

### Diagnostic accuracy of urinary prostate protein glycosylation profiling in prostatitis diagnosis

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#### Abstract

**Introduction:** Although prostatitis is a common male urinary tract infection, clinical diagnosis of prostatitis is difficult. The developmental mechanism of prostatitis is not yet unraveled which led to the elaboration of various biomarkers. As changes in asparagine-linked-(N)-glycosylation were observed between healthy volunteers (HV), patients with benign prostate hyperplasia and prostate cancer patients, a difference could exist in biochemical parameters and urinary N-glycosylation between HV and prostatitis patients. We therefore investigated if prostatic protein glycosylation could improve the diagnosis of prostatitis.

**Materials and methods:** Differences in serum and urine biochemical markers and in total urine N-glycosylation profile of prostatic proteins were determined between HV (N = 66) and prostatitis patients (N = 36). Additionally, diagnostic accuracy of significant biochemical markers and changes in N-glycosylation was assessed.

**Results:** Urinary white blood cell (WBC) count enabled discrimination of HV from prostatitis patients ( $P < 0.001$ ). Urinary bacteria count allowed for discriminating prostatitis patients from HV ( $P < 0.001$ ). Total amount of biantennary structures (urinary 2A/MA marker) was significantly lower in prostatitis patients compared to HV ( $P < 0.001$ ). Combining the urinary 2A/MA marker and urinary WBC count resulted in an AUC of 0.79, 95% confidence interval (CI) = (0.70–0.89) which was significantly better than urinary WBC count (AUC = 0.70, 95% CI = [0.59–0.82],  $P = 0.042$ ) as isolated test.

**Conclusions:** We have demonstrated the diagnostic value of urinary N-glycosylation profiling, which shows great potential as biomarker for prostatitis. Further research is required to unravel the developmental course of prostatic inflammation.

**Key words:** diagnostic marker; prostatitis; urinary asparagine-linked glycosylation

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#### Introduction

Prostatitis is a common male urinary tract infection with global prevalence ranging from 2.2% to 9.7% (1). The term prostatitis describes a combination of infectious diseases which have been classified into 4 syndromes by the National Institutes of Health (2,3): acute bacterial prostatitis, chronic bacterial prostatitis, chronic non-bacterial prostatitis (inflammatory and non-inflammatory chronic pelvic pain syndrome), and, asymptomatic inflammatory prostatitis.

Clinical presentation of prostatitis is heterogeneous as it is based on patients symptoms, mainly pain at various locations and different degrees of lower urinary tract symptoms (LUTS, e.g. pain at the lower urinary tract, pain in the lower abdomen, pain at the perineal region, difficult voiding, frequent need to void, pain on urination or pain that increases with urination), and on presence of leukocyturia and bacteriuria (3,4). The diagnosis may however be difficult due to several reasons:

non-specific symptoms can arise (3), bacteria are only detected in 5 to 10% of cases (3,5), and another underlying pathology, e.g. benign prostate hyperplasia (BPH), prostate cancer (PCa) and bladder carcinoma, could be present (3,6).

As the developmental mechanism of prostatitis is still unraveled, other inflammatory markers, such as interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$  (7), and non-classic biomarkers, namely serum creatinine, urinary  $\alpha$ 1-microglobulin / creatinine ratio, urinary  $\alpha$ <sub>2</sub>-macroglobulin/creatinine ratio and urinary  $\alpha$ <sub>2</sub>-macroglobulin / albumin ratio (8,9), have been examined for their potential use in prostatitis diagnosis. Currently, none of these parameters have been implemented in clinical routine laboratories.

Glycosylation studies have pointed out that infection and/or inflammation may alter the glycosylation patterns of host proteins. Two major hypotheses on the mechanisms of these glycosylation alterations are: changes in metabolic activity, thus altering the availability of the carbohydrate donor molecules needed for the formation of complex asparagine-linked (N-) glycan structures by the Golgi enzymes; and/or; increased and rapid proliferation during an immune response which overcomes the limits of the glycosylation pathway, restricting the relative amount of modification and complexity (10).

We have recently developed a technique to determine the N-glycosylation profile of urinary prostate proteins (11). By means of this technique, we have proven that differences in N-glycans enabled distinction between BPH and PCa and that N-glycosylation profiling could have an added value to PCa diagnosis, especially in the diagnostic gray zone of serum prostate specific antigen (sPSA) concentrations between 4 and 10  $\mu$ g/L (12).

Because changes in N-glycosylation were observed between healthy volunteers (HV), patients with BPH and PCa patients (12), we hypothesized that a difference could exist in biochemical parameters and urinary N-glycosylation between HV and prostatitis patients. As the use of N-glycomics has never been tested in prostatic inflammation, our objective in this diagnostic accuracy study is to de-

termine whether changes in N-glycosylation patterns can differentiate between healthy volunteers (HV) and prostatitis patients and if these differences can improve prostatitis diagnosis.

