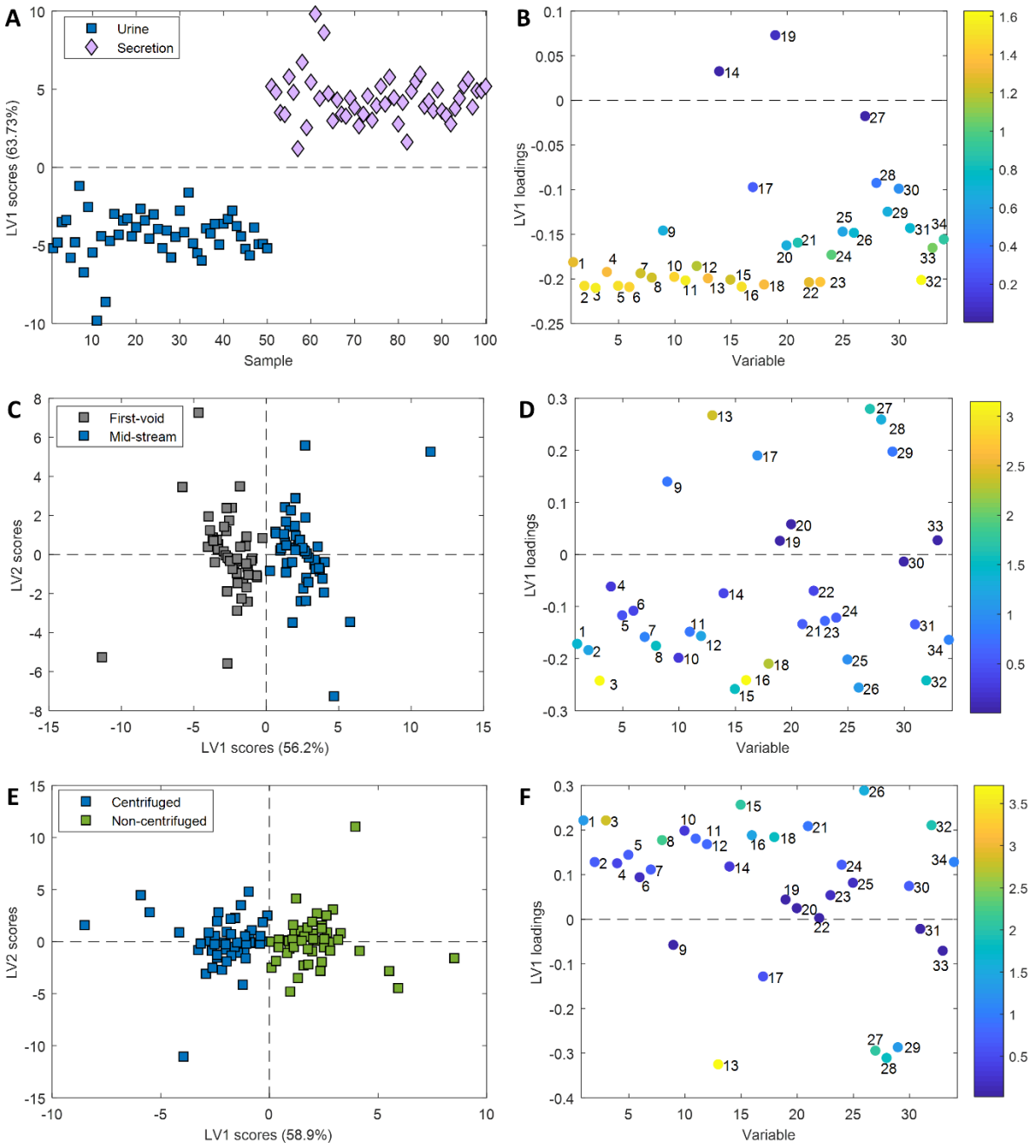

Figure S4: Metabolic changes in urine related to sampling and sample processing procedures. A,B) Orthogonalized multilevel PLSDA score and loading plot showing differences between urine with and without prostate secretion. C,D) Orthogonalized multilevel PLSDA score and loading plot showing differences between first-void and mid-stream urine. E,F) Orthogonalized multilevel OPLSDA score and loading plot showing differences between centrifuged and non-centrifuged urine. 1. Valine; 2. 3-

