## Title

Urinary cytokine profile to predict response to intravesical BCG with or without HS-410 therapy in patients with non-muscle invasive bladder cancer



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# Urinary cytokine profile to predict response to intravesical BCG with or without HS-410 therapy in patients with non-muscle invasive bladder cancer

A thesis submitted in partial satisfaction of the requirement for the degree Master of Science in Clinical Research

by

Amirali Salmasi

2018

ABSTRACT OF THE THESIS

Urinary cytokine profile to predict response to intravesical BCG with or without HS-410 therapy in patients with non-muscle invasive bladder cancer

by

Amirali Salmasi

Master of Science in Clinical Research

University of California, Los Angeles, 2018

Professor Robert M. Elashoff, Chair

Abstract:

Background: Despite extensive research to identify biomarkers of response in patients with non-muscle invasive bladder cancer (NMIBC), there is no biomarker to date that can serve this purpose. Herein, we sought to determine if a large panel of urinary cytokines measured at different time points can predict response to treatment in patients with NMIBC.

Methods and Material: Serial urine samples were collected from 50 patients with intermediate or high-risk NMIBC enrolled in a prospective, phase II randomized study evaluating intravesical BCG +/- intradermal HS-410 therapy. Urines were collected at baseline, week-7, week-13, week-28 and at end of treatment. Using a multiplex

immunoassay, 105 cytokines were analyzed. To predict outcome of time to event (either recurrence or progression), univariate and multivariable Cox analyses were performed.

Results: 15 patients (30%) developed recurrence and 4 patients (8%) progressed during the follow-up. Among clinicopathologic variables, only smoking status, ever-smoker vs. non-smoker status, was associated with an improved response rate (HR 0.38, 95% CI 0.14 - 0.99, p=0.0483). In the most relevant multivariable model, the percent change (for 100 units) of IL-18 binding protein-a (HR 1.995, 95% CI 1.157-3.438, p=0.01), IL-23 (HR 1.116, 95% CI 1.012-1.23, p=0.03), IL-8 (HR 0.273, 95% CI 0.069-1.082, p=0.06), and interferon gamma-induced protein-10 (HR 0.955, 0.914-0.997, p=0.04) at week-13 from baseline best predicted time to event.

Conclusion: In a time-dependent manner, urinary cytokines provided additional value to clinicopathologic features to predict response to immune modulating agents in patients with intermediate and high risk NMIBC. Further studies are needed to validate these findings.

The thesis of Amirali Salmasi is approved.

Karim Chamie

David Elashoff

Robert M. Elashoff, Committee Chair

University of California, Los Angeles

2018

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List of Abbreviations

BCG: Bacillus Calmette-Guérin

C-index: Concordance index

CUETO: Club Urologico Espano de Tratamiento

EORTC: European Organization for Research of Cancer

EOT: End of treatment

FDR: False discovery rate

IGFBP: Insulin-like growth factor-binding protein

IL18BPa: IL-18 binding protein-a

IP10: Interferon gamma-induced protein 10

ITAC: Interferon-inducible T-cell alpha chemoattractant

MCP-3: Monocyte-chemotactic protein 3

MIP-1a/MIP1-b: Macrophage inflammatory protein

NB: Naïve Bayes

NMIBC: Non-muscle invasive bladder cancer

SHBG: Sex hormone binding- globulin

Th1: T helper-1

Th2: T helper-2

TRAIL: TNF-related apoptosis-inducing ligand

TURBT: Transurethral resection of bladder tumor

VEGF: Vascular endothelial growth factor

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1. Introduction:

Non-muscle invasive bladder cancer (NMIBC) includes a heterogeneous group of tumors with a different risk of recurrence or progression.[1-3] While transurethral resection is sufficient for most low-risk tumors, adjuvant intravesical treatment is recommended for intermediate and high-risk cancers.[4] In fact, intravesical bacillus Calmette-Guérin (BCG) has been utilized for more than four decades for high-risk NMBIC with favorable outcomes. However, approximately 50% of patients treated with intravesical BCG will experience disease recurrence or progression, which may have significant impact on a patient's cancer specific outcomes and quality of life. [2, 3] Therefore, there is an urgent need for development of response predictive biomarkers in these patients.[5] Response predictive biomarkers could readily be used to identify potentially unresponsive patients before they are exposed to treatment-related toxicities and high out-of-pocket costs with little or no benefit. Biomarkers have also the potential to increase our understanding of the mode of action of drugs, and thereby identify potential combination therapies.

The BCG-induced immune response is complex and not fully understood.[6] It involves both humoral and cell-mediated components.[6, 7] Clinicopathologic features, tumor molecular biomarkers, tumor immune profile, genetic testing, and urinary cytokines have been applied as risk stratification tools to predict response to BCG treatment with mixed results.[5] Currently, there is no single predictive biomarker which can be used to screen patients for BCG treatment.[5, 8]

Cytokines are key mediators of immune responses that allow recruitment, activation, and differentiation of a variety of immune cells.[9] It has been shown that urinary cytokine levels increase after BCG instillation[8, 10], therefore, it has been proposed that a panel of urinary cytokines could be a useful tool to assess the BCG-induced immune response. Urinary levels of IL-1, IL-2, IL-6, IL-10, IL-8, IL18, IFN-gamma, TNF-alpha, and TNF-related apoptosis-inducing ligand (TRAIL) have been evaluated as predictors for treatment response after BCG.[5, 8] Recently, Kamat et al. reported a panel of nine urinary cytokines (IL-2, IL-6, IL-8, IL-18, IL-1ra, TRAIL, IL-12[p70], and TNF-alpha), which predicted the likelihood of recurrence after BCG treatment with 85.5% accuracy (95% confidence interval 77.9–93.1%)[11]. In each study, a limited number of cytokines was analyzed, thus limiting the scope of the work. Therefore, we hypothesize that a large panel of 105 urinary cytokines measured longitudinally at different time points during BCG treatment, will provide the information needed to predict response to treatment. In this study, we sought to determine if clinicopathologic data in addition to longitudinal urinary cytokines collected during intravesical BCG treatment with or without concomitant HS-410 vaccine are predictors of time-to-treatment failure in patients with intermediate or high risk NMIBC.

