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## **Metabolomics of biofluids : from analytical tools to data interpretation**

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# Chapter

# 5

Integrating study design and clinical data into  
metabolic profiling of urinary tract infection

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## ABSTRACT

Urinary Tract Infection (UTI) encompasses a variety of clinical syndromes that can range from mild to life-threatening conditions. As such, it represents an interesting model for the development of an analytically based scoring system of disease severity and/or host response. Here we test the feasibility of this concept using  $^1\text{H}$  NMR based metabolomics as the analytical platform. Using an exhaustively clinically characterized cohort and taking advantage of the multi-level study design, which opens possibilities for case-control and longitudinal modeling, we were able to identify molecular discriminators that characterize UTI patients. Moreover, we show that using such a design allows not only a better validation of the statistical models, but also helps dissecting various biological processes and, most importantly, significantly improves biological interpretation of the obtained results.

## INTRODUCTION

Despite the progress made in understanding the mechanistic basis of many diseases in the last century, medicine is still essentially “more an art than a science”.(1) Specific and sensitive biological markers are important contributors to the improved diagnostic methods as well as to patient care and drug discovery. Advanced “-omics” technologies, such as genomics, proteomics and metabolomics, enable identification of such markers. Of our particular interest is metabolomics that focuses on the analysis of metabolites present in biological fluids. Metabolites are end-points of all the biochemical processes of the organism and thus their collection – the metabolome is the closest approximation of the physiological phenotype and as such has a great potential for uncovering the biology underlying diseases and providing valuable markers of pathology.(2;3)

The biological interpretation of results from metabolomics studies is rather complex and still in an early phase of development(4). The human body is a “super-organism” that unites its own network of interconnected tissues and organs with multiple colonies of microorganisms.(5) Interpretation of changes in concentration of metabolites found in biological fluids can readily be performed based on the underlying metabolic pathway; however, it is not always possible to link the observed change in systemic metabolite concentrations to a specific tissue or organ.(6) Especially in the case of disruption of highly abundant metabolites, *e.g.* from energy or amino acid metabolism, additional information would be required in order to interpret the data in respect to the tissue of origin. In addition, a change of such metabolites does not always improve the knowledge about the underlying cellular mechanisms and biology. A way to facilitate the interpretation of clinical metabolomics data is to integrate a plethora of available clinical parameters and to utilize a multilevel study design that should provide the opportunity to access the various levels of biological processes.

One of the examples of a complex and heterogeneous clinical entity, for which current diagnostic methods are not straightforward, is Urinary Tract Infection (UTI)(7). Clinical manifestations of UTI can cover the range from mild cystitis to advanced pyelonephritis potentially leading to urosepsis and multiple-organ failure. Physical symptoms may vary from patient to patient and be similar to a number of other diseases, mainly of infectious origin. Thus, the presence of bacteria and leucocytes in urine can not be considered as a sole common denominator for UTI and even if it was, the criterion for the colony count is variable and anyway considered insensitive(8). The correct and timely diagnosis relies on effective joint work of clinicians and microbiologists(8). All of this explains the considerable interest in providing new, specific and sensitive markers for UTI and for the uropathogen involved. The focus of the available metabolomics studies on UTI in the literature has so far

been on the identification of pathogens: in the work of Gupta *et al.* a beautiful method with the use of  $^1\text{H}$  NMR was proposed.(9-11) However, regrettably the method is not quantitative nor does it provide any information about the localization of the infection within the urinary tract, morbidity and preferred strategy of treatment.

In the current study we investigated possibilities of using urinary metabolic profiles to monitor the health state of UTI patients, the degree of infection and the recovery process of UTI patients in the context of febrile, complicated UTI. We used a selection of samples from an exhaustively characterized cohort, with multiple urine samples available per individual and with the main pathogen identified as *Escherichia coli*, which is the most common pathogen for UTI. Samples from a group of age- and gender- matched UTI symptom-free subjects were included as control. The longitudinal design allowed studying various biological processes: not only the difference between the patients and controls, but also the recovery process, using each patient as its own control.

## MATERIALS AND METHODS

**Samples.** The study protocol was approved by the ethical committee of the Leiden University Medical Center and all included patients gave written informed consent.

Urine samples were collected at the Emergency Department and Primary Care Department. The sampling was carried out at several time points: the first urine samples were collected at the day of enrolment as baseline samples ( $t=0$ ). Clean midstream-catch urine cultures were obtained and were analyzed using local standard microbiological methods. Three-four ( $t=4$ ) and thirty days ( $t=30$ ) after the day of enrolment, urine samples of the same patients were collected and new bacterial culture tests were performed (Supplementary Materials, Figure S1).

For the current study, from a database of about 700 subjects enrolled, 40 subjects, for which urine culture confirmed *E.coli*-positive complicated febrile urinary tract infection that recovered after antibiotic treatment, were selected. Samples from age- and gender-matched subjects with low bacterial culture in urine and without evidence of inflammatory diseases were used as controls (Table 1). A number of samples were missing, a few removed from the analysis due to either insufficient spectra quality or high glucose content (Supplementary Materials, Figure S1). In the end the study included four classes of samples originating from UTI symptom-free ( $N = 35$ ) at day 0 (baseline control), UTI patients ( $N = 32$ ) at day 0 (baseline), UTI patients ( $N= 29$ ) at day 4 and UTI patients after recovery from infection ( $N = 37$ ) at day 30 (Supplementary Materials, Figure S1).