## Materials and methods

### Subjects

From July 2012 to May 2015, a cohort of 36 prostatitis patients presenting at the Department of Urology at the Ghent University Hospital were continuously recruited and prospectively analyzed in our diagnostic accuracy study. Inclusion/exclusion criteria consisted of: patients with at least one of the following symptoms of prostatitis: pain at the lower urinary tract, pain in the lower abdomen, pain at the perineal region, difficult voiding, frequent need to void, pain on urination or pain that increases with urination; but did not suffer from PCa. Acute or chronic bacterial prostatitis was confirmed on urinalysis: urinary white blood cell (WBC) count > 25 cells/ $\mu$ L and/or urinary bacteria count > 11.4 bacteria/ $\mu$ L (reference test). sPSA concentration was not used as inclusion criteria for prostatitis patients. Patients were included within 6 weeks of onset of symptoms at which time a digital rectal examination (DRE) was performed (no DRE was performed at time of onset in order to prevent urosepsis (3)). Prostatitis patients consisted of 16 acute bacterial prostatitis patients (of which 8 received antibiotic treatment); 9 chronic bacterial prostatitis patients (of which 4 received antibiotic treatment); and 11 chronic non-bacterial prostatitis patients (non-inflammatory chronic pelvic pain syndrome).

Healthy volunteers (N = 66) were enrolled as a reference group for comparison with the patient cohort and consisted of personnel from the Ghent University Hospital and family members of patients presenting at the Department of Urology at the Ghent University Hospital. Criteria used to enroll HV were: aged between 18 and 50 years, not suffering from any prostate pathology (BPH / PCa / LUTS or history of LUTS) and no first- or second-line family member (father or brother) with PCa diagnosed before the age of 65.

Informed consent was given by all participants and the study was approved by the local Ethics Committee (Belgian registration number: B670201214356).

## Methods

One Venosafe® VF-106SAS plastic serum silicone coated gel tube (Terumo Medical Corporation; Somerset, NJ, USA) was collected before DRE. The blood sample was allowed to coagulate during 30 min and centrifuged at 3000 RPM for 10 minutes. The samples were analyzed for sPSA within 1 hour following sampling. Urine samples were collected directly after DRE in 60 mL urine containers (International Medical Products n.v.; Brussels, Belgium). A 5 mL portion was transferred to 5 mL ultraclear polypropylene storage tubes (Deltalab, S.L.; Barcelona, Spain) and centrifuged at 3000 RPM for 10 minutes. The centrifuged urine was aliquoted into 2 portions: 3 mL was transferred to 5 mL ultraclear polypropylene storage tubes for urinary biochemical analysis and 500 µL was transferred to 1.5 mL Eppendorf® Safe-Lock microcentrifuge tubes (Novolab NV; Geraardsbergen, Belgium) for N-glycosylation analysis. Aliquot for urine N-glycosylation analysis was preserved at -20 °C until assayed (within 1 week after preservation of the urine samples).

We used Cobas® 8000 modular P analyzer series (Roche Diagnostics GmbH, Mannheim, Germany) to measure total urinary protein concentrations and gamma-glutamyltranspeptidase (GGT) activity in urine. Total urinary protein was assayed by means of a robust pyrogallol-based dye binding method (13) using the Total Protein Reagent Pyrogallol Red Method with SDS reagent standardized according to Total Protein Standard Concentration 1000 mg/L (within run coefficient of variation (CV) = 2.1%, between run CV = 2.1%). Upper reference values limit is taken at 0.2 g/L. All reagents and controls are commercially available (Instruchemie BV, Delfzijl, The Netherlands).

Urinary GGT activity was assessed to determine the presence of acute kidney injury (14,15). This assay was performed to determine the possible in-

fluence of highly glycosylated liver proteins on the urine N-glycosylation profile. Urinary GGT activity was measured through kinetic photometric determination (16) using the commercially available GGT-2 kit (Roche Diagnostics GmbH, Mannheim, Germany) with within run CV = 1.1% and between run CV = 1.3%. Normal male values range between 12 and 64 U/L.

Immunonephelometry on a BN Nephelometer II analyzer (Siemens Healthcare, Marburg, Germany) was used to determine the albumin concentration in urine using commercially available N Antiserum to human albumin with N Protein standard SL as control (within run CV = 2.2%, between run CV = 8.9%). Reference values range from 0 to 20 mg/L. All reagents and controls are commercially available (Siemens Healthcare, Marburg, Germany).

sPSA, urinary total PSA (tPSA) and urinary free PSA (fPSA) were assayed by means of electrochemiluminescence immunoassay on a Modular E170 analyzer series (Roche Diagnostics GmbH, Mannheim, Germany) and standardized against the PreciControl Tumor marker 1 and 2. sPSA and urinary tPSA were assessed by means of the total PSA kit (within run CV for PreciControl Tumor marker 1 and 2 = 1.9% and = 0.8%, respectively; between run CV = 4.2% and = 4.6%, respectively). Urinary fPSA was determined using the free PSA kit (within run CV for PreciControl Tumor marker 1 and 2 = 1.9% and = 0.8%, respectively; between run CV = 2.0% and = 1.9%, respectively). All reagents and controls are commercially available (Roche Diagnostics GmbH, Mannheim, Germany).