Hydroxyisobutyrate; 3. 4-Deoxyerythronic acid; 4. 3-Aminoisobutyrate; 5. 4- Deoxythreonic acid ; 6. 3-Hydroxyisovalerate; 7. 2-Hydroxyisobutyrate; 8. Alanine; 9. Acetate; 10. Acetone; 11. Phenylacetylglutamine; 12. p-Cresol sulfate; 13. Succinate*; 14. Citrate; 15. Dimethylamine; 16. Dimethylglycine; 17. Creatine; 18. Creatinine: 19. Singlet**; 20. Carnitine ; 21. Trimethylamine-N-oxide ; 22. Taurine ; 23. Glycine ; 24. Hippurate; 25. Tartaric acid; 26. Ascorbate; 27. Xylose; 28. Unknown1***; 29. Allantoin; 30. 2-Furoylglycine; 31. 4-Hydroxyphenylacetate; 32. Tyrosine; 33. Formate; 34. Trigonelline.).*in some spectra, the succinate peak is overlapping with an unknown peak at 2.41/32.5 in HSQC. **Singlet at 3.11 ppm consisting of signals from spermine, proline betaine, cis-aconitic acid, 3-aminoisobutyrat and an unknown compound. ***Unknown1: multiplet at 5.25 ppm.

Supplementary Tables:

Table S1: Quantified serum metabolites. Metabolites were quantified by integration of spectral peaks and normalized to total area. Mean signals were used for metabolites with more than one resonances. d , doublet; dd, double doublet; m, multiplet, q, quadruplet; s, singlet.

Metabolite	**ppm (multiplicity)**
Leucine	0.95 (t)
Valine	0.97 (d), 1.03 (d), 3.61 (d)
Isoleucine	0.99 (d)
Dimethyl glutarate	1.06 (d)
Alanine	1.47 (d)
Acetate	1.91 (s)
3-hydroxybutyrate	2.30 (m), 2.39 (m)
Glutamate	2.34 (m)
Pyruvate	2.37 (s)
Glutamine	2.45 (m)
Citrate	2.53 (d)
Methionine	2.64 (t)
Lysine	3.02 (t)
Creatine	3.03 (s), 3.93 (s)
Creatinine	4.05 (s)
Proline betaine	3.10 (s)
Dimethyl sulfone	3.15 (s)
Unknown1	3.36 (s)

Glucose	3.24 (dd), 3.41 (m), 3.46 (m), 3.49 (m), 3.53 (dd), 3.72 (m), 3.83 (m), 3.9 (dd), 5.25 (d)
Glycine	3.56 (s)
Lactate	4.11 (q)
Tyrosine	6.90 (m), 7.20 (m)
Histidine	7.06 (s), 7.79 (s)
Phenylalanine	7.33 (d), 7.37 (m), 7.43 (m)
Formate	8.47 (s)

Table S2: Quantified urine metabolites. Metabolites were quantified by integration of normalized spectra. Mean signals were used for metabolites with more than one resonances. d, doublet; dd, double doublet; m, multiplet, q, quadruplet; s, singlet. *in some spectra, the succinate peak is overlapping with an unknown peak at 2.41/32.5 in HSQC. ** peak consisting of signals from spermine, proline betaine, cis-aconitic acid, 3-aminoisobutyrat and an unknown compound.