## 2. Methods and Material:

### 2.1. Study population

Urine samples were collected from 50 patients with intermediate- or high-risk NMIBC enrolled in a phase II, randomized study to evaluate the safety, immune response and clinical activity of HS-410 treated individuals with NMIBC who have undergone

transurethral resection of bladder tumor (TURBT) from 2013 to 2017 (clinicaltrials.gov Identifier NCT02010203). In this trial, patients with high or intermediate-risk NMIBC received weekly intradermal injections of either $10^6$ or $10^7$ cells/dose of HS-410 or placebo in combination with intravesical induction BCG for 6 weeks followed by 6 weekly injection of vaccine or placebo. HS-410, Vesigenurtacel-L, was derived from a cancer cell line, and modified to express HLA-A1 protein and secrete gp96-Ig fusion protein, which activates CD8+ cytotoxic T cells.[10] Patient continuing on trial received maintenance treatment consisting of 3-weekly treatments of vaccine and BCG approximately 3, 6, and 12 months after initiating induction treatment. Demographic and clinical data were collected. Mid-stream voided urine samples were collected from patients on study at baseline, week 7, 13, 28 and at end of treatment (EOT). EOT evaluations was completed about 4 weeks after last dose of vaccine or upon early discontinuation of treatment. Thus, a total of 200 urine samples; 50 at baseline, 46 at week 7, 50 at week 13, 36 at week 28, and 18 at EOT were available for analysis. Cystoscopy and urinary cytology were performed per standard of care (every 3 months for 2 years and then every 6 months for up to a year). Patients who were found to have an abnormality on cystoscopy or abnormal urinary cytology received standard of care treatment per discretion of the investigator, which typically included cystoscopy, bladder biopsy or TURBT. The primary endpoint of the study was time to recurrence and/or time to progression, which is based upon the pathologic interpretation of the bladder biopsy and/or TURBT samples.

## 2.2. Urine biomarker measurement

Freshly voided urine samples were collected and centrifuged at 1000-1500 x g for 10 minutes. The supernatant was collected and stored at -80°C until biomarker analysis. All experiments were performed using a multiplex immunoassay-based cytokine array (R&D Systems Proteome Profiler Human XL Cytokine Array Kit, Minneapolis, MN) and were detected by a LI-COR Odyssey CLx imager (Lincoln, Nebraska) system and quantified using the Quick Spots array analysis software by Western Vision Software. For each marker, the data is the average of the analyte duplicates minus the average of negative controls. Normalization was not performed since no single urinary marker has been identified for urinary protein normalization. [11]

## 2.3. Statistical analysis

Student's t-test and Wilcoxon rank sum test were used to compare quantitative characteristics between response groups. Categorical variables were compared using Chi-square or Fisher's exact test. To adjust for multiple testing, the false discovery rate (FDR) approach was used. [12] The Skillings–Mack test was used to evaluate whether cytokine levels changed over time. [13] To predict outcome of time to event (either recurrence or progression), univariate and multivariable Cox models using clinicopathologic variables and urinary cytokines were constructed (Figure 3). All event and late event predictive models (excluding events prior to day 120) were constructed using either baseline cytokines, week 13 biomarkers or the change in cytokines from baseline to week 13. An additional model was constructed treating cytokines as time dependent covariates using all the time points (BL, week 7, 13, 28, and EOT). To

predict early events (at first cystoscopic evaluation at 3 months), univariate and multivariable logistic regression modeling were applied to cytokine levels at base line or week 13. For the model construction, first we created the best possible model using clinical parameters. Then, we included the cytokines of interest (with p-value ≤ 0.15 in univariate Cox model) in the model in a stepwise manner to see if value added to prior model. The concordance index (C-index) was used to evaluate the performance of these models.[14]

As an exploratory analysis, we evaluated the predictive value of urinary cytokines at week 13 to predict treatment response (all event model) using the Naïve Bayes (NB) classification technique. Naïve Bayes is a classifier based on applying Bayes' theorem, which relates a strong independence assumption between features within the classifier. [14] Briefly, the top five cytokines with highest correlation with response status were selected using univariate logistic regression and applied to multivariable analysis. To avoid model over-fitting and to test the generalizability of the results, these performance measures were assessed by applying 10-fold cross validation.

Analyses were generated with Stata statistical software version 15 (StataCorp, College Station, TX), SAS software version 9.4 (SAS Institute, Inc., Cary, NC, USA), and Waikato Environment for Knowledge Analysis (Weka). P-values <0.05 were considered statistically significant.

## 3. Results:

### 3.1. Patient demographics and Urine biomarkers

Demographic and clinical data from participants is summarized in Table 1 as it relates to treatment response. The mean age of participants was 70 years old. The majority of the cohort was white (48 patients) and male (42 patients). Twelve patients (24%) had no history of smoking. Median time of follow up was 349 days (IQR 121 -421 days). Twenty-seven patients (46%) had a history of bladder cancer and had received prior intravesical BCG (16 patients) or Mitomycin (6 patients). High-grade disease was found in 47 patients: 13 CIS, 14 high grade Ta, 6 CIS + Ta, 12 T1, and 2 CIS + T1. Of the 50 patients on study, 15 patients (30%) developed recurrence (defined as disease that has recurred to the same stage/severity or lower stage/severity of disease compared to screening) and 4 patients (8%) progressed (defined as an increase in T stage from CIS or Ta to T1 disease, development of T2 or greater or lymph node (N+) disease or distant metastasis, or an increase in grade from low to high) during the follow up. There was no significant difference in clinical variables between patients with and without recurrence/progression (Table 1). Table 2 depicts univariate analysis to predict recurrence free and progression free survival. There was no association between response rate and gender, ethnicity (white versus others), recurrent versus newly diagnosed disease, tumors stage (T1 versus CIS, Ta versus CIS, Ta + CIS versus CIS), tumor grade, and previous treatments with intravesical BCG or Mitomycin. Only smoking status, ever-smoker status vs. non-smoker, was associated with improved response rate (HR 0.38, 95% CI 0.14 - 0.99, p=0.0483), specifically among the patients who did not respond to the treatment, 8 patients were non-smoker (57% of non-smokers) and 11 patients had a positive history of smoking (31% of ever-smokers).

Event free survival estimates in non-smoker versus ever-smokers are further illustrated in Figure 1.

Table 1. Baseline characteristics by response status.

