Impact of Molecular Androgen and Estrogen Response Scores on Outcomes Following Neoadjuvant Pembrolizumab in Muscle-Invasive Bladder Cancer

Published in:
Clinical Cancer Research : an official journal of the American Association for Cancer Research

Publication status and date:
Published: 15/08/2025

DOI (link to publisher):
10.1158/1078-0432.CCR-24-4312

Document Version
Publisher's PDF, also known as Version of record

Impact of Molecular Androgen and Estrogen Response Scores on Outcomes Following Neoadjuvant Pembrolizumab in Muscle-Invasive Bladder Cancer

Valentina Tateo[1], Joep J. de Jong[2], Antonio Cigliola[1,3], Brigida A. Maiorano[1], Chiara Mercinelli[1,3], Emanuele Crupi[1,3], Patrizia Giannatempo[4], Maurizio Colecchia[3,5], Marco Moschini[6], James A. Proudfoot[7], Daniele Santini[8], Elai Davicioni[7], Alberto Briganti[3,6], Francesco Montorsi[3,6], and Andrea Necchi[1,3]

ABSTRACT

Purpose: Androgen and estrogen influence bladder cancer pathogenesis, but their role in modulating treatment response, especially immunotherapy, remains unclear. This *post hoc* analysis of the PURE-01 trial evaluated the impact of androgen and estrogen signatures on outcomes in patients with muscle-invasive bladder carcinoma treated with neoadjuvant pembrolizumab followed by radical cystectomy.

Experimental Design: Pretreatment transurethral resection of bladder tumor samples from 102 PURE-01 patients was analyzed using the Decipher Bladder whole-transcriptome assay to classify genomic subtypes. Hallmark androgen response (ARS), early estrogen response (ERS-early), and late estrogen response (ERS-late) scores were derived and visualized with boxplots. Subgroup differences were assessed using Mann–Whitney U, χ^2, and Kruskal–Wallis tests. Event-free survival (EFS) was the primary endpoint, whereas pathologic response was the secondary endpoint. Cox proportional hazard models and Kaplan–Meier analyses evaluated the association of molecular scores with outcomes. Immune190 and FGFR3 signatures were also analyzed to explore their interactions with ARS and ERS.

Results: Luminal subtypes had the highest ARS and ERS scores across molecular classifications. Negative correlations were observed between Immune190 scores and ERS and ARS scores. ERS-late scores were highest in nonresponders and significantly associated with worse EFS. Conversely, ARS was not significantly linked to EFS. FGFR3 signature positively correlated with ARS and ERS and negatively with Immune190 scores.

Conclusions: The strong association of ERS-late scores with both pathologic response and EFS suggests that this signature could guide patient stratification and treatment selection. These findings support future trials combining immunotherapy with antiestrogens in both neoadjuvant and advanced urothelial carcinoma settings.

Introduction

In 2020, nearly 600,000 cases of bladder cancer were diagnosed worldwide, revealing a notable sex imbalance (1, 2), with bladder cancer ranking the sixth most common cancer in males and the 17th in females (1). Of note, this imbalance is also evident in disease stage and patient prognosis, with women often having more advanced and aggressive cancers at presentation, which are related to worse outcomes (2, 3). These sex-specific differences have led to speculations about the role of sex steroid hormones in the pathogenesis and treatment response of bladder cancer (3), which have been corroborated by emerging evidence highlighting the crucial roles of both androgen and estrogen pathways in driving bladder cancer. For example, the expression of androgen receptors (AR) and estrogen receptors (ER) in bladder cancer specimens have been assessed in several studies, showing their presence in most cases (4–6). Clinical and preclinical observations suggest a role for androgens in facilitating tumorigenesis in the bladder by stimulating urothelium and suppressing immunity (3, 4, 7). Furthermore, loss of AR expression and increase of ER expression were observed in more advanced disease stages, suggesting a dynamic and complex role for sex hormones throughout bladder cancer development (3). Lastly, transcriptome-wide expression analyses of 1,000 MIBC specimens revealed sex-based differences: tumors from female patients showed higher expression of basal and immune-related genes, while those from male patients exhibited higher levels of luminal marker gene expression (8). Moreover, androgen and estrogen signaling pathways exhibited significantly higher activity in luminal subtypes, with androgen signature enrichment being more pronounced in males compared with females exclusively within the luminal subtypes (8).

Another important factor to consider in the gender disparities in bladder cancer is FGFR3, which also plays a role in both pathogenesis and treatment response. *FGFR3* alterations are more prevalent in women, although they are globally more frequent in males

[1]Department of Medical Oncology, IRCCS Ospedale San Raffaele, Milan, Italy. [2]Department of Urology, Erasmus University Medical Center, Rotterdam, the Netherlands. [3]Vita-Salute San Raffaele University, Milan, Italy. [4]Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy. [5]Department of Pathology, IRCCS Ospedale San Raffaele, Milan, Italy. [6]Department of Urology, IRCCS Ospedale San Raffaele, Milan, Italy. [7]Veracyte Inc., South San Francisco, California. [8]Dipartimento Scienze e Biotecnologie Medico-Chirurgiche, Medical Oncology, La Sapienza Università di Roma, Rome, Italy.

V. Tateo and J.J. de Jong are co-first authors of this article.

Corresponding Author: Valentina Tateo, Department of Medical Oncology, IRCCS San Raffaele Hospital, Via Olgettina 60, Milan 20132, Italy. E-mail: tateo.valentina@hsr.it

Clin Cancer Res 2025;31:3503–11

doi: 10.1158/1078-0432.CCR-24-4312

Translational Relevance

Muscle-invasive bladder cancer is a highly lethal disease, with standard treatment consisting of neoadjuvant cisplatin-based chemotherapy followed by radical cystectomy. However, ~50% of patients are ineligible for cisplatin, and recurrence risk after surgery remains high. PURE-01, a phase II trial, evaluated neoadjuvant pembrolizumab followed by radical cystectomy, achieving a 42% pathologic complete response rate with excellent safety. However, ~40% of patients are nonresponders, highlighting the need for predictive biomarkers to refine patient selection. In this study, we investigated androgen and estrogen signatures, uncovering the complex interplay between sex–hormonal pathways and immunotherapy efficacy. Our results demonstrate that high estrogen response signature expression negatively impacts pembrolizumab efficacy. These findings pave the way for future trials combining immunotherapy with next-generation antiestrogens in biomarker-selected patients, aiming to reverse resistance, in both neoadjuvant and metastatic settings.

due to the higher incidence of bladder cancer in men (9). Preclinical evidence indicates that FGFR3 interacts with AR and ER activities, suggesting how these pathways may reciprocally influence the biological course of bladder cancer (10).

