Development and validation of urine-based peptide biomarker panels for detecting bladder cancer in a multi-center study

Maria Frantzi[1,2*], Kim E. van Kessel[3], Ellen C. Zwarthoff[3], Mirari Marquez[4], Marta Rava[4], Núria Malats[4], Axel S. Merseburger[5], Ioannis Katafigiotis[6], Konstantinos Stravodimos[6], William Mullen[7], Jerome Zoidakis[2], Manousos Makridakis[2], Martin Pejchinovski[1], Elena Critselis[2], Ralph Lichtinghagen[8], Korbinian Brand[8], Mohammed Dakna[1], Maria G. Roubelakis[9], Dan Theodorescu[10,11], Antonia Vlahou[2], Harald Mischak[1,7] and Nicholas P. Anagnou[9,12]

[1]Mosaiques diagnostics GmbH, Hannover, Germany; [2]Biotechnology Division, Biomedical Research Foundation Academy of Athens, Athens, Greece; [3]Department of Pathology, Erasmus MC Cancer Institute, Erasmus Medical Center, Rotterdam, The Netherlands; [4]Genetic and Molecular Epidemiology Group, Spanish National Cancer Research Center (CNIO), Madrid, Spain; [5]Department of Urology, University of Lübeck, Lübeck, Germany; [6]Department of Urology, Laikon Hospital, Medical School of Athens, Athens, Greece; [7]Institute of Cardiovascular and Medical Sciences, University of Glasgow, Glasgow, United Kingdom; [8]Institute of Clinical Chemistry, Hannover Medical School, Hannover, Germany; [9]Laboratory of Biology, Department of Basic Medical Sciences, University of Athens School of Medicine, Athens, Greece; [10]University of Colorado, Department of Surgery and Pharmacology, Aurora, Colorado, USA; [11]University of Colorado Comprehensive Cancer Center, Aurora, Colorado, USA; [12]Laboratory of Cell and Gene Therapy, Biomedical Research Foundation of the Academy of Athens, Athens, Greece;

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Corresponding Author:
Maria Frantzi
Mosaiques diagnostics GmbH
Rotenburger Str. 20
D-30659 Hannover, Germany
E-mail: frantzi@mosaiques-diagnostics.com
Phone: +49 (0)511 55 47 44 29
Fax: +49 (0)511 55 47 44 31

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Translational relevance

Urothelial bladder cancer (UBC) remains the second most frequent cause of mortality among genitourinary cancers. Due to high relapse rates, frequent patient monitoring is required, leading to augmented associated healthcare costs and diminished patient compliance. A prevailing need for non-invasive biomarkers which will facilitate the timely diagnosis of primary and recurrent UBC remains unmet to date. This study is focused on the investigation of biomarker panels based on urinary peptides, as screening tools for the diagnosis of urothelial bladder cancer. Two biomarker panels were developed for primary and recurrent UBC, by employing 1357 urine samples. Further validation of the peptide panels in patients representing primary and surveillance settings resulted in AUCs (area under the Receiver Operating Characteristic curve) of 0.87 and 0.75, respectively. In reference to the peptide biomarker model for detection of recurrence, combination with cytology increased the AUC to 0.87.

Abstract

Purpose: Urothelial bladder cancer (UBC) presents high recurrence rates, mandating continuous monitoring via invasive cystoscopy. The development of non-invasive tests for disease diagnosis and surveillance remains an unmet clinical need. In this study, validation of two urine-based biomarker panels for detecting primary and recurrent UBC was conducted.

Experimental Design: Two studies (total n=1357) were performed for detecting primary (n=721) and relapsed UBC (n=636). Cystoscopy was applied for detecting UBC, while patients negative for recurrence had follow-up for at least one year to exclude presence of an undetected tumor at the time of sampling. Capillary electrophoresis coupled to mass spectrometry (CE-MS) was employed for the identification of urinary peptide biomarkers. The candidate urine-based peptide biomarker panels were derived from nested cross-sectional studies in primary (n=451) and recurrent (n=425) UBC.

Results: Two biomarker panels were developed based on 116 and 106 peptide biomarkers using support vector machine algorithms. Validation of the urine-based biomarker panels in independent validation sets, resulted in AUC values of 0.87 and 0.75 for detecting primary (n=270) and recurrent UBC (n=211), respectively. At the optimal threshold, the classifier for detecting primary UBC exhibited 91% sensitivity and 68% specificity, while the classifier for recurrence demonstrated 87% sensitivity and 51% specificity. Particularly for patients undergoing surveillance, improved performance was achieved when combining the urine-based panel with cytology (AUC of 0.87).

Conclusions: The developed urine-based peptide biomarker panel for detecting primary UBC exhibits good performance. Combination of the urine-based panel and cytology resulted in improved performance for detecting disease recurrence.

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105 1. Introduction

106 Urinary bladder cancer (UBC) remains the second most frequent cause of mortality among
107 genitourinary cancers, including approximately 430,000 incident cases and 165,000
108 attributable deaths annually worldwide (1). The striking majority of malignant bladder
109 tumours are of epithelial origin. Depending on the degree of tumour infiltration in the vesical
110 wall, 80% of neoplasms are classified as non-muscle invasive (NMIBC), while the remainder
111 are muscle invasive (MIBC) tumours (2). Following initial treatment, up to 70% of NMIBC
112 patients experience disease recurrence (3, 4). Current approaches for detecting both primary
113 and recurrent disease rely on invasive cystoscopy. However, due to high UBC relapse rates
114 (4) frequent patient monitoring is required (3), leading to diminished patient compliance and
115 augmented associated healthcare costs (5). In an effort to reduce the frequency of
116 cystoscopies conducted, several non-invasive biomarkers have been approved by the U.S.
117 Food & Drug Administration, albeit with performance rates remaining insufficient to replace
118 current diagnostic and monitoring practices relying on cystoscopy (6). Therefore, a prevailing
119 need for non-invasive biomarkers which will facilitate the timely diagnosis of primary and
120 recurrent UBC (6), including low-grade NMIBC (7, 8), remains unmet to date. Such
121 biomarkers are likely to reduce the frequency of diagnostic cystoscopies conducted,
122 particularly among patients undergoing monitoring for recurrent UBC.
123 Capillary electrophoresis coupled to mass spectrometry (CE-MS) has been applied for the
124 investigation of peptides and low molecular weight proteins (≤20kDa) as urinary biomarkers
125 (9, 10). The biomarkers can be subsequently sequenced and identified by employing CE-
126 MS/MS and LC-MS/MS proteomics platforms (11). Taking advantage of existing ample sized
127 peptide databases available for conducting in-depth biomarker assessment (10), support vector
128 machines (SVM), shown to be more efficient in the analysis of such complex proteomics

datasets in comparison to unsupervised methods (12), are used to generate high-dimensional biomarker classifiers (namely biomarker panels) which exhibit superior accuracy to single protein markers (9, 11, 13-15).

Particularly for UBC, CE-MS based biomarker panels have been previously described, exhibiting discriminatory capabilities for primary UBC (15), nevertheless at advanced stages (13). The present multi-center study aims to expand upon these initial efforts targeting to discover and validate urinary biomarker panels for detecting primary and recurrent UBC.