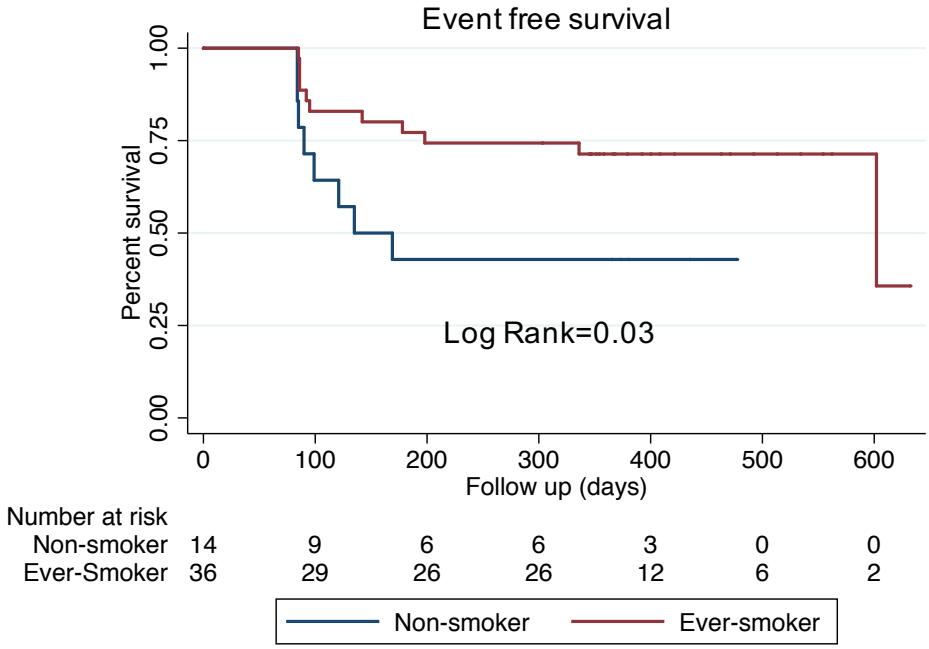
| Variable | Recurrence or Progression | | p Value |
|---|---|---|---|
| | Yes (N=19) | No (N=31) | |
| **Age, mean (SD)** | 72 (11.3) | 69 (11.3) | 0.33 |
| **Sex (%)** | | | 0.23* |
| **Female** | 5 (26) | 3 (10) | |
| **Male** | 14 (74) | 28 (90) | |
| **Ethnicity (%)** | | | 0.62* |
| **White** | 18 (95) | 30 (97) | |
| **Other** | 1 (5) | 1 (3) | |
| **Smoker (%)** | | | 0.10* |
| **Ever** | 11 (61) | 25 (83) | |
| **Never** | 7 (39) | 5 (17) | |
| **Diagnosis of cancer (%)** | | | 0.19 |
| **Newly** | 8 (42) | 19 (61) | |
| **Recurrent** | 11 (58) | 12 (39) | |
| **Stage (%)** | | | 0.16* |
| **CIS only** | 3 (16) | 10 (32) | |
| **Ta** | 9 (47) | 8 (26) | |
| **Ta + CIS** | 4 (21) | 2 (6) | |
| **T1** | 3 (16) | 9 (29) | |
| **T1 + CIS** | 0 | 2 (6) | |
| **Grade (%)** | | | 0.55* |
| **High** | 17 (89) | 30 (97) | |
| **Low** | 2 (11) | 1 (3) | |
| **Previous Mitomycin (%)** | | | 0.66* |
| **Yes** | 3 (17) | 3 (10) | |
| **No** | 15 (83) | 28 (90) | |
| **Previous BCG (%)** | | | 0.19 |
| **Yes** | 4 (21) | 12 (39) | |
| **No** | 15 (79) | 19 (61) | |

* Fisher exact test

Table 2. Univariate analysis to predict recurrence free and progression free survival

| Variable | HR | 95% Confidence Interval | p Value |
|---|---|---|---|
| **Age** | 1.03 | 0.98-1.07 | 0.238 |
| **Sex** | | | |
| **Female** | Referent | | |
| **Male** | 0.472 | 0.17-1.35 | 0.161 |
| **Smoking** | | | |
| **Non-smoker** | Referent | | |
| **Ever-smoker** | 0.38 | 0.14-0.99 | 0.048 |
| **Diagnosis of cancer** | | | |
| **Newly diagnosed** | Referent | | |
| **Recurrent** | 2.21 | 0.85-5.71 | 0.102 |
| **Previous Mitomycin** | | | |
| **No** | Referent | | |
| **Yes** | 1.29 | 0.36-4.64 | 0.700 |
| **Previous BCG** | | | |
| **No** | Referent | | |
| **Yes** | 0.60 | 0.20-1.85 | 0.374 |
| **Stage** | | | |
| **CIS** | Referent | | |
| **T1** | 0.68 | 0.11-4.07 | 0.672 |
| **Ta** | 2.80 | 0.75-10.36 | 0.124 |
| **Ta + CIS** | 2.81 | 0.63-12.60 | 0.176 |
| **Grade** | | | |
| **High** | Referent | | |
| **Low** | 0.47 | 0.09-2.44 | 0.370 |
| **Ethnicity** | | | |
| **White** | Referent | | |
| **Other** | 1.12 | 0.15-8.44 | 0.911 |

Figure 1. Event free survival in non-smokers versus ever-smokers.



One hundred and five cytokines were measured for each urine sample. Summary of baseline urine cytokines by response status was reported in Table 3. At FDR of 0.15, there was no significant difference in baseline cytokine levels in responders vs. non-responders. The median levels of Apolipoprotein A1, Angiopoietin-1, Chitinase-3-like 1, Complement C5-C5a, dickkopf-related protein 1 (DKK1), growth related protein-alpha (GRO-alpha), IL8, macrophage inflammatory protein-3 beta (MIP- 3beta), interferon gamma-induced protein 10 (IP10), interferon-inducible T-cell alpha chemoattractant (ITAC), monokine induced by gamma interferon (MIG), matrix metallopeptidase 9 (MPP-9), Myeloperoxidase, Serpin E1, sex hormone binding- globulin (SHBG), and vascular endothelial growth factor (VEGF) significantly changed over time in patients with or without recurrence/progression (Figure 2).

Table 3. Summary of baseline urine cytokines by response status. At FDR of 0.15, there was no significant difference in baseline cytokine levels in responders versus non-responders.

