Chapter 3

Fibrinogen alpha chain *O*-glycopeptides as possible markers of urinary tract infection

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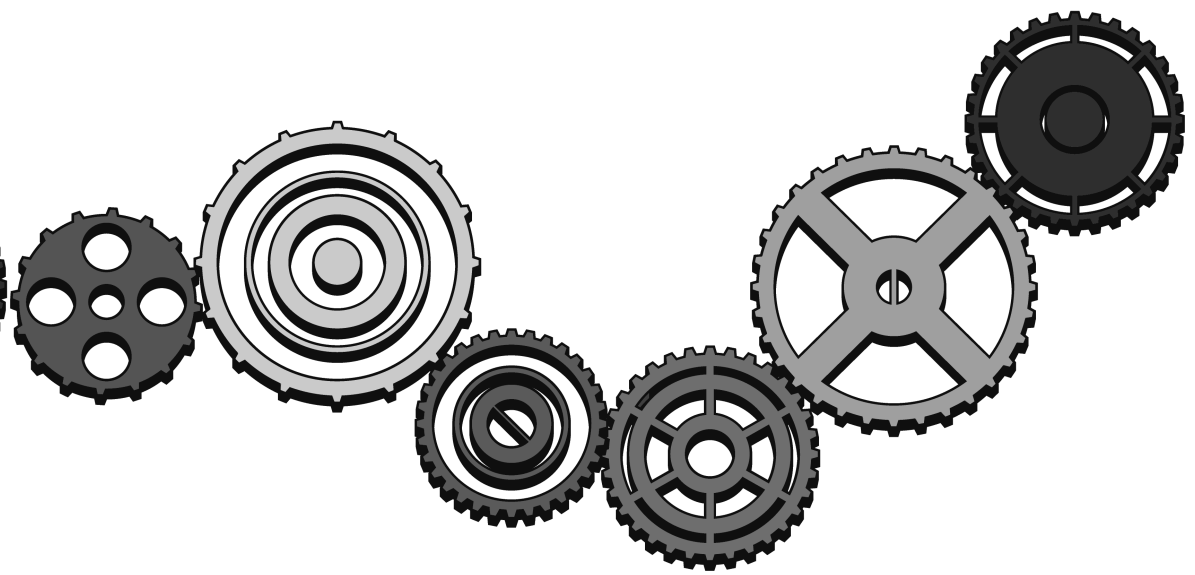

ABSTRACT

Urinary tract infection (UTI) is the most common bacterial infection leading to substantial morbidity and considerable health care expenditures across all ages. Here we present an exploratory UPLC–MS study of human urine in the context of febrile, complicated urinary tract infection aimed to reveal and identify possible markers of a host response on infection. A UPLC–MS based workflow, taking advantage of Ultra High Resolution (UHR) Qq-ToF-MS, and multivariate data handling were applied to a carefully selected group of 39 subjects with culture-confirmed febrile Escherichia coli UTI. Using a combination of unsupervised and supervised multivariate modeling we have pinpointed a number of peptides specific for UTI. An unequivocal structural identification of these peptides, as O-glycosylated fragments of the human fibrinogen alpha 1 chain, required MS^2 and MS^3 experiments on two different MS platforms: ESI-UHR-Qq-ToF and ESI-ion trap, a blast search and, finally, confirmation was achieved by matching experimental tandem mass spectra with those of custom synthesized candidate-peptides.

In conclusion, exploiting non-targeted UPLC-MS based approach for the investigation of UTI related changes in urine, we have identified and structurally characterized unique *O*-glycopeptides, which are, to our knowledge, the first demonstration of *O*-glycosylation of human fibrinogen alpha 1-chain.

INTRODUCTION

Urinary tract infection (UTI) is the most common bacterial infection leading to substantial morbidity and considerable health care expenditures across all ages [1]. The term 'urinary tract infection' encompasses a variety of clinical syndromes with the common denominator of a positive urine culture (i.e., significant bacteriuria ≥ 10^3 CFU/ml) [2]. Pathogens most commonly associated with UTI are *Enterobacteriacaea* such as *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus sp.* and *Staphylococcus saprophyticus*. In a first approximation, UTI can be grouped into acute uncomplicated UTI (non-pregnant young women with cystitis but no evidence of urological abnormalities) and complicated UTI (UTI in males, all febrile UTI syndromes and those with urological abnormalities) [3, 4]. In this respect, fever reflects the presence of a tissue invasive disease such as pyelonephritis, prostatitis or the urosepsis syndrome. A clinical management of UTI is rather straightforward and implementation of modern analytical technologies such as mass spectrometry for diagnostic purposes will have little impact on the clinical decision making. However, mass spectrometry might provide new, highly needed input in scaling the morbidity inflicted to, for example, prostate or kidney or obtaining guidance for duration of the treatment.