2. Patients and Methods

2.1 Patient enrollment and urine collection

Two multi-center cross-sectional studies were conducted to investigate the study objectives according to the REMARK Reporting Recommendations (16) and the recommendations for biomarker identification and reporting in clinical proteomics (17). A schematic representation of the study design is depicted in **Figure 1**. The study was performed in accordance with the Declaration of Helsinki and ethical approval was obtained by local Ethics Committees. Proper informed consent procedures under Institutional Review Board approved protocols were followed. Urine samples were collected in the period 2003-2014 from eligible outpatients visiting the Hospital del Mar in Barcelona, Spain (n=526), Erasmus Medical Center in Rotterdam, The Netherlands (n=456), University Hospital of Virginia, USA (n=304), Laikon Hospital in Athens, Greece (n=47) and Hannover Medical School, Germany (n=24). Voided midstream urine was collected at outpatient visit and prior to any treatment, according to the standard protocol for urine collection defined by the European Kidney and Urine Proteomics (EuroKUP) and Human Kidney and Urine Proteome Project (HKUPP) networks. All urine samples were collected prior to cystoscopy and stored immediately following sample collection at -20°C until CE-MS analysis was performed. Presence of UBC was confirmed

with on-site cystoscopy. Among UBC patients, tumour stage and grade was defined according to the TNM (tumour nodes metastases) classification (18), following histological examination of biopsied tumor specimens. To avoid misclassification bias, all patients were re-evaluated for at least one year following baseline assessment.

2.2 Study cohort for Primary UBC

For assessing primary UBC, 721 eligible participants, including 509 primary UBC patients and 212 urological controls were evaluated. The latter included patients presenting with hematuria and patients suffering from other disorders of the genitourinary tract, such as acute cystitis and nephrolithiasis (**Supplementary Table S1**). Exclusion criteria were presence of adenocarcinoma and papillary carcinoma (n=5). Forty-seven urine samples (random-catch) were collected at Laikon Hospital in Athens, Greece; 304 urine samples were from patients enrolled in the Department of Urology of University of Virginia, Charlottesville, USA. Eighty-five urine samples were from patients undergoing cystoscopy at the Erasmus MC, the Netherlands; 267 from Hospital del Mar in Barcelona, Spain, and 18 samples were from patients visiting the Hannover Medical School, Germany. All patients were primary referrals, with no prior history of any urinary tract malignancy. The UBC patients received the following treatment: radical cystectomy (MIBC patients) and TUR-B (NMIBC patients). The primary cohort of 721 patients (mean age of 66±13 years) was randomly separated in discovery and validation sets of 451 (mean age of 66±13 years) and 270 (mean age of 68±12 years) patients, respectively. The frequencies of the various stages were similar in the discovery and validation sets.

2.3 Study cohort for patients undergoing surveillance

For evaluating recurrent UBC, 763 patients undergoing UBC recurrence monitoring, according to the EORTC risk assessment and EAU guidelines (3), were evaluated

(**Supplementary Table S2**). A total of 447 voided urine samples were collected from patients undergoing cystoscopy in at the Erasmus MC, the Netherlands. Similarly, 310 urine samples were derived from patients attending the Hospital del Mar in Barcelona and 6 urine samples from Hannover Medical School, Germany. Among the 763 patients undergoing UBC recurrence monitoring, UBC was confirmed by cystoscopy and histological diagnosis in 164 cases. Out of these 164 relapses, 136 were NMIBC and 28 MIBC cases. Exclusion criteria were presence of adenocarcinoma and papillary carcinoma (n=9). Negative cystoscopy (n=599) was used to exclude recurrence and define controls in this population under surveillance. Controls with follow-up for less than 1 year or relapse within 1 year from sampling were excluded to rule out false negatives at the time of sampling. In total, 127 patients had to be excluded based on the above criteria and the remaining 472 urine samples were included in the analysis. The surveillance cohort (164 confirmed UBC cases and 472 eligible negative for recurrence controls) presented a mean age of 68 years (SD=±12) and was separated in a discovery set (n=425; mean age of 69±12) and a validation set (n=211; mean age of 68±13).

The distribution of the different disease stages was similar in the discovery and validation sets. In addition, 55 urine samples (out of the 211 validation set samples) originated from UBC positive cases, of which 14 or 6.6% were confirmed with MIBC.

Information on supplementary treatment [intravesical Bacillus Calmette-Guerin treatment (BCG), Epirubicin (Epi) or Mitomycin C (MMC)] prior to the urine collection was available for 371 patients undergoing UBC recurrence monitoring from those attending the Erasmus MC (**Supplementary Table S2**). The distribution of these treatment modalities was similar in the discovery and validation sets [discovery set included 21.1% treated (out of which 1.6% was BCG, 1.6% MMC, and 17.9% EPI) and 78.9% non-treated; validation set included 21.0% treated (out of which 1.4% was BCG, 0.4 MMC, and 18.1% Epi) and 79.0% non-treated, p=0.6229, Chi-squared test], as shown in the **Supplementary Table S2**.

Of the 211 subjects that were included in the validation phase, voided urinary cytology (VUC) results were available for 96 urinary samples.

2.4 Sample preparation and CE-MS analysis

Sample preparation was performed according to a standardized protocol (19). Data acquisition was performed by employing a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, USA) coupled on-line to a Micro-TOF MS (Bruker Daltonic, Bremen, Germany), following the previously described sample injection and acquisition protocol (15). Accuracy, precision, selectivity, sensitivity, reproducibility, and stability have been previously reported (15, 20). Mass spectral ion peaks representing identical molecules at different charge states were de-convoluted into single masses using the MosaiquesVisu software (21). Normalization of the CE-MS data was performed by using 29 internal peptide standards (10, 13). Detected peptides were deposited, matched, and annotated in a Microsoft-SQL database.

2.5 Statistical Analysis

Discovery Phase: Statistical analysis was performed for identifying discriminatory biomarkers for primary and recurrent UBC, by analysing the two discovery sets separately. Peptide intensities were log-transformed, and their difference between cases and controls was evaluated by using the Wilcoxon rank-sum test. To eliminate potential center bias, the biomarkers were further analyzed for their correlation with primary or recurrent UBC across the various participating clinical centers. All the peptides fulfilling at least one of the two following criteria: a) significant after multiple testing adjustment using Benjamini-Hochberg and/or b) consistent in regulation in at least two clinical centers, were shortlisted. Considering that the significant biomarkers should display differences associated with UBC disease as a whole, and in order to increase the SVM statistical power, a pool of significant UBC

associated peptides were shortlisted. Using these later shortlisted peptides, two urine-based biomarker panels were optimized in the two separate training sets, using the SVM-based MosaCluster software (version 1.7.0) (12, 22). The MosaCluster software tool allows for the classification of samples in the high dimensional parameter space by using SVM algorithms, previously shown to be particularly effective in analyzing high dimensional proteomics datasets (22, 26). The software generates a classifier, based on predefined peptides. Each of these peptides allegorizes one dimension in the *n*-dimensional parameter space (23, 24). Specifically, the following SVM parameters were defined and further applied during validation: for the Primary UBC classifier: C=5.04494, gamma=0.008269, epsilon=0.001; and for the Recurrent UBC classifier: C=6.40000, gamma=0.008000, epsilon=0.001.