In the context of systemic immunotherapy, understanding interactions between elements of the tumor microenvironment (TME) and cancer cells is crucial for identifying biomarkers for predicting response to immune checkpoint inhibitors (ICI) and guiding treatment strategies (11–22). Moreover, sex hormones may play a critical role in determining the TME and sensitivity to treatments, which has already been shown in other tumor types (13–16). In MIBC, neoadjuvant immunotherapy—either alone or combined with other agents—has shown promising results, with pathologic complete response (pCR) rates comparable with or even higher than those of standard chemotherapy regimens (23–29). In the present study, we sought to explore the intricate relationship between the expression of AR- and ER-related molecular signatures and the response to ICI through a *post hoc* analysis of the PURE-01 trial, a single-arm phase II trial, evaluating three cycles of neoadjuvant pembrolizumab followed by radical cystectomy (RC) in patients with clinically (c) T2-4aN0M0 MIBC (23). We aimed to examine their implications in outcomes and response to ICI to provide biomarkers for patient stratification, treatment selection, and therapeutic development in the era of precision medicine.

Materials and Methods

Patient cohort

We analyzed transurethral resection of bladder tumor (TURBT) tissue samples from patients with MIBC included in the phase II, single-arm PURE-01 trial (NCT02736266). This trial evaluated the effect of three cycles of neoadjuvant pembrolizumab 200 mg every 3 weeks followed by RC in patients with cT2-4aN0M0 MIBC (23). The *post hoc* analysis presented in this article is part of a large biomarker project on MIBC (Associazione Italiana per la Ricerca sul Cancro Investigator Grant IG 2022 27746; NCT06341478). The study was performed after approval by an Institutional Review Board in accordance with Good Clinical Practice guidelines and the provisions of the Declaration of Helsinki. All patients provided written informed consent before enrollment.

Tissue sampling and gene expression profiling

Specimen collection and sample processing were conducted as previously described (30). In brief, transcriptome-wide analysis was performed on formalin-fixed, paraffin-embedded TURBT specimen using Decipher Bladder, a clinical-grade assay conducted in a Clinical Laboratory Improvement Amendments–certified laboratory (Veracyte). The test uses RNA extracted (RNeasy Kit, Qiagen) from formalin-fixed primary tumor (at least 0.5 mm^2 of high-grade tumor obtained from TURBT tissue samples). About 50 ng of RNA is used to generate a cDNA library (Ovation FFPE Kit, Tecan) that is hybridized to an oligonucleotide microarray (Human Exon 1.0 ST, Thermo Fisher Scientific). Microarrays were normalized, and genes were summarized using single-channel array normalization (31–36).

Gene expression analyses

Decipher Bladder genomic subtyping classifier (GSC) was used to generate subtypes [luminal, basal, claudin-low, infiltrated luminal, and neuroendocrine (NE)-like], based on previously validated signatures (31–35).

R package consensusMIBC was applied to classify tumors among The Cancer Genome Atlas (TCGA) 2017 and consensus molecular subtypes (31, 32). A previously validated, long noncoding RNA (lncRNA)-based genomic classifier was applied separately for identifying higher FGFR3 activity among luminal-type bladder cancer (34, 37). Of note, the classification of this model involves a separate transcriptomic risk score, meaning tumors could harbor higher lncRNA-based genomic classifier scores while not being classified as luminal by other classifiers. Boxplots were used to visualize differences between subgroups using hallmark gene sets from the MSigDB hallmark gene set collection (RRID: SCR_016863; ref. 38), including hallmark signature scores for androgen response (ARS), estrogen response early (ERS-early), and estrogen response late (ERS-late). ARS and both ERS (early and late) scores were computed by taking the mean expression of each gene in the set from the hallmarks of cancer gene sets in the MSigDB as previously described (38, 39).

Statistical analyses

Patient and tumor characteristics were compared between subgroups by Mann–Whitney U and χ^2 tests for continuous and categorical factors, respectively. Kruskal–Wallis tests were applied when comparing continuous variables across more than two groups. The primary endpoint was event-free survival (EFS), defined as the time from the initiation of pembrolizumab until radiographic disease progression preventing curative surgery, initiation of neoadjuvant chemotherapy (NAC), treatment-related side effects hindering RC, inability to complete curative surgery at the time of RC, local or distant recurrence after RC, or death from any cause. Secondary endpoints included pathologic response at RC categorized as no pathologic response, major pathologic response, or pCR. Univariable (UVA) and multivariable (MVA) Cox proportional hazard models evaluated the association of molecular scores, with covariable adjustment for patient age, sex, and clinical stage with HR and their associated confidence intervals reported. The Kaplan–Meier method was used to estimate differences in survival outcomes between subgroups of interest. The results of the correlation analyses were displayed using scatter plots.

Data availability

The data supporting the findings of this study have been deposited in the Gene Expression Omnibus data repository under accession number GSE298296. Additional raw data are available from the corresponding author (V. Tateo) upon reasonable request.

Results

Study cohort

The clinicopathologic characteristics of the cohort are reported in **Table 1**. The present *post hoc* analysis included patients with MIBC from the PURE-01 trial, for which complete transcriptomic and clinical data were available, resulting in a cohort size of 102 patients, including 15 females (14.7%). The study population comprised 50 (49%) cT2, 49 (48%) cT3, and three (3%) cT4a MIBC cases, in which cT is clinical stage, and the median patient age at TURBT was 67.3 years (IQR, 61.4–73.5 years). At RC, pCR was observed in 41 (40%) of patients, and 17 (16.7%) patients achieved a major pathologic response, which was defined as downstaging to at least pT1N0. The median follow-up after TURBT was 1.7 years (IQR, 1.2–2.3 years). Comparing patient sex revealed no significant differences in clinical or molecular variables. Interestingly, no differences in tumor-based ARS or ERS scores and tumor mutational burden (TMB) score were observed according to patient sex (**Table 1**).

Table 1. Clinicopathologic characteristics of the patient cohort.