So far, the most successful application of mass spectrometry in the context of UTI was the identification of a pathogen. Ferreira *et al* [5] have shown that matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-ToF MS) in combination with pattern recognition algorithms can provide a correct pathogen identity in more than 90% of cases with bacteriuria cut-off at 10^5 CFU/ml. In general, MALDI-ToF MS has quickly evolved as a method of pathogen identification and took its place in microbiological laboratories alongside with more classical techniques [6]. However, for an unbiased exploratory study, which is needed for a first evaluation of the host response to UTI, a hyphenated technique as, for example, liquid chromatography–mass spectrometry (LC–MS) is probably a better choice. Those techniques cover a broad range of chemical entities and most importantly a very special group of compounds such as small molecules and naturally occurring urinary peptides. The clinical relevance of the last ones was proven in a large scale study of Good *et al* [7]. Here, we present a first exploratory LC–MS study of human urine in the context of febrile, complicated urinary tract infection. Using a carefully matched selection of patients with infection due to *E. coli*, we have evaluated the applicability of a reverse phase (RP)UPLC-ToF-MS approach for UTI in a clinical study and identified a glycopeptide representing a unique proteolytic fragment of the fibrinogen alpha 1 chain as a possible marker of active UTI.

EXPERIMENTAL SESSION

Chemicals

Ethanol, methanol (HPLC grade) and formic acid were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Sodium hydroxide was purchased from Fluka (Zwijndrecht, The Netherlands). Peptides were synthesized at the LUMC-facility as described previously [8].

Clinical samples

Urine samples were collected in a prospective observational multicenter cohort study

of eight emergency departments (ED) of seven hospitals and 35 affiliating primary health care providers. From January 2004 through November 2008, patients who presented with a diagnosis of febrile UTI, were considered for enrolment in the study. The study was approved by the local ethics committees and all included patients gave written informed consent. Inclusion criteria and exclusion criteria have been described in detail elsewhere [9,10]; in short, patients of 18 years or older, with fever and presenting at least one symptom of UTI (dysuria, frequency, of urination, perineal pain, flank pain or costovertebral tenderness) and a positive nitrite dipstick test or a positive leukocyte esterase dipstick test were enrolled. Exclusion criteria were current treatment for urolithiasis or hydronephrosis, pregnancy, hemo- or peritoneal dialysis, a history of kidney transplantation or known presence of polycystic kidney disease. Details with respect to empiric therapy and follow-up are provided elsewhere [11]. Urine samples were collected at enrolment as baseline samples (t=0). After three (t=3) and thirty days (t=30) urine samples of the same patients were collected for follow-up. Clean midstream-catch urines were collected and stored, within 2 h after the collection, at -80° C until further analysis. Bacterial cultures were obtained at all time points and were analyzed using standard microbiological methods. A positive urine culture was defined as bacterial growth of over 10^3 colony-forming units per ml^2.

For the current study, from a database of about 700 subjects enrolled, a group of 39 subjects with culture-confirmed *E. coli* in the urine was selected. Samples from volunteers without UTI or symptoms of infection were enrolled as negative control (a negative bacterial culture; no evidence of inflammatory disease) and in this study used as references. Baseline characteristics of the samples are presented in Table 1. The study design includes the control samples and samples of UTI patients collected at baseline (t=0) as well as patient samples collected after the antibiotic treatment (t=30).

Table 1. Baseline characteristics. Data are presented as n (%) unless otherwise stated. IQR: interquartile range.

Characteristics	Cases n = 39	Controls n = 39	*p*
Age, years, median [IQR]	59 [42-70]	58 [49-67]	0.900
Male sex	18 (46)	18 (46)	1.000
Smoking	5 (13)	5 (13)	1.000
Co-morbidity			
Urinary tract disorder	4 (10)	4 (10)	1.000
Malignancy	4 (10)	1 (3)	0.166
Heart failure	5 (13)	3 (8)	0.456
Renal insufficiency	1 (3)	0 (0)	0.134
Diabetes mellitus	6 (15)	2 (5)	0.136
Immunocompromised	1 (3)	1 (3)	1.000
Urine dipstick results			
Nitrate	26/36 (72)*	0/36 (0)*	< 0.001
Leucocyte esterase	35/36 (97)*	5/36 (14)*	< 0.001