Urinary red blood cell (RBC) count (/µL), urinary white blood cell (WBC) count (/µL) and urinary bacteria count (/µL) were determined using a Sysmex UF-1000i® urinary flow cytometer (Sysmex Corporation, Kobe, Japan). The system uses a semiconductor laser instrument to perform automated microscopic analysis and to automatically detect, identify and count RBCs, WBCs and bacteria (17,18) with UF-Control as quality control (within run CV = 2.2%, = 6.6% and = 9.2%, respectively; between run CV = 3.0%, = 2.0%, and = 3.0%, respectively). All reagents and controls used are commercially available (Sysmex Corporation, Kobe, Japan).

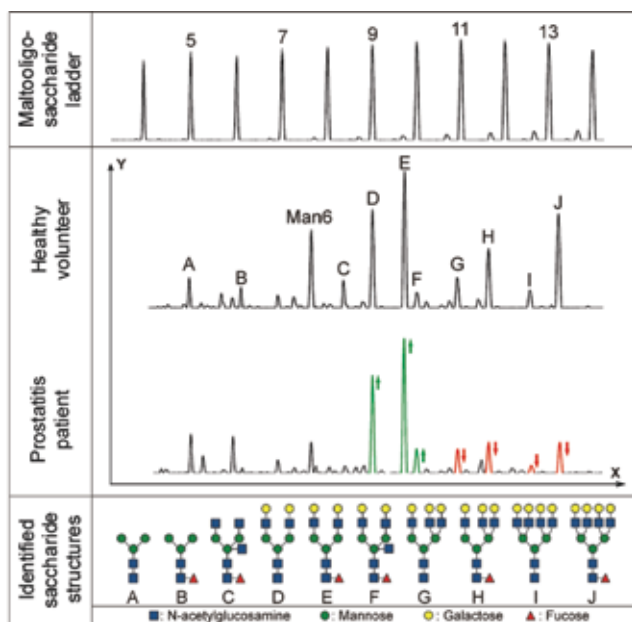
Upper reference limits implemented are 25 RBC/ $\mu$ L, 25 WBC/ $\mu$ L and 11.4 bacteria/ $\mu$ L.

As previously described, urinary prostate protein N-glycans were determined using a multicapillary electrophoresis-based ABI3130 sequencer. Urinary prostate protein N-glycans were released using an on-membrane deglycosylation method and labeled with 8-aminopyrene-1,3,6-trisulphonic acid (Molecular Probes, Eugene, OR, USA). Subsequently, the glycans were desialylated overnight at 37 °C by the addition of 2  $\mu$ L of 10 mM ammonium acetate pH 5.0 containing 40 mU of *Arthrobacter urea-*

*faciens*  $\alpha$ -2,3/6/8-sialidase (provided by the laboratory of Prof. Nico Callewaert, Unit for Medical Biotechnology, Inflammation Research Center, VIB—Ghent University, Ghent, Belgium). The desialylated N-glycan samples and a reference maltooligosaccharide ladder (dextran from *Leuconostoc mesenteroides*, Sigma-Aldrich, St. Louis, MO, USA) were analyzed with a multicapillary electrophoresis-based ABI3130 sequencer. The peaks were analyzed with GeneMapper version 3.7 software (Applied Biosystems, Foster City, CA, USA) and peak height intensities were normalized to the total intensity of the measured peaks (11). The method showed analytical variability of less than 5%. The difference in total amount of biantennary structures / multiantennary structures was named the ‘urinary 2A/MA marker’, the difference in total amount of triantennary structures / multiantennary structures was named the ‘urinary 3A/MA marker’, and, the difference in total amount of tetraantennary structures / multiantennary structures was named the ‘urinary 4A/MA marker’. The different markers used for distinction of prostatitis patients from HV are given in Figure 1.

**Statistical analysis**

All participants were eligible for statistical analysis. Normal distribution of the subject groups was verified by D’Agostino-Pearson test. Overall differences between HV and prostatitis patients were analyzed by means of unpaired Student’s t-test (for normally distributed groups), Mann-Whitney U-test (for non-normally distributed groups), or Fischer exact test for categorical data. Receiver operating characteristic (ROC) curve analysis was used to determine criterion value of the urinary 2A/MA marker (index test) and to calculate area under the curve (AUC), sensitivity and specificity of the marker with their respective 95% confidence interval (CI). Further analysis compared the ROC curve of the urinary 2A/MA marker with that of urinary WBC count (criterion  $\geq$  25 cells/ $\mu$ L) and urinary bacteria count (criterion  $\geq$  11.4 bacteria/ $\mu$ L). Criterion values for urinary WBC count and urinary bacteria count were based on reference values currently used in our laboratory. *Post-hoc* calculation of criterion values was used to compare sensitivity