Validation Phase: The urine-based biomarker panels were subsequently validated for detecting primary or recurrent UBC in the independent validation sets of 270 and 211 samples, respectively. Sensitivity and specificity of the SVM-based classifier were estimated based on the number of correctly classified samples, as defined by cystoscopy (12). The biomarker score was calculated via SVM-based software, MosaCluster (version 1.7.0). Confidence intervals (95% CI) were based on exact binomial calculations and were calculated in MedCalc® Version 12.1.0.0 (Mariakerke, Belgium), as were the receiver operating characteristic (ROC) plots. The area under the ROC curve (AUC) was evaluated for estimating the overall accuracy (25). Statistical comparisons of the validation classification scores between the UBC patients and the control groups, was performed by the Kruskal-Wallis test using MedCalc®. For the UBC patients, the classification scores according to the tumor stages were also investigated. Negative and positive predictive values (NPV/PPV), were calculated accounting for the specific prevalence rates for primary and recurrent disease in this study.

2.6 Sequencing of peptides

Urine samples were analyzed on a Dionex Ultimate 3000 RSLS nanoflow system (Dionex, Camberly UK) coupled to an Orbitrap Velos instrument (Thermo Scientific) (26). The data files were analyzed using Proteome Discoverer 1.2 (activation type: HCD; min-max precursor mass: 790-6000 Da; precursor mass tolerance: 10 ppm; fragment mass tolerance: 0.05 Da; S/N threshold: 1) and were searched against the Uniprot human non-redundant database without enzyme specificity. No fixed modifications were selected, oxidation of methionine and proline were selected as variable modifications. The criteria for accepting sequences were high confidence (Xcorr≥1.9), absence of unmodified cysteine and absence of oxidized proline in protein precursors other than collagens or elastin. For further validation of obtained peptide identifications, the strict correlation between peptide charge at the working pH of 2.0 and CE-migration time was used to prevent false identifications (27).

3. Results

3.1 Identification of significant urinary biomarkers for UBC

For assessing primary UBC, a case-control comparison was conducted in the 451 urine samples of the discovery set, between 341 primary UBC cases and 110 urological controls. The statistical comparison enabled the identification of 329 apparently significant peptides ($p<0.05$, Wilcoxon rank-sum test), shown in **Supplementary Table S3.** Among those 329, 9 peptides were statistically significant after adjustment for FDR by using Benjamini Hochberg test (**Supplementary Table S3**). To further increase the validity of the findings, the 329 biomarkers were assessed for concordant regulation across the different clinical centers. For the primary cohort, 62 potential biomarkers were identified as being concordant in the regulation trend in at least two clinical centers (**Supplementary Table S3**). Combination of the 9 peptides significant after multiple testing adjustment and the 62 peptides that were identified with concordant regulation in at least two clinical centers resulted in 66 unique

peptide biomarkers, apparently significantly associated with the disease. These were included in a peptide panel using the SVM-based software. The biomarker panel exhibited 76% diagnostic accuracy and an estimated AUC value of 0.77 after complete take-one out cross-validation in the training set of 451 samples. For investigating recurrent UBC, comparison was performed between the 109 recurrent UBC cases and 316 negative for recurrence controls. In detail, 327 biomarkers were identified as apparently significantly altered. Among the latter, 51 were significant after multiple adjustment using the Benjamini Hochberg test. As described above, the regulation of the 327 potential biomarker peptides was subsequently examined for their regulation in the different clinical cohorts (**Supplementary Table S4)**. In total, 25 peptides were identified as concordantly regulated in the different clinical centers, as shown in **Supplementary Table S4.** Among the 25 with concordant regulation in the recurrent cohort, 13 had already been shortlisted as remaining statistical significant after multiple adjustment using Benjamini Hochberg. Therefore, the combination of the 25 consistently regulated peptides with the 51 peptides remaining significant after multiple comparison adjustment resulted in 63 unique peptide biomarkers. These were subsequently combined in a multiple-peptide SVM-based panel. The diagnostic accuracy was initially assessed using cross-validation in the training set of 425 urine samples. The estimated AUC value was 0.70 after complete take one out cross validation. Both the biomarker panels apparently exhibited satisfactory performance when cross-validated in the two separate training sets. Considering that classifiers based on a higher number of biomarkers regularly show increased stability and performance (12), in the next step all possible available biomarkers (pooling all potential peptide biomarkers defined above, 66 for primary and 63 for recurrent UBC (including 4 overlapping peptides) were employed aiming to establish SVM-based UBC-specific classifiers with superior performance. Using the pool of these 125 biomarkers and the SVM-based MosaCluster software, two biomarker panels were generated and optimised using a take-one-out procedure and the two separate training sets of 451

(primary) and 425 (surveillance) urine samples, respectively. In the former (discovery set of 451 urine samples from patients with primary UBC), 116 peptides were employed to form an optimized SVM-based biomarker classifier (**Supplementary Table S5).** Similarly, during the optimization of the SVM model for recurrence, out of the 125 peptides, a peptide panel based on 106 peptides (coinciding with 116-peptide model for primary UBC minus ten hemoglobin peptide sequences that were proven to carry no value in detecting recurrence) was developed. Both the aforementioned peptide panels (116-peptide model for primary and 106-peptide model for recurrence) exhibited better diagnostic accuracies that the initially developed panels (66-peptide model for primary and 63-peptide model for recurrence). This was indicated by AUC value of 0.88 for detection of primary UBC after cross-validation in the training set and AUC value of 0.76 after cross-validation in the recurrent training cohort. Based on these results, the 116-peptide panel for detecting primary UBC and the 106-peptide panel for detecting recurrent UBC were chosen for further validation in the independent validation sets.

3.2 Validation of the UBC biomarker panel for detecting primary UBC

Subsequent validation of the 116 peptide biomarker panel was conducted in an independent set of 270 samples (including 168 primary UBC cases and 102 controls) resulting in an AUC of 0.87, (95% CI: 0.83-0.91). At a cut-off level of -0.27, which was selected to allow for high sensitivity in UBC detection, the biomarker panel's sensitivity was estimated at 91% with specificity of 68%, respectively (**Figure 2A)**. Considering a prevalence rate of 63.5%, as estimated based on the participating centers, NPV was estimated at 81.3% (68-91%; 95% CI) and PPV at 83.2 (75-89%; 95% CI). Notably, the 116 biomarker panel enabled significant discrimination of UBC cases from controls regardless UBC TNM stage (**Figure 2B**; $p<0.001$, Kruskal-Wallis test, **Table 2**).

Out of the 116 CE-MS derived peptide biomarkers, sequence could be obtained for 105 peptides (i.e. 90.5%) listed in **Supplementary Table S5)**. Most of the peptide sequences

(48/116 or 41%), were collagen fragments, possibly attributed to cancer-related processes (e.g. increased protease activity, extracellular matrix remodeling and increased collagen cleavage). The second most frequent sequences (14/116 or12%) originated from hemoglobin chains probably due to the presence of hematuria. Additional prominent peptides were derived from apolipoprotein A (5%), CD99 antigen (3%), fibrinogen A (2%), beta-2-microglobulin (2%), and single peptides from small proline-rich protein 3, insulin and histidine-rich glycoprotein.