*: 3 missing values

Sample processing

Samples were prepared for non-targeted analysis of small molecules and natively occurring urinary peptides. Protein precipitation was carried out by adding 75 µl of cold ethanol to 25 µl of urine followed by 20 min incubation on ice. Subsequently, the samples

were centrifuged at 3660 g for 10 min and the supernatant was collected and evaporated until dryness overnight. The samples were then reconstituted with 100 µl of water and 10 µl was used for injection. The injection scheme was randomized and it included quality control samples (fresh voided urine samples prepared in the same way as the clinical samples) to ensure the robustness of the workflow and to evaluate the analytical variability. The total number of 117 clinical samples was organized in 4 sequences (4 injection days); two quality control samples were injected at the beginning, at the end and after every six biological samples. In total, 165 samples (48 quality controls plus 117 clinical samples) were analyzed by UPLC-ESI-Qq-TOF MS (Ultra High Resolution ToF, maXis, Bruker Daltonics, Bremen, Germany). The UPLC (Ultimate 3000 RS tandem LC system, Dionex, Amsterdam, The Netherlands) was equipped with a pre-column (Acclaim 120 C18, 5 µm, 120 Å, and 2.1 x 10 mm,) and two analytical columns (Acclaim RSLC 120 C18, 2.2 µm, 120 Å, and 2.1 x 100 mm) working alternatively to speed up the acquisition series. The UPLC flow was set at 400 µl/min and the mobile phases were water + 0.1 % formic acid v/v (Phase A) and methanol + 0.1% formic acid v/v (Phase B). The gradient was as follows: 1 min 0 % phase B, then in 1 min to 10% phase B, held for 1 min at 10% phase B, and subsequently in 6.5 min to 100% phase B and held for 3 min at 100% phase B. Before each chromatographic run, a calibrant solution of sodium formate was injected in flow injection mode.

The mass spectrometer was operated in positive ionization mode and acquired data in the mass range from *m/z* 50 to 1000 with a spectra rate of 1 Hz. The capillary was set at 4500 V, the end plate offset at -500 V, the nebulizer gas at 2 bars and the dry gas at 8 L/min at 200°C.

For compound identifications MS^2 and MS^3 experiments were performed using ESI-ultra high resolution UHR-Qq-ToF and ESI-ion trap MS (HCTultra, Bruker Daltonics). The data were acquired in a wider scan range from *m/z* 50 to 2000 in auto MS/MS mode and by using an inclusion list of precursor ions of interest (the ones relevant after statistical data analysis). Collision energies were the following: *m/z* 500 (singly charged) 50 V, *m/z* 500 (doubly charged) 30 V and *m/z* 1500 (singly charged) 35 V, and *m/z* 500 (doubly charged) 25 V. All the *m/z* values within this interval were fragmented with interpolated values of collision energy.

MS^3 experiments were performed using a reverse phase LC-ESI-ion trap MS system as described previously [12] with slight modifications. For electrospray (1100–1250 V), stainless steel capillaries with an inner diameter of 30 mm (from Proxeon, Odense, Denmark) were used. The solvent was evaporated at 170°C employing a nitrogen stream of 7 L/min and ions from *m/z* 500 to 1800 were registered. Automatic fragment ion analysis was enabled resulting in MS/MS spectra of the most abundant peaks. The two most intense ions of each MS/MS spectrum were subjected to an additional ion isolation/fragmentation cycle, resulting in MS^3 fragment ion spectra.

Data analysis

The LC–MS data files were exported as mzXML files and aligned by using the in-house developed alignment algorithm msalign2 tool [13] (http://www.ms-utils.org/msalign2/); peak picking was performed using XCMS package (The Scripps Research Institute, La Jolla,USA) using the default settings except for bandwidth (bw parameter) for grouping of features set to 10 [14].

The generated data matrix was imported to the SIMCA-P 12.0 software package (Umetrics, Umeå, Sweden). The data were mean centered and unit variance-scaled prior to statistical analysis. The validity and the degree of overfitting of the PLS-DA models were checked using a 200 permutations test.

To identify metabolites of interest, rational chemical formulas were generated based on internally calibrated monoisotopic masses within 10 mDa mass error, using the SmartFormula tool within the DataAnalysis software package (Bruker Daltonics).

RESULTS

Considering the fact that the presented study is the first exploratory LC–MS study of human urine in the context of febrile urinary tract infection, a general overview of the analytical consistency of the method was the starting point of the data analysis. To evaluate the effect of analytical variability, we performed Principal Component Analysis (PCA) using the whole data set of clinical and quality control samples (165 samples). Figure 1 shows the PCA score plot for the first two principal components. No analytical trends, such as day of injection, injection sequence or influence of the column, were represented in the first three principal components, which altogether cover 46% of the total variation. Thus, the analytical variability within our data set had negligible influence on the data matrix.

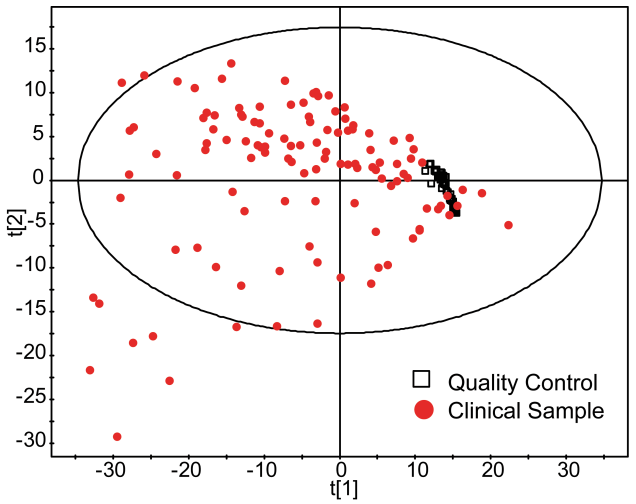

Figure 1. Scores plot of the PCA analysis of the entire data set (including quality control and clinical samples).