	Female (n = 15)	Male (n = 87)	P value
Age (years); median (IQR)	68.3 (59.8–76.1)	67.3 (62.0–72.7)	0.98
Clinical stage; *n* (%)			
cT2	8 (53%)	42 (48%)	0.57
cT3	6 (40%)	43 (49%)	
cT4a	1 (7%)	2 (2%)	
GSC; *n* (%)			
Basal	7 (47%)	31 (36%)	0.67
Claudin-low	3 (20%)	11 (13%)	
Inf. luminal	3 (20%)	19 (22%)	
Luminal	2 (13%)	23 (26%)	
NE-like	0 (0%)	3 (3%)	
Consensus; *n* (%)			
Ba/Sq	8 (53%)	30 (34%)	0.64
LumNS	1 (7%)	16 (18%)	
LumP	3 (20%)	13 (15%)	
LumU	2 (13%)	18 (21%)	
NE-like	0 (0%)	3 (3%)	
Stroma-rich	1 (7%)	7 (8%)	
TCGA; *n* (%)			
Ba/Sq	6 (40%)	30 (34%)	0.52
Luminal	2 (13%)	16 (18%)	
Luminal-inf	6 (40%)	21 (24%)	
Luminal-pap	1 (7%)	16 (18%)	
Neuronal	0 (0%)	4 (5%)	
Pathologic response; *n* (%)			
Complete	7 (47%)	34 (39%)	0.71
Major	3 (20%)	14 (16%)	
No	5 (33%)	39 (45%)	
ARS; median (IQR)	0.90 (0.70–1.02)	0.89 (0.66–1.07)	0.91
ERS-early score; median (IQR)	−0.55 (−0.74 to −0.36)	−0.52 (−0.64 to −0.35)	0.41
ERS-late score; median (IQR)	−0.38 (−0.56 to −0.27)	−0.33 (−0.47 to −0.20)	0.31
TMB; median (IQR)	11.4 (6.1–13.6)	10.3 (6.1–15.0)	0.74

Abbreviations: Ba/Sq, basal/squamous subtype; Inf. luminal, infiltrated luminal; Luminal-inf, luminal infiltrated; Luminal-pap, luminal papillary; LumNS, luminal nonspecified; LumP, luminal papillary; LumU, luminal unstable; n, number; TCGA, TCGA molecular classification.

Molecular subtyping and androgen/estrogen response scores

Application of GSC, consensus, and TCGA subtyping models revealed a GSC luminal subtype in 24.5% (25/102), a consensus luminal-papillary subtype in 15.6% (16/102), and a TCGA luminal-papillary subtype in 16.7% (17/102) of patients (**Table 1**). Notably, no tumors in female subjects were classified as belonging to the neuronal/NE-like molecular subtype. Evaluating molecular ARS and ERS scores, we found the highest median scores for luminal subtypes across all three major molecular classifications, whereas the lowest median response scores were observed for basal/squamous (consensus/TCGA), claudin-low (GSC), and neuronal/NE-like molecular subtypes (**Fig. 1**).

ERS scores negatively correlate with immune scores

Initial analyses from PURE-01 revealed a significant association between favorable outcomes and molecular Immune190 signature scores—a signature composed of 190 immune-related genes providing insights into tumor immunogenicity (30). Based on this, we correlated Immune190 scores with androgen/estrogen response scores, revealing a moderate but significant negative correlation between the Immune190 scores and both the ERS scores (ERS-early: correlation = −0.64, $P < 0.001$; ERS-late: correlation = −0.6, $P < 0.001$). Furthermore, we found a significant but weak, negative correlation between Immune190 and ARS scores (correlation = −0.22, $P = 0.027$). Supplementary Figure S1 depicts scatter plots for correlation between Immune190 scores and ARS, ERS-early, and ERS-late scores.

pCR at RC following neoadjuvant pembrolizumab

Comparison of pathologic response categories following neoadjuvant pembrolizumab revealed no association between baseline molecular subtyping classifications and pathologic response (Supplementary Table S1). Of interest, we found that ARS and ERS scores were highest in patients who did not achieve a pCR, revealing a significant association for ERS-late scores when comparing complete responders, major responders, and nonresponders (**Fig. 2**). On MVA adjusting for baseline clinical tumor stage, patient age, and sex, we also found ERS-late scores to be significantly associated with pCR ($P = 0.01$), whereas ARS scores were associated with pCR on UVA ($P = 0.042$) but not MVA ($P = 0.13$; **Table 2**). We repeated the MVA with TMB adjustment, but no significant changes were observed (Supplementary Table S2).

Higher ERS scores are associated with worse EFS following neoadjuvant pembrolizumab

Next, we evaluated the association between EFS outcomes and androgen/estrogen response scores. We found that ARS scores were not significantly associated with EFS outcomes, whereas both ERS-early and ERS-late continuous scores were significantly associated with EFS on MVA ($P = 0.014$ and 0.02, respectively, **Table 3**). To depict survival differences using Kaplan–Meier curves, we evaluated quantile/tertile splits among response signatures for which the continuous score was significantly associated with EFS (**Fig. 3**) on MVA, illustrating worse