3.3 Validation of UBC biomarker panel for detection of recurrent UBC

The 106 peptide biomarker panel for detection of recurrence was validated in 211 independent samples (including 55 UBC recurrent cases and 156 recurrent controls), with an AUC of 0.75, (95% CI: 0.68-0.80) (**Figure 3A).** At the ideal cut-off of -0.63, sensitivity and specificity values were 88% and 51%, respectively, while NPV was estimated at 93.6% (85-98%; 95% CI) and PPV at 32.32% (23-43%; 95% CI), accounting for a prevalence of 21.2% in the population investigated.

The classification scores of patients with recurrent UBC significantly differed from the negative for recurrence controls (p<0.001, Kruskal-Wallis test) (**Figure 3B)**. The biomarker panel for detecting UBC recurrence also presented significant discriminatory ability between patients presenting with NMIBC (Ta, T1 and CIS: n=41, p<0.0001) and MIBC (T2-T4: n=14, p<0.0001), respectively (**Table 2)**.

For a substantial fraction of patients undergoing surveillance, data from the cytological examination of urine samples to evaluate presence of malignant cells, were available. Out of 211 patients included in the validation phase, cytology had been performed in 96. Sensitivity and specificity of cytology for detecting recurrence was estimated at 31% and 100% respectively, while sensitivity of the classifier was 92% with a specificity of 53% in this subset of samples. Multivariate analysis, accounting for the available demographical variables

(age and gender) showed a superior AUC value of 0.80 for the classifier for detection of recurrence, compared to an AUC value of 0.69 obtained for cytology. Combination of both tests increased the performance, as assessed by an AUC of 0.87, compared to the performances of any single test alone (0.69 for cytology and 0.80 for the classifier - **Supplementary Table S6**).

The 106 peptide biomarker panel was further evaluated for detecting low risk recurrent UBC, as this represents a highly relevant biomarker context-of-use during disease surveillance of major anticipated added value to regular clinical practice (potentially decrease in number of cystoscopies [8]). For this assessment, 182 samples were available, including 26 UBC recurrent cases and 156 recurrent controls. An AUC of 0.72 (95% CI: 0.65-0.78) was observed (**Supplementary Table S6).** Cytological examination was available for 88 out of 182 samples. The 106 peptide biomarker panel outperformed cytology, with an AUC of 0.79 compared to an AUC of 0.64 for cytology. Combination of both tests increased the performance, as assessed by the given AUC of 0.90, compared to the performances of any single test alone, 0.64 for cytology and 0.79 for the classifier.

Data on supplementary treatment (e.g. BCG or chemotherapy) administered prior to the urine collection were available for a total of 123 patients included in the validation set. Logistic regression analysis indicated that the classification score based on the 106 peptide biomarker was not affected by supplementary treatment (**Supplementary Table S7**).

Out of 106 peptides included in the biomarker panel, 95 (89.6%) sequences were obtained (**Supplementary Table S5**). The majority (57%) were collagen fragments, while Apolipoprotein A-I peptides accounted for the second most frequent peptide sequences (6%). Other peptide biomarkers corresponded to fragments of basement membrane-specific heparan sulfate proteoglycan core protein (HSPG2), a disintegrin and metalloproteinase domain-containing protein 22 (ADAM22), disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1).

4. Discussion

The present multi-center study optimized and validated two unique urinary peptide-based biomarker panels for detecting primary and recurrent UBC. The findings presented demonstrate the value of a multiple-marker approach for facilitating UBC diagnosis, particularly considering the increased variability which is likely caused in part by biological variability and by the high intra-tumor heterogeneity.

The two classifiers were developed based on a pool of statistically significant different peptides. Even though these initial sets of shortlisted peptides did not largely overlap between the two cohorts, likely due to the applied stringent thresholds in each case (e.g. significant after BH adjustment, being consistent among centers), the final selected peptides forming the two classifiers are identical, with the exception of 10 hemoglobin fragments included solely in the "primary" classifier. Interestingly, out of these 116 peptide biomarkers, 56 were significantly correlated with disease stage and 32 with disease grade (**Supplementary Table S8)**. Moreover, both peptide biomarker panels exhibited superior discriminatory ability in detecting MIBC compared to NMIBC (AUC of 0.94 for MIBC versus 0.84 for NMIBC for the 116 primary panel; AUC of 0.90 for MIBC and 0.70 for NMIBC for the 106-recurrent panel; **Supplementary Table S8**). Collectively these data suggest that the peptide biomarkers in their vast majority are reflective of changes associated with cancer progression, regardless whether that corresponds to a primary or relapsed event.

Several of the biomarkers included in the classifiers were previously reported as biomarkers for the detection of UBC. Apolipoprotein A-I peptide fragments were found up-regulated in both primary and recurrent disease in line with earlier reported findings on this protein in UBC following application of two-dimensional electrophoresis (2-DE), or iTRAQ/LC-MS/MS-based proteomics analysis (28-35). In addition, Fibrinogen chains α and β, well known urinary biomarkers for the detection of bladder cancer (30, 31, 33-35), were also

found at increased levels in urine from patients with UBC and included in the classifiers. Beta-2-macroglobulin and Basement membrane-specific heparan sulfate proteoglycan core protein (HSPG2) were also confirmed with decreased excretion levels in urine from patients with UBC, in line with previously reported proteomics data (28, 31, 33). Moreover, PGMRC1 (Membrane-associated progesterone receptor component 1) has been previously included as a peptide biomarker in a CE-MS classifier discriminating between NMIBC and MIBC patients (13). In the presented study, the same PGMRC1 peptide was detected at increased levels in urine of UBC patients, in comparison to controls.

Of note, in the two biomarker panels for detection of primary and recurrent UBC, most peptide sequences were derived from collagen fragments. This likely reflects increased extracellular matrix (ECM) turnover, related to the activation of collagen-degrading proteases during tumor invasion (36). Several hemoglobin fragments were significantly associated with primary UBC, but not found to be significantly altered in the urine from patients presenting recurrence. This observation is in accordance with the hypothesis that hemoglobin fragments most likely indicate hematuria, which is frequently present in the urine of patients with primary tumours, but rarely in recurrence.

Several peptide biomarkers included in the classifiers originate from proteins reported as associated with cancer initiation and/or progression. Small proline-rich protein 3 (SPPR3) was detected at higher levels in the urine of both primary and recurrent UBC patients, in comparison to controls. SPRR3 protein has not been characterized in the context of bladder cancer yet, however, up-regulation of SPRR3 protein levels promotes colorectal tumorigenesis (37) and is associated with tumour cell proliferation and invasion in glioblastoma (38). Additionally, 14-3-3 sigma protein is frequently down-regulated in a variety of human cancers including invasive UBC tumours, particularly in lesions undergoing Epithelial to Mesenchymal transition (39) and this down-regulation is attributed to increased methylation of its promoter (40). In the present study, fragments of 14-3-3

sigma were detected at lower urinary levels in patients with either primary or recurrent UBC in comparison to controls. Similarly, CD99 low protein expression levels, likely due to gene promoter hyper-methylation, have been also reported in UBC (41). In the present study, several peptides of CD99 protein were detected at lower levels in urine from patients with UBC, compared to controls.

