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Meeting Report

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Abstract. The Fourth Annual Albert Institute Bladder Cancer Care and Research Symposium was held from September 14th–16th in Houston, Texas. The symposium covered a range of topics relevant to bladder cancer, including basic science aspects of immunology and immunotherapy that inform clinical management; intravesical therapy for non-muscle invasive disease; understanding the nuances of carcinoma *in situ*; and optimizing patient care and outcomes following therapy. The moving landscape of bladder cancer from an industry perspective was also discussed.

In the following sections we discuss intrinsic and extrinsic factors, including the immune microenvironment and sex bias, in the context of bladder cancer; how these influence tumor development, progression, and treatment strategies; and how the interpretation of immune features in relation to molecular subtypes informs both treatment decisions and response. We conclude with a summary of key points that will need to be addressed to ensure best use of new knowledge in this area for improved clinical management of patients with bladder cancer.

INTRODUCTION

The past few years have seen an explosion of interest in the use of immunotherapy with checkpoint inhibitors for treatment of advanced bladder cancer (reviewed in [1]). For a subset of patients with locally advanced or metastatic disease, immunotherapy in the form of inhibitory anti-PD-L1 or anti-PD-1 antibodies, has yielded durable responses [2, 3], representing a major advance in disease management. This clinical success has meshed with pre-existing basic and translational research investigations into how the immune system influences and is influenced by the development and progression of urothelial cancers. At the fourth Annual Albert Institute Bladder

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Cancer Care and Research Symposium, we integrated these ideas in a session addressing the topics of basic immunology, preclinical evaluation of novel immunotherapy strategies, and potential modulators of response including sex differences, heat, and molecular subtypes. This perspective piece summarizes recent findings, highlighting the extent to which preclinical and early clinical work supports innovative research into microenvironmental modulators of bladder cancer development, progression and therapy.

SEX DIFFERENCES IN THE DEVELOPMENT OF BLADDER CANCER AND RESPONSE TO THERAPY

Men are 3–5 times more likely than women to develop bladder cancer (BCa) [4–6]. Sex disparities in BCa risk and mortality persist even after accounting for known risk factors, including cigarette smoking, occupational hazards, and urinary tract infection, are taken into consideration [7]. The mechanisms underlying sex differences, however, remain poorly defined. A better understanding of the underlying reasons for male dominance in BCa is essential for developing effective strategies to reduce cancer risk and mortality. Generally speaking, males may have a higher risk and/or females are better protected from cancer. Many confounding variables contribute to the sex disparities in cancer. The challenge has been to dissect the relative significance of each contributor and to uncover the major causative and protective mechanisms. For example, the XX chromosome complement is linked to ovary differentiation and female-specific phenotypes; and the XY chromosome complement is linked to testis differentiation and male-specific phenotypes. As the sex chromosomes co-vary with gonadal differentiation, potential biasing effects of the sex chromosomes (X and Y) can be masked by effects of other biasing factors such as sex hormones produced by the gonads (ovary and testis) [8].

Women with Turner syndrome are characterized by partial or complete loss of one X chromosome. The overall risk of solid tumors, including BCa, in Turner patients is significantly higher compared to the general population [9, 10]. Conversely, men with Klinefelter syndrome, who have two or more copies of the X chromosome, display an overall reduction in the risk of developing solid tumors [10]. While changes in cancer risk of patients harboring non-diploid chromosome complements are often attributed to changes in the sex hormone milieu, these observations also suggest that sex chromosomes may contribute to sex differences in BCa, independently of hormone status.