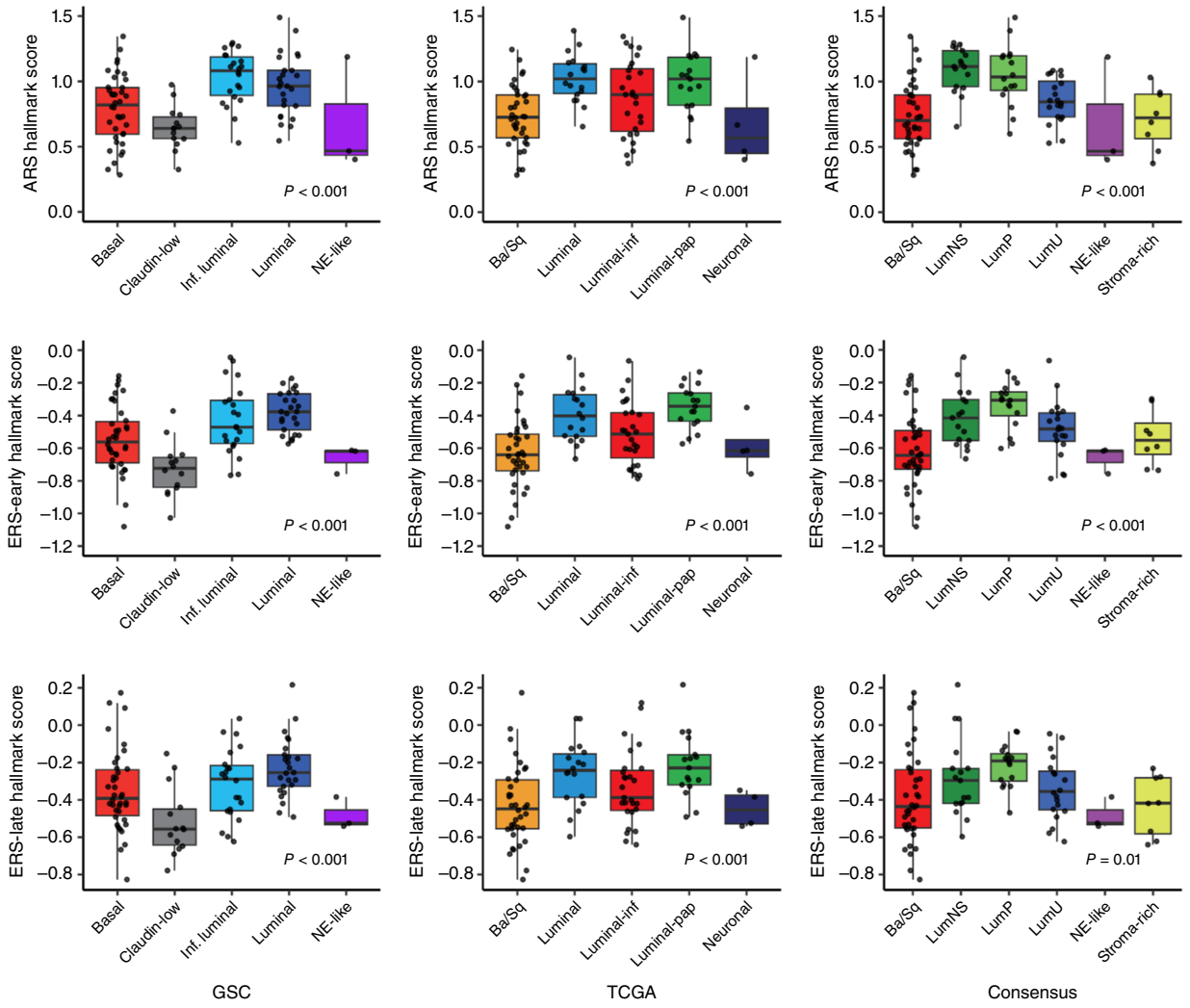

Figure 1.

Evaluation of molecular ARS and ERS scores across molecular subtyping models. From top to bottom, box plots display the scores for the ARS, ERS-early, and ERS-late signatures across molecular subtypes. From left to right, the molecular classification systems used are GSC, TCGA, and consensus models. Across all three classification systems, luminal subtypes consistently exhibit the highest median scores for ARS, ERS-early, and ERS-late signatures. Ba/Sq, basal/squamous subtype; Inf. luminal, infiltrated luminal; Luminal-inf, luminal infiltrated; Luminal-pap, luminal papillary; LumNS, luminal nonspecified; LumP, luminal papillary; LumU, luminal unstable.

outcomes for higher molecular scores. Notably, the effect of AR on EFS seems to be nonlinear, suggesting a potential violation of the proportional hazards assumption. We repeated the MVA and Kaplan–Meier analyses with TMB adjustment, but no significant changes were observed (Supplementary Fig. S2; Supplementary Table S3). Furthermore, we explored the association between molecular subtypes, specifically luminal versus nonluminal, and EFS. Whereas the difference was not statistically significant ($P = 0.66$), we observed a trend toward worse outcomes in patients with luminal tumors treated with neoadjuvant pembrolizumab (Supplementary Fig. S3).

lncRNA-based FGFR3 activity is correlated with androgen/estrogen response scores

To interrogate FGFR3 activity, we applied a previously developed signature to quantify FGFR3 activity (37, 40). Whereas in initial PURE-01 analyses, we did not find an association with response to pembrolizumab (41), present analyses with more follow-up data did find a significant association with EFS outcomes on UVA but not MVA (Supplementary Table S4). Notably, we repeated the MVA adjusting for TMB also, but no significant changes were observed (Supplementary Table S5). As such, we used quantile/tertile splits illustrating higher scores were associated with worse outcomes following pembrolizumab, and correlating FGFR3 activity with ARS and ERS found significant positive correlations (ARS: correlation = 0.61, $P < 0.001$; ERS-early: correlation = 0.44, $P < 0.001$; ERS-late: correlation = 0.22, $P = 0.026$), whereas FGFR3 activity was inversely correlated with Immune190 scores (correlation = −0.37, $P < 0.001$; Supplementary Fig. S4).

Discussion

Identifying biomarkers to predict treatment response is essential in the context of neoadjuvant immunotherapy and remains an area

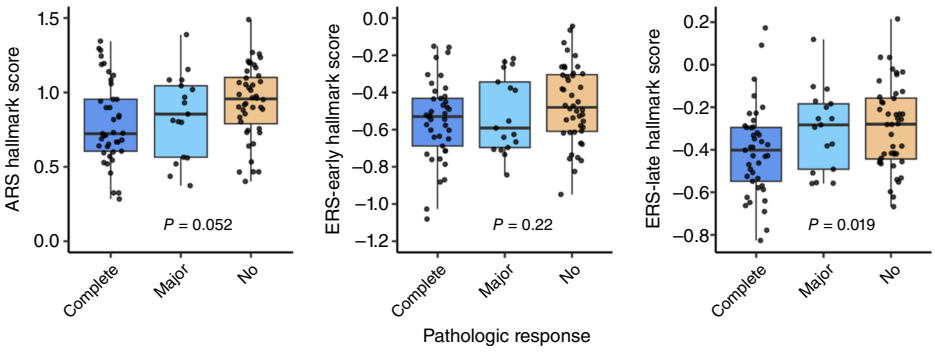

Figure 2.
Evaluation of molecular ARS and ERS scores across pathologic response categories. From left to right, box plots display the scores for the ARS, ERS-early, and ERS-late signatures according to the pathologic response. Higher scores for both ARS and ERS signatures are observed in patients who did not achieve a pCR, with a statistically significant association noted for the ERS-late signature.

of ongoing research. Considering the epidemiology and clinical behavior of bladder cancer and drawing on emerging evidence from other cancer types, we hypothesized that sex hormones might significantly modulate the TME and influence immunotherapy efficacy in MIBC. Specifically, we explored whether sex steroid hormones play a role in MIBC response to neoadjuvant pembrolizumab. This *post hoc* analysis of the PURE-01 trial focused on the role of AR and ER signaling pathways in predicting clinical outcomes after neoadjuvant treatment.

The population analyzed in this study reflects the typical distribution of MIBC (2, 3), showing a marked sex imbalance, with males comprising approximately 85% of the sample. Notably, no differences in ARS and ERS-early and ERS-late scores were observed between sexes, reinforcing previous evidence that androgen and estrogen pathways may contribute to bladder cancer pathogenesis in both males and females (42, 43). In contrast to previous observations (8), we did not find significant differences in the distribution of molecular subtypes based on gender. Notably, the reduced total number of female patients enrolled in our study may indicate reduced power to confirm this finding. However, significant differences regarding ARS, ERS-early, and ERS-late scores emerged among molecular subtypes, with luminal tumors displaying higher median response scores compared with other subtypes across all three major molecular classification systems. This finding further supports existing evidence of molecular similarities between luminal subtypes in breast and bladder cancers (31). Additionally, we observed a negative correlation between androgen and estrogen signaling and the Immune190 signature. Of particular interest were the findings for ERS-early and ERS-late, which exhibited a significant moderate negative correlation with the Immune190 signature, indicating that higher ERS scores were associated with lower Immune190 scores. A significant association between favorable outcomes with neoadjuvant immunotherapy and molecular Immune190 signature scores was already reported (30). In this context, tumors expressing higher levels of ARS and, especially, ERS scores—which are associated with lower levels of Immune190 scores—may demonstrate reduced responsiveness to neoadjuvant pembrolizumab. This hypothesis was supported by our analyses of association with pathologic response and EFS. Whereas we did not find an association between baseline molecular subtype classifications and pathologic response, we observed that ARS and ERS scores were highest in patients who did not achieve a pCR. Specifically, significantly lower ERS-late scores were seen in tumors from patients who achieved a pCR after neoadjuvant pembrolizumab compared with those with major or no response. This finding was further validated after adjusting for confounders in the MVA. Additionally, we found that both ERS-early and ERS-late score levels affected survival outcomes, with higher scores correlating with poorer EFS. These observations were further confirmed at the MVA.