A small number (about 10%) of the CE-MS ion peaks included in the classifiers could not be identified by MS/MS. Failure to obtain sequence from these peptides is generally due to either peptides not fragmenting well, or due to unknown post-translational modifications that prevent mapping to the available sequence databases (42). Since a higher number of biomarkers confers increased stability of the test (12), the presented biomarker panels include all significant biomarkers identified, irrespective of whether the sequence was obtained, or not. Efforts are ongoing to obtain sequences from all peptides included in the discriminatory panels.

For primary UBC, CE-MS derived urinary peptide biomarker panels were previously reported and assessed for detecting UBC (13). These studies, however, included lower number of samples and mostly MIBC cases (13). When investigating the performance of the previously reported classifier in additional cohorts mainly composed of NMIBC patients (13), the classifier exhibited 71% sensitivity and 40% specificity (data not shown), insufficient for clinical implementation.

In our study, the source population and recruitment procedures very closely represent typical clinical situations. In such settings, high risk patients (e.g. with hematuria at primary diagnosis and/or under surveillance) are most likely to potentially benefit from the adoption of a non-invasive urine test, with a high NPV value, which could accurately guide cystoscopy (3, 8). Considering the prevalence rates for the specific cohorts, the developed biomarker panels present an NPV value of 81.3% (68-91%; 95% CI) and PPV value of 83.2 (75-89%; 95% CI) for the primary and NPV of 93.6% (85-98%; 95% CI) and PPV of 32.32% (23-43%; 95% CI),

for the follow-up cohorts. These performance rates are at least as good as those of other UBC molecular markers which are FDA approved and/or are currently under investigation (6). A direct comparison of different markers and their performance, as reported in various studies is difficult, mainly due to differences in the clinical design of the respective studies. A 10-biomarker ELISA-based assay (IL8, MMP9, MMP10, SERPINA1, VEGFA, ANG, CA9, APOE, SDC1, and SERPINE1) provided an overall AUC of 0.85 (95% CI, 0.80-0.91) in discriminating UBC patients from healthy and benign controls (45), slightly lower than the rates received from the CE-MS classifier for primary UBC (AUC=0.87; 95% CI 0.83-0.91). A three-gene methylation panel (*OTX1*, *ONECUT2*, and *OSR1)* detected low/ intermediate risk UBC with a sensitivity of 74% and specificity of 90% (43). In the present study, when investigating the sub-population of low/intermediate risk patients (NMIBC G1/G2; n=25), the CE-MS based classifier provided a sensitivity of 89% in UBC detection at the pre-selected cut-off (AUC of 0.72). For those low-intermediate risk patients where information on cytology was available (n=7), an increased sensitivity upon combination of the classifier with cytology could be obtained (AUC of 0.90; 100% sensitivity, 63% specificity). Even though promising, this result is from a small subset of samples, therefore, its further validation is required.

The strengths of the present investigation include that it is the largest multi-center study conducted to date for identifying and validating biomarker panels for primary and recurrent UBC. The presented urine-based biomarker panels hold promise for facilitating UBC diagnosis non-invasively in outpatient settings. The proteomics approaches applied for the biomarker panel development including use of an analytically validated platform (19) represent the current state-of-the-art, securing optimal diagnostic performance. The study design employed has diminished the potential effects of both source population and selection biases. Additionally, patient classification according to the standard of care, cystoscopy,

deters misclassification bias whilst enhancing the external validity and translational potential of study findings.

However, several limitations are present in this study and warrant further consideration. Adjustment for potential confounding effects, including patient characteristics, clinical, and/or treatment variables, upon biomarker panel performance could not be conducted due to data limitations. In detail, clinical variables such as tumour size, multiplicity, presence of hematuria were not available for all samples.

Moreover, multivariable regression analyses to predict UBC could not be performed, since known risk factors for UBC, including smoking history, previous upper tract cancer, and history or presence of macroscopic hematuria were also not recorded for all patients.

Collectively, through the present study we aimed to meet a very clear clinical need in bladder cancer management: the development of biomarker assays to be used for diagnosis of bladder cancer and detection of disease recurrence, particularly among non-muscle invasive bladder cancer patients. Non-muscle invasive bladder cancer patients represent the largest bladder cancer subtype and also the group that would benefit most from improvement in recurrence monitoring procedures, as existing approaches are invasive. The specific impact of the non-invasive biomarker classifiers would primarily be to guide cystoscopy and in combination with cytology as suggested by our results, and/or other molecular assays, reduce the number of surveillance cystoscopies. Moreover, in view of a positive test, urologists may be alerted to perform a more thorough investigation of the bladder hence increasing the overall accuracy in disease detection.

Due to the applied cross-sectional study design, the presented findings should be confirmed in a prospective study. Further longitudinal investigations, accounting for potential confounding effects on biomarker performance could confirm the value of the present findings, and possibly allow detecting additional benefits (e.g. value in prognosis of progression).

5. Conclusions

Two urine-based biomarker panels for detecting primary and recurrent UBC were developed to support patient screening and monitoring. The urine-based biomarkers for primary UBC hold promise for facilitating UBC diagnosis non-invasively in outpatient settings. The urine-based panel for detecting recurrence in combination to cytology resulted in improved non-invasive UBC detection. Additional prospective investigations accounting for potential confounding effects are planned to evaluate potential clinical implementation.

Figure Legends

Figure 1: Schematic representation of the study design and the analytical workflow for the development of urine-based biomarker panels. As shown in the schema, a discovery and validation phase was followed for the urine-based biomarker panels, for detecting a) primary UBC, as shown in the left arm of the schema and b) recurrent UBC, as displayed in the right arm of the schema. *UBC= urothelial bladder cancer.*

Figure 2: A) Receiver operating characteristics (ROC) analysis performed in the independent validation cohort, displaying the performance of the UBC model for classifying the primary UBC cases and **B)** the respective classification scores, presented in Box-and-Whisker plots according to the NMIBC (n=115) and MIBC (n=53) stages of the primary UBC cases. The UBC model was constructed based on 116 bladder cancer specific peptides. Other ROC characteristics, such as area under the curve (AUC), 95% confidence intervals (CI), and *p* value are provided for the classification of UBC patients. *AUC, area under the curve; CI, confidence intervals, ROC, receiver operating characteristics; UBC, Urothelial bladder cancer.*

Figure 3: A) Receiver operating characteristics (ROC) curve for the urinary biomarker panel, consisting of 106 peptides as performed in the independent validation cohort. The area under the curve (AUC), 95% confidence intervals (CI), and *p* value are also provided for the classification of UBC patients. **B)** Classification scores presented in Box-and-Whisker displaying the level of discrimination between the recurrent UBC cases and negative for UBC control and the distribution of classification values for NMIBC (n=41) and MIBC cases (n=14). A post-hoc rank-test was performed using Kruskal-Wallis test. The average rank differences were significantly different ($p<0.05$) between the controls and the recurrent UBC case group. *UBC, Urothelial bladder cancer.*

568 **Table 1 -** Patient cohorts and samples sizes involved in the different study phases