| Biomarker | Recurrent/progression | No Recurrent/progression | P value |
|---|---|---|---|
| **Adiponectin** | 18624.89 (4316.84 - 29291.15) | 14972.91 (3941.77 - 29424.29) | 0.30 |
| **Angiogenin** | 12796.64 (2548.77 - 27722.56) | 13929.24 (4374.14 - 28526.65) | 0.63 |
| **Angiopoietin-1** | 1126.38 (-191.13 - 9843.11) | 1277.73 (-1315.24 - 2670.03) | 0.81 |
| **Angiopoietin-2** | 2360.53 (173.01 - 10418.25) | 2304.01 (-299.44 - 6111.79) | 0.87 |
| **Apolipoprotein-AI** | 7370.47 (2177.49 - 17078.49) | 7206.13 (2772.48 - 24177.64) | 0.75 |
| **BAFF** | 2171.92 (100.66 - 8891.9) | 1740.12 (-1462.44 - 5469.64) | 0.36 |
| **BDNF** | 2207.45 (189.46 - 6337.18) | 2222.58 (-102.4 - 6017.31) | 0.70 |
| **CD14** | 5524.78 (3196.79 - 11765.97) | 5776.6 (412.86 - 16641.78) | 0.67 |
| **CD30** | 5768.26 (2867.71 - 11621.47) | 5508.47 (2383.04 - 11900.91) | 0.55 |
| **CD31** | 6301.87 (3800.33 - 9454.91) | 6719.84 (3340.62 - 21275.18) | 0.76 |
| **CD40 ligand** | 2815.63 (1282.32 - 5074) | 2736.26 (733.85 - 8014.74) | 0.65 |
| **C Reactive Protein** | 2881.59 (563.88 - 13468.35) | 3283.02 (-697.82 - 15032.16) | 0.84 |
| **Chitinase-3-like-1** | 13464.06 (2038.6 - 27030.07) | 12752.83 (3182.76 - 24880.19) | 0.18 |
| **Complement C5-C5a** | 4195.71 (898.15 - 15854.11) | 3802.93 (-709 - 21248.75) | 0.55 |
| **Complement Factor-D** | 5303.36 (765.12 - 10365.04) | 5481.43 (872.31 - 12891.95) | 1.00 |
| **Cripto1** | 1555.19 (-226.74 - 13530.51) | 1742.85 (-810.26 - 6178.01) | 0.40 |
| **Cystatin-C** | 8178.09 (3111.74 - 18445.24) | 9529.4 (1738.72 - 26978.46) | 0.35 |
| **DPPIV** | 14009.84 (4258.53 - 26459.85) | 13466.45 (5532.18 - 32085.13) | 0.72 |
| **Dkk1** | 3001.05 (233.93 - 6609.29) | 2712.04 (615.39 - 7001.3) | 0.78 |
| **EGF** | 27213.93 (20575.78 - 34960.27) | 27363.44 (18050.05 - 36185.52) | 0.89 |
| **EMMPRIN** | 14659.34 (7472.87 - 21870.95) | 13533.9 (7727.02 - 29171.21) | 0.65 |
| **ENA78** | 3173.9 (1720.13 - 7201.14) | 3203.3 (591.71 - 15129.01) | 0.67 |
| **Endoglin** | 5086.93 (2317.09 - 10495.56) | 5574.47 (610.7 - 16032.81) | 0.76 |
| **FGF-19** | 3025.08 (1034.35 - 5881.33) | 3454.51 (-149.29 - 10024.06) | 0.35 |
| **FGF-7** | 907.1 (-399.68 - 4328.24) | 1008.25 (-1812.28 - 3384.04) | 0.84 |
| **FGF-basic** | 1648.2 (98.28 - 15423.03) | 2042.94 (-490.93 - 7449.73) | 0.25 |
| **Fas Ligand** | 1333.3 (555.4 - 5460.75) | 1663.91 (-106.77 - 5326.21) | 0.66 |
| **Flt3 Ligand** | 1832.04 (-347.91 - 4241.68) | 2080.93 (-179.26 - 5818.64) | 0.22 |
| **GCSF** | 1952.65 (-191.9 - 4248.49) | 2106.2 (63.39 - 4702.21) | 0.47 |
| **GDF-15** | 17909.87 (11338.27 - 29012.95) | 19263.86 (9703.38 - 31591.12) | 0.78 |
| **GMCSF** | 2003.52 (931.67 - 4886.25) | 2547.38 (954.56 - 4176.43) | 0.19 |
| **GRO-alpha** | 2875.41 (887.17 - 8254.78) | 2735.34 (-233.95 - 9705.87) | 0.73 |
| **Growth Hormone** | 873.22 (144.81 - 2928.06) | 944.68 (-493.61 - 3414.82) | 0.84 |
| **HGF** | 1419.46 (458.55 - 3468.96) | 1914.67 (-33.25 - 6311.73) | 0.10 |
| **ICAM1** | 1004.52 (-19.88 - 2883.99) | 1339.7 (-186.2 - 6615.71) | 0.25 |
| **IFN-gamma** | 2235.3 (-16.22 - 5254.71) | 2365.65 (-267.64 - 7290) | 0.81 |
| **IGFBP2** | 7875.99 (4953.93 - 22299.33) | 10055.23 (3999.14 - 29552.21) | 0.73 |
| **IGFBP3** | 975.51 (-614.18 - 5405.67) | 1730.63 (-560.95 - 7205.76) | 0.08 |
| **IL10** | 859.71 (-377.67 - 3992.44) | 1452.59 (-1431.86 - 4002.76) | 0.23 |
| **IL11** | 1428.73 (-532.45 - 4324.59) | 1858.96 (-207.22 - 5713.61) | 0.09 |
| **IL12p70** | 1191.04 (-686.12 - 5322.42) | 1517.92 (-373.09 - 4740.86) | 0.17 |
| **IL13** | 589.22 (-892.19 - 3436.57) | 1109.84 (-1055.77 - 2826.49) | 0.09 |
| **IL15** | 1327.06 (-624.32 - 3653.12) | 1739.72 (-651.76 - 6840.59) | 0.10 |