**FIGURE 1.** Desialylated urinary N-glycan profiles of prostatic proteins from HV and prostatitis patients. X-axis depicts elution time; Y-axis represents relative fluorescence units. On top, a maltooligosaccharide ladder (dextran) is shown to determine the amount of structural units in each glycan structure. Identified glycosylation structures shown in the figure. Glycan symbols are those suggested by the Consortium for Functional Glycomics (<http://glycomics.scripps.edu/CFGnomenclature.pdf>). Man6 stands for a mannose-rich structure that consists of 6 mannose and 2 N-acetylglucosamine units. The concept of the figure was adapted from previously reported N-glycan profiles determined by means of the same technique (11). An increase was observed in the urinary 2A/MA marker (ratio [peaks D+E+F] / [peaks D till J]) of prostatitis patients compared to HV (green arrows) while the urinary 3A/MA marker (ratio [peaks G+H] / [peaks D till J]), and, the urinary 4A/MA marker (ratio [peaks I+J] / [peaks D till J]) were decreased in prostatitis patients compared to HV (red arrows).

and specificity of the *post-hoc* calculated criterion with those of the reference criterion currently used. Multivariate logistic regression was performed to assess the additive diagnostic value of the urinary 2A/MA marker next to urinary WBC and bacteria count, and to calculate odds ratio (OR). Next, all prostatitis patients were subdivided into acute *versus* chronic prostatitis patients and into bacterial *versus* non-bacterial prostatitis patients in order to assess differences in biochemical markers and N-glycans between these subgroups. P-values < 0.050 were considered statistically significant. Statistical analyses were performed with MedCalc Statistical Software version 13.3.1.0 (MedCalc Software, Ostend, Belgium) and GraphPad Prism version 4.7 (GraphPad Software Inc., La Jolla, CA, USA).

## Results

### Baseline characteristics

The subjects' baseline characteristics are summarized in Table 1. Overall median sPSA concentration was significantly higher in prostatitis patients compared to HV (P < 0.001; Figure 2A), as was the urinary albumin concentration (P < 0.001; Figure 2B). Next, urinary WBC count and urinary bacteria count was higher in prostatitis patients compared to HV (both P < 0.001; Figure 2C-D). Furthermore, urinary WBC count was significantly different when comparing acute *versus* chronic prostatitis (P = 0.029; Figure 2E) and in the comparison between bacterial *versus* non-bacterial prostatitis (P < 0.001; Figure 2F) whereas urinary bacteria count differed

**TABLE 1.** Baseline characteristics of the population enrolled in the study.

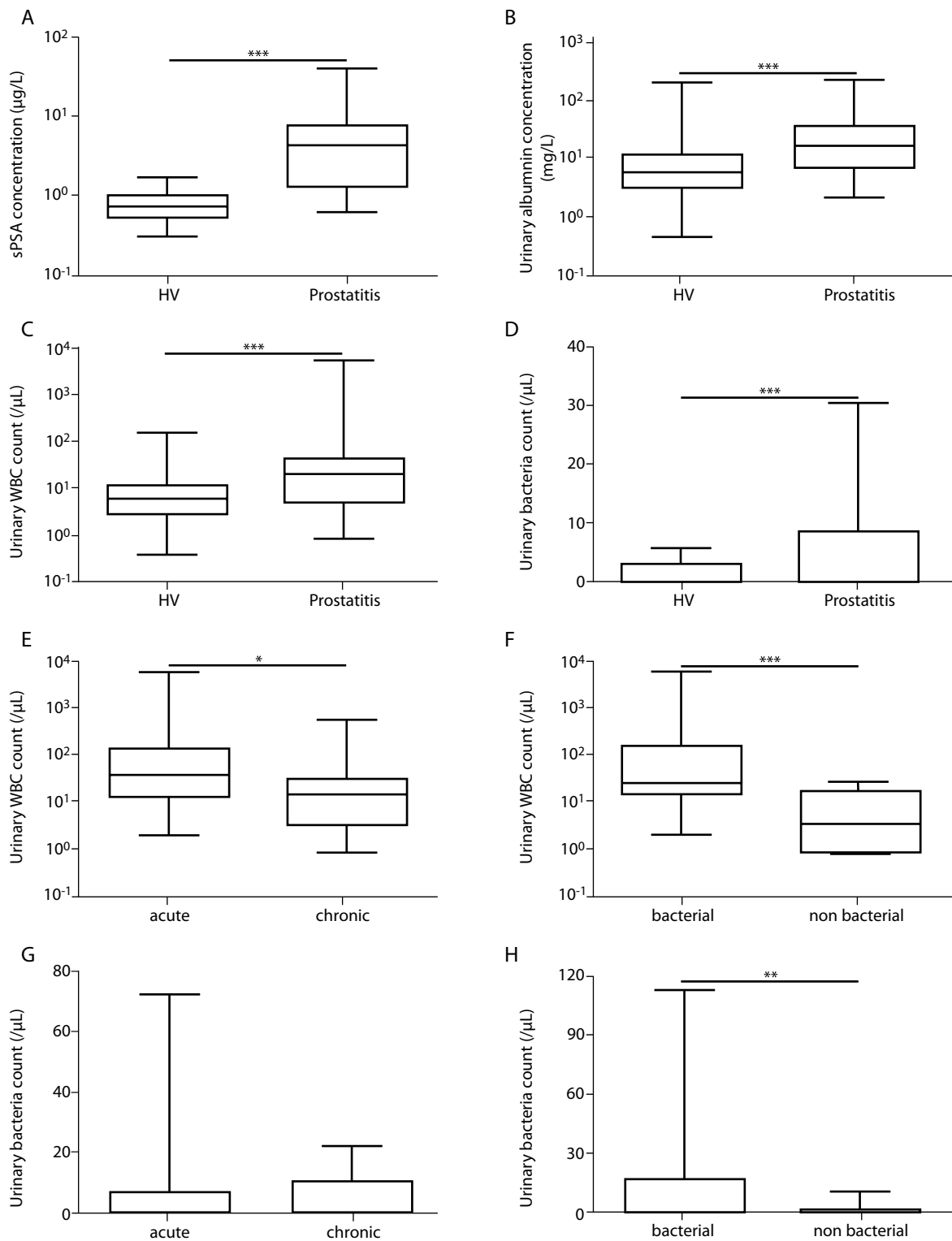
Characteristics	Prostatitis patients	HV	P-value
Participants (%)	36 (35)	66 (65)	N/A
Age (years)	55 (45–63)	29 (24–38)	< 0.001
sPSA (µg/L)	4.1 (1.2–7.6)	0.7 (0.5–1.0)	< 0.001
Total urinary protein (g/L)	0.09 (0.03–0.17)	0.06 (0.03–0.10)	0.056
Urinary albumin (mg/L)	15.9 (7.2–38.3)	5.8 (3.2–12.4)	< 0.001
Ratio urinary albumin / total protein (%)	25 (12–37)	13 (8–26)	0.014
Urinary GGT activity (U/L)	28 (11–46)	16 (5–45)	0.216
Urinary total PSA (tPSA) (µg/L)	548 (180–2145)	340 (74–1417)	0.186
Ratio urinary tPSA / total protein (%)	0.9 (0.2–2.9)	0.6 (0.1–2.9)	0.839
Urinary free PSA (fPSA) (µg/L)	512 (153–1893)	278 (62–1114)	0.266
Ratio urinary fPSA / tPSA (%)	83 (74–89)	87 (79–92)	0.115
pH	6.5 (5.3–7.0)	6.5 (6.0–7.0)	0.980
Urinary RBC count (/µL)	7.9 (4.1–13.5)	5.6 (2.9–9.4)	0.124
Urinary WBC count (/µL)	21.8 (5.2–47.0)	5.9 (2.7–12.5)	< 0.001
Participants with urinary WBC count > 25.0 cells/µL (%)*†	17 (47)	10 (15)	< 0.001
Urinary bacteria count (/µL)	8.7 (2.8–30.5)	3.3 (1.8–5.6)	< 0.001
Participants with urinary bacteria count > 11.4 cells/µL*†	17 (47)	3 (5)	< 0.001