Our findings in this study are supported by a substantial body of evidence highlighting the critical role of sex hormones in carcinogenesis, tumor progression, and therapeutic response (13–16). This influence extends beyond classic hormone-dependent cancers, such as breast and prostate cancers, to encompass other malignancies, including lung and bladder cancers (4, 13). Androgens and estrogens exert their oncogenic effects by enhancing the transcription of genes related to cell survival and proliferation. More intriguingly, they also impact the TME, promoting immunosuppressive actions that facilitate tumor growth and contribute to treatment resistance (4, 13, 16). Previous studies have investigated the role of molecular subtypes on

Table 2. UVA and MVA logistic regression model results for pCR following neoadjuvant pembrolizumab for androgen and estrogen responses.

	UVA		MVA					
	OR (95% CI)	***P* value**	**OR (95% CI)**	***P* value**	**OR (95% CI)**	***P* value**	**OR (95% CI)**	***P* value**
AR (per 0.1)	0.85 (0.73–0.99)	0.04*	0.88 (0.75–1.04)	0.13	—	—	—	—
ERS-early (per 0.1)	0.85 (0.70–1.03)	0.10	—	—	0.87 (0.71–1.06)	0.17	—	—
ERS-late (per 0.1)	0.75 (0.60–0.93)	0.009*	—	—	—	—	0.74 (0.59–0.93)	0.01*
Female vs. male	1.36 (0.45–4.11)	0.58	1.35 (0.42–4.33)	0.61	1.23 (0.38–4.01)	0.73	1.10 (0.33–3.72)	0.88
Age at TURBT (per 5 years)	1.07 (0.86–1.33)	0.54	1.09 (0.86–1.38)	0.46	1.09 (0.86–1.38)	0.48	1.12 (0.88–1.42)	0.36
Clinical stage cT3/4 vs. cT2	0.31 (0.14–0.72)	0.006*	0.35 (0.15–0.83)	0.02*	0.33 (0.14–0.75)	0.009*	0.31 (0.13–0.73)	0.007*

Abbreviation: CI, confidence interval.
*Indicates statistical significance.

Table 3. UVA and MVA Cox proportional hazard model results for EFS following neoadjuvant pembrolizumab for androgen and estrogen responses.

	UVA		MVA					
	HR (95% CI)	***P* value**	**HR (95% CI)**	***P* value**	**HR (95% CI)**	***P* value**	**HR (95% CI)**	***P* value**
AR (per 0.1)	1.10 (0.95–1.26)	0.19	1.05 (0.91–1.21)	0.52	—	—	—	—
ERS-early (per 0.1)	1.29 (1.07–1.55)	0.008*	—	—	1.27 (1.05–1.54)	0.01*	—	—
ERS-late (per 0.1)	1.21 (1.03–1.43)	0.02*	—	—	—	—	1.22 (1.03–1.45)	0.02*
Female vs. male	0.38 (0.09–1.61)	0.19	0.38 (0.09–1.62)	0.19	0.39 (0.09–1.67)	0.20	0.40 (0.09–1.70)	0.21
Age at TURBT (per 5 years)	1.08 (0.87–1.34)	0.48	1.09 (0.88–1.34)	0.42	1.12 (0.91–1.38)	0.30	1.09 (0.89–1.35)	0.40
Clinical stage cT3/4 vs. cT2	3.27 (1.38–7.75)	0.007*	3.04 (1.26–7.35)	0.01*	3.08 (1.30–7.31	0.01*	3.45 (1.45–8.21)	0.005*

Abbreviation: CI, confidence interval.
*Indicates statistical significance.

treatment sensitivity; however, to our knowledge, this is the first report providing data on response and survival in MIBC based on the expression levels of estrogenic and androgenic activity signatures. As previously described (44) and confirmed in our dataset, luminal tumors are enriched in ERS and ARS. Prior studies reported that molecular subtypes are not associated with pathologic response in patients treated either with NAC and neoadjuvant pembrolizumab (30, 33). In terms of survival, luminal tumors had better overall survival compared with nonluminal tumors, and the addition of NAC did not significantly impact outcomes (33), whereas nonluminal tumors derived greater benefit from NAC, with a 10% improvement in overall survival at 2 years (45). Conversely, in patients treated with neoadjuvant pembrolizumab, claudin-low tumors were associated with improved survival, as already reported (30). In this study, we analyzed differences in EFS by stratifying the target population into luminal and nonluminal tumors. We observed a worse outcome for luminal MIBC in the PURE-01 trial, although the differences were not statistically significant, whereas ERS-late was found to significantly influence EFS.

To explore additional factors that may influence responsiveness to neoadjuvant pembrolizumab and their potential interactions with androgen and estrogen pathways, we also examined the FGFR3 activity signature. We found that higher FGFR3 scores were associated with poorer EFS, although this association was not confirmed in the MVA. Additionally, FGFR3 activity exhibited a positive correlation with androgen and estrogen scores, particularly with ARS, whereas it demonstrated an inverse correlation with Immune190 scores. The association between FGFR3 activity and response scores, in particular ARS, suggests that these pathways may share common molecular mechanisms driving tumor progression and treatment resistance. Preclinical evidence further supports this observation; murine models have shown that FGFR3-altered tumors, both in non-MIBC (NMIBC) and MIBC, exhibit higher AR and lower ESR1 regulon activity (10).

To account for other potential confounding factors, we repeated the MVA and survival analyses including TMB. In PURE-01 pretreatment, tumors with TMB scores ≥15 mut/Mb were associated with higher rates of pT0 responses to pembrolizumab. Additionally, TMB is an agnostic biomarker of immunotherapy benefit (46). However, in our analyses, adjusting for TMB did not significantly change the results, suggesting that the influence of hormonal signatures on immunotherapy efficacy is independent of TMB status.