**Table 1. Characteristics of the studied patients and controls groups at baseline (t=0).**

| Characteristics         | UTI patients | Controls   | p       |
|-------------------------|--------------|------------|---------|
|                         | n = 40       | n = 40     |         |
| Age, years, median (sd) | 59 (14.6)    | 58 (17.9)  | 0.9     |
| Female, n (%)           | 22 (55)      | 22 (55)    | 1       |
| Smoking, n (%)          | 5 (12)       | 5 (12)     | 1       |
| Co-morbidity, n (%)     |              |            |         |
| Urinary tract disorder  | 4 (10)       | 4 (10)     | 1       |
| Malignancy              | 4 (10)       | 1 (3)      | 0.17    |
| Heart failure           | 5 (13)       | 3 (8)      | 0.46    |
| Renal insufficiency     | 1 (4)        | 0 (0)      | 0.13    |
| Diabetes mellitus       | 6 (15)       | 2 (5)      | 0.14    |
| Immunocompromised       | 1 (3)        | 1 (3)      | 1       |
| Urine dipstick results  |              |            |         |
| Nitrate                 | 26/37 (75)*  | 0/37 (0)*  | < 0.001 |
| Leucocyte esterase      | 35/37 (95)*  | 5/37 (14)* | < 0.001 |

\* 3 missing values

**Sample preparation.** Samples were thawed, transferred into 96 deep-well plates and centrifuged at 3000g for 15 minutes at 4°C to remove any precipitate. For sample preparation 520 µL urine were mixed with 60 µL of pH 7.0 phosphate buffer (1.5 M) in 100% D<sub>2</sub>O containing 4 mM sodium 3-trimethylsilyl-tetraduteriopropionate (TSP) and 2mM NaN<sub>3</sub> in a 96 deep-well plate using a Gilson 215 liquid handler controlled by a Bruker Sample Track LIMS system (Bruker BioSpin, Karlsruhe, Germany).

**NMR experiments and processing.** <sup>1</sup>H NMR data were collected using a Bruker 600 MHz AVANCE II spectrometer equipped with a 5 mm TCI cryogenic probehead and a z-gradient system; a Bruker BEST (Bruker Efficient Sample Transfer) system was used in combination with a 120 µL CryoFIT™ flow insert for sample transfer. One-dimensional (1D) <sup>1</sup>H NMR spectra were recorded at 300 K using the first increment of a NOESY pulse sequence(12) with presaturation ( $\gamma B_1=50$  Hz) during a relaxation delay of 4 s and a mixing time of 10 ms for efficient water suppression(13). Eight scans of 65,536 points covering 12,335 Hz were recorded and zero filled to 65,536 complex points prior to Fourier transformation, an exponential window function was applied with a line-broadening factor of 1.0 Hz. The spectra were manually phase and baseline corrected and automatically

referenced to the internal standard (TSP = 0.0 ppm). Phase offset artifacts of the residual water resonance were manually corrected using a polynomial of degree 5 least square fit filtering of the free induction decay (FID) (14). In order to monitor proper filling of the NMR flow cell and for quality control 1D gradient profiles (15) along the z-axis were recorded for each sample prior and post data acquisition. Duration of 90 degree pulses were automatically calibrated for each individual sample using a homonuclear-gated nutation experiment(16) on the locked and shimmed samples after automatic tuning and matching of the probe head.

**Statistical analysis.** Each spectrum was integrated (binned) using 0.014 ppm integral regions between 10 and 1 ppm, the residual water and urea region between 6 and 4.5 ppm was excluded, resulting in 550 data points used for the analysis. To account for any difference in concentration between the samples, each spectrum was normalized to a total area of 1. Absolute values were log-transformed. All pre-processing was done using in-house developed routines in R statistical environment (<http://www.r-project.org/>). Variables were centered and unit variance scaled prior to statistical analysis in SIMCA-P+ (version 12.0; Umetrics, Sweden) software package. For initial analysis and outlier detection, principal component analysis (PCA) was performed using 10 components. After the initial PCA analysis the following regions corresponding to paracetamol and its metabolites were excluded from the analysis: 7.5 – 6.75, 3.95 – 3.8, 3.7 – 3.45, 2.2 – 2.14 and 1.84-1.88 ppm according to (17). For partial least squares-discriminant analysis (PLS-DA) (18) samples were categorized based on classes as defined by the study design. PLS model was built using 5 categories according to logarithm of bacterial count as a Y variable. Statistical models from supervised multivariate data analysis were validated by random permutation of the response variable and comparison of the goodness of fit ( $R^2Y$  and  $Q^2$ ) (19;20). For random permutation tests 100 models were calculated and the goodness of fit was compared with the original model in a validation plot. Spectral regions responsible for the separation between classes in supervised models were identified based on the Variable Influence on Projection (VIP) values, which correspond to the importance of the variables (bins) for the model. The variables with a VIP value larger than 1.8 were considered significant and used for further analysis and identification of the responsible peak(s) within the spectrum. Prediction of class membership of samples by PLS-DA model was based on the predicted Y variable with the cut-off of 0.5.

For multilevel components analysis (MCA) using an in-house developed script in R as described by Jansen *et al.*(21) data were not log-transformed.

Univariate tests were performed to assess the statistical significance of the spectroscopic regions found using multivariate analysis: unpaired t-test was performed for the regions

found as discriminating between UTI patients and controls by PLS-DA; ANOVA was performed on the regions that showed association with bacterial count in PLS; paired t-test was carried out on the regions identified in multilevel analysis. All the corresponding p-values were adjusted for multiple testing using Benjamini-Hochberg correction.

**Identification of compounds of interest.** Annotation of identified peaks was performed based on reference spectra from the Bruker Bioref database and in-house reference data. Confident identification was facilitated by the use of Statistical Total Correlation Spectroscopy method (STOCSY)(22).

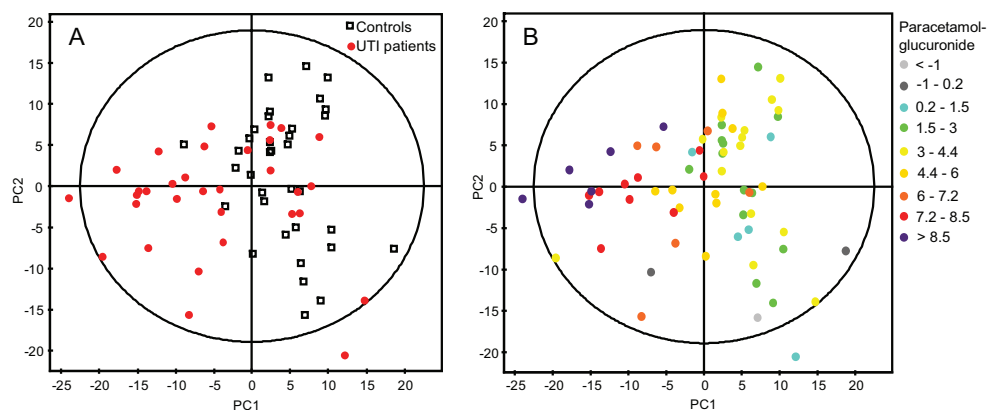
**Quantification of paracetamol.** Quantification was performed by deconvolution and subsequent integration of paracetamol-glucuronide resonance at 5.10 ppm (d, 7.1 Hz) using an in-house developed automation routine. The absolute concentrations were calculated based on internal reference TSP. Values were not corrected for differential attenuation of the signals caused by relaxation during the mixing time and rapid-pulsing saturation effects.