569

	Sample size n=1357 (%)	Age (SD)‡
Study Arm I: Discovery and validation of biomarker panel for primary UBC	**721**	**66(±13)**
Overall primary UBC patients	509 (71%)	68(±12)
Overall urological controls	212 (29%)	63(±15)
Discovery Phase	451	66(±13)
Primary UBC patients	341 (76%)	67(±12)
Urological controls	110 (24%)	60(±16)
Validation Phase	270	68(±12)
Primary UBC patients	168 (62%)	69(±11)
Urological Controls	102 (38%)	65(±14)
Study Arm II: Discovery and validation of biomarker panel for recurrent UBC	**636**	**68 (±12)**
Overall UBC patients with recurrent disease	164 (26%)	70± (11)
Overall UBC patients without recurrent disease ("recurrent controls")	472 (74%)	68± (12)
Discovery Phase	425	69(±12)
UBC patients with recurrent disease	109 (26%)	71± (10)
UBC patients without recurrent disease	316 (74%)	68± (12)
Validation Phase	211	68(±13)
UBC patients with recurrent disease	55 (26%)	69± (13)
UBC patients without recurrent disease	156 (74%)	68± (13)

UBC; Urothelial Bladder Cancer
‡Mean Value

570

571

572 **Table 2 -** Classification scores of the biomarker panels for NNMIBC and MIBC, as obtained
573 during the independent validation phase. The urinary biomarker panel of 116 peptides was
574 developed for the detection of primary UBC patients and the urinary biomarker panel of 106
575 was optimized for detecting recurrent UBC patients.

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578

(diagnosis†)	Urinary biomarker panel for the detection of Primary UBC			Urinary biomarker panel for the detection of Recurrent UBC		
	Sample Size n=270, (%)‡	Classification score mean value (SD)	*p* value¥	Sample Size n=211 , (%)	Classification score mean value (SD)	*p* value) ¥
NMIBC (Ta, T1, CIS††)	115 (42.6%)	0.29(0.54)	<0.0001	41 (19.4%)	-0.18 (0.48)	*<0.0001*
MIBC	53 (19.6%)	0.58(0.32)	*<0.0001*	14 (6.7%)	0.10 (0.50)	*<0.0001*
Controls‖	102 (37.8%)	-0.69(0.82)		156 (73.9%)	-0.56 (0.48)	

‖ For the primary UBC, the controls are referring to urological controls (hematuria, benign urological diseases), while in the recurrent group, the controls included are samples derived from patients negative for recurrence.
† According to the 2004 TNM system (18); †† According to EAU guidelines (3, 44); ‡ Percentage over the number of UBC cases; ¥ Independent samples T-Test.
MIBC = muscle invasive bladder cancer; NMIBC = non muscle invasive bladder cancer; UBC = urothelial bladder cancer.