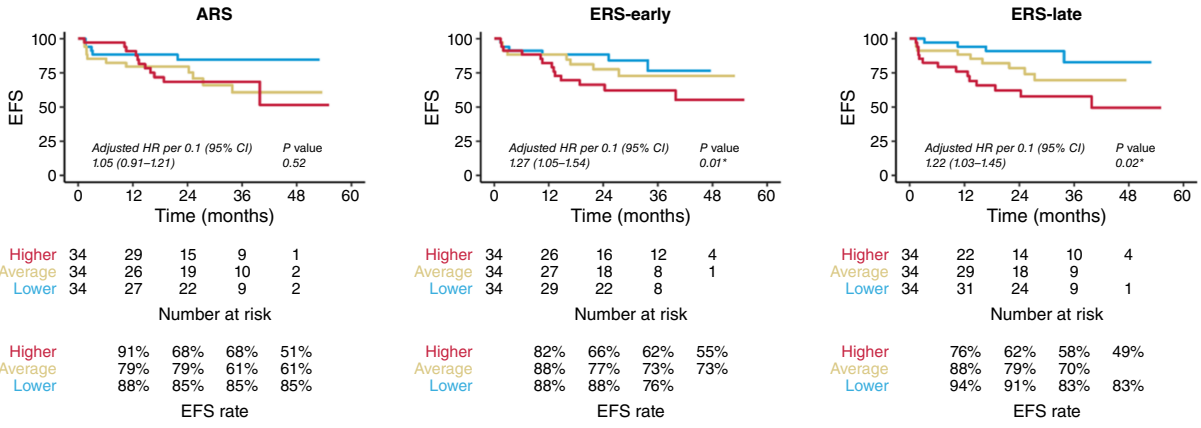

Figure 3.
Kaplan–Meier plots for EFS with ARS and ERS signatures split into tertiles. From left to right, the plots show the survival curves according to the scores of the ARS, ERS-early, and ERS-late signatures. Higher molecular signature scores are associated with worse EFS, reaching statistical significance for both ERS-early and ERS-late signatures. CI, confidence interval.

Our findings suggest that androgen and estrogen signaling significantly influence pathologic response and survival outcomes in patients treated with neoadjuvant pembrolizumab and RC. ERS, especially ERS-late, has emerged as a potential predictive biomarker for the benefits of neoadjuvant immunotherapy in MIBC. Whereas ARS scores showed some association with pathologic response in UVA, they lost significance in MVA and did not correlate with EFS. The lack of a significant association between ARS and EFS could be partially attributed to the limited cohort size but also suggests that AR activity alone is not a dominant driver of ICI resistance in MIBC. This finding is further supported by its low negative correlation with Immune190 scores, indicating a weak link between androgen signaling and ICI resistance. Androgen activity seems more closely linked to FGFR3 activity, which also shows a modest negative correlation with Immune190 scores. Whereas FGFR3 activity was significantly associated with EFS in UVA, this was not confirmed in MVA, suggesting that FGFR3 and AR activities may have a less dominant role in ICI resistance or that their influence is modulated by other molecular pathways. Conversely, estrogen activity, particularly ERS-late, seems to play a more central and independent role in influencing ICI efficacy in MIBC. This is supported by its significant association with both pCR and EFS, as well as a stronger negative correlation with Immune190 scores, indicating a more pronounced immunosuppressive effect. These results highlight a complex interplay between hormonal signaling and immune evasion in bladder tumors, consistent with findings from other cancer types.

Antiandrogen therapies are currently under investigation in bladder cancer. A phase I/Ib trial of enzalutamide in combination with gemcitabine and cisplatin tested in 10 patients with metastatic bladder cancer showed no toxicity issues, four partial responses, and a complete response in a female with strong AR expression (47). The phase II BicaBCa trial (NCT05327647) is currently active and recruiting patients with NMIBC who are randomized to receive Bacillus Calmette–Guérin with or without bicalutamide for the six cycles of induction. The TASUC-Neo trial (NCT05839119) combines degarelix with neoadjuvant standard-of-care chemotherapy in AR-expressing muscle-invasive disease. The TASUC-Neo trial is currently active and enrolling patients. Preclinical evidence supports the role of antiestrogen drugs in enhancing the efficacy of Bacillus Calmette–Guérin *in vitro* and in mouse models with NMIBC (48). Two phase 2 trials are investigating ER signaling in bladder cancer. One (NCT02197897) tested tamoxifen in patients with primary or recurrent low/intermediate-risk papillary urothelial carcinoma of the bladder, but no efficacy results have been reported. Another study (NCT00710970) assessed tamoxifen monotherapy in patients with pretreated metastatic urothelial carcinoma but showed limited activity (49).

To our knowledge, no trials combining antihormonal therapies with immunotherapy are currently ongoing in bladder cancer. In our opinion, the findings of our study support the design of future trials exploring the combination of immunotherapy with hormonal therapies, particularly new antiestrogen drugs, in urothelial carcinoma, both in neoadjuvant and advanced settings.

The limitations of this study include its unplanned retrospective exploratory design, the limited number of patients included, particularly the very small number of female patients enrolled—reflecting both the epidemiology of bladder cancer and the historic underrepresentation of females in clinical trials—and the omission of other possible confounders that could affect the activity and efficacy of pembrolizumab in MIBC. Furthermore, we acknowledge another limitation as the unavailability of histologic material prevents us from evaluating AR and ER protein expression by IHC, although the signatures analyzed in this study have already been biologically validated (38).

Conclusions

In summary, our study suggests an intricate relationship between sex hormone receptor signaling, molecular subtypes, and treatment outcomes in MIBC. The robust associations of ERS-late scores with both pathologic response and EFS suggest that this signature may serve as a valuable biomarker for patient stratification and treatment selection in the era of precision medicine. Further research into the interactions between sex hormones, the TME, and immunotherapy responses is essential for optimizing therapeutic strategies and improving outcomes in MIBC. Continued exploration in this field may lead to innovative treatment approaches that enhance the efficacy of immunotherapy in bladder cancer, such as the combination of sex hormone–modulating agents with immunotherapy.