## RESULTS

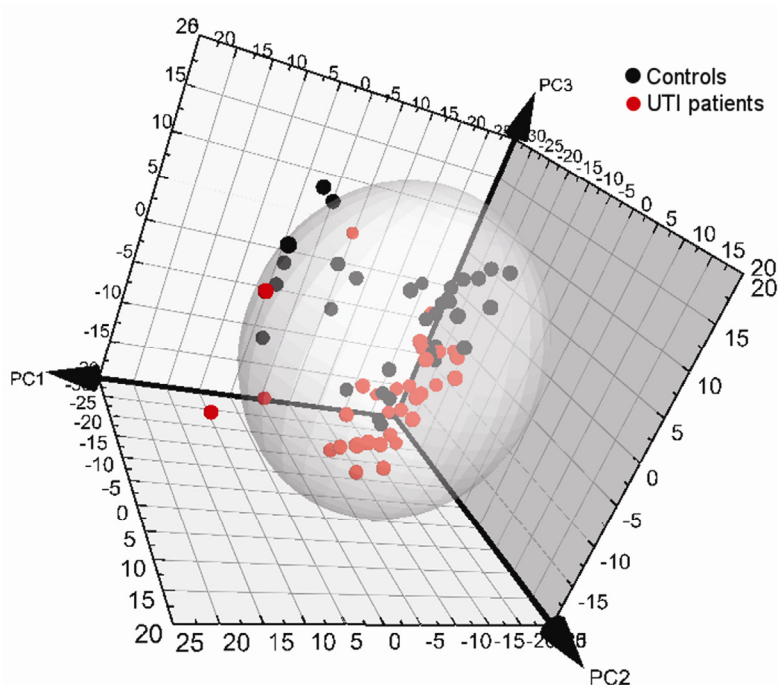
The initial PCA on baseline samples revealed a trend in separation between UTI patients and controls in the scores plot of the first two principal components as shown in Figure 1A. The loadings plot of this model was dominated by the spectral regions that belonged to one of the most commonly used over-the-counter analgesic, paracetamol (Supplementary Materials, Figure S2). The absolute concentration of paracetamol-glucuronide was used to stratify samples in the PCA plot: the direction of increase of paracetamol-glucuronide was found to match the direction of controls-patients separation (Figure 1B). As paracetamol is not an infection or morbidity marker, the further analysis was performed after the exclusion of the regions corresponding to the drug and its metabolites.

The PCA analysis of the baseline samples after the removal of spectral regions of paracetamol and its metabolites did not show separation between UTI patients and controls within the scores plot of the first two principal components; however, a clear trend was identified along the third principal component (Figure 2), which means that inter-individual variability is to a certain extent more prominent than the disease effect. No outliers were detected based on distance to the model (DModX).



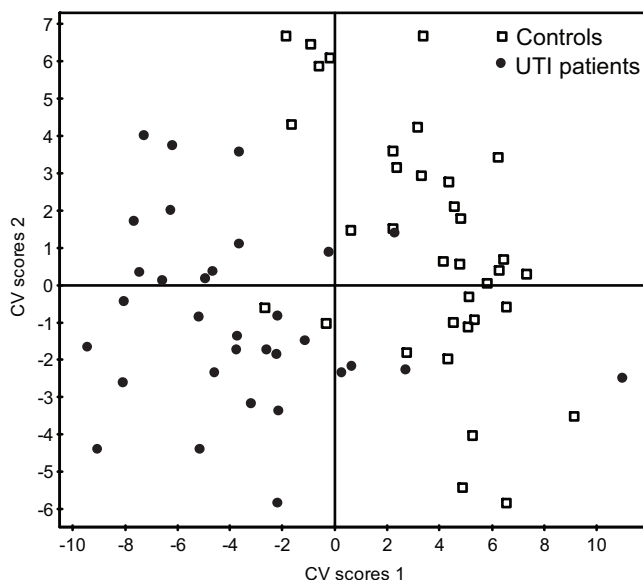


**Figure 1.** PCA scores plot of  $^1\text{H}$  NMR data from controls and UTI patients urine samples at baseline, first two principal components covering 14.5 and 10.2% of variation respectively. (A) Colored according to controls (□) and UTI patients (●). (B) Colored according to the logarithm of absolute concentration of paracetamol-glucuronide.



**Figure 2.** PCA scores plots of  $^1\text{H}$  NMR data from controls (black) and UTI patients (red) urine samples at baseline after removal of the regions corresponding to paracetamol and its metabolites. First principal component covers 11.7%, second 11.2% and third 9.8% of variation.

In the next step a supervised PLS-DA model was built for  $t=0$  using UTI/controls as a response variable. In the scores plot of the resulting model the two groups were well separated (Figure 3). Cumulative explained variance ( $R^2Y$ ) of 0.88 and cross validated predictive fraction ( $Q^2$ ) of 0.63 were calculated for the model; the model validation plot showed intercepts of the  $R^2Y$  and  $Q^2$  regression lines with the vertical axis at 0.63 and -0.11, respectively, indicating a valid model. Molecular discriminators were identified based on relevant regions as identified by the corresponding VIP. A list of those regions, along with the p-values based on t-test (corrected for multiple testing), the direction of change and identities of the corresponding metabolites are summarized in Table 2.