Subsequently, we removed the quality control samples from the model and we focused the data analysis on clinical samples. We observed a clear separation of UTI patients and UTI free subjects (Controls and t=30 days) in the PCA model (Supplementary Figure 1A). A two-class PLS-DA model built on baseline samples (controls and patients at t=0 days) had satisfactory values of goodness of fit and prediction ability (Supplementary Figure 1 B). The analysis of Variables Importance on the Projection values (VIP) higher than 1.5 revealed that the separation was related to the presence of acetaminophen metabolites in the patients

affected by febrile UTI. Taking advantage of the intrinsic properties of the UHR-Qq-ToF instrument such as the high mass accuracy and the high-confidence resolution of isotopic distribution we could identify glucuronide ($C_{14}H_{17}NO_8$), acetaminophen mercapturate ($C_{11}H_{14}N_2O_4S$) and N-acetylacetaminophen mercapturate ($C_{13}H_{16}N_2O_5S$) (Supplementary Figure 1C, Supplementary Table1). These data were confirmed by our internal database in which the use of fever reducers was reported for those subjects.

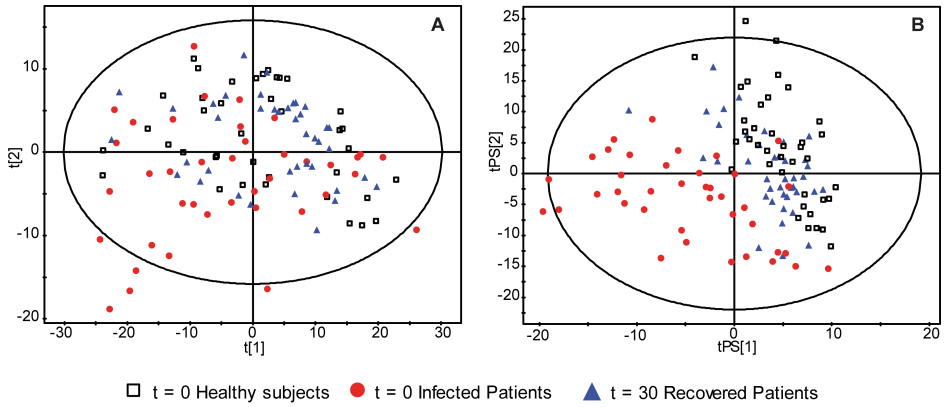

Figure 2. Statistical models of the LC-Qq-ToF-MS data. A) PCA and B) PLS-DA with Q2 (cum)=0.520 and R2Y (cum)=0.647. Samples are coloured according to the disease status.

It is evident that the metabolites of acetaminophen introduce a bias in the data concealing the effects relevant for the study. Therefore, we removed all variables related to acetaminophen metabolites from our dataset and re-analyzed the data. The PCA model built on this neatened data matrix did not show such a clear separation between infected and uninfected patients as in the previous model (Figure 2A). However, a visual trend for such a separation was evident. The goodness of fit and prediction values for the new two-class PLS-DA model built on baseline samples (t=0 days and controls) were lower (Figure 2B), but for human studies still within the statistically acceptable limits. The 30 days after enrolment samples were used to test the predictive power of a model: 34 of 39 samples were predicted as recovered patients, clustering with the group of not infected subjects. An overview of the VIP values > 1.5 revealed 40 features. After closer examination, half of them could be organized in three groups according to their retention times (Table 2).

Table 2 List of major classifiers expressed as *m/z* with their retention times and VIP>1.5 in bracket.

Group	Retention time (s)	*m/z* (VIP Value)
1	58.3	747.274(1.79) 675.239(1.81) 676.248(1.81) 672.174(2.21) 657.232(1.55) 292.103(1.67)
2	382.4	875.361 (1.78) 876.365(1.78) 877.37(1.77) 584.266(2.12) 375.211(1.74) 366.139(2.07) 292.103(2.11) 274.093(2.12)
3	410.3	816.86(2.16) 817.865(1.69)

Within the MS spectra of groups 1 and 2, we observed ions corresponding to some putative in source fragmentation of glycan moieties. Classifier 1 appeared to be a free sugar moiety composed of a sialic acid, one hexose and one N-acetylhexosamine (Figure 3A). Classifier 2 (Figure 3B) contains a similar glycan moiety most probably on a peptide backbone of 218.127 Da which can represent either the dipeptide threonine–valine or the dipeptide serine–(iso)leucine.