| | | | |
|---|---|---|---|
| **IL16** | 1686.61 (-392.51 - 4192.89) | 1936.91 (-337.82 - 8361.94) | 0.13 |
| **IL17A** | 3124.78 (1583.43 - 5880.29) | 4310.9 (1621.67 - 11332.45) | 0.02 |
| **IL18BPa** | 10950.06 (2485 - 18784.04) | 11848.36 (8249.62 - 29335.99) | 0.44 |
| **IL19** | 943.18 (126.97 - 20051.47) | 788.12 (-912.92 - 2747.26) | 0.36 |
| **IL1alpha** | 2371.19 (-51.52 - 4192.79) | 2332.22 (611.39 - 7035.92) | 1.00 |
| **IL1beta** | 1180.3 (-505.67 - 4181.87) | 1903.09 (-803.06 - 5034.87) | 0.03 |
| **IL1ra** | 8799.87 (6593.83 - 13726.74) | 8176.44 (2123.85 - 16823.83) | 0.33 |
| **IL2** | 1157.73 (-74.35 - 4472.91) | 1814.78 (213.15 - 6005.06) | 0.13 |
| **IL22** | 1708.19 (466.18 - 5303.32) | 2194.9 (146.48 - 4535.44) | 0.52 |
| **IL23** | 682.02 (35.2 - 2897.18) | 1289.82 (-616.79 - 4115.06) | 0.11 |
| **IL24** | 1396.78 (-94.98 - 3541.69) | 2041.01 (418.45 - 6418.04) | 0.04 |
| **IL27** | 1599.09 (-466.52 - 5094.03) | 2454.53 (500.56 - 7188.91) | 0.05 |
| **IL3** | 1018.05 (5.23 - 3849.94) | 1817.9 (491.93 - 7062.15) | 0.08 |
| **IL31** | 951.02 (-794.24 - 3321.39) | 1419.91 (-1118.91 - 4100.59) | 0.25 |
| **IL32** | 1601.7 (-706.11 - 3954.85) | 2093.71 (132.27 - 4186.69) | 0.14 |
| **IL33** | 938.28 (-733.97 - 2908.84) | 1375.36 (-901.71 - 4043.85) | 0.04 |
| **IL34** | 1082.65 (-690.84 - 2618.98) | 1690.48 (-752.11 - 6141.3) | 0.02 |
| **IL4** | 1516.13 (351.97 - 17159.49) | 1717.09 (-128.76 - 4455.15) | 0.87 |
| **IL5** | 1350.35 (496.01 - 3801.19) | 1390.1 (-517.1 - 4565) | 0.63 |
| **IL6** | 2092.28 (1302.53 - 8096.71) | 2597.04 (188.23 - 11042.32) | 0.10 |
| **IL8** | 8998.87 (673.96 - 16948.26) | 7207.83 (-283.22 - 23758.54) | 0.17 |
| **IP10** | 2377.3 (-55.54 - 12900.81) | 2715.62 (-525.92 - 26185.22) | 0.45 |
| **ITAC** | 1429.87 (-276.3 - 3036.57) | 1785.12 (-278.19 - 10882.81) | 0.19 |
| **Kallikrein3** | 3224.23 (601.72 - 29045.45) | 7102.94 (2262.53 - 29835.4) | 0.01 |
| **LIF** | 1350.79 (365.29 - 4843.7) | 1373.14 (-163.6 - 5495.08) | 0.76 |
| **Leptin** | 991.23 (47.9 - 8468.96) | 1237.67 (-311.49 - 3884.5) | 0.78 |
| **Lipocalin2** | 10254.21 (3808.77 - 15880.03) | 10713.13 (5042.53 - 23981.82) | 0.59 |
| **MCP1** | 2157.54 (751.25 - 9332.38) | 3231.16 (794.97 - 11063.84) | 0.05 |
| **MCP3** | 1274.82 (-504.74 - 3520.09) | 1836.79 (83.63 - 4037.29) | 0.26 |
| **MCSF** | 2724.51 (-200.55 - 4127.27) | 3081.05 (-409.13 - 8061.79) | 0.25 |
| **MIF** | 6619.56 (1065.29 - 12992.92) | 7022.52 (369.59 - 19407.99) | 0.55 |
| **MIG** | 2084.94 (-268.46 - 4404.45) | 2127.64 (481.37 - 15786.9) | 0.48 |
| **MIP-1alpha/MIP1beta** | 1391.76 (-571.61 - 3057.44) | 1391.79 (81.12 - 4606.14) | 0.22 |
| **MIP-3alpha** | 2561.54 (-393.8 - 10403.33) | 2821.54 (109.88 - 8362.31) | 0.41 |
| **MIP-3beta** | 1980.85 (-374.38 - 4333.75) | 2280.02 (846.81 - 6045.94) | 0.28 |
| **MMP9** | 8363.26 (2273.17 - 18109.04) | 9420.06 (3209.73 - 22970.75) | 0.98 |
| **Myeloperoxidase** | 4766.7 (1861.64 - 33167.42) | 4492.98 (1235.11 - 12761.56) | 0.50 |
| **Osteopontin** | 15608.19 (9465.35 - 33994.09) | 15894.46 (5204.31 - 30831.62) | 0.75 |
| **PDGFAA** | 2316.44 (1251.81 - 4963.49) | 2805.37 (447.83 - 5311.86) | 0.12 |
| **PDGFABBB** | 1530.07 (182.89 - 2920.3) | 1829.22 (-41.28 - 4738.34) | 0.18 |
| **PF4** | 1489.96 (-192.15 - 4033.83) | 1860.17 (-821.14 - 4991.17) | 0.78 |
| **Pentraxin3** | 3475.01 (987.46 - 6535.68) | 3547.92 (1483.18 - 8820.68) | 1.00 |
| **RAGE** | 4411.79 (1685.99 - 7023.25) | 4472.82 (1984.88 - 12736.07) | 0.89 |
| **RANTES** | 2991.32 (569 - 7902.15) | 2420.64 (1025.46 - 7023.06) | 0.70 |
| **RBP4** | 9386.94 (2532.61 - 23813.47) | 11472 (2754.42 - 26495.73) | 0.18 |
| **Relaxin2** | 2090.34 (-255.67 - 7798.54) | 2770.35 (1118.04 - 7418.58) | 0.39 |
| **Resistin** | 7203.83 (2863.45 - 13776.72) | 7622.8 (2703.57 - 25114.59) | 0.67 |
| **SDF1alpha** | 5437.52 (2563.19 - 7692.14) | 5400.49 (3493.19 - 19856.26) | 0.42 |
| **SHBG** | 4461.96 (2169.69 - 8830.45) | 3654.37 (1415.49 - 8550.39) | 0.12 |
| **ST2** | 1172.2 (479.8 - 4446.31) | 1463.95 (79.05 - 5502.79) | 0.79 |
| **SerpinE1** | 5340.81 (2187.02 - 29638.76) | 4369.67 (1151.43 - 20561.24) | 0.11 |
| **TARC** | 2225.32 (1017.78 - 21906.91) | 2500.88 (1008.5 - 6720.02) | 0.48 |
| **TFF3** | 15924.66 (10220.83 - 26642.46) | 15279.78 (8316.21 - 30013.68) | 0.65 |

| | | | |
|---|---|---|---|
| **TGF-alpha** | 1504.92 (-108.34 - 5004.37) | 1943.2 (111.97 - 8168.12) | 0.95 |
| **TIM3** | 23561.22 (15471.65 - 32225.16) | 22492.43 (14714.98 - 34828.29) | 1.00 |
| **TNF-alpha** | 1447.59 (-244.79 - 5203.43) | 1503.74 (-175.58 - 5009.89) | 0.69 |
| **TfR** | 2316.85 (-94.04 - 3918.54) | 2055.1 (-491.69 - 7072.56) | 0.94 |
| **Thrombospondin1** | 4363.51 (1822.82 - 10482.45) | 3910.25 (1161.13 - 10886.06) | 0.72 |
| **VCAM1** | 23953.58 (14596.6 - 32280.43) | 22515.27 (8112.31 - 35893.66) | 0.66 |
| **VEGF** | 4289.55 (1800.81 - 7682.77) | 4734.84 (2054.4 - 10689.64) | 0.60 |
| **Vitamin DBP** | 10978.05 (3452.96 - 22953.48) | 8969.45 (2504.26 - 26460) | 0.55 |
| **uPAR** | 8844.3 (4889.69 - 12774.83) | 9047.54 (4861.75 - 25763.51) | 0.90 |

Figure 2. The median values of cytokines with significant changes over time.