**Figure 3. Cross-validated PLS-DA scores plot of urine  $^1\text{H}$  NMR spectra of controls (□) and UTI patients at baseline (●),  $R^2Y = 0.88$ ,  $Q^2 = 0.63$ .**

The advantage of PLS-based models is that they can easily be used to predict the class membership of new samples. Data of the UTI patients at  $t=4$  were predicted using the two-class PLS-DA model that was built as described above. Of a total of 29 urine samples included in the prediction set, 19 (65.5%) were classified as controls, whereas 10 (34.5%) samples were classified as UTI (Figure 4). Besides using data from the 4-days time point as prediction set, we also performed a separate analysis for the 30-days time point (Figure 4). In this case, out of 37 samples collected, 32 (86.5%) were attributed to the group of controls and 5 (13.5%) were categorized as UTI.

**Table 2. Spectroscopic regions that appear as influential in various statistical models and statistical significance of the corresponding univariate tests adjusted for multiple testing using Benjamini-Hochberg method.**

| ppm region    | Identity                 | Controls vs.<br>UTI patients <sup>a</sup> |        | Bacteria<br>concentration <sup>b</sup> |        | Recovery from<br>t=0 to t=30 <sup>c</sup> |        |
|---------------|--------------------------|-------------------------------------------|--------|----------------------------------------|--------|-------------------------------------------|--------|
|               |                          | t-test<br>p-value                         | change | ANOVA<br>p-value                       | change | paired<br>t-test<br>p-value               | change |
| 9.291 - 9.277 | 1-methylnicotinamide     | <0.0001                                   | -      | <0.001                                 | -      |                                           |        |
| 9.277 - 9.264 | 1-methylnicotinamide     | <0.01                                     | -      |                                        |        |                                           |        |
| 8.977 - 8.964 | 1-methylnicotinamide     | <0.01                                     | -      |                                        |        |                                           |        |
| 4.491 - 4.477 | 1-methylnicotinamide     | <0.01                                     | -      | <0.01                                  | -      |                                           |        |
| 1.941 - 1.927 | Acetic acid              | <0.01                                     | +      | <0.01                                  | +      |                                           |        |
| 1.927 - 1.914 | Acetic acid              | <0.0001                                   | +      | <0.0001                                | +      |                                           |        |
| 3.196 - 3.182 | Acetylcarnitine          | <0.01                                     | +      |                                        |        |                                           |        |
| 2.568 - 2.555 | Citric acid              | <0.01                                     | -      |                                        |        |                                           |        |
| 2.541 - 2.527 | Citric acid              | <0.01                                     | -      |                                        |        |                                           |        |
| 4.082 - 4.068 | Creatinine               | 0.03                                      | -      |                                        |        |                                           |        |
| 3.073 - 3.059 | Creatinine               | <0.01                                     | -      | 0.07                                   | -      |                                           |        |
| 3.059 - 3.045 | Creatinine               | 0.09                                      | -      |                                        |        |                                           |        |
| 7.709 - 7.696 | Furoylglycine            |                                           |        |                                        |        | <0.01                                     | +      |
| 7.696 - 7.682 | Furoylglycine            | <0.01                                     | -      | <0.01                                  | -      |                                           |        |
| 3.959 - 3.946 | Glycolic acid derivative | <0.001                                    | -      | <0.01                                  | -      | <0.0001                                   | +      |
| 7.859 - 7.846 | Hippuric acid            | <0.01                                     | -      | <0.01                                  | -      |                                           |        |
| 7.668 - 7.655 | Hippuric acid            | <0.001                                    | -      | <0.01                                  | -      |                                           |        |
| 7.655 - 7.641 | Hippuric acid            | 0.01                                      | -      | 0.02                                   | -      |                                           |        |
| 7.586 - 7.573 | Hippuric acid            | <0.01                                     | -      | 0.05                                   | -      |                                           |        |
| 3.973 - 3.959 | Hippuric acid            | 0.01                                      | -      | 0.03                                   | -      |                                           |        |
| 8.555 - 8.541 | Hippuric acid (amide)    | <0.01                                     | -      |                                        |        |                                           |        |
| 8.541 - 8.527 | Hippuric acid (amide)    | <0.001                                    | -      | <0.01                                  | -      |                                           |        |
| 1.341 - 1.327 | Lactic acid              | <0.01                                     | +      | <0.01                                  | +      |                                           |        |
| 7.764 - 7.75  | Para-aminohippuric       |                                           |        |                                        |        | <0.001                                    | +      |
| 3.332 - 3.318 | Scyllo-inositol          |                                           |        |                                        |        | <0.01                                     | +      |
| 3.455 - 3.441 | Taurine                  | <0.0001                                   | +      | <0.001                                 | +      | <0.0001                                   | -      |
| 3.441 - 3.427 | Taurine                  | <0.0001                                   | +      | <0.001                                 | +      | <0.0001                                   | -      |
| 3.427 - 3.414 | Taurine                  | <0.0001                                   | +      | <0.01                                  | +      |                                           |        |
| 3.264 - 3.250 | Taurine                  | <0.001                                    | +      |                                        |        |                                           |        |
| 8.855 - 8.541 | Trigonelline             |                                           |        |                                        |        | 0.01                                      | +      |
| 4.45 - 4.436  | Trigonelline             |                                           |        |                                        |        | <0.01                                     | +      |
| 2.896 - 2.881 | Trimethylamine           | <0.0001                                   | +      | <0.0001                                | +      |                                           |        |
| 8.486 - 8.473 | Unknown                  |                                           |        |                                        |        | <0.01                                     | +      |
| 7.968 - 7.955 | Unknown                  |                                           |        |                                        |        | <0.001                                    | +      |
| 7.75 - 7.736  | Unknown                  |                                           |        |                                        |        | <0.01                                     | +      |
| 7.518 - 7.505 | Unknown                  |                                           |        | <0.01                                  | +      |                                           |        |
| 6.686 - 6.673 | Unknown                  |                                           |        |                                        |        | <0.0001                                   | +      |
| 6.509 - 6.496 | Unknown                  |                                           |        |                                        |        | 0.04                                      | +      |
| 3.168 - 3.155 | Unknown                  |                                           |        | <0.01                                  | -      |                                           |        |