Data are median (interquartile range) except for participants: N (%).

P-values are given for all parameters (Mann Whitney U-test).

\*Categorical data were assessed using Fisher Exact test; †percentages are given for group.

sPSA - serum prostate specific antigen; GGT - gamma-glutamyl transpeptidase; tPSA - total PSA; fPSA - free PSA; RBC - red blood cell; WBC - white blood cell; N/A - not applicable.



**FIGURE 2.** Differences in biochemical parameters between HV (N = 66) and prostatitis patients (N = 36). X-axis indicates the patient cohorts; Y-axis shows different biochemical parameters. Comparisons are illustrated for A: sPSA concentration (µg/L), B: urinary albumin concentration (mg/L), C: overall urinary WBC count(/µL), D: overall urinary bacteria count (/µL), E: urinary WBC count (/µL) in acute versus chronic prostatitis, F: urinary WBC count (/µL) in bacterial versus non bacterial prostatitis, G: urinary bacteria count (/µL) in acute versus chronic prostatitis, H: urinary bacteria count (/µL) in bacterial versus non bacterial prostatitis. Significance is depicted in the plots: P < 0.05 (\*), P < 0.01 (\*\*), or P < 0.001 (\*\*\*).

only between bacterial and non-bacterial prostatitis ( $P = 0.002$ ; Figure 2H) and was not significant between acute versus chronic prostatitis ( $P = 0.610$ ; Figure 2G). None of the other biochemical markers was significantly different between prostatitis patients and HV ( $P > 0.050$ ).