Rodents also exhibit sex differences in cancer. For example, when mice are exposed *ad libitum* to N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN), a bladder-specific chemical carcinogen [11], male mice develop and die from BCa significantly faster than female mice [12, 13]. BBN-induced BCa in mice exhibits similar histopathology to human BCa [14, 15], and recapitulates the molecular alterations of human muscle invasive bladder cancer (MIBC) [16]. Both sexes consume equal amounts of BBN in the drinking water [12]; and more importantly, DNA mutation rates resulting from BBN exposure are the same between sexes [17]. Nevertheless, the mean tumor induction time is 63 days shorter in male mice compared to female animals [12]. Castrated male mice adopt the female pattern of response to BBN, which can be reversed by testosterone treatment. Conversely, testosterone-treated female mice exhibit the male pattern of response to BBN [12]. Moreover, genetic deletion of the androgen receptor reduces BBN-induced BCa incidence and mortality in male mice [18, 19]. Collectively, these findings provide strong evidence suggesting that sex differences in BCa are not simply the consequence of differential exposure and metabolic response to carcinogens. Instead these differences in BCa appear to be a conserved feature of cancer biology in mice and humans, and are tightly associated with sex biology including sex chromosomes and sex hormones.

In addition to influencing cancer incidence, sex differences are also evident in the response to treatment in certain tumor types (reviewed in [20]), including response to the immune checkpoint inhibitors [21]. While some disparities can be explained by metabolic and pharmacokinetic differences between men and women, responses to therapy also likely reflect differences in tumor biology. For example, in patients with small cell lung cancer, the extent of response to chemotherapy, as well as associated toxicity are increased in female patients compared to male patients [22]. Conversely, in the context of non-small cell lung cancer, the addition of bevacizumab to a chemotherapeutic regimen of paclitaxel and carboplatin improved survival in male, but not female, patients [23]. Notably, in individuals with B cell lymphoma treated with rituximab-containing immunochemotherapy, female patients responded more favorably, with male patients showing poorer

prognosis [24]. Recent meta-analyses of clinical trials evaluating immune checkpoint inhibitors to CTLA-4, PD-1, and PD-L1 across a range of tumor types suggests that differences in the effectiveness of immunotherapy between male and female patients exists, although they seem to be restricted to treatment with anti-CTLA-4 inhibitors, and not those targeting the PD-1/PD-L1 axis [25, 26]. Together, these findings suggest that sex differences in response to treatment, including immunotherapy are a significant influence on patient outcome. As immune checkpoint inhibitors are used more broadly in bladder cancer treatment, differences may also emerge in male versus female patients in this setting, as well.

ROLE OF MACROPHAGES IN RESPONSE TO IMMUNOTHERAPY IN CANCER

Research addressing the role of macrophage populations in the context of bladder cancer has lagged behind studies of their roles in other malignancies [27]. Indeed, in other tumor types, a vast majority of work supports that the presence of macrophages within the tumor environment signals a poor prognosis for the patient [27]. This is because, rather than engaging in tumor cell killing, macrophages induce vascularization, tumor cell growth, and even metastasis [28–31]. These activities are attributed to the activation state assumed by the macrophage within the tumor microenvironment, and may also reflect their origins. For example, macrophages can be polarized towards an immunosuppressive phenotype by cytokines such as IL-4, IL-13, or IL-10, leading to expression of M2-like cell surface markers, such as scavenger receptor (CD204, SR-A) and mannose receptor (CD206) [28, 32]. Importantly, however, the M1-M2 paradigm, meant to describe activation states similar to the Th1-Th2 paradigm for T cells, is likely overly simplistic to describe tumor-associated macrophage phenotypes, as macrophages can express a mixture of M1- and M2-associated gene products, which likely influence their behavior in the tumor microenvironment [33].

A handful of studies have addressed the impact of tumor-associated macrophages in bladder cancer, however methods used to detect macrophages and stratify patients are highly diverse, and at times poorly defined. A survey of tumors from 103 patients with muscle invasive or lymph node metastatic bladder cancer failed to find a correlation between macrophage infiltration and disease-specific death, except in the case of a subgroup analysis only considering tumors with "weak" or "strong" anti-CD163 antibody staining [34]. Interestingly, a second study reported that "high" CD163 expression is associated with reduced recurrence-free survival in 68 patients evaluated [35]. The underlying reasons for these divergent findings are unclear, however in the second study, earlier stage tumors were evaluated (Ta-T2) as compared to tumors from more advanced disease (T1-T4) assessed in the first analysis. Additional studies of tumor macrophages and outcome in muscle invasive (MIBC) or nonmuscle invasive BCa (NMIBC) have measured cell surface proteins such as CD68 (all macrophages), MSC387 (recently infiltrated monocyte-derived macrophages), CD204 (M2-associated), CD163 (scavenger receptor), and CD169 (sialoadhesin), and in each case, tumor macrophages are associated with a negative patient prognosis [36–40]. Specifically in NMIBC, a long-term study of patients treated with BCG intravesical immunotherapy reported that recurrence free survival was worse for patients with tumor samples with "high" numbers of tumor-associated macrophages [41]. Additional studies have also identified a correlation between macrophage presence and an increased risk for recurrence after BCG therapy [42–44].