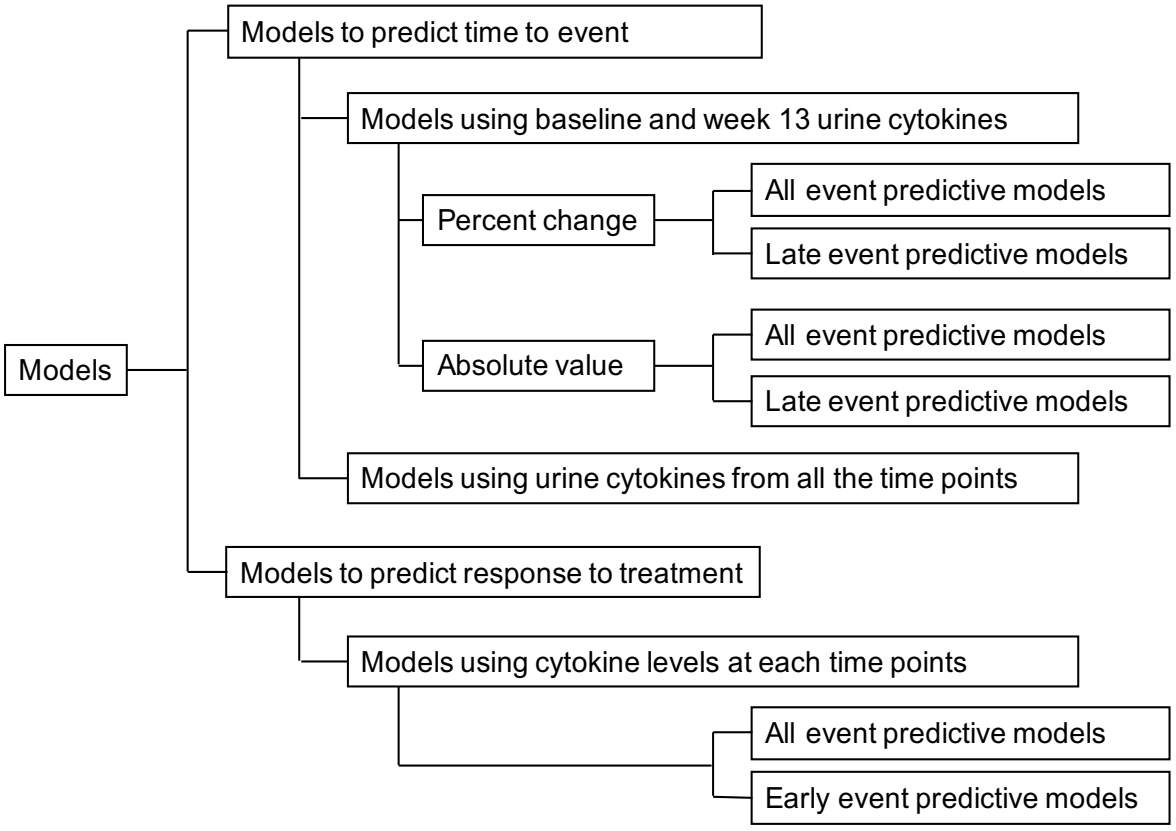
3.2. Models to predict time to event (Figure 3)

3.2.1. Models using baseline and week 13 urine cytokines

All event predictive model

Simple cox models using percent change of cytokines at week 13 from baseline were tested. A multivariable Cox model was constructed from smoking status and percent change of the following cytokines: insulin-like growth factor-binding protein (IGFBP), IL-18 binding protein-a (IL18BPa), IP-10, IL-3, platelet-derived growth factor AB/BB (PDGF-AB/BB), complement factor D, angiopoietin-1, IFN-gamma, IL-8, IL-15, IL-34, IL-23, TNF-alpha, and IL-13. In a multivariable Cox model (C-index 0.70), the percent change (for 100 units) of IL-18BPa (HR 1.995, 95% CI 1.157-3.438, p=0.01), IL-23 (HR 1.116, 95% CI 1.012-1.23, p=0.03), IL-8 (HR 0.273, 95% CI 0.069-1.082, p=0.06), and IP10 (HR 0.955, 0.914-0.997, p=0.04) at week 13 was predictors of time to failure. An additional model (C-index 0.74), was constructed using absolute values of cytokines at week 13 and baseline, which showed ever-smoker versus non-smoker status (HR 0.24, 95%CI 0.086-0.669) and urinary level (100 units) of ITAC (HR 0.949, 95% CI 0.915-0.985, p=0.005) and SHBG (1.056, 95% CI 1.018-1.096, p=0.004) as predictors of response.

Figure 3. Schematic illustration of models constructed to predict time to event or event.



Late event predictive models

To predict future events (recurrence/progression after 120 days), prognostic models were constructed using absolute values of cytokines at baseline and week 13 or percent changes of cytokines at week 13 from baseline. No marker remained significant predictor of response in multivariable Cox models.

3.2.2. Models using urine cytokines from all the time points

To explore the association between urine cytokine levels and event free survival, we treated cytokine levels as a time dependent covariates in simple and multivariable Cox model. Urine levels of IL-4, IL17A, Cystatin-C, IP10, ITAC, Myeloperoxidase, retinol binding protein-4 (RBP-4), resistin, SHBG, and VEGF were significant predictors of event free survival. In multivariable cox model (C-index 0.82), ever-smoker versus non-smoker status (HR 0.208, 95% CI 0.068-0.689, p=0.006) and higher urine levels (100 units) of IP10 (HR 0.978. 95% CI 0.963-0.995, p=0.01) and resistin (HR 0.984, 95% CI 0.968-0.999, p=0.04) were associated with improved event free survival. Higher levels of SHBG (HR 1.097, 95% CI 1.048-1.148, p<0.01) were associated with worse event free survival.

3.3 Models to predict response to treatment

Early event predictive models

Urinary cytokines at week 13 and baseline or percent change of cytokines at week 13 were used to predict the occurrence of recurrence or recurrence at first cystoscopic evaluation. In multivariable regression models, ever-smoker versus non-smoker (OR

0.16, 95%CI 0.02-1.03, p=0.053) and percent change (for 100 units) of IGFBP-2 (OR 4.44, 95%CI 1.13-17.44, p=0.033), and IL-8 (OR 0.19, 95%CI 0.03-1.34, p=0.096) were predictors of early events (ROC 0.79). Additional modeling using absolute values of urinary cytokines at week 13 and baseline showed ever-smoker versus non-smoker status (OR 0.10, 95%CI 0.01-0.84, p=0.034) and week 13 levels (for 100 units) of IGFBP-2 (OR 1.03, 95%CI 1.003-1.06, p=0.027), monocyte-chemotactic protein 3 (MCP-3, OR 0.84, 95%CI 0.72-0.97, p=0,02), and SHBG (OR 1.12, 95%CI 0.998-1.263, p=0.54) are associated with response status at first cystoscopic evaluation (ROC 0.88).