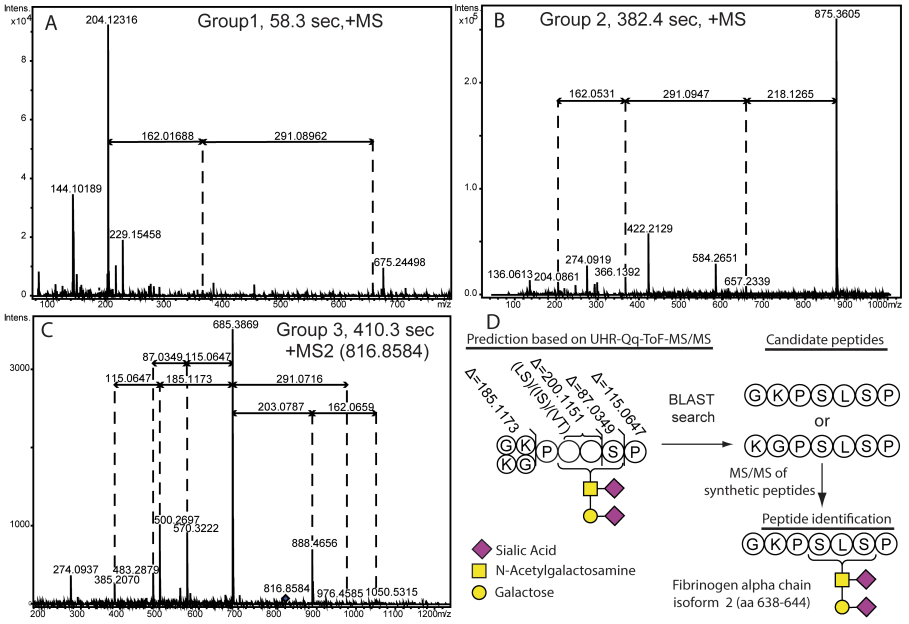

Figure 3. Mass spectrometric characterization of the major classifiers. MS spectra of the classifiers from group 1 (**A**) and 2 (**B**). MS^2 spectrum of *m/z* 816.86 belonging to group 3 (**C**) with the work flow for its identification (**D**) combining information from the fragmentation spectrum, BLAST searches and data from synthetic peptides.

To identify classifier 3, we performed MS/MS experiments using two different mass analyzers: UHR-Qq-ToF and ion trap to take advantage of the characteristics of each respective analyzer. We started the structural assignment form the largest identified precursor. MS/MS data were acquired selecting as precursor the double charged ion at *m/z* 816.86 (Table 2, Group 3) on the UHR-Qq-ToF (Figure 3C). The identification workflow is shown in Figure 3D. The fragmentation pattern revealed characteristics of a glycopeptide based on the sequential losses of two sialic acids (NeuAc; *m/z* 1050.5315), one hexose (Hex; *m/z* 888.4656) and one *N*-acetylhexosamine (HexNAc; *m/z* 685.3869). According to the literature [15-18] this most probably reflects a typical core-1 type O-glycan structure composed of $Gal_1GalNAc_1NeuAc_2$. The peptide backbone was represented by the ion at *m/z* 685.3869. Apparently, some peptide backbone cleavages also occurred, resulting in the ions at *m/z* 570.3222 and 483.2897. Using the mass accuracy of the UHR-Qq-ToF-analyzer, we assigned these ions as b-type ions generated after the sequential loss of a terminal proline (exact mass difference 115.0633 Da) and a serine, and not as y-type ions resulting from a

terminal aspartic acid (exact mass difference 115.0269 Da) and a serine. The loss of proline was also observed after the initial loss of 185.1173, resulting in an internal fragment ion. In this case, the accurate mass points towards the loss of the dipeptide glycine–lysine (GK/KG, exact mass 185.1164). To further corroborate that the above mentioned ions were indeed the result of peptide backbone fragmentation, an MS^3 spectrum from the ion at *m/z* 685.4 was recorded using ESI-ion trap MS (Supplementary Figure 2A). Assuming that the loss of the glycine–lysine dipeptide was the result of a cleavage N-terminal of a proline, we predicted that the peptide backbone should have the sequence (GK/KG)P(200.1151 Da)SP. The total mass of the two remaining amino acids (200.1151 Dalton) matches with a dipeptide consisting of (iso)leucine–serine or threonine–valine (which have the same elemental composition with an exact mass of 200.1161 Dalton). Therefore, we performed BLAST searches against the human NCBI database, taking all the above possibilities into account. This resulted in two positive identifications; one (GKPSLSP) corresponding to a peptide from the C-terminus of fibrinogen alpha 1chain (GI 11761629) and one (KGPSLSP) to an unnamed protein product (GI 22760358). No positive matches were found when identical BLAST searches were performed against the *E. coli* database. To discriminate between the two above mentioned possibilities we synthesized both peptides, performed MS/MS experiments on the ion trap and compared the fragmentation patterns with the MS^3 spectrum that we obtained from the urinary glycopeptide (Supplementary Figure 2B and C). This unambiguously showed that the glycopeptide we have identified corresponded to the C-terminal peptide from fibrinogen alpha 1 chain (GKPSLSP). At this stage we can relate group 2 (see above) as putative derivative of this same peptide containing the dipeptide SL/LS carrying the glycan moiety. Finally, using integrated peak areas for the ion at *m/z* 816.86 we constructed a box plot, which clearly demonstrated the differences between the patients with an active UTI status, the recovered group (t=30) and the control group (Figure 4).