Surprisingly, a majority of reports rely upon immunostaining in tissues to identify macrophages and other tumor characteristics; however, staining is often described as low, medium, or high, with representative images rather than specific quantitative values. Given that the evidence suggests that tumor associated macrophages are associated with a worse prognosis in bladder cancer, development of standardized assessments and definitions for identifying macrophages would help the field to make comparisons and draw conclusions across clinical studies. Defining the polarization state, localization within the tumor environment, expression of macrophage specific pro-and anti-tumor factors, and the critical cut off of different macrophage populations will be of paramount importance to implement new macrophage-targeted immunotherapeutic approaches on the horizon [45–47].

HEAT-TARGETED THERAPIES FOR BLADDER CANCER

Hyperthermia (HT) is a treatment in which a mild temperature increase is induced in a tumor or organ [48, 49]. Different from thermal ablation, where

temperatures reach 60–90°C and effectively destroy tissue, fever-range heating at 42–44°C has many different properties. This mild form of thermal therapy is not new and dates back to William Coley who noted in 1891 that injecting patients with unresectable and metastatic sarcomas with a cocktail of bacterial toxins led to substantial tumor regression and even cure in some cases [50]. Over many years of observation, Coley found that for his toxin to work, a high fever had to be induced and the treatment repeated many times. This fractionated fever-like therapy represented one of the first effective systemic cancer treatments and was a combination of HT and immunotherapy. The modern understanding of how HT works as a cancer therapy has improved greatly since Coley [51, 52], and it is now clear that fever-range HT can be used to (1) improve drug delivery to cancer cells, (2) improve cancer cell sensitivity to therapeutic agents and radiotherapy, and (3) trigger anti-cancer immune responses [51, 53, 54].

Vascular modifications occur when a tumor is heated to 42–44°C [49]. Heat initially induces vasodilation, which results in increased blood flow to the tumor. The warmer environment renders the lipid-protein membrane bilayer more permeable, resulting in easier penetration of drugs through the cell membrane. These two factors cooperate to make an already leaky tumor vasculature even more permeable, a phenomenon known as the enhanced permeability and retention (EPR) effect [55]. By increasing the EPR effect, HT results in increased drug delivery to tumors [56]. More efficient drug delivery to the tumor is postulated to lead to better tumor cell killing. Hyperthermia also leads to increased leukocyte trafficking and tumor infiltration [57], which may lead to enhanced immune responses.

For a variety of mechanistic reasons, many cancer drugs are more efficacious when administered in a heated environment [58]. The extent to which heat modulates the efficacy of a drug is quantified by the thermal enhancement ratio (TER), or the ratio of cytotoxicity at 43°C to that at 37°C. Drugs that have a TER >1 work better with heat, and several chemotherapeutic agents presently used in bladder cancer have been shown to have a TER >1.3, including cisplatin, mitomycin C, gemcitabine, and doxorubicin [58]. Thus, many of the drugs currently used to treat bladder cancer actually kill tumor cells more effectively when combined with heat.

Body temperature is a well-known regulator of immune function. A fever-like milieu in the surrounding tumor microenvironment can induce or enhance various immune–relevant processes, including heat-shock protein release from tumor cells, changes in the number and phenotype of tumor-infiltrating leukocytes, and improved tumor-infiltrating leukocyte function and cytokine release [59]. The consequence of these heat-mediated immune effects is that heated tumors actively participate in their own demise through a form of self-vaccination. As the activity of several cell types involved in innate and adaptive immune responses is promoted by mild temperature increases, targeted HT may enhance the effectiveness of immunotherapy. Notably, immunotherapies have emerged as one of the most promising modalities to treat bladder cancer, however, the role of heat in modulating these drugs is not known.