All event predictive models

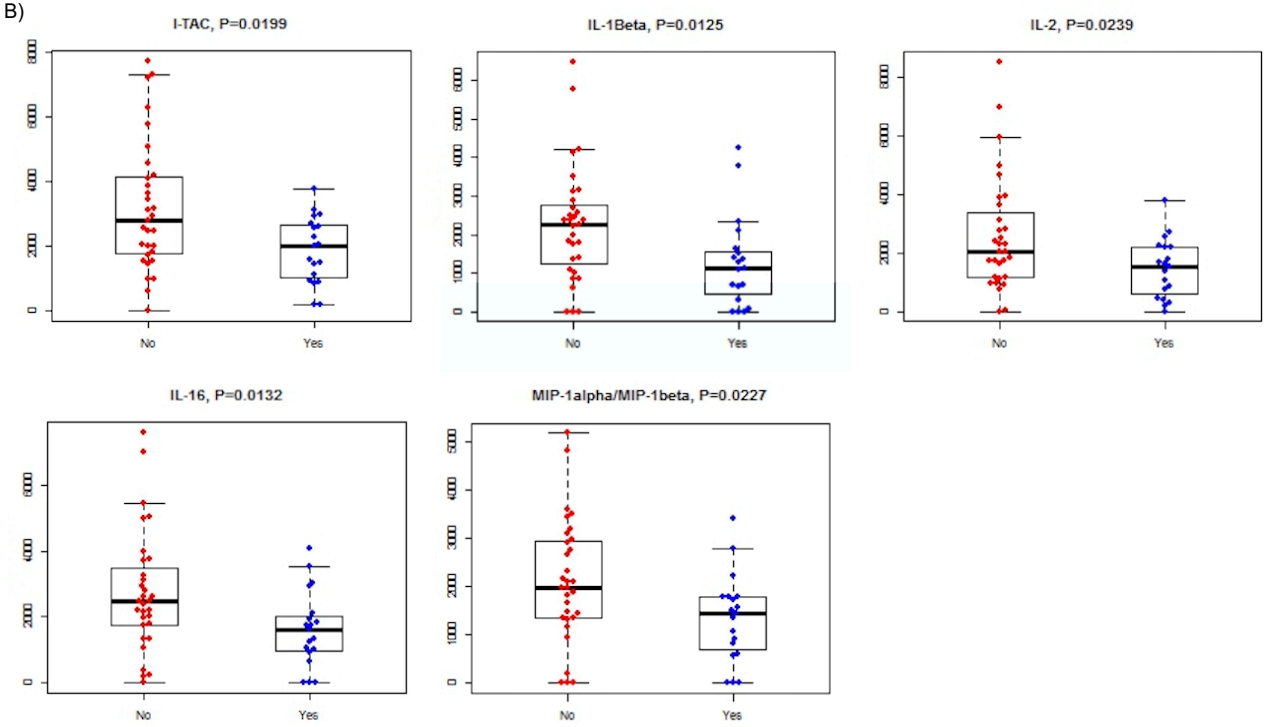
To adjust for modulatory effects of cytokines on each other, we used multiple classification analysis to report the performance of urinary cytokines at week 13. We found that lower levels of ITAC, IL-1b, IL2-, IL-16, and macrophage inflammatory protein (MIP-1a/MIP1-b) at week 13 were predictors of higher rate of recurrence or failure. Among classification modules, NB showed the most reliable results. NB allows us to analyze each marker separately if it can predict the class outcomes with high confidence.[15] AUC of this model was 0.76. Figure 4 shows discriminatory characteristics of this model (A) and distribution of these cytokines by event status (B).

Figure 4. Classification analysis of urinary cytokine at week 13 (ITAC, IL16, IL1b, IL2, MIP-1alpha/MIP-1beta). A. The discriminatory features of model using Naïve Bayes technique. B) Distribution of cytokines at week 13 by failure status. The negative values were imputed to zeros for these analyses. The results from dataset with and without imputation were similar.

A)

| Cohort | Sensitivity | Specificity | PPV | NPV | AUC |
|---|---|---|---|---|---|
| Full training dataset | 0.842 | 0.516 | 0.516 | 0.842 | 0.756 |
| 10-fold cross validation | 0.842 | 0.548 | 0.533 | 0.85 | 0.727 |

B)



## 4. Discussion:

In the current study, we evaluated the predictive value of a large panel of longitudinal urinary cytokines to predict response and disease outcome to immune treatment with intravesical BCG with or without HS-410 therapy in a cohort of patients with intermediate or high risk NMIBC. Our study has several important findings. First, we conclude that patients with a history of smoking responds better to immune treatment compared to non-smokers. Second, urinary cytokine levels of ITAC and SHBG at week 13 or change percent of IP10, IL-8, IL-23, and IL-18BP at week 13 from baseline could predict disease recurrence and/or progression. Third, urinary levels of IP10, resistin, and SHGB were associated with time to treatment failure. Finally, a panel of urinary cytokines (ITAC, IL-1b, IL-2, IL-16, and MIP-1a/MIP1-b for all events and IGFBP-2, MCP-3, and SHBG for early events) at week 13 was predictors of all events and events at first cystoscopic evaluation.

Among clinical and pathologic features, only positive history of smoking was associated with an improved response to therapy. This could be explained by an increased number of mutations and neo-antigens in the tumors of smokers, which is associated with a better response to immunotherapy in other organs.[16] Two large groups, Club Urologico Espano de Tratamiento (CUETO) and European Organization for Research of Cancer (EORTC), have reported prediction models of response using clinicopathologic data.[2, 3] In the CUETO study, female gender, recurrent disease, tumor multiplicity, and presence of concomitant CIS were associated with an increased risk of recurrence; high grade

tumors, T1 disease, and recurrence at 3-months cystoscopy were predictors of progression.[3] The EORTC group found tumor multiplicity and grade as predictors of recurrence, and tumor grade and stage as predictors of disease progression.[2] The discrepancy in our findings could be secondary to differences in sample size, study population, or treatment protocols among these studies. Moreover, Xylinas et al. evaluated the accuracy of these models and demonstrated a poor discrimination for disease recurrence and progression (0.597 and 0.662, and 0.523 and 0.616, respectively, for the EORTC and CUETO models).[17]

Immune response after intravesical BCG instillation involves recruitment and activation of various immune cells resulting in a cascade of cytokine secretion that favors a robust cytotoxic T helper-1 (Th1) response suppressing a less favorable T helper-2 (Th2) response.[8, 18] Therefore, urinary cytokines levels may reflect the local immune microenvironment after BCG and have been used in multiple studies as predictors of recurrence or progression.[5, 8] For example, urinary levels of IL-2 and IL-10 were used as indirect indicators of Th1 and Th2 responses, respectively.[19] Despite all these efforts, there is no validated urinary cytokine panel to predict response to BCG. In this study, for the first time, we evaluated the predictive value of a large panel of urinary cytokines at different time points to predict treatment failure in patients with intermediate and high risk NMIBC receiving immune treatment. Notably, we focused on cytokines at week 13 to assess the use of urinary cytokines to identify patients who may not benefit from further maintenance treatment.

In this cohort, we found that at week 13, the increased percent change of IL-18BPa and IL-23 from baseline in addition to decreased percent change of IP10 and IL-8 were predictors of treatment failure. Additionally, higher urinary levels of SHBG with lower levels of ITAC at week 13 were associated with worse failure free survival. In an alternate model, urinary levels of IP10, Resistin, and SHGB as a time dependent variables were associated with treatment failure. Moreover, smoking status in addition to urinary levels of IGFBP-2, MCP-3, and SHBG at week 13 were predictors of early events in this study. These markers are directly or indirectly involved in the generation of Th1 type or innate immune responses.