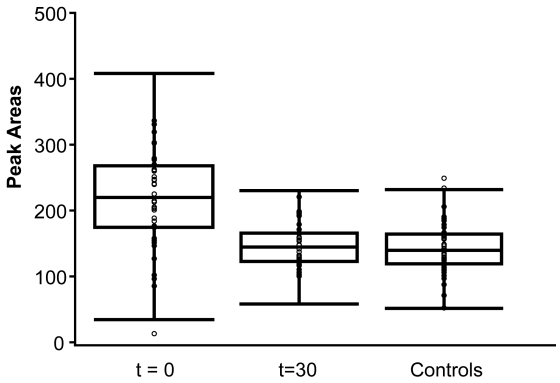

Figure 4. Box plot displaying the differences of peak area related to *m/z* 816.86 in the model classes.

DISCUSSION

Here we present the first exploratory UPLC-MS based study of UTI. As clinical entity UTI includes a number of syndromes in which the only common feature is a positive urine culture (bacteriuria). Several pathogens are most commonly associated with UTI:

Enterobacteriacaea such as *E. coli, K. pneumoniae, Proteus sp.* and *S. saprophyticus*. Thus, to minimize sample variability we have selected for the current study only the patients with culture-confirmed febrile *E. coli* UTI (Table 1).

Using a combination of unsupervised and supervised multivariate modeling we have selected forty most significant variables according to their VIP values. Approximately half of them could be grouped into three chromatographic peaks. A close examination of the spectra revealed the possible structural similarity of these three peaks with the peak at 410 s containing the most complete structure. The identification of this peak (*m/z* 816.86) as a product of proteolytic degradation of one of the most abundant serum proteins (human fibrinogen alpha chain isoform 2) may not seem encouraging at the first glance. The increased release of fibrinogen fragments in urine as a response to an infection or infection related kidney damage has little novelty. It was reported for the first time in the seventies and confirmed many times afterwards [7, 19-22]. Yet, the fact that this C-terminal peptide of fibrinogen alpha chain is carrying an O-glycan turns it into a rather unusual finding. Human fibrinogen alpha 1-chain was considered to be free of glycosylation, and only the alpha 2 isoform which is a splice variant with an elongated C-terminus has been shown to be *N*-glycosylated upon expression in monkey cells (COS cells) [23]. The proposed glycan structure corresponds to a T-antigen — core 1 type disaccharide Galβ1-3GalNAcα1 — which may carry 0, 1, or 2 sialic acid moieties. Such (partially) sialylated T-antigen structures are commonly found on various glycoproteins such as, for example, apolipoproteins [15-18]. There is only a single report by L'Hôte *et al*. which indicated a similar modification on porcine fibrinogen alpha chain on the basis of analysis with a combination of lectins [24]. However, neither the structure of the glycan nor the site of glycosylation was identified. In our case a combination of the data sets generated with the help of two mass analyzers and an additional confirmation using a synthetic peptide helped us to identify the exact sequence of the peptide backbone, although the question whether serine 641 or serine 643 carry the glycan moiety remains open.

Thus, our findings lead to a modification of the existing concept of fibrinogen structure. As an essential part of one of the most important physiological defense systems in the human organism, fibrinogen has been studied rather well. Nevertheless till now the alpha 1 chain was considered to be free of glycosylation. One might argue that the bulk of the work was done on the hepatic fibrinogen, which represents approximately 70% percent of total fibrinogen [25]. The peripheral tissues are capable to produce the 'localized' isoforms of fibrinogen as well; however, data on their structure and physiological significance remain scarce. An unusual *O*-glycosylation might be interpreted as an indication of the extra-hepatic origin of the fragments. However our data do not provide evidence for tracing of the physiological origin of this fragment and the question whether it is derived from hepatic or peripheral fibrinogen remains open.