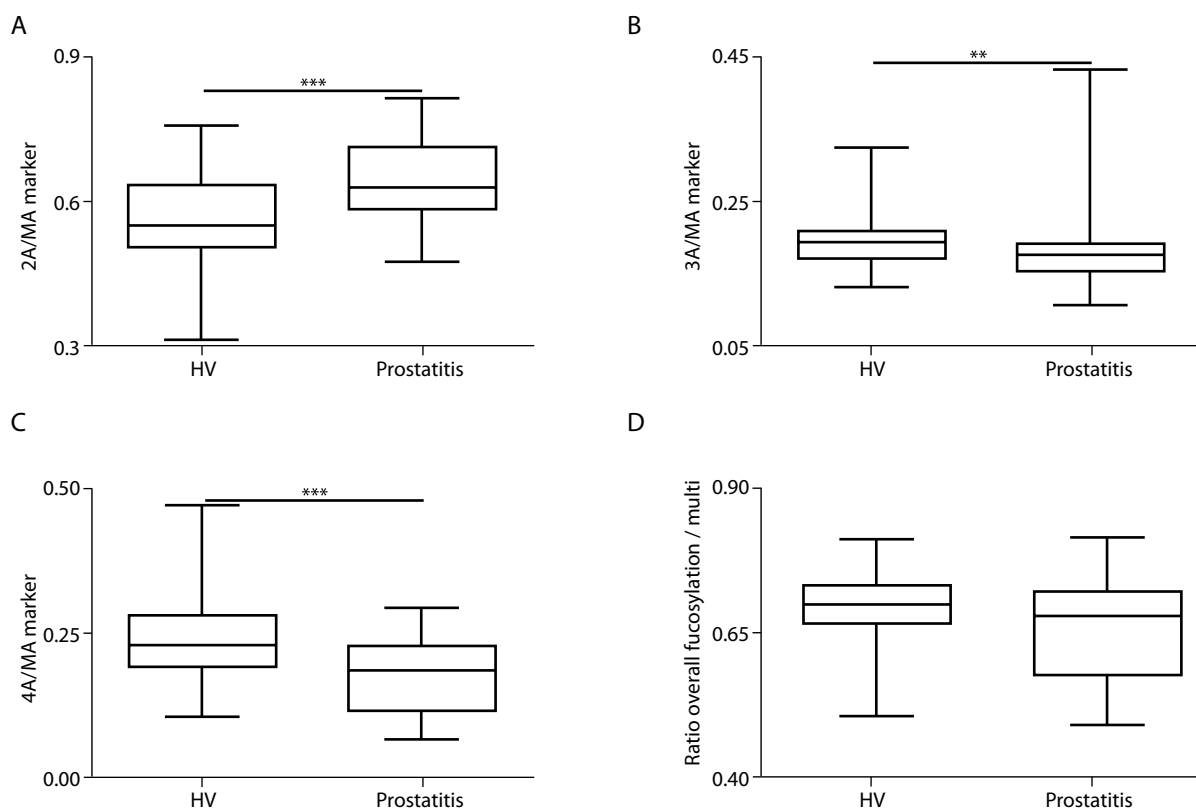
### Changes in glycosylation patterns

All obtained N-glycan structures were examined. Normalization and analysis of the relative peak heights showed a difference between the N-glycan profiles of HV and prostatitis patients (Figure 1). A significant increase of the urinary 2A/MA marker was found in the N-glycan profile of prostatitis patients compared to HV ( $P < 0.001$ ; Figure 3A), which was the direct result of a decrease of the urinary 3A/MA and 4A/MA marker in prostatitis patients ( $P = 0.008$  and  $P < 0.001$ , respectively;

Figure 3B-C). No difference was observed in the percentage of overall core- $\alpha$ -1,6-fucosylation ( $P = 0.051$ ; Figure 3D). Also, no differences in N-glycosylation were found between all types of prostatitis or between bacterial and non-bacterial prostatitis ( $P > 0.050$ ).

### Diagnostic accuracy of urinary WBC count, urinary bacteria count and the urinary 2A/MA marker in differentiating HV from prostatitis patients

The urinary 2A/MA marker showed fair diagnostic accuracy (AUC = 0.73, 95% CI [0.63-0.81]), comparable to urinary WBC count (AUC = 0.70, 95% CI [0.59-0.82]) and urinary bacteria count (AUC = 0.70, 95% CI [0.58-0.82]; Figure 4). These tests did not differ significantly from each other ( $P = 0.508$  and  $P = 0.586$ , respectively). Table 2 summarizes the



**FIGURE 3.** Differences in biochemical parameters between HV (N = 66) and prostatitis patients (N = 36). X-axis indicates the patient cohorts; Y-axis shows different glycosylation parameters. Comparisons are illustrated for A: urinary 2A/MA marker (ratio [peaks D+E+F] / [peaks D till J]; Figure 1), B: urinary 3A/MA marker (ratio [peaks G+H] / [peaks D till J]; Figure 1), and, C: urinary 4A/MA marker (ratio [peaks I+J] / [peaks D till J]; Figure 1), D: Ratio overall fucosylation / multi (ratio [peaks E+F+H+J] / [peaks D till J]; Figure 1). Significance is depicted in the plots:  $P < 0.01$  (\*\*) or  $P < 0.001$  (\*\*\*).

**TABLE 2.** Comparison of the discriminative power of several parameters for HV versus prostatitis patients and multivariate logistic regression model.

Parameter	Criterion	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	Logistic regression OR (95% CI)	P
Urinary WBC count	> 14.6 / $\mu$ L †	0.64 (0.46–0.79)	0.80 (0.69–0.89)	0.70 (0.59–0.82)	1.02 (1.01–1.03)	<b>0.017</b>
Urinary WBC count	> 25.0 / $\mu$ L *	0.44 (0.28–0.62)	0.85 (0.74–0.92)			
Urinary bacteria count	> 6.5 / $\mu$ L †	0.64 (0.46–0.79)	0.85 (0.74–0.92)	0.70 (0.58–0.82)	1.00 (0.99–1.01)	0.078
Urinary bacteria count	> 11.4 / $\mu$ L *	0.47 (0.30–0.65)	0.95 (0.87–0.99)			
Urinary 2A/MA marker	$\leq$ 0.588 †	0.75 (0.58–0.88)	0.60 (0.47–0.72)	0.73 (0.63–0.81)	2.0 x 10 <sup>4</sup> (62.9–6.5 x 10 <sup>6</sup> )	<b>&lt;0.001</b>

Combining urinary WBC count and urinary 2A/MA marker resulted in an AUC of 0.79, 95% CI (0.70–0.87) with sensitivity = 0.69, 95% CI (0.52–0.84) and specificity = 0.78, 95% CI (0.67–0.88).

