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REVIEW

Immune checkpoint inhibitors in urothelial cancer: recent updates and future outlook

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Abstract: Bladder cancer is the sixth most common cancer in the US and most tumors have urothelial (transitional cell) histology. Platinum-based chemotherapy has long been the standard of care in advanced disease, but long-term outcomes have largely remained poor. Since the peak incidence of bladder cancer is in the eighth decade of life and beyond, medical comorbidities may often limit the use of chemotherapy. Immune checkpoint inhibitors with their favorable toxicity profiles and notable antitumor activity have ushered in a new era in the treatment of advanced urothelial cancer (UC) with five agents targeting the PD-1/PD-L1 pathway being recently approved by the US Food and Drug administration. A plethora of clinical trials are ongoing in diverse disease settings, employing agents targeting PD-1/PD-L1 and related immune checkpoint pathways. While reactivating anti-tumor immunity, these agents may lead to a unique constellation of immune-related adverse events, which may warrant discontinuation of therapy and potential use of immunosuppression. Novel combinations with various treatment modalities and optimal sequencing of active therapies are being investigated in prospective clinical trials and retrospective registries. At the era of precision molecular medicine, and since patients do not respond uniformly to these agents, there is a growing need for identification and validation of biomarkers that can accurately predict treatment response and assist in patient selection. This review discusses current updates and future directions of immunotherapy in advanced UC.

Keywords: immunotherapy, urothelial carcinoma, bladder cancer, PD-1, PD-L1, CTLA-4, biomarkers, immune-related adverse events

Introduction

Bladder cancer is the sixth most common cancer overall and fourth most common among males in the US, with more than 79,000 new cases and close to 17,000 deaths predicted in 2017.[1,2] The vast majority of bladder cancers are of urothelial (transitional cell) histology, which can also arise from upper urinary tract and urethra. Death rates from urothelial cancer (UC) have remained stable for the past three decades.[3] Long-term survival rates for locally advanced (with extravesical and/or node-positive disease) and metastatic disease remain dismal, with an overall survival (OS) of 9–15 months and 5-year OS rate of 5% for the latter, even with platinum-based chemotherapy, which is the standard of care.[4–6] The prognosis is particularly poor in patients who are refractory to cisplatin-based chemotherapy, with median OS with salvage chemotherapy, such as taxanes or vinflunine ranging from 5–7 months.[7] Moreover, with an average age at diagnosis of 73 years (~45% patients are older than 75 years), medical comorbidities often prevent these patients from receiving cisplatin-based therapy.[1] Immune checkpoint inhibitors (ICIs), with their favorable safety and antitumor activity profiles, have

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heralded a new era in the treatment of advanced UC. Clinical trials in metastatic UC include patients with upper and/or lower urinary tract UC.

The primary molecular targets for ICIs are the programmed cell death-1 (PD-1), programmed death-ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) checkpoints, which act as co-inhibitory signals blocking anti-tumor effector T-cell responses, thereby down-regulating anti-tumor response.[8] PD-1 is a transmembrane protein expressed on activated T cells that interacts with PD-L1 (B7-H1 or CD274), which is expressed on antigen presenting cells and many types of tumor cells (TC).[9,10] This interaction leads to suppression of T-cell receptor (TCR)-mediated effector functions and inhibits proliferation of antigen-specific $CD8^+$ T cells.[10] Cells of immunologically responsive tumors often upregulate PD-L1 expression, thereby facilitating immune escape. By blocking either PD-1 or PD-L1, ICIs reinvigorate anti-tumor T-cell-mediated immune responses.[11] CTLA-4 is structurally related to CD28, a co-stimulatory signal that plays a vital role in T-cell activation.[12] By competitively binding to its ligands B7.1 and B7.2, CTLA-4 blocks CD28-mediated co-stimulatory signaling and thus inhibits T-cell activation.[13,14] ICIs that block CTLA-4 will thus lead to reactivation of anti-tumor effector T-cell response mechanisms.[15]

PD-L1 inhibitors

Atezolizumab

Atezolizumab (MPDL3280A) is a fully humanized monoclonal antibody of IgG1 isotype that selectively binds to PD-L1 leading to the blockade of its interaction with PD-1 and CD80 (B7.1) and thereby enabling T cells to overcome peripheral tolerance against TC. It has an engineered Fc domain that prevents T-cell depletion via antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).[16,17]

In 2014, Powles et al first reported results from the UC cohort of a large phase Ia multicenter study with adaptive design (NCT01375842) and subsequently a 2-year clinical update from the study was presented at the American Society of Clinical Oncology (ASCO) Genitourinary Cancers Symposium in 2017.[17,18] Although the cohort was initially selected based on positive PD-L1 immunoreactivity of tumor-infiltrating immune cells (ICs), it was subsequently expanded to include patients regardless of PD-L1 status. To be included in the study, patients needed to have measurable disease per Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1), Eastern Cooperative Oncology Group (ECOG) performance status 0–1 and a representative tissue sample. PD-L1 expression on ICs (macrophages, dendritic cells and lymphocytes) was scored using immunohistochemistry (IHC) assay into 4 levels – IC0 (<1%), IC1 (≥1% and <5%), IC2 (≥5% and <10%) and IC3 (≥10%) – based on the highest score among available tissue specimens in a patient. At 2-year follow-up, 94 patients were available for efficacy and 95 for safety analysis. The patients in this cohort were heavily pretreated (69% had received prior platinum-based therapy), 78% had visceral metastases and 46% were either IC0 or IC1. At the time of data cutoff, patients had received atezolizumab for a median of 3 months (range 0–35) and a median of 5 doses (range 1–46) with a median follow-up duration of 29.2 months (range 0.7–35.5 months). Overall, atezolizumab was well tolerated; treatment-related adverse events (TRAEs) were reported in 57% of patients with only 9% grade 3–4 TRAEs and 1% treatment withdrawal, with no treatment-related deaths. The most common TRAEs were fatigue (18%), asthenia (14%) and decreased appetite (13%). Most TRAEs occurred within the first year following initiation of therapy and no