HT has been used clinically to treat bladder cancer for many years [60]. The main limitation to the use of bladder HT in the US has been the lack of availability of an FDA-approved bladder heater. Three methods of heating the bladder in a clinical context currently exist [61]: (1) deep regional HT administered via an external array of tunable radiofrequency antennae (*e.g.*, BSD-2000 device); (2) intravesical radiofrequency antennae (*e.g.*, Synergo SB-TS 101.1 device); and (3) intravesical circulating fluid convection heating (*e.g.*, Combat BRS device). Most bladder HT clinical trials to date have tested HT in combination with intravesical mitomycin C (HT-MMC) for NMIBC and most have demonstrated positive outcomes [60].

The first clinical trial of HT-MMC using deep regional hyperthermia was performed at Duke University using the BSD-2000 device to treat BCG-unresponsive NMIBC [62]. This trial showed that excellent thermal treatment plans were possible to safely and effectively heat the bladder and approximately half of participants were able to forgo radical cystectomy [63, 64]. This positive outcome has been confirmed in a second cohort of patients [65]. While effective, deep regional hyperthermia HT-MMC treatment method has numerous limitations. For example, the BSD-2000 device is expensive, requires a radiofrequency-shielded room, is very large in size, requires a medical physics team to operate, and cannot be safely used in certain patients (*e.g.*, severe obesity and hip prostheses). Therefore, deep regional hyperthermia technology is not currently generalizable to the broader urological community treating NMIBC.

The sole intravesical radiofrequency antenna that is currently available clinically, the Synergo SB-TS

device, has been tested in multiple clinical trials in Europe for NMIBC [60]. This device is moderately expensive with high operating costs, is moderately large, requires the supervision of a nurse to operate, can heat the bladder unevenly and cause burns in the bladder, and requires a large 20 F Foley catheter. For these reasons, the Synergo device has only been used by a handful of European centers despite being available for over a decade [60]. Despite these caveats, randomized trials have found that Synergo-based HT-MMC can be used to successfully treat many patients whose tumors are unresponsive to BCG treatment [60]. One recent randomized trial has even found Synergo-based HT-MMC to be superior to BCG immunotherapy [66].

The most recent bladder heating method to be tested in clinical trials is the convection bladder heating method, typified by the Combat BRS device. This device costs significantly less, is small, portable, simple to operate, and does not require supervision during operation. Importantly, this device heats the bladder with negligible toxicity, and is easily generalizable to the broader urological community, since it can be used in any center where intravesical therapy is administered. Currently, the first clinical trial of this device for HT-MMC treatment of NMIBC is published [67] and two large randomized trials of HT-MMC using the Combat BRS device have completed accrual in Europe for intermediate risk NMIBC (EudraCT: 2013-002628-18, 2014-005001-20).

Finally, to take full advantage of the potential of HT, researchers at Duke University have developed a new class of drugs, which are triggered by heat and consist mainly of thermally-sensitive liposomes that release their pharmaceutical contents only when they are subject to heating above a certain temperature (*e.g.* 41°C) [68, 69]. These liposomes can be used in combination with HT to safely direct drug delivery to heated tissues and consequently increase intratumoral drug levels by 10–30 fold [68]. Due to their selective targeting of heated tissues, thermally-sensitive liposomes can be administered at lower systemic levels than free drug, which minimizes toxicity and still achieve very high local tissue levels of the active agent to maximize efficacy [70]. Notably temperature-sensitive liposomes can be loaded with a variety of anti-neoplastic agents. Currently, doxorubicin (ThermoDox), cisplatin, and gemcitabine containing liposomes have been generated. ThermoDox has been licensed and is in phase III trials for liver cancer (NCT00617981). Since doxorubicin, cisplatin, and gemcitabine are drugs that are well established for the treatment of advanced bladder cancer, heat-targeted versions of these drugs are desirable tools to add to the bladder cancer armamentarium. Studies conducted in swine, both at Duke University and the NCI, have shown that ThermoDox can be targeted to the bladder using intravesical conductive hyperthermia, minimizing systemic toxicity and maximizing local drug delivery to the bladder [71]. Altogether, heat therapy has great potential to improve treatment efficacy for BCa without increasing the risk for adverse effects.