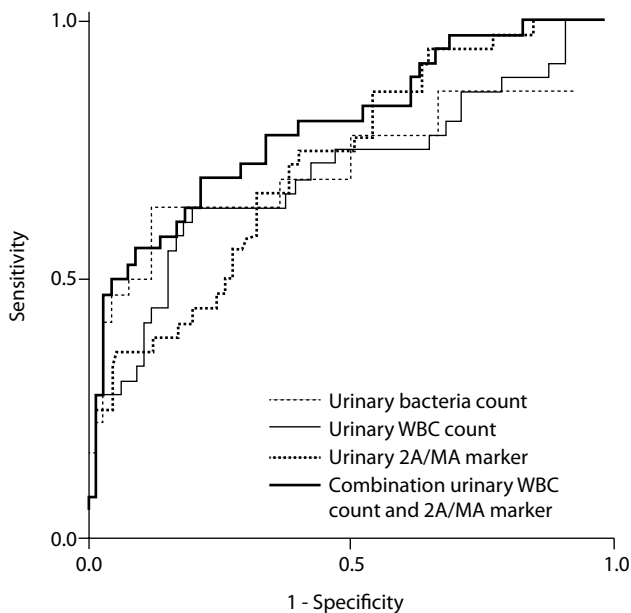
\*Criterion values based on references values applied in our laboratory.; †criterion values calculated *post-hoc*.

WBC - white blood cell; N/A - not applicable.

discriminative power with matching sensitivity and specificity for these parameters in discriminating HV from prostatitis patients. Use of reference criterion values for urinary WBC count and urinary bacteria count resulted in a higher specificity while the use of the post-hoc calculated criterion value gave a higher sensitivity. Next, using multivariate logistic regression, urinary WBC and bacteria count were combined with the urinary 2A/MA marker (Table 2) which favored the combination of urinary WBC count and the urinary 2A/MA marker (urinary bacteria count was not included in the model). This combination of tests achieved fair diagnostic accuracy (AUC = 0.79, 95% CI [0.70-0.89]) which was significantly better compared to urinary WBC as isolated test (P = 0.042) but not to urinary bacteria count (P = 0.156; Figure 4).

### Discussion

In our research, we assessed if differences existed between the urinary N-glycosylation profile of HV compared to prostatitis patients and if these differences could assist in the diagnosis of prostatitis. Here we reported that changes in several serum and urinary biochemical markers occurred when



**FIGURE 4.** ROC curves analysis for the detection of prostatitis compared to HV. Analysis demonstrated AUC of 0.70, 95% CI [0.58-0.82]; 0.70, 95% CI [0.59-0.82]; and 0.73, 95% CI [0.63-0.81]; for urinary bacteria count (dashed line), urinary WBC count (thin full line) and the urinary 2A/MA marker (dotted line), respectively. No significant differences were observed between the isolated tests (P > 0.050). Combining urinary WBC count and the urinary 2A/MA marker (bold full line) resulted in an AUC of 0.79, 95% CI [0.70-0.89]; which was significantly better compared to urinary WBC count (P = 0.042) as isolated test but not to urinary bacteria count (P = 0.156). Diagonal segments are produced by ties.



comparing HV to prostatitis patients. The biochemical parameter usable for the distinction between HV and prostatitis patients were sPSA concentration, urinary WBC count and urinary bacteria count; which were increased in prostatic inflammation. Next, we observed a lower amount of urinary tri- and tetraantennary N-glycans (3A/MA and 4A/MA marker, respectively) which resulted in an increase of biantennary structures (2A/MA marker). Combining urinary WBC count and the 2A/MA marker proved favorable for prostatitis diagnosis compared to urinary WBC count as isolated test.

The most important parameter for the distinction between HV and prostatitis patients was urinary WBC count. Notable was that the reference criterion ( $> 25$  cells/ $\mu\text{L}$ ) was higher than the *post-hoc* calculated criterion ( $> 14.6$  cells/ $\mu\text{L}$ ). The same was noticed for the criterion value of urinary bacteria count (routinely used criterion  $> 11.4$  bacteria/ $\mu\text{L}$ ; calculated criterion  $> 6.5$  bacteria/ $\mu\text{L}$ ). This is probable due to the bias that prostatitis patients were enrolled in the study until 6 weeks after onset of their symptoms and that some of them had already received antibiotic treatment. The urinary bacteria count proved also significantly to differentiate prostatitis patients and HV. Again, this result is hard to interpret due to the design of our study and the fact that some of the prostatitis patients already received antibiotic treatment.