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Fig.1

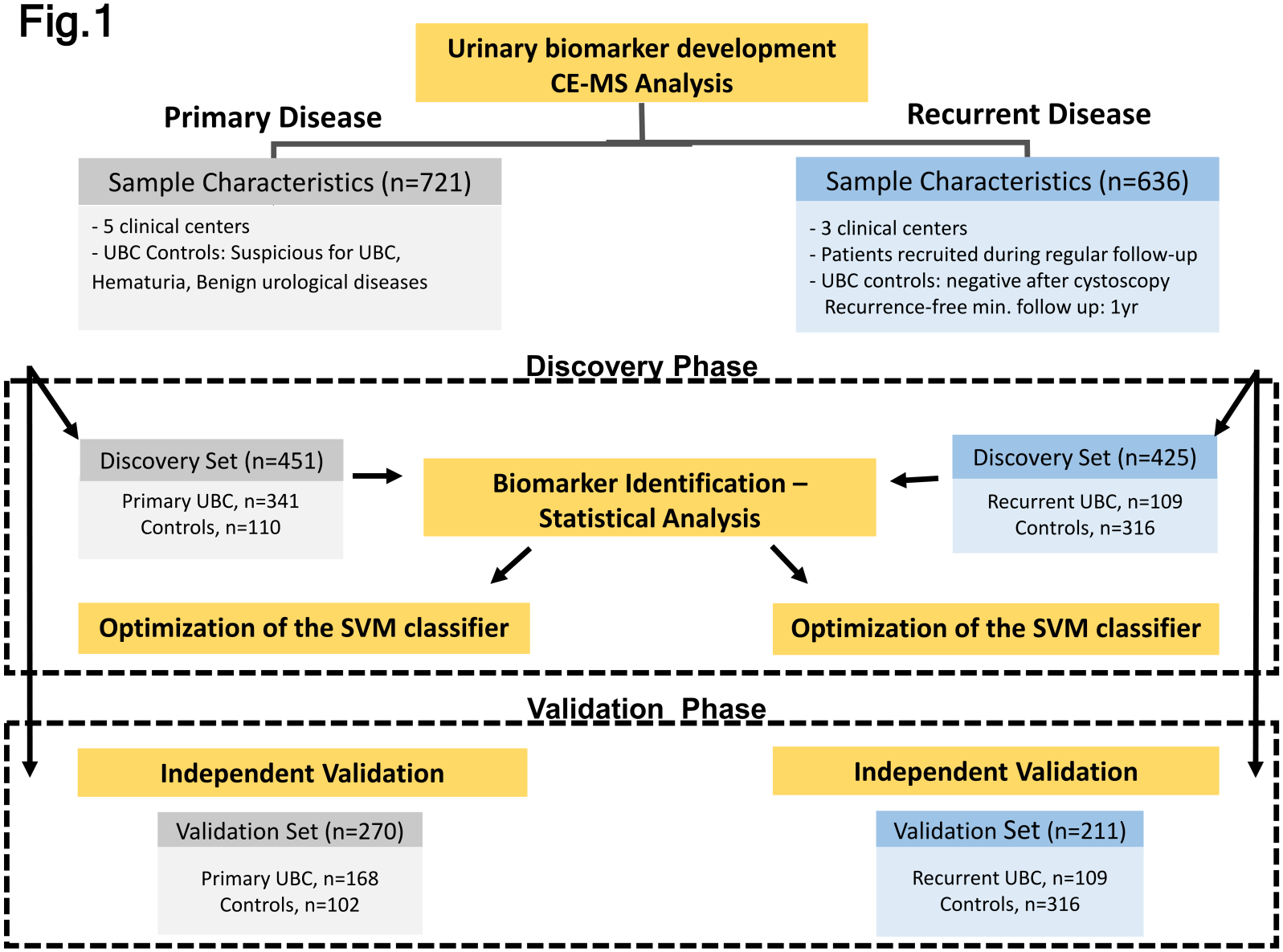

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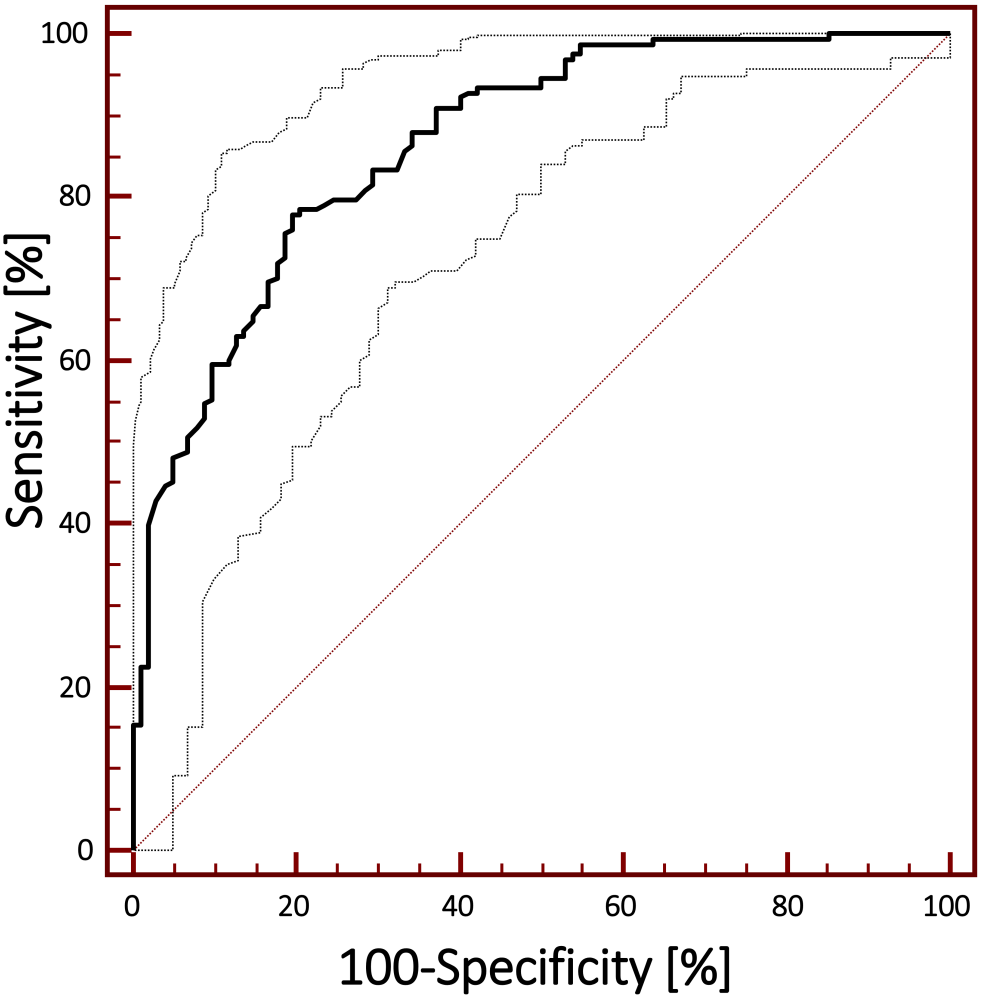
Fig. 2A

Biomarker Model for detection of Primary UBC



Urinary UBC model

ROC characteristics	Performance
Number of Peptides	116
Sample size (n)	270
Case/Control (n)	168 / 102
Area under curve (AUC)	0.87
95% Confidence Interval	0.83 to 0.91
Significance P	<0.0001

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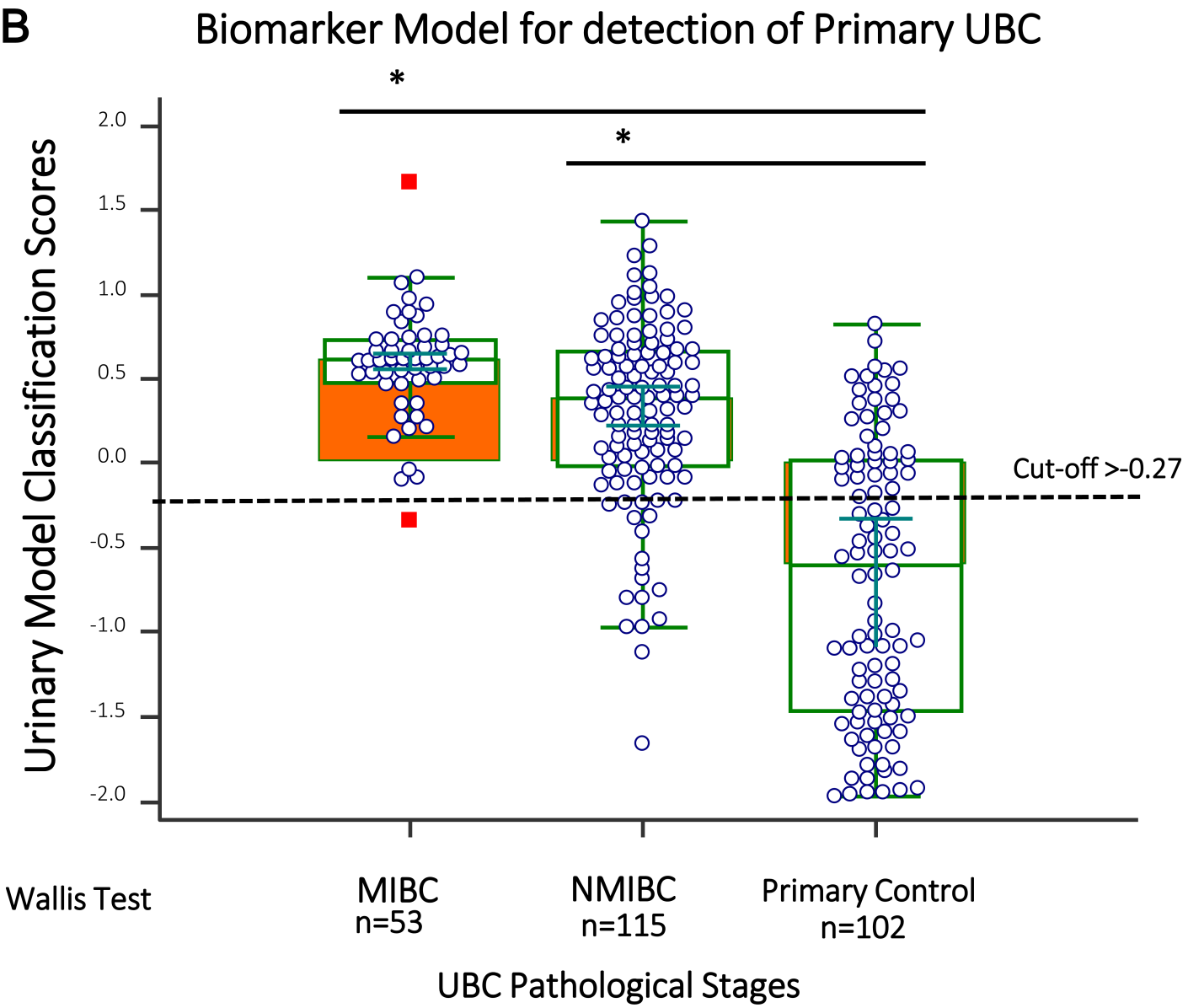
Fig. 2B