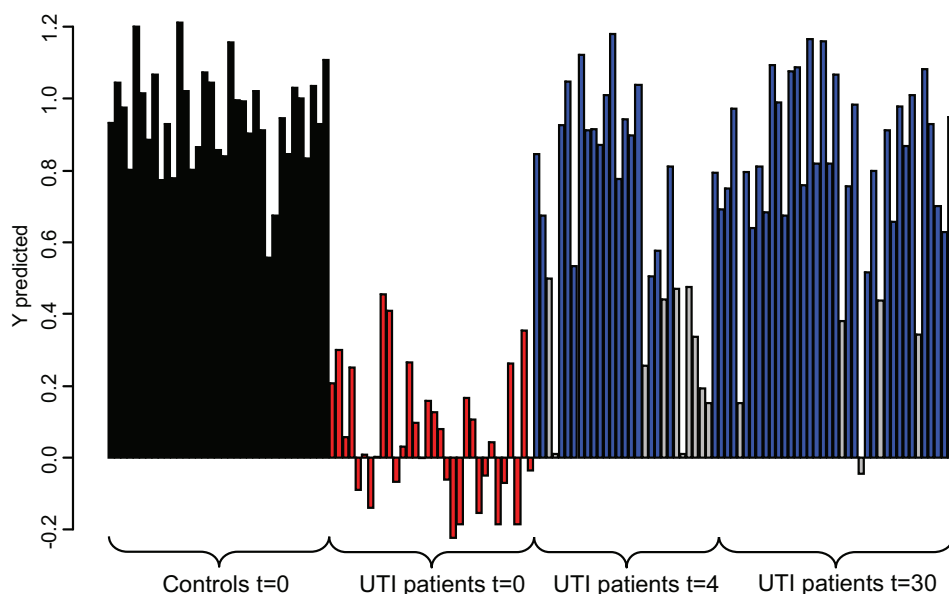
<sup>a</sup> two-group t-test for the healthy controls and UTI patients at baseline; positive direction of change corresponds to intensity of the region being higher in UTI patients compared to controls, negative - region intensity is lower in UTI patients compared to controls

<sup>b</sup> ANOVA analysis for the number of bacteria present in urine; direction corresponds to the correlation to the number of bacteria: positive corresponds to the raise of the region intensity with the increase of the number of bacteria, negative - to the decrease of the region intensity with the increase of the number of bacteria

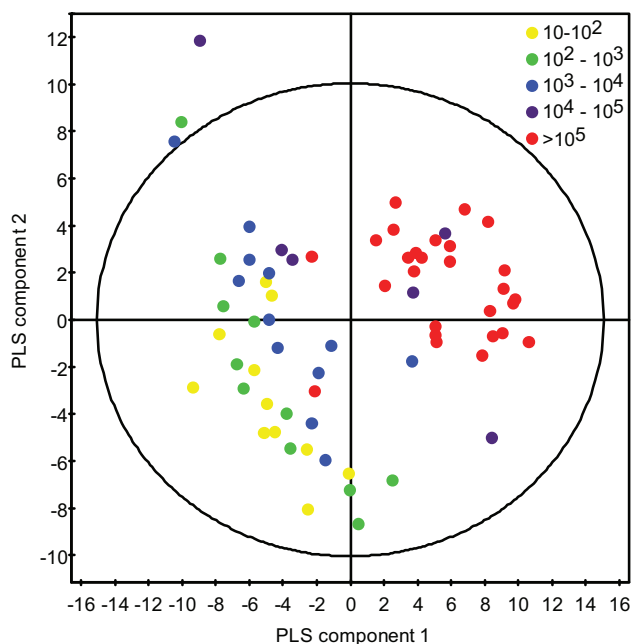
<sup>c</sup> paired t-test for the UTI patients at baseline and 30 days; positive direction of change corresponds to intensity of the region being higher at 30 days compared to baseline, negative - region intensity is lower at 30 days compared to baseline

An important parameter characterizing UTI patients is the number of bacteria in urine; however, bacteria can also be present in urine of the individuals, who do not exhibit any symptoms of UTI(25). We built a PLS regression model from NMR data of urine at baseline using the result of bacterial culture as response variable. Since bacterial count and UTI classification do not fully correlate we expected to obtain a slightly different model as compared to the model built based on UTI classification for this timepoint. Using 2 components a cumulative  $R^2Y = 0.78$  and  $Q^2 = 0.44$  were obtained and model validation showed intercepts of the  $R^2Y$  and  $Q^2Y$  regression lines with the vertical axis at 0.63 and -0.12, respectively, in the model validation plot. As can be seen from the PLS scores plot (Figure 5) the samples with the highest bacteria concentration in urine were very distinct from the rest forming a separate cluster, whereas the rest of the samples were overlapping. The spectral regions responsible for the correlation of the  $^1H$  NMR data and bacterial count were chosen on the basis of the corresponding VIP. A list of those regions, along with the p-values derived from ANOVA (corrected for multiple testing), the direction of change and identities of the corresponding metabolites are summarized in Table 2.

To better understand the process of patient recovery and to find the spectroscopic regions that correlate with this process, we took advantage of the longitudinal study design. One of the statistical methods suitable for such analysis is multilevel component analysis (MCA) that separates variation present in the data into two levels: between-individual and within-individual. We performed this analysis on the 29 patients for which both the data from the baseline and from the 30-days time point were available and concentrated on the within-individual information. This should best reflect the recovery from the baseline, when patients are diagnosed as infected, to 30 days, when they are considered UTI symptom-free. PCA scores plot of the first two principle components that cover 15.8 and 14.8% of the variation, respectively, showed good separation between baseline and  $t=30$  time points (data not shown). The PLS-DA model of this data had high quality parameters ( $R^2Y = 0.98$ ,  $Q^2 = 0.78$  for four components), performs significantly better than random models ( $p < 10^{-15}$ ) and perfectly separated the two time points (data not shown). The NMR spectral regions responsible for the separation between baseline and the  $t=30$  time point were identified based on VIP values. The underlying metabolites as well as the p-values from paired t-test (corrected for multiple testing) and the direction of change are summarized in Table 2.