IMMUNOTHERAPY AND CHECKPOINT INHIBITORS - THE FUTURE BEYOND CTLA-4 AND PD-1/PD-L1

In many laboratories and clinical settings, efforts are underway to improve the therapeutic potential of immune-based monotherapies by designing rational combinations of those agents. In prior preclinical studies, the monotherapy antitumor effects of two such immunotherapeutic molecules, an immunocytokine, NHS-muIL12, and an anti-programmed cell death protein-1 ligand (PD-L1) antibody, avelumab, have been reported [72, 73]. NHS-muIL12 is a novel immunocytokine delivery system whose antitumor actions are based on the *in vivo* tumor delivery of potent T_h1 cytokine, IL-12, to necrotic portions of tumors through recognition by the NHS76 antibody. NHS-muIL12 increases serum IFN-γ levels, upregulates MHC class I protein expression on dendritic cells (DCs), and induces proliferation of $CD49b^+$ natural killer (NK) cells and $CD8^+$ T cells [72], all consistent with the known properties of recombinant murine IL-12 [74, 75]. Antitumor effects of NHS-muIL12 are dose-dependent over a 50-fold range and superior to those of recombinant muIL-12. Thus, tumor targeting with NHS-muIL-12 could potentially deliver significant antitumor effects while mitigating some of the dose-limiting toxicity reported in patients treated with rIL-12 [76]. A recently completed first-in-human phase I dose escalation clinical trial of NHS-IL12 showed the agent to be well-tolerated and reported preliminary evidence of clinical benefit in patients diagnosed with late-stage cancers [77].

Avelumab, which interrupts the PD-1/PD-L1 interaction, overcomes immune resistance in preclinical models [78, 79] and has led to remarkable clinical responses in a variety of cancer patients [80, 81]. Combining NHS-muIL12 and avelumab

brings together different immune-associated actions to enhance the antitumor effects above those achieved by either monotherapy. The antitumor actions of NHS-muIL12 are IFN-γ dependent and upregulate mPD-L1 expression on mouse tumors, an action that could be construed as exacerbating immunosuppression. Yet, concurrent therapy with NHS-muIL12 and an anti-PD-L1 antibody results in additive/synergistic antitumor effects in PD-L1–expressing subcutaneously transplanted tumors and in an intravesical bladder tumor model [72]. Antitumor efficacy correlates with a higher frequency of tumor antigen-specific splenic $CD8^+$ T cells and enhanced T cell activation over a wide range of NHS-IL12 concentrations. The PD-1/PD-L1 axis is associated with suppressing antitumor effects at the tumor-T cell interface. With disruption of that axis, resident cytotoxic T cells can then exert their control over tumor growth. Studies in $Rag2^{-/-}$ mice and mice depleted of $CD4^+$ and $CD8^+$ T cells clearly show the need for an intact immune system to elicit the antitumor effects of the anti-PD-L1 antibody [72, 73]. PD-L1 is also expressed on antigen-presenting cells (APC) and its binding to PD-1 on T cells recruits phosphatase SHP-2 to the T cell membrane [82–84], which inhibits both TCR and CD-28 downstream signaling, thus limiting T cell activation [85]. Disruption of the APC (PD-L1):T cell (PD-1) interaction renders T cells more sensitive to antigen presentation and costimulation. Delivery of IL-12 via a tumor necrosis–targeting human IgG1 (NHS76) provides an immediate source of a T_h1 proinflammatory cytokine within the tumor microenvironment. By binding to the IL-12 receptor (IL-12R) on T cells, NHS-muIL12 could provide an additional third signal to direct differentiation along the T_h1 pathway. IL-12R activation induces Jak2/Tyk2 signaling, which in turn promotes STAT4 phosphorylation and enhances T_h1 gene transcription [75]. Another potential source of IL-12 within the tumor microenvironment might come from "newly" activated, mature CD8α+ DCs, which are enhanced following parenteral administration of NHS-muIL12 [72]. By combining PD-L1 blockade with NHS-muIL12, complementary signaling pathways for $CD8^+$ T cells and NK cells could become fully activated, which would explain the increases seen in cytotoxic effector functions and, ultimately, in better immunotherapy. The combined therapeutic potential of NHS-IL12 and avelumab in those preclinical studies has provided the rationale for the design of an ongoing phase I clinical study (NCT01417546).