*Kruskal Wallis Test
p<0.05

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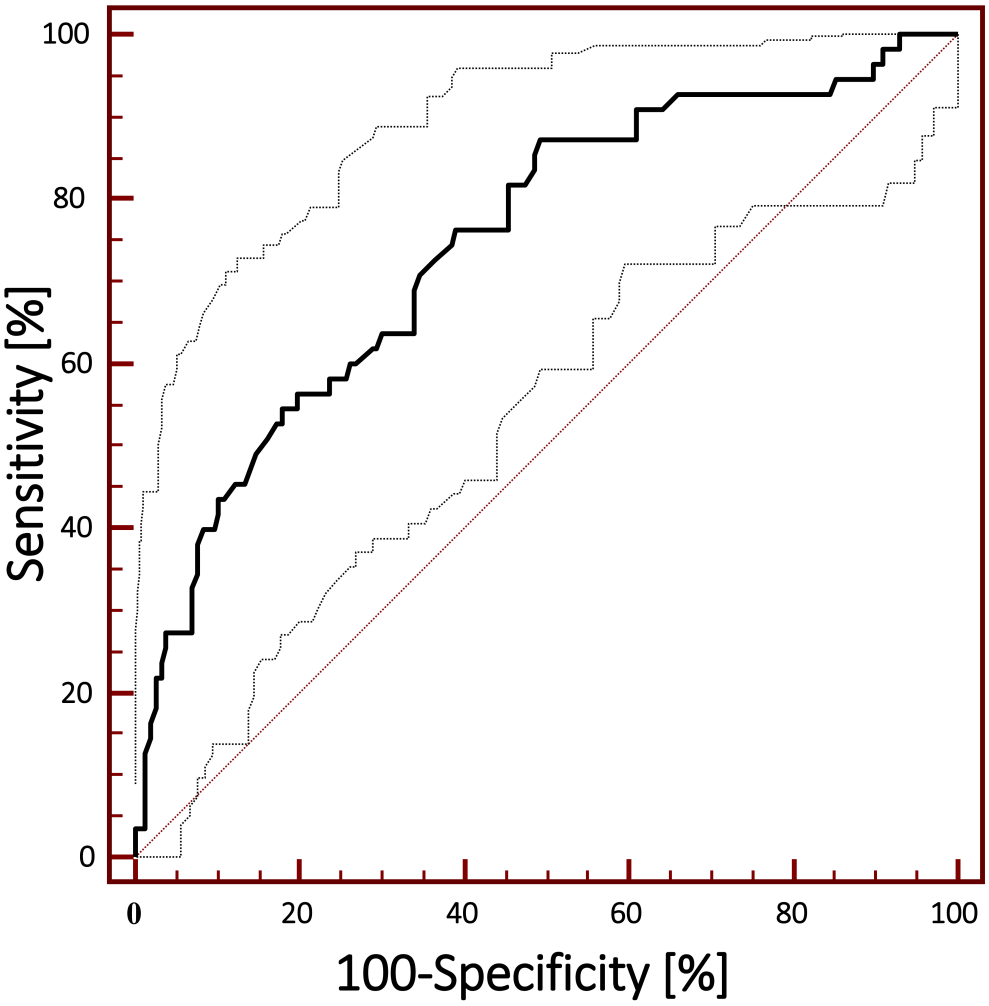
Fig. 3A

Biomarker Model for detection of Recurrent UBC



Urinary UBC model

ROC characteristics	Performance
Sample size (n)	211
Case/Control (n)	55 / 156
Area under curve (AUC)	0.75
95% Confidence Interval	0.68 to 0.80
Significance P	<0,0001

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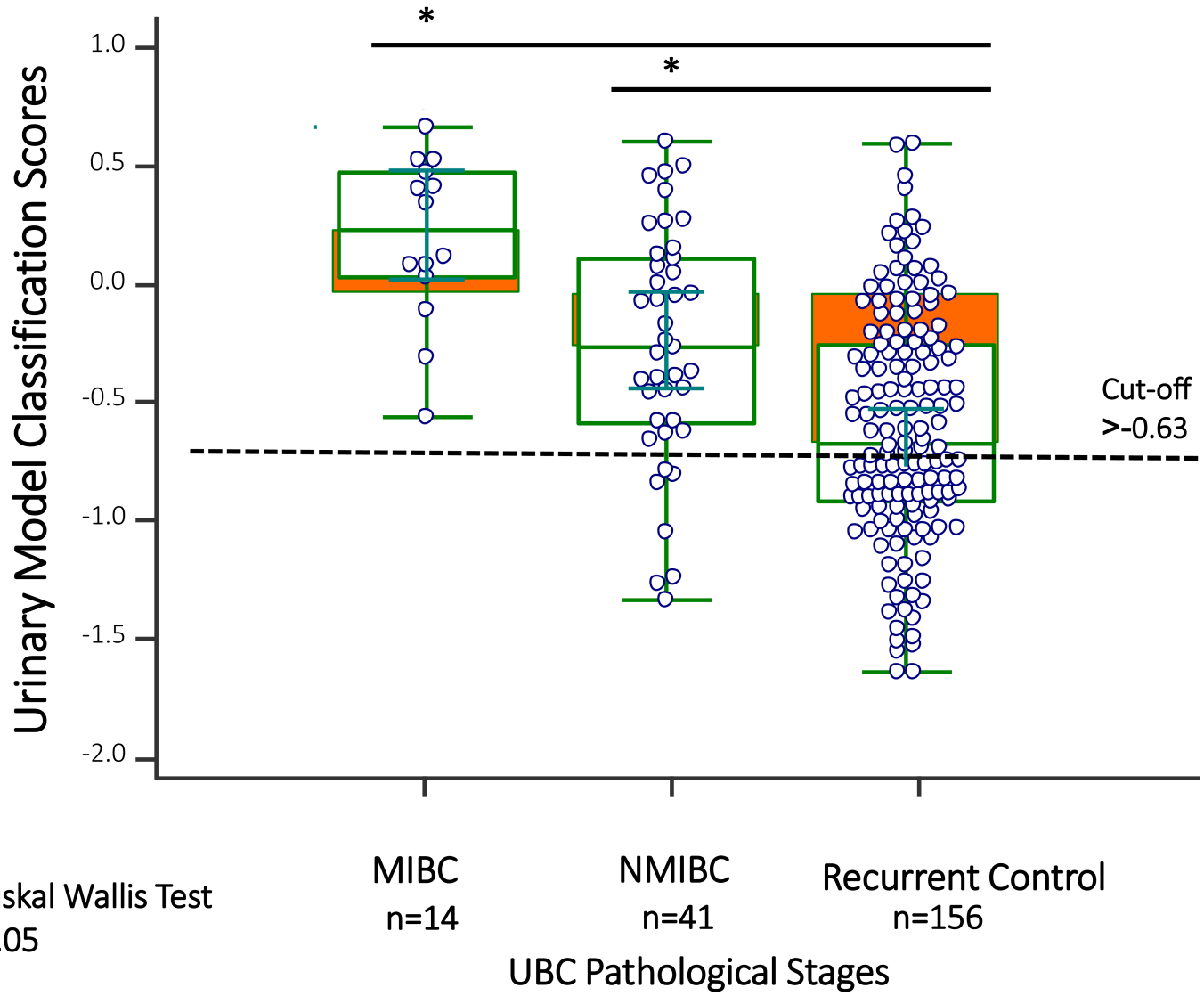
Fig. 3B Biomarker Model for detection of Recurrent UBC



*Kruskal Wallis Test
p<0.05

AACR American Association for Cancer Research

Clinical Cancer Research

Development and validation of urine-based peptide biomarker panels for detecting bladder cancer in a multi-center study

Maria Frantzi, Kim E Van Kessel, Ellen C. Zwarthoff, et al.

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