**Figure 4.** Predicted response value for two-class PLS-DA model based on controls (black bars) and UTI patients (red bars) at baseline: blue bars are the t=4 and t=30 classified as controls, grey are the t=4 and t=30 samples classified as UTI patients at t=0.



**Figure 5.** Scores plot of the PLS model of urine  $^1\text{H}$  NMR spectra at baseline vs. the number of bacteria (CFU/mL) found in urine ( $R^2_Y = 0.78$ ,  $Q^2 = 0.44$ ). Colored by the number of bacteria.

## DISCUSSION

UTI represents a complex clinical entity, for which diagnostics is not straightforward and based on consensus criteria (7). In the current paper we identified metabolites that characterize UTI and its pathology with the use of  $^1\text{H}$  NMR. We demonstrate how the use of clinical data and multiple samples per individual can enrich the biological interpretation of the findings. To reduce the heterogeneity typically posed by UTI research, as a first attempt the smaller selection of UTI subjects from a bigger cohort was used, with similar diagnosis and with the major pathogen being *E.coli*. A set of matched controls was also available.

Unlike in animal experiments, in clinical research assigning people to certain groups is not always unconditional. The diagnosis of a disease can be fuzzy and defining the “healthy” group is even more difficult, as there is hardly a definition of healthy. Thus, it may be very advantageous to supplement a traditional “case-control” design with a more complex study design and the use of additional clinical data. When used without extra information, “case-control” analysis might even be misleading. For example, the separation of the control and UTI groups was seen in the first two principal components of PCA; however, this discrimination was not disease-related, but the result of patients taking the antipyretic and analgesic drug paracetamol. An analysis strategy for such type of data is to identify all of the spectroscopic regions that contain signals from drug-related compounds and to exclude them prior to further analysis. However, it is not feasible to account for the whole range of the medication used and, more importantly within the context of clinical metabolomics studies in general, to account for drug-related shifts in metabolism, especially in the case of long-term treatment regimes of chronic conditions. It is essential to consider such effects when developing the study design in order to minimize or control such influences.

Samples from 4 days after admission, when the patients were still under therapy, but on the way to recovery, were used to check if the modeled differences were related to the effect of medication or not. The fact that the majority of those samples were classified as healthy by the model built on baseline samples is an indication that the model is not reflecting therapy/drug intake, but is indeed related to the clinical difference between the groups.

The samples from the 30-days time point, when UTI patients were symptom-free, could also be used to gain additional information on the performance of the model as well as to get insight into the underlying biology. When predicted using the PLS-DA model built on the baseline UTI infected and UTI symptom-free samples, most of the 30-days samples (86.5%) were projected to the control group. Those few, which were still predicted as infected UTI patients, may have another condition (as we do not know at this point how

specific our model is) or have asymptomatic UTI. On the other hand, they can be healthy and be false positives, as the predictive ability of our model, estimated by cross-validation was 63%. Despite that, considering the prediction of 30-days samples as an independent statistical test for our model, it gives very satisfactory results.

Pair-wise analysis for baseline and 30-days samples from the same individuals was conducted in order to monitor the recovery process. It revealed a number of classifiers and improved their statistical significance. The identified metabolites overlapped with the compounds from the model discriminating healthy and UTI subjects, however a few of them were unique (para-aminohippuric acid, scyllo-inositol and a few unidentified compounds).

Besides the multilevel design, the advantage of the current study was the exhaustive clinical characterization of the patients. Among the variety of clinical parameters available, the number of bacteria in urine was of specific importance. We performed regression-based analysis of the relation between the  $^1\text{H}$  NMR data and the bacterial load in urine as determined by bacterial culture. The classifiers that emerged from this analysis were to a certain extent overlapping with the classifiers derived from the discriminative model on baseline samples. This was no surprise, since UTI is generally characterized by the presence of bacteria in urine.

When comparing the lists of discriminators obtained from the different models (discriminating UTI patients from controls, modeling the recovery process and modeling the data against the degree of bacterial contamination of urine) it is evident that there is a large overlap which makes biological interpretation of the results feasible. For instance, some of the overlapping metabolites were already known from the literature to be related to the bacterial contamination of urine: acetate, lactate and trimethylamine (9). Others, if they were found only in the comparative analysis of the two groups, could be attributed based on previous studies to certain phenomena. Hippuric acid, for example, is often associated with the gut microflora (26) and taurine with liver toxicity (27). However, our findings suggest that they are also associated with the bacterial contamination of urine, which obviously does not mean that they are not related to the mentioned physiological processes as well, but that a complex network of interconnected factors is involved. The metabolites that appear to be related to the recovery process might be considered as potential morbidity markers. One of them, para-aminohippuric acid, is a well-established diagnostic marker for renal plasma flow and glomerular filtration.(28) The recovery from the complicated, tissue-invasive UTI is associated with the resumption of the kidneys' function, so the positive change in para-aminohippuric acid corroborates our assumption that some of the markers discovered in the paired analysis are the markers of morbidity.

## CONCLUSIONS

In the current paper we used a metabolomics approach to profile Urinary Tract Infection, which is on the one hand one of the most common infectious diseases among the adults, and on the other hand a disease that still lacks markers of morbidity. Using  $^1\text{H}$  NMR profiles of urine we generated various statistical models: a) discriminating UTI patients and control subjects, b) following the recovery process of UTI patients and c) associating urine metabolic content with bacterial contamination. The discriminative model was able to classify most of the independent samples correctly according to their diagnosis, which indicates its high predictive ability. Comparing the sets of molecules derived from different analyses, we concluded that some of the compounds (*e.g.* trimethylamine and acetate) can be attributed to the effect of bacterial contamination of urine, others (*e.g.* para-aminohippuric acid, scyllo-inositol) can be considered markers of morbidity.