	Metabolite	**ppm (multiplicity)**
1	Valine	1.04 (d)
2	3-Hydroxyisobutyrate	1.07 (d)
3	4-deoxyerythronic acid (4-DEA)	1.11 (d)
4	3-Aminoisobutyrate	1.20 (d)
5	4- deoxythreonic acid (4-DTA)	1.23 (d)
6	3-Hydroxyisovalerate	1.27 (s)
7	2-Hydroxyisobutyrate	1.36 (s)
8	Alanine	1.48 (d)
9	Acetate	1.92 (s)
10	Acetone	2.23 (s)
11	Phenylacetylglutamine	2.27 (t)
12	p-cresol sulfate	2.34 (s)
13	Succinate*	2.41 (s)
14	Citrate	2.54 (d)
15	Dimethylamine	2.72 (s)
16	Dimethylglycine	2.93 (s)
17	Creatine	3.03 (s), 3.93 (s)

18	Creatinine	3.04(s), 4.05 (s)
19	Singlet**	3.11 (s)
20	Carnitine	3.23 (s)
21	Trimethylamine-N-oxide (TMAO)	3.27 (s)
22	Taurine	3.43 (t)
23	Glycine	3.57 (s)
24	Hippurate	3.97 (d), 7.55 (m), 7.64 (m)
25	Tartaric acid	4.34 (s)
27	Ascorbate	4.53 (d)
28	Xylose	5.20 (d)
29	Unknown1	5.25 (m)
30	Allantoin	5.39 (s)
31	2-Furoylglycine	6.64 (dd)
32	4-Hydroxyphenylacetate	6.85 (m)
33	Tyrosine	6.89 (m)
34	Formate	8.47 (s)
26	Trigonelline	8.84 (m), 9.12 (s)

Table S3: Quantified lipoprotein subfractions. Lipoprotein subfractions were quantified from fasting serum samples using quantification procedures from Bruker Biospin (Bruker IVDr Lipoprotein Subclass Analysis (B.I.LISA)). Intraclass correlation coefficients (ICC) for each parameter are shown.

Matrix	**Analyte**	**Name**	**ICC**
Total Plasma	Triglycerides	TPTG	0.860
Total Plasma	Cholesterol	TPCH	0.927
Total Plasma	Free Cholesterol	TPFC	0.900
Total Plasma	Apo-A1	TPA1	0.921
Total Plasma	Apo-A2	TPA2	0.696
Total Plasma	Apo-B	TPAB	0.932
VLDL	Triglycerides	VLTG	0.869
VLDL	Cholesterol	VLCH	0.895
VLDL	Free Cholesterol	VLFC	0.889
VLDL	Phospholipids	VLPL	0.876
VLDL	Apo-B	VLAB	0.901
IDL	Triglycerides	IDTG	0.822
IDL	Cholesterol	IDCH	0.841
IDL	Free Cholesterol	IDFC	0.851

IDL	Phospholipids	IDPL	0.836
IDL	Apo-B	IDAB	0.845
LDL	Triglycerides	LDTG	0.729
LDL	Cholesterol	LDCH	0.918
LDL	Free Cholesterol	LDFC	0.895
LDL	Phospholipids	LDPL	0.927
LDL	Apo-B	LDAB	0.909
HDL	Triglycerides	HDTG	0.583
HDL	Cholesterol	HDCH	0.940
HDL	Free Cholesterol	HDFC	0.944
HDL	Phospholipids	HDPL	0.936
HDL	Apo-A1	HDA1	0.946
HDL	Apo-A2	HDA2	0.834
VLDL-1	Triglycerides	V1TG	0.861
VLDL-1	Cholesterol	V1CH	0.874
VLDL-1	Free Cholesterol	V1FC	0.893
VLDL-1	Phospholipids	V1PL	0.874
VLDL-2	Triglycerides	V2TG	0.866