Authors' Disclosures

V. Tateo reports personal fees from Bayer S.p.A., personal fees and nonfinancial support from Johnson & Johnson, nonfinancial support from Ipsen S.p.A., and nonfinancial support from Merck Serono outside the submitted work. J.J. de Jong reports personal fees from Veracyte during the conduct of the study. B.A. Maiorano reports grants and personal fees from Merck and personal fees from Bayer, Johnson & Johnson, and Bristol Myers Squibb outside the submitted work. C. Mercinelli reports personal fees from Astellas and nonfinancial support from Ipsen and Johnson & Johnson outside the submitted work. P. Giannatempo reports personal fees from MSD, Pfizer, Merk, Astellas, and Janssen and grants from AstraZeneca and Ipsen during the conduct of the study. J.A. Proudfoot reports other support from Veracyte outside the submitted work. E. Davicioni reports other support from Veracyte during the conduct of the study. A. Necchi reports grants and personal fees from Merck; nonfinancial support from Veracyte during the conduct of the study; grants and personal fees from Gilead and Bristol Myers Squibb; and personal fees from EMD Serono, Johnson & Johnson, Pfizer, AstraZeneca, Astellas, Bicycle Therapeutics, and Daiichi Sankyo outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

V. Tateo: Conceptualization, writing-original draft, project administration. **J.J. de Jong:** Data curation, formal analysis, writing–original draft. **A. Cigliola:** Writing–review and editing. **B.A. Maiorano:** Writing–review and editing. **C. Mercinelli:** Writing–review and editing. **E. Crupi:** Writing–review and editing. **P. Giannatempo:** Writing–review and editing. **M. Colecchia:** Writing–review and editing. **M. Moschini:** Writing–review and editing. **J.A. Proudfoot:** Software, formal analysis. **D. Santini:** Conceptualization. **E. Davicioni:** Supervision, project administration, writing–review and editing. **A. Briganti:** Writing–review and editing. **F. Montorsi:** Writing–review and editing. **A. Necchi:** Conceptualization, supervision, project administration, writing–review and editing.

Acknowledgments

A. Necchi was supported by Associazione Italiana per la Ricerca sul Cancro Investigator Grant IG 2022 27746; NCT06341478.

Note

Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Received December 22, 2024; revised February 26, 2025; accepted June 13, 2025; posted first June 20, 2025.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021;71:209–49.
2. Dyrskjøt L, Hansel DE, Efstathiou JA, Knowles MA, Galsky MD, Teoh J, et al. Bladder cancer. Nat Rev Dis Primers 2023;9:58.
3. Dobruch J, Daneshmand S, Fisch M, Lotan Y, Noon AP, Resnick MJ, et al. Gender and bladder cancer: a collaborative review of etiology, biology, and outcomes. Eur Urol 2016;69:300–10.
4. Chen J, Huang C-P, Quan C, Zu X, Ou Z, Tsai Y-C, et al. The androgen receptor in bladder cancer. Nat Rev Urol 2023;20:560–74.
5. Goto T, Miyamoto H. The role of estrogen receptors in urothelial cancer. Front Endocrinol (Lausanne) 2021;12:643870.
6. Sato N, Ise K, Hata S, Yamashita S, Ito A, Sasano H, et al. Clinicopathological significance of estrogen receptor β and estrogen synthesizing/metabolizing enzymes in urothelial carcinoma of urinary bladder. Pathol Oncol Res 2021;27:589649.
7. Xiang P, Du Z, Hao Y, Guan D, Liu D, Yan W, et al. Impact of androgen suppression therapy on the risk and prognosis of bladder cancer: a systematic review and meta-analysis. Front Oncol 2021;11:784627.
8. de Jong JJ, Boormans JL, van Rhijn BWG, Seiler R, Boorjian SA, Konety B, et al. Distribution of molecular subtypes in muscle-invasive bladder cancer is driven by sex-specific differences. Eur Urol Oncol 2020;3:420–3.
9. Guercio BJ, Sarfaty M, Teo MY, Ratna N, Duzgol C, Funt SA, et al. Clinical and genomic landscape of FGFR3-altered urothelial carcinoma and treatment outcomes with erdafitinib: a real-world experience. Clin Cancer Res 2023;29:4586–95.
10. Shi MJ, Fontugne J, Moreno-Vega A, Meng X-Y, Groeneveld C, Dufour F, et al. FGFR3 mutational activation can induce luminal-like papillary bladder tumor formation and favors a male sex bias. Eur Urol 2023;83:70–81.
11. Abramenko N, Vellieux F, Veselá K, Kejík Z, Hajduch J, Masařík M, et al. Investigation of the potential effects of estrogen receptor modulators on immune checkpoint molecules. Sci Rep 2024;14:3043.
12. Ide H, Miyamoto H. Sex hormone receptor signaling in bladder cancer: a potential target for enhancing the efficacy of conventional non-surgical therapy. Cells 2021;10:1169.
13. Anobile DP, Salaroglio IC, Tabbò F, La Vecchia S, Akman M, Napoli F, et al. Autocrine 17-β-Estradiol/Estrogen receptor-α loop determines the response to immune checkpoint inhibitors in non-small cell lung cancer. Clin Cancer Res 2023;29:3958–73.
14. Oner G, Broeckx G, Van Berckelaer C, Zwaenepoel K, Altintas S, Canturk Z, et al. The immune microenvironment characterisation and dynamics in hormone receptor-positive breast cancer before and after neoadjuvant endocrine therapy. Cancer Med 2023;12:17901–13.
15. Hargrove-Wiley E, Fingleton B. Sex hormones in breast cancer immunity. Cancer Res 2023;83:12–9.
16. Rothenberger NJ, Somasundaram A, Stabile LP. The role of the estrogen pathway in the tumor microenvironment. Int J Mol Sci 2018;19:611.
17. Lutz CT, Livas L, Presnell SR, Sexton M, Wang P. Gender differences in urothelial bladder cancer: effects of natural killer lymphocyte immunity. J Clin Med 2021;10:5163.
18. Korpal M, Puyang X, Jeremy Wu Z, Seiler R, Furman C, Oo HZ, et al. Evasion of immunosurveillance by genomic alterations of PPARγ/RXRα in bladder cancer. Nat Commun 2017;8:103.
19. Tripathi A, Gupta S. Androgen receptor in bladder cancer: a promising therapeutic target. Asian J Urol 2020;7:284–90.
20. Sun A, Luo Y, Xiao W, Zhu Z, Yan H, Miao C, et al. Androgen receptor transcriptionally inhibits programmed death ligand-1 expression and influences immune escape in bladder cancer. Lab Invest 2023;103:100148.
21. Besançon M, Gris T, Joncas F-H, Picard V, Bergeron A, Fradet Y, et al. Combining antiandrogens with immunotherapy for bladder cancer treatment. Eur Urol Open Sci 2022;43:35–44.
22. Liu Q, You B, Meng J, Huang CP, Dong G, Wang R, et al. Targeting the androgen receptor to enhance NK cell killing efficacy in bladder cancer by modulating ADAR2/circ_0001005/PD-L1 signaling. Cancer Gene Ther 2022;29:1988–2000.
23. Necchi A, Anichini A, Raggi D, Briganti A, Massa S, Lucianò R, et al. Pembrolizumab as neoadjuvant therapy before radical cystectomy in patients with muscle-invasive urothelial bladder carcinoma (PURE-01): an open-label, single-arm, phase II study. J Clin Oncol 2018;36:3353–60.
24. Powles T, Kockx M, Rodriguez-Vida A, Duran I, Crabb SJ, Van Der Heijden MS, et al. Clinical efficacy and biomarker analysis of neoadjuvant atezolizumab in operable urothelial carcinoma in the ABACUS trial. Nat Med 2019;25:1706–14.
25. van Dijk N, Gil-Jimenez A, Silina K, Hendricksen K, Smit LA, de Feijter JM, et al. Preoperative ipilimumab plus nivolumab in locoregionally advanced urothelial cancer: the NABUCCO trial. Nat Med 2020;26:1839–44.
26. Galsky MD, Daneshmand S, Izadmehr S, Gonzalez-Kozlova E, Chan KG, Lewis S, et al. Gemcitabine and cisplatin plus nivolumab as organ-sparing treatment for muscle-invasive bladder cancer: a phase 2 trial. Nat Med 2023;29:2825–34.
27. Rose TL, Harrison MR, Deal AM, Ramalingam S, Whang YE, Brower B, et al. Phase II study of gemcitabine and split-dose cisplatin plus pembrolizumab as neoadjuvant therapy before radical cystectomy in patients with muscle-invasive bladder cancer. J Clin Oncol 2021;39:3140–8.
28. Funt SA, Lattanzi M, Whiting K, Al-Ahmadie H, Quinlan C, Teo MY, et al. Neoadjuvant atezolizumab with gemcitabine and cisplatin in patients with muscle-invasive bladder cancer: a multicenter, single-arm, phase II trial. J Clin Oncol 2022;40:1312–22.
29. Powles T, Catto JWF, Galsky MD, Al-Ahmadie H, Meeks JJ, Nishiyama H, et al. Perioperative durvalumab with neoadjuvant chemotherapy in operable bladder cancer. N Engl J Med 2024;391:1773–86.
30. Necchi A, Raggi D, Gallina A, Ross JS, Farè E, Giannatempo P, et al. Impact of molecular subtyping and immune infiltration on pathological response and outcome following neoadjuvant pembrolizumab in muscle-invasive bladder cancer. Eur Urol 2020;77:701–10.
31. Kamoun A, de Reyniès A, Allory Y, Sjödahl G, Robertson AG, Seiler R, et al. A consensus molecular classification of muscle-invasive bladder cancer. Eur Urol 2020;77:420–33.
32. Robertson AG, Kim J, Al-Ahmadie H, Bellmunt J, Guo G, Cherniack AD, et al. Comprehensive molecular characterization of muscle-invasive bladder cancer. Cell 2017;171:540–56.e25.
33. Seiler R, Ashab HAD, Erho N, van Rhijn BWG, Winters B, Douglas J, et al. Impact of molecular subtypes in muscle-invasive bladder cancer on predicting response and survival after neoadjuvant chemotherapy. Eur Urol 2017;72:544–54.
34. de Jong JJ, Proudfoot JA, Daneshmand S, Svatek RS, Narayan V, Gibb EA, et al. A luminal non-coding RNA-based genomic classifier confirms favourable outcomes in patients with clinically organ-confined bladder cancer treated with radical cystectomy. BJU Int 2025;135:648–56.
35. Batista Da Costa J, Gibb EA, Bivalacqua TJ, Liu Y, Oo HZ, Miyamoto DT, et al. Molecular characterization of neuroendocrine-like bladder cancer. Clin Cancer Res 2019;25:3908–20.
36. Piccolo SR, Sun Y, Campbell JD, Lenburg ME, Bild AH, Johnson WE. A single-sample microarray normalization method to facilitate personalized-medicine workflows. Genomics 2012;100:337–44.
37. De Jong JJ, Liu Y, Robertson AG, Seiler R, Groeneveld CS, van der Heijden MS, et al. Long non-coding RNAs identify a subset of luminal muscle-invasive bladder cancer patients with favorable prognosis. Genome Med 2019;11:60.
38. Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst 2015;1:417–25.
39. Spratt DE, Alshalalfa M, Fishbane N, Weiner AB, Mehra R, Mahal BA, et al. Transcriptomic heterogeneity of androgen receptor activity defines a de novo low AR-active subclass in treatment Naïve primary prostate cancer. Clin Cancer Res 2019;25:6721–30.
40. Sjödahl G, Lauss M, Lövgren K, Chebil G, Gudjonsson S, Veerla S, et al. A molecular taxonomy for urothelial carcinoma. Clin Cancer Res 2012;18:3377–86.
41. Necchi A, Raggi D, Giannatempo P, Marandino L, Farè E, Gallina A, et al. Can patients with muscle-invasive bladder cancer and fibroblast growth factor receptor-3 alterations still Be considered for neoadjuvant pembrolizumab? A comprehensive assessment from the updated results of the PURE-01 study. Eur Urol Oncol 2021;4:1001–5.
42. Miyamoto H, Yao JL, Chaux A, Zheng Y, Hsu I, Izumi K, et al. Expression of androgen and oestrogen receptors and its prognostic significance in urothelial neoplasm of the urinary bladder. BJU Int 2012;109:1716–26.