IDENTIFYING RESPONDERS TO IMMUNOTHERAPY AND IMMUNOLOGIC DIFFERENCES BETWEEN MOLECULAR SUBTYPES

The success of immune checkpoint blockade in a subset of patients with metastatic urothelial carcinoma highlights the potential for these therapies to induce potent anti-cancer immune responses (reviewed in [86]). The lack of response in most patients, however, is indicative of a need to better understand the underlying mechanisms of treatment resistance. To reach the full clinical utility of immunotherapy for treatment of MIBC, we require a comprehensive understanding of the mechanisms of immune evasion and their relationship to bladder tumor biology.

The identification of molecular subtypes of MIBC, based on patterns of gene expression [87–91], has provided a critical framework to study the heterogeneity of bladder cancer. Each subtype corresponds to a biologically distinct tumor type. Patterns of immune infiltration and expression of immune-related genes vary markedly by subtype, to the extent that immune signatures alone may be able to recapitulate the subtypes [92]. In addition, two early reports indicate that response to checkpoint blockade in metastatic bladder cancer depends on molecular subtypes [93, 94].

Bladder tumors can generally be classified into basal and luminal subtypes, with further classification varying among the different classification systems [87–91]. Kardos et al. defined a subset of basal tumors that are highly mesenchymal and immune infiltrated, which they label claudin-low due to similarities to the corresponding subtypes of breast cancers [95]. The cluster IV from the original TCGA classification had some overlap with claudin-low tumors, but this group was dropped from the updated TCGA classification [88, 91, 95, 96]. The Lund and TCGA classifications highlight a subset of luminal tumors defined primarily by a high level of stromal cell infiltration ("infiltrated" or "luminal infiltrated", respectively) [87, 88, 91]. These differ markedly from the rest of the luminal tumors (TCGA cluster I or luminal papillary), which are characterized by high rates of altered PPARγ and FGFR3 and an "immune desert" phenotype.

Seiler and colleagues, building on the work of the MD Anderson group [97, 98], determined that basal tumors have the largest survival benefit from cisplatin-based neoadjuvant chemotherapy

[96]. In that study, luminal tumors had the best outcome regardless of chemotherapy, while the luminal infiltrated tumors did poorly regardless of chemotherapy and the claudin-low tumors had only a modest benefit from chemotherapy [96]. Rosenberg et al. reported that cluster II (luminal infiltrated) and IV (claudin-low) tumors have the highest response rate to atezolizumab [93], while Sharma et al. reported that cluster II (luminal infiltrated) and III (basal) tumors responded best to nivolumab [94]. Nonetheless these studies underline important differences between subtypes that may be important for patient selection for systemic therapy (Table 1). The updated TCGA report subsequently suggested a similar subtype-directed treatment approach [91] (Table 2).

Defining differences in the immune cell component of each subtype appears to be critical to understanding both response and resistance to therapy. It may be that each molecular subtype of MIBC employs unique mechanisms of immune evasion, and that specific subtypes actively suppress effector T cell responses through therapeutically targetable mechanisms. The luminal tumors (cluster I) have the most obvious difference in immune infiltration, with a relative absence of immune cells within the tumor. At the same time, they are characterized by high rates of genomic alteration or over-expression of FGFR3 and PPARγ, both of which are potential regulators of immune cell exclusion.