VLDL-2	Cholesterol	V2CH	0.881
VLDL-2	Free Cholesterol	V2FC	0.892
VLDL-2	Phospholipids	V2PL	0.874
VLDL-3	Triglycerides	V3TG	0.874
VLDL-3	Cholesterol	V3CH	0.901
VLDL-3	Free Cholesterol	V3FC	0.891
VLDL-3	Phospholipids	V3PL	0.882
VLDL-4	Triglycerides	V4TG	0.866
VLDL-4	Cholesterol	V4CH	0.863
VLDL-4	Free Cholesterol	V4FC	0.869
VLDL-4	Phospholipids	V4PL	0.874
VLDL-5	Triglycerides	V5TG	0.836
VLDL-5	Cholesterol	V5CH	0.800
VLDL-5	Free Cholesterol	V5FC	0.880
VLDL-5	Phospholipids	V5PL	0.819
VLDL-6	Triglycerides	V6TG	0.493
VLDL-6	Cholesterol	V6CH	0.883
VLDL-6	Free Cholesterol	V6FC	0.642

VLDL-6	Phospholipids	V6PL	0.876
LDL-1	Triglycerides	L1TG	0.402
LDL-1	Cholesterol	L1CH	0.937
LDL-1	Free Cholesterol	L1FC	0.872
LDL-1	Phospholipids	L1PL	0.820
LDL-1	Apo-B	L1AB	0.931
LDL-2	Triglycerides	L2TG	0.722
LDL-2	Cholesterol	L2CH	0.906
LDL-2	Free Cholesterol	L2FC	0.919
LDL-2	Phospholipids	L2PL	0.843
LDL-2	Apo-B	L2AB	0.828
LDL-3	Triglycerides	L3TG	0.748
LDL-3	Cholesterol	L3CH	0.825
LDL-3	Free Cholesterol	L3FC	0.863
LDL-3	Phospholipids	L3PL	0.912
LDL-3	Apo-B	L3AB	0.914
LDL-4	Triglycerides	L4TG	0.728
LDL-4	Cholesterol	L4CH	0.829

LDL-4	Free Cholesterol	L4FC	0.748
LDL-4	Phospholipids	L4PL	0.824
LDL-4	Apo-B	L4AB	0.883
LDL-5	Triglycerides	L5TG	0.872
LDL-5	Cholesterol	L5CH	0.870
LDL-5	Free Cholesterol	L5FC	0.791
LDL-5	Phospholipids	L5PL	0.871
LDL-5	Apo-B	L5AB	0.874
LDL-6	Triglycerides	L6TG	0.867
LDL-6	Cholesterol	L6CH	0.681
LDL-6	Free Cholesterol	L6FC	0.595
LDL-6	Phospholipids	L6PL	0.696
LDL-6	Apo-B	L6AB	0.744
HDL-1	Triglycerides	H1TG	0.763
HDL-1	Cholesterol	H1CH	0.919
HDL-1	Free Cholesterol	H1FC	0.921
HDL-1	Phospholipids	H1PL	0.926
HDL-1	Apo-A1	H1A1	0.915

HDL-1	Apo-A2	H1A2	0.858
HDL-2	Triglycerides	H2TG	0.569
HDL-2	Cholesterol	H2CH	0.937
HDL-2	Free Cholesterol	H2FC	0.877
HDL-2	Phospholipids	H2PL	0.916
HDL-2	Apo-A1	H2A1	0.917
HDL-2	Apo-A2	H2A2	0.826
HDL-3	Triglycerides	H3TG	0.621
HDL-3	Cholesterol	H3CH	0.936
HDL-3	Free Cholesterol	H3FC	0.879
HDL-3	Phospholipids	H3PL	0.833
HDL-3	Apo-A1	H3A1	0.861
HDL-3	Apo-A2	H3A2	0.749
HDL-4	Triglycerides	H4TG	0.838
HDL-4	Cholesterol	H4CH	0.822
HDL-4	Free Cholesterol	H4FC	0.707
HDL-4	Phospholipids	H4PL	0.859
HDL-4	Apo-A1	H4A1	0.863

HDL-4	Apo-A2	H4A2	0.811