IL-18BPa is induced by IFN-gamma and has an inhibitory effect on IL18. IL18 increases expression of IL-8 and plays a central role in the Th1 induced immune response.[20] Likewise, IL-8 participates in innate and acquired immunity. It has been shown that elevated IL-8 or IL-18 expression in the first hours after BCG treatment is associated with longer disease-free survival in patients with NMIBC.[21] The cytokine IL-23 predominantly expressed by activated dendritic cells, has a proinflammatory role. It promotes tumor development and metastases by suppressing natural or cytokine-induced innate immunity.[22] Urothelial cells and endothelial cells also secrete IP10 in response to BCG, which acts as a chemoattractant for T cells, specifically for regulatory T cells (Tregs).[23] It has been shown that both increased and decreased urinary IP10 was associated with poor recurrence free survival.[24, 25] Further studies are needed to understand the role of IP10 in patients with bladder cancer.[26] ITAC has a pivotal role in mediating effector T cells and induce Th1 type immune responses.[27] Moreover, It has

been shown that ITAC-modified tumor cell vaccines can enhance anti-tumor immunity and reduce the incidence of disseminated metastasis.[27] Resistin, an adipokine, has been suggested as a prognostic biomarker for breast and colorectal cancer with conflicting results. [28, 29] It is secreted from monocytes and macrophages and is involved in insulin resistance, inflammation, and cell signaling. Likewise, IGFBP2, has been reported as an oncogenic marker in various cancers including bladder cancer. [30] It has been suggested that blockage of IGFBP2 may increase the sensitivity of bladder cancer cells to chemotherapy.[31]

SHBG modulates the bioavailability of sex hormones. There are few reports that have investigated the association between SHBG and cancers. For example, Cheng et al. reported that plasma levels of SHBG are significantly increased in patients with gastric cancer, or Huang et al. suggested that higher expression of SHBG in ovarian cancer is a poor prognostic factor. [32, 33] Being female gender has been reported as a poor prognostic factor in patients with NMIBC.[3, 34] These findings may suggest a diagnostic role for SHBG and sex hormones in patients with bladder cancer.

In exploratory analysis, we used classification technique (Figure 4) to adjust for modulatory effects of cytokines on each other, and found that lower levels of ITAC, IL-1b, IL-2, IL-16, and MIP-1a/MIP1-b at week 13 were predictors of higher recurrence rates or failure. IL-2 is secreted by activated $CD4^+$ T cells and stimulates growth, differentiation, and survival of cytotoxic lymphocytes among others. In multiple studies, higher levels of urine IL-2 after BCG administration were an indication of longer

recurrence free survival. [8, 19] Further studies are needed to investigate the role of IL-1b, MIP-1a/MIP-1b, and IL-16 in the immune response after BCG. Briefly, IL-16 is a lymphocyte chemoattractant factor for $CD4^+$ T cells, which primes these cell for IL-2 responsiveness.[35] Likewise, macrophage inflammatory proteins, MIP-1a and MIP-1b, attract and activate CD4+ and CD8+ T lymphocytes.[36] IL-1b is a proinflammatory cytokine, which is secreted by innate immune cells such as macrophages.[37]

This study has several limitations. First, though prospective, it is a relatively small study, 50 patients. Second, the large number of cytokines in addition to small sample size increase false discovery rate. Therefore, these models need to be externally validated. Third, tumor immune profile of patient with recurrence may be different from patients who progress. Third, there is a complex modulatory effect between cytokines and many cytokines have multiple roles in immune response. Additional pathway analysis may be needed to find the next generation of predictive biomarkers. Finally, some of patients in this cohort received the HS-410 vaccine in addition to intravesical BCG to boost the immune response. Therefore, our finding may not be applicable to patients with NMIBC receiving BCG only. Despite these limitations, this is the first study to suggest multiple models incorporating urinary cytokines to predict treatment responses to immune modulating agents in patients with intermediate and high risk NMIBC.

## 5. Conclusion:

Urinary cytokines provided additional value to clinicopathologic features to predict response to immune modulation in patients with intermediate and high risk NMIBC. Moreover, the predictive value of urinary cytokines was time dependent. Notably, a panel of cytokines measured at week 13 can be used to identify patients who will recur, and thus, has no benefit from further maintenance treatment. Further studies are needed to validate these findings.

APPENDICES

Statistical analysis:

Baseline characteristics and multiple measurements:

We used student T test and Wilcoxon rank sum test to compare quantitative characteristics between response groups. We used Chi-square or Fisher's exact test to compare categorical data. Ever-smoker status was defined as history of current or past smoking. We compared 105 urinary cytokine levels at baseline between patients with and without recurrence/progression. To adjust for multiple testing, the false discovery rate (FDR) approach was used. We used FDR level of 0.15 to control for erroneous findings from multiple measurements.

Cytokine changes over the time:

We used the Skillings–Mack test to evaluate whether cytokine levels changed over time. The Skillings–Mack test is similar to Friedman test when there are missing data. This test is suitable for non-parametric data, repeated measurement, and missing data. The median values of cytokines with significant changes over time in Figure 2. We used median since the urinary cytokines were not normally distributed.

Primary analysis:

The primary endpoint was time to the event: defined as progression or recurrence. We used multiple Cox models using urinary cytokines at different time points to predict time to the event. Schematic illustration of models is shown in Figure 3. For the model

construction, first we created the best possible model using clinical parameters. Then, we included the cytokines of interest (with p-value ≤ 0.15 in univariate Cox model) in the model in a stepwise manner to see if it significantly adds value to the prior model. The model selection depends on the questions needed to answered. We focused on week 13 time point since it is the time of first response evaluation after a course of induction therapy. Likewise, the week 13 data biomarkers would help to identify a population who may not respond to additional treatment (maintenance treatment). We constructed models to predict early (in first cystoscopic evaluation at 3 months), late (excluding events prior to day 120), and all events. We analyzed the urinary cytokines at different time points (base line, week 7, 13, 28, and end of treatment) as time-dependent variables. The concordance index (C-index) was used to evaluate the performance of these models. The C-index, similar to the area under a ROC curve, is a measure of goodness of fit for regression models.

Exploratory analysis using classification techniques:

As an exploratory analysis, we evaluated the predictive value of urinary cytokines at week 13 to predict treatment response (all event model) using classification techniques: logistic regression, Naïve Bayes (NB), decision tree, and random forests. In brief, top five cytokines with highest correlation with response status were selected using univariate and multivariable logistic regression. The negative values were imputed to zeros for these analyses. The performance of different classification models to predict events were calculated. To avoid model over-fitting and to test the generalizability of the

results, these performance measures were assessed by applying 10-fold cross validation.

The discriminatory features of model using logistic regression, Naïve Bayes, decision tree, and random forests are shown below.

| **ROC analysis** | | | | | |
|---|---|---|---|---|---|
| I-TAC, IL-16, IL-1beta, IL-2, MIP-1alpha/MIP-1beta | | | | | |
| **Full training dataset** | **Sensitivity** | **Specificity** | **PPV** | **NPV** | **AUC** |
| **Logistic regression** | 0.474 | 0.774 | 0.563 | 0.706 | 0.744 |
| **Naïve Bays** | 0.842 | 0.516 | 0.516 | 0.842 | 0.756 |
| **Decision Tree (J48)** | 0 | 1 | n/a | 0.62 | 0.5 |
| **Random forests** | 1 | 1 | 1 | 1 | 1 |
| | | | | | |
| **10-fold cross validation** | **Sensitivity** | **Specificity** | **PPV** | **NPV** | **AUC** |
| **Logistic regression** | 0.421 | 0.71 | 0.471 | 0.667 | 0.603 |
| **Naïve Bays** | 0.842 | 0.548 | 0.533 | 0.85 | 0.727 |
| **Decision Tree (J48)** | 0.263 | 0.806 | 0.455 | 0.641 | 0.52 |
| **Random forests** | 0.526 | 0.581 | 0.435 | 0.667 | 0.615 |

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