It is well known that in healthy individuals the loss of fibrinogen through the coagulation cascade accounts only for 2% or 3% of total plasma fibrinogen [26]. Consequently, the peptides described in our study might well be filtrated from blood or proteolytically converted in the lower urinary tract or even be a result of urinary fibrinolytic activity. It has been shown that granulocytes possess a set of neutral proteases capable to use fibrinogen as a substrate [27]. In view of the involvement of granulocytes in antibacterial host defense the appearance of such proteolytic fragment in urine might then be considered as an indication of an active UTI. Regardless of the exact physiological interpretation, which at the moment

can only be speculative, a simple comparison of peak areas in each of three groups shows the difference between the group of patients with clear UTI symptoms (t=0) and the groups of the symptom free patients (controls and t=30).

In conclusion, using non-targeted exploratory UPLC–MS based approach for the investigation of UTI related changes in urine, we have identified and structurally characterized a unique C-terminal glycopeptide of the human fibrinogen alpha-chain, which to our knowledge is the first demonstration of glycosylation of alpha-chain. Of course, clinical significance of this finding should be evaluated on the larger cohort. Indeed, on the basis of the presented results we are now analyzing Leiden prospective UTI cohort of 700 samples.

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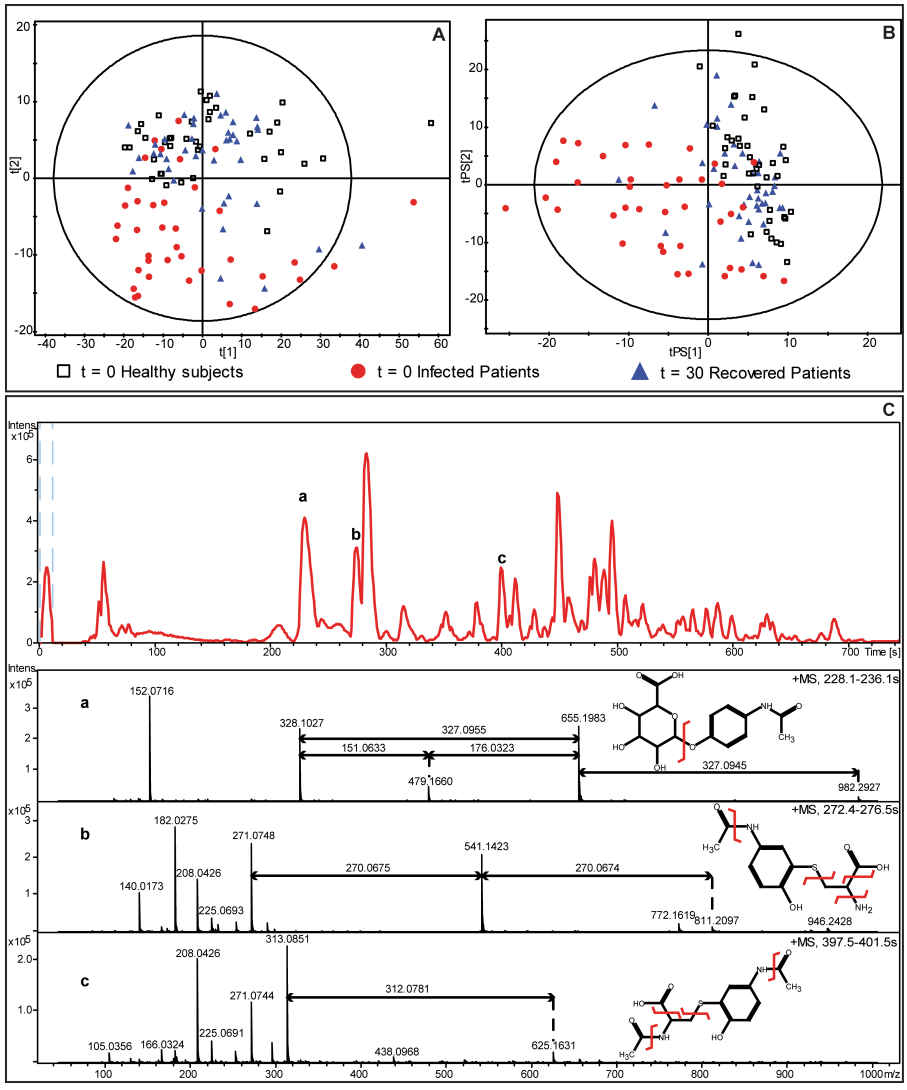
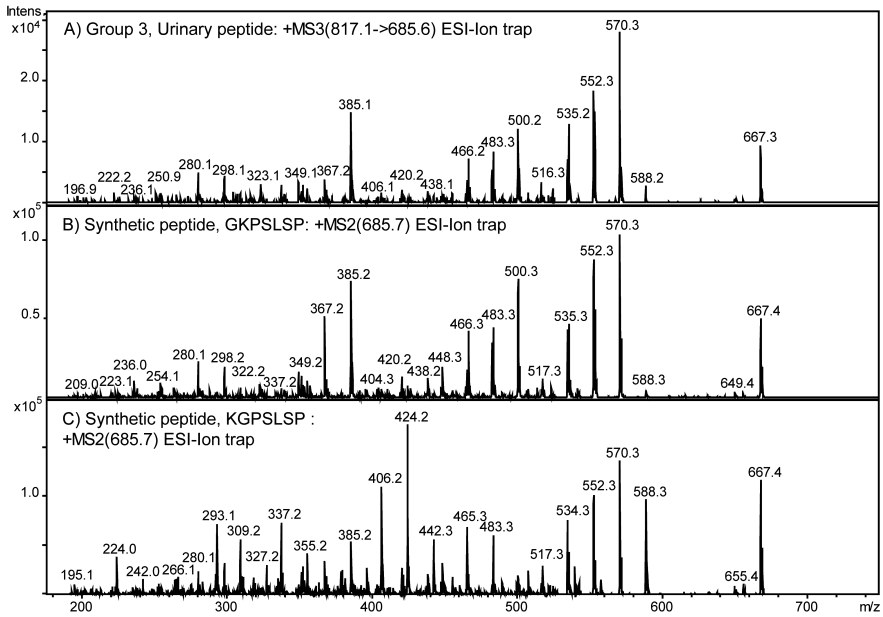
Supplementary Table 1. Assignment of acetaminophen metabolites fragments in urine of UTI patients.