Next, the use of sPSA as a marker for prostatitis is contradictory as an elevated sPSA concentration can be the result of underlying subclinical disease such as BPH or PCa (6). Furthermore, Pansadoro *et al.* (19) found that in 72 prostatitis patients under 50 year of age only 71% of patients with acute bacterial prostatitis, 15% of patients with chronic bacterial prostatitis and 6% of patients with chronic non-bacterial prostatitis had an elevated sPSA concentration ( $> 4$   $\mu\text{g/L}$ ) because of increased vascular permeability and disrupted prostate gland epithelium. Nadler *et al.* (20) even stated that sPSA, although slightly increased in patients with chronic non-bacterial prostatitis (mean sPSA concentration = 1.97  $\mu\text{g/L}$ , standard deviation = 2.87  $\mu\text{g/L}$ ), is not usable as biomarker for prostatitis due to the low sensitivity and specificity. Another study that

evaluated the diagnostic performance of sPSA in prostatitis reported a decrease of the ratio free sPSA / total sPSA in acute bacterial prostatitis. We were unable to observe this difference for urinary PSA concentrations indicating that no error occurs in PSA synthesis in the prostate gland during inflammation of the prostate gland (21).

In contrast, we noticed that urinary albumin concentration was higher in prostatitis patients compared to HV. This could be the consequence of leakage through the prostate gland. However, this parameter is of no significance for the differentiation of prostatitis patients as it was within normal reference parameters (0-20 mg/L) (22).

Furthermore, we investigated the clinical utility of urinary N-glycosylation analysis in prostatitis diagnosis. As a post-translational modification, N-glycosylation is highly sensitive to its environment and can be affected by disease status (23). The urinary 2A/MA marker enabled distinction between prostatitis patients and HV, with similar diagnostic accuracy to urinary WBC and bacteria count. Combining this urinary 2A/MA marker with urinary WBC count even improved the diagnostic performance for prostatitis detection compared to urinary WBC as isolated test.

The proposed combination of markers could show high potential for use in prostatitis diagnosis with some great advantages. Firstly, a model for prostatitis detection combining urinary WBC count and the urinary 2A/MA marker improved diagnostic accuracy by 9% which would greatly decrease the number of false-negatives derived by urinary WBC count and facilitate the difficult diagnosis of prostatitis patients.

Secondly, our test uses a non-invasive method to obtain the urine samples. As the combination of urinary WBC count and the urinary 2A/MA marker achieved a fair diagnostic accuracy, this could possible decrease the need for invasive serum sampling to determine other inflammatory markers, e.g. C-reactive protein.

Thirdly, as this technique is easy to use without prior need for extensive training, easily repeatable, and affordable, implementation of this test in

centers with high number of urological specimens could be advisable.

An important remark is that HV are not age-matched with the patient groups. As reported in previous publications (11,12), this was done deliberately to exclude presence of subclinical BPH or PCa in the HV group. However, this study design did not render the results less powerful because no differences were observed in N-glycosylation profiling when HV were subdivided into 3 age-increasing categories (12).

Surprisingly, we were unable to notice a difference between the N-glycosylation profile of acute bacterial prostatitis, chronic bacterial prostatitis and chronic non-bacterial prostatitis, nor when prostatitis patients were subdivided into acute *versus* chronic prostatitis patients or into bacterial *versus* non-bacterial prostatitis patients. This is probable due to the design of our study and as a direct effect thereof, the low number of patients in the prostatitis cohort.

Finally, an interesting finding is that no difference in overall core- $\alpha$ -1,6-fucosylation was found between HV and prostatitis patients, while previously this glycosylation change proved significant in the differentiation between HV, patients with BPH and PCa patients (12). However both prostatitis and BPH, which is characterized as an immune-mediated inflammatory disease with characteristics of chronic inflammation and significantly more infiltrated T-lymphocytes, macrophages and B-lymphocytes (24-27), showed a decrease in triantennary structures. This finding would indicate that the change in total amount of tri- and tetraantennary structures / multiantennary structures could

be associated with an inflammatory responses while the change in overall core- $\alpha$ -1,6-fucosylation is more likely an indicator of cancer progression. Both changes could possibly be linked as it has been stated that an inflammatory environment can be mutagenic and promote cancer progression, although there is still no evidence of a causal relation between inflammation and PCa (24,28-30).

In summary, we have demonstrated the diagnostic value of N-glycosylation profiling from urinary prostate proteins, as a possible biomarker for differentiating HV from prostatitis patients. Further research is however warranted. An increased number of prostatitis patients could allow validating differences in N-glycan profiles between acute bacterial prostatitis, chronic bacterial prostatitis, chronic pelvic pain syndrome and asymptomatic prostatic inflammation which would have a great benefit for prostatitis diagnosis. Furthermore, comparison of N-glycosylation at time of disease onset and post treatment could help unravel the developmental course of prostatic inflammation indicate which patients are sensitive to chronification of prostatitis. Lastly, comparison between prostatitis, BPH and PCa is needed as it could further indicate the possible role of inflammation in the development of PCa.

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### Potential conflict of interest

None declared.

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