43. Shrivastava N, Jena R, Choudhary GR, Bhargava P, Vishwajeet V, Elhence P, et al. Correlation between androgen and estrogen receptor expression and clinicopathologic features in carcinoma urinary bladder. J Cancer Res Clin Oncol 2023;149:15795–804.
44. Choi W, Porten S, Kim S, Willis D, Plimack ER, Hoffman-Censits J, et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell 2014;25:152–65.
45. Lotan Y, De Jong JJ, Liu VYT, Bismar TA, Boorjian SA, Huang HC, et al. Patients with muscle-invasive bladder cancer with nonluminal subtype derive greatest benefit from platinum based neoadjuvant chemotherapy. J Urol 2022;207:541–50.
46. Marabelle A, Fakih M, Lopez J, Shah M, Shapira-Frommer R, Nakagawa K, et al. Association of tumour mutational burden with outcomes in patients with advanced solid tumours treated with pembrolizumab: prospective biomarker analysis of the multicohort, open-label, phase 2 KEYNOTE-158 study. Lancet Oncol 2020;21:1353–65.
47. Gupta S, Dhillon J, Magliocco A, Puskas J, Caceres G, Masawi F, et al. Results from a phase I/Ib trial of enzalutamide and gemcitabine and cisplatin in metastatic bladder cancer (mBC). J Clin Oncol 2019;37(suppl 7):471.
48. Shang Z, Li Y, Hsu I, Zhang M, Tian J, Wen S, et al. Targeting estrogen/estrogen receptor alpha enhances Bacillus Calmette-Guérin efficacy in bladder cancer. Oncotarget 2016;7:27325–35.
49. Lerner SP, Sternberg CN, Younes M, Calabro F, Cerbone L, Chandrashekar A, et al. Phase II trial of tamoxifen for progressive advanced urothelial carcinoma following prior chemotherapy. J Clin Oncol 2012;30(suppl 15):e15003.