Retention time (s)	Compound		Experimental *m/z*	Theoretical *m/z*	Error (mDalton)
230.1	Acetaminophen glucuronide $C_{14}H_{16}NO_8$	$[3M+H]^+$	982.2927	982.2935	-0.8
		$[2M+H]^+$	655.1983	655.1981	+0.2
		$[2M+H\text{-Glucoronide}]^+$	479.1660	479.1660	0.0
		$[M+H]^+$	328.1027	328.1027	0.0
		$[M+H\text{-Glucuronide}]^+$	152.0716	152.0706	+1.0
374.5	Acetaminophen mercapturate $C_{11}H_{14}N_2O_4S$	$[3M+H]^+$	811.2097	811.2096	+0.1
		$[2M+H]^+$	541.1423	541.1413	+1.0
		$[M+H]^+$	271.0748	271.0747	+0.1
		$[M+H\text{-}OH]^+$	254.0457	254.0720	-2.63
		$[M+H\text{-}OH\text{-}CO]^+$	225.0693	225.0692	+0.1
		$[M+H\text{-}OH\text{-}CO\text{-}NH_3]^+$	208.0426	208.0427	-0.1
		$[M+H\text{-}OH\text{-}CO\text{-}NH_3\text{-}C_2H_2]^+$	182.0275	182.0270	+0.5
		$[M+H\text{-}OH\text{-}CO\text{-}NH_3\text{-}C_2H_2\text{-}C_2H_2O]^+$	140.0173	140.0165	+0.8
399.5	N-acetylacetaminophen mercapturate $C_{13}H_{16}N_2O_5S$	$[2M+H]^+$			
		$[M+H]^+$	625.1631	625.1633	-0.2
		$[M+H\text{-}H_2O]^+$	313.0851	313.0852	-0.2
		$[M+H\text{-}C_2H_2O]^+$	295.0745	295.0747	-0.2
		$[M+H\text{-}H_2O\text{-}C_2H_2O]^+$	271.0744	271.0747	-0.3
		$[M+H\text{-}H_2O\text{-}C_2H_2O\text{-}CO]^+$	253.0639	253.0641	-0.2
		$[M+H\text{-}H_2O\text{-}C_2H_2O\text{-}CO\text{-}NH_3]^+$	225.0691	225.0692	-0.1
			208.0426	208.0427	-0.1
		$[M+H\text{-}H_2O\text{-}C_2H_2O\text{-}CO\text{-}NH_3\text{-}C_2H_2O]^+$	166.0324	166.0321	+0.3



Supplementary Figure 1. **Models of LC-Qq-ToF-MS data**: A) PCA and B) PLS-DA, two class model built using baseline patients (t=0) and control (Q2 (cum) = 0.598 and R2Y (cum) = 0.686) with t=30 group used as a validation set. Samples are colored according to the disease status. C) A representative base peak chromatogram of urine sample collected at t=0; the peaks marked as a, b and c correspond to acetaminophen metabolites. In details: a) $[C14H17NO8+H]^+$, b) $[C11H14N2O4S+H]^+$ and c) $[C13H16N2O5S+H]^+$.



Supplementary Figure 2. Peptides MS^n spectra. A: Group 3, MS3 of urinary glycopeptide on ESI-Ion trap. B and C: MS^2 on ESI-Ion trap at m/z 685.7 of two synthetic peptides. The spectrum B resembles better the urinary peptide fragmentation showed in the spectrum A.