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## REFERENCES

1. Woodcock, J. 2007. The prospects for "personalized medicine" in drug development and drug therapy. *Clinical Pharmacology & Therapeutics* 81:164-169.
2. Lindon, J.C., Holmes, E., Bollard, M.E., Stanley, E.G., and Nicholson, J.K. 2004. Metabonomics technologies and their applications in physiological monitoring, drug safety assessment and disease diagnosis. *Biomarkers* 9:1-31.
3. Holmes, E., Wilson, I.D., and Nicholson, J.K. 2008. Metabolic phenotyping in health and disease. *Cell* 134:714-717.
4. Mendes, P., Camacho, D., and de la, F.A. 2005. Modelling and simulation for metabolomics data analysis. *Biochem. Soc. Trans.* 33:1427-1429.
5. Nicholson, J.K., Holmes, E., and Wilson, I.D. 2005. Gut microorganisms, mammalian metabolism and personalized health care. *Nat. Rev. Microbiol.* 3:431-438.
6. Adourian, A., Jennings, E., Balasubramanian, R., Hines, W.M., Damian, D., Plasterer, T.N., Clish, C.B., Stroobant, P., McBurney, R., Verheij, E.R. *et al* 2008. Correlation network analysis for data integration and biomarker selection. *Molecular Biosystems* 4:249-259.
7. Wilson, M.L., and Gaido, L. 2004. Laboratory diagnosis of urinary tract infections in adult patients. *Clinical Infectious Diseases* 38:1150-1158.
8. Johnson, J.R. 2004. Laboratory diagnosis of urinary tract infections in adult patients. *Clinical Infectious Diseases* 39:873.
9. Gupta, A., Dwivedi, M., Mahdi, A.A., Gowda, G.A., Khetrapal, C.L., and Bhandari, M. 2009.  $^1\text{H}$ -nuclear magnetic resonance spectroscopy for identifying and quantifying common uropathogens: a metabolic approach to the urinary tract infection. *BJU. Int.* 104:236-244.



10. Gupta,A., Dwivedi,M., Nagana Gowda,G.A., Ayyagari,A., Mahdi,A.A., Bhandari,M., and Khetrapal,C.L. 2005. <sup>1</sup>H NMR spectroscopy in the diagnosis of *Pseudomonas aeruginosa*-induced urinary tract infection. *NMR Biomed.* 18:293-299.
11. Gupta,A., Dwivedi,M., Gowda,G.A., Mahdi,A.A., Jain,A., Ayyagari,A., Roy,R., Bhandari,M., and Khetrapal,C.L. 2006. <sup>1</sup>H NMR spectroscopy in the diagnosis of *Klebsiella pneumoniae*-induced urinary tract infection. *NMR Biomed.* 19:1055-1061.
12. Kumar,A., Ernst,R.R., and Wuthrich,K. 1980. A two-dimensional nuclear Overhauser enhancement (2D NOE) experiment for the elucidation of complete proton-proton cross-relaxation networks in biological macromolecules. *Biochem. Biophys. Res. Commun.* 95:1-6.
13. Price,W.S. 1999. Water signal suppression in NMR spectroscopy. *Annual Reports on Nmr Spectroscopy*, Vol 38 38:289-354.
14. Coron,A., Vanhamme,L., Antoine,J.P., Van,H.P., and Van,H.S. 2001. The filtering approach to solvent peak suppression in MRS: a critical review. *J. Magn Reson.* 152:26-40.
15. Vanzijl,P.C.M., Sukumar,S., Johnson,M.O., Webb,P., and Hurd,R.E. 1994. Optimized Shimming for High-Resolution Nmr Using 3-Dimensional Image-Based Field-Mapping. *Journal of Magnetic Resonance Series A* 111:203-207.
16. Wu,P.S., and Otting,G. 2005. Rapid pulse length determination in high-resolution NMR. *J. Magn Reson.* 176:115-119.
17. Bales,J.R., Nicholson,J.K., and Sadler,P.J. 1985. Two-dimensional proton nuclear magnetic resonance "maps" of acetaminophen metabolites in human urine. *Clin. Chem* 31:757-762.
18. Nocairi,H., Qannari,E.M., Vigneau,E., and Bertrand,D. 2005. Discrimination on latent components with respect to patterns. Application to multicollinear data. *Computational Statistics & Data Analysis* 48:139-147.
19. Westerhuis,J.A., Hoefsloot,H.C.J., Smit,S., Vis,D.J., Smilde,A.K., van Velzen,E.J.J., van Duijnhoven,J.P.M., and van Dorsten,F.A. 2008. Assessment of PLSDA cross validation. *Metabolomics* 4:81-89.
20. Lindgren,F., Hansen,B., Karcher,W., Sjostrom,M., and Eriksson,L. 1996. Model validation by permutation tests: Applications to variable selection. *Journal of Chemometrics* 10:521-532.
21. Jansen,J.J., Hoefsloot,H.C.J., van der Greef,J., Timmerman,M.E., and Smilde,A.K. 2005. Multilevel component analysis of time-resolved metabolic fingerprinting data. *Analytica Chimica Acta* 530:173-183.
22. Cloarec,O., Dumas,M.E., Craig,A., Barton,R.H., Trygg,J., Hudson,J., Blancher,C., Gauguier,D., Lindon,J.C., Holmes,E. et al 2005. Statistical total correlation spectroscopy: An exploratory approach for latent biomarker identification from metabolic H-1 NMR data sets. *Analytical Chemistry* 77:1282-1289.
23. Lin,K., and Fajardo,K. 2008. Screening for asymptomatic bacteriuria in adults: evidence for the U.S. Preventive Services Task Force reaffirmation recommendation statement. *Ann. Intern. Med.* 149:W20-W24.
24. Swann,J., Wang,Y., Abecia,L., Costabile,A., Tuohy,K., Gibson,G., Roberts,D., Sidaway,J., Jones,H., Wilson,I.D. et al 2009. Gut microbiome modulates the toxicity of hydrazine: a metabonomic study. *Mol. Biosyst.* 5:351-355.
25. Nicholson,J.K., Lindon,J.C., and Holmes,E. 1999. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 29:1181-1189.
26. REUBI,F.C. 1953. Glomerular filtration rate, renal blood flow and blood viscosity during and after diabetic coma. *Circ. Res.* 1:410-413.