To date, analyses of the tumor-associated immune system in the molecular subtypes of MIBC have primarily utilized gene expression data from whole tissue, as well as IHC analysis [99, 100]. Accurate identification of subsets of immune cells requires the positive expression and often exclusion of a combination of markers, and many of these markers overlap among different types of immune cells. Consequently, neither gene expression analysis of whole tissue nor IHC is particularly well suited for characterizing immune responses as stand-alone methods. Therefore, a comprehensive analysis of the composition of the tumor-associated immune compartment is currently lacking and is critical for the investigation of mechanisms of immune evasion in MIBC. Comparing tumor-infiltrating immune cells to peripheral blood and "normal" adjacent tissue-infiltrating immune cells may help to distinguish among inter-patient differences in immune states and tumor-specific differences in immune composition and activation. Analysis of immune cells in peripheral blood will also determine whether these cells can be used as a surrogate marker for altered tumor-associated immune responses.

Table 1
Treatment response by subtype (2014 TCGA classification)

Subtype	*Localized MIBC* Cystectomy alone	*Localized MIBC* Chemo + Cystectomy	*Metastatic MIBC* Immunotherapy
Luminal (≈Cluster I)	Best	Same as RC	Poor
Luminal infiltrated (≈Cluster II)	Poor	Poor	Best
Basal (≈Cluster III)	Poor	Best	???
Claudin-low (≈Cluster IV)	Poor	Intermediate	Intermediate

RC = radical cystectomy; MIBC = muscle invasive bladder cancer.

Table 2
Treatment response by subtype (2017 TCGA classification)

Subtype	Key biological features	Predicted therapy
Luminal-papillary	FGFR3 mutations SHH-positive Low risk for progression	FGFR3 kinase inhibitors
Luminal-infiltrated	EMT markers miR-200 family PD-L1, CTLA4	Immunotherapy
Luminal	Luminal markers	Not defined
Basal/squamous	Squamous differentiation PD-L1, CTLA4 Immune infiltrates	Immunotherapy Cisplatin-based chemotherapy
Neuronal	Neuroendocrine markers Neuronal markers	Etoposide-cisplatin chemotherapy

Mass cytometry, also known as cytometry by time of flight (CyTOF), makes the analysis of up to 40 parameters on each immune cell possible, including expression of surface proteins and intracellular molecules such as transcription factors. When studying tumor tissue, antibodies can also be selected to measure tumor and stromal cell proteins. Mass cytometry is similar in principle to flow cytometry in that it utilizes tagged antibodies to identify specific proteins. However, in mass cytometry the antibodies are conjugated to heavy-metal isotopes, which are then detected and quantified by time of flight mass spectrometry. The advantage of mass cytometry is the ability to quantify up to 40 markers simultaneously on a per cell level basis. By contrast, due to spectral overlap among fluorophores, flow cytometry is typically limited to about 16 fluorophores. Flow cytometry, however, makes it possible to capture specific cell subpopulations, expand them in culture, and perform functional analyses such as T cell proliferation assays. The optimal approach to profiling bladder cancer should, therefore, incorporate both mass and flow cytometry.

In summary, a comprehensive analysis of the immune milieu in each subtype of MIBC will require a large multi-level study that assesses the cellular phenotypes of immune, stromal, and tumor cells, as well as their spatial localization within tumors, together with gene expression and *ex vivo* functional data, which will improve our ability to predict patient response to therapy and ultimately to develop personalized approaches for the treatment of bladder cancer.

CONCLUSIONS AND PERSPECTIVES

In conclusion, investigation of the normal and tumor-associated immune microenvironment has provided novel insights into modulators of disease burden and the response to treatment. Preclinical models continue to enhance our understanding of both intrinsic and extrinsic influences on bladder cancer development, progression and treatment, including sex, the immune cell milieu, and treatment modulators such as heat. Moreover, syngeneic animal models enable the testing and optimization of novel immunotherapy, chemotherapy, and hyperthermia combinations. Key areas of need include extensive validation of molecular subtypes at the protein level, assessment of immune profiles in terms of immune system function, determination of the extent to which subtypes inform treatment response and choices, beyond mere association. Finally, the demonstration that sex differences influence not only susceptibility to bladder cancer development, but also disease progression and response to treatment emphasizes the need for both preclinical and clinical studies to incorporate male and female subjects.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to report.

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