## SUPPLEMENTARY MATERIALS

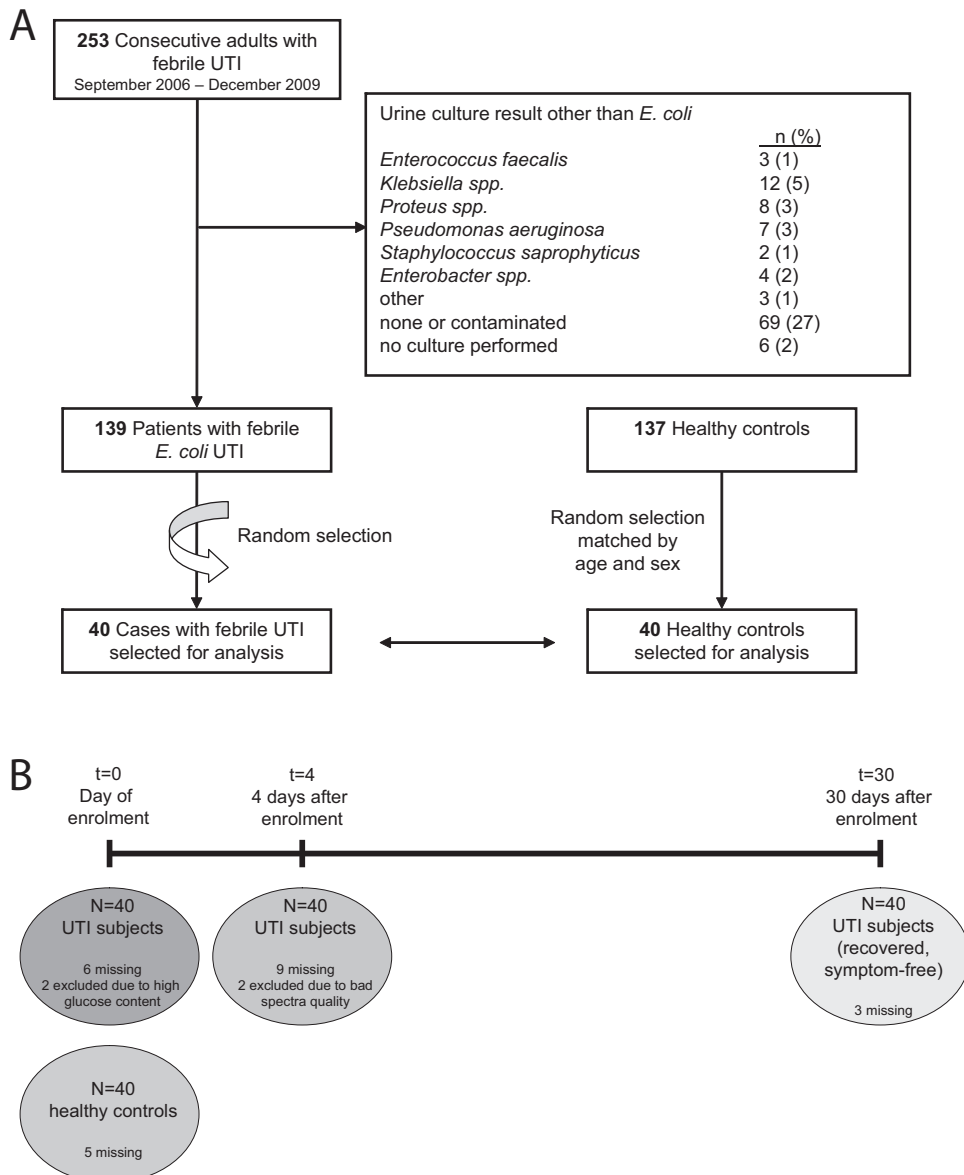
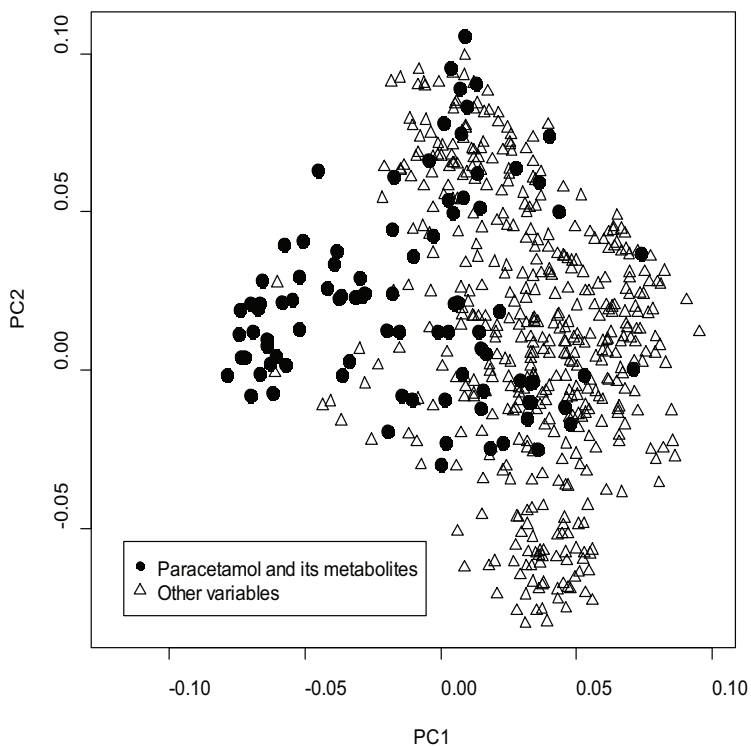


Figure S1. Design of the study.



**Figure S2.** Loadings plot of the PCA model created using urine spectra of samples at baseline. Dots indicate variables that correspond to the spectral regions of paracetamol and its metabolites, triangles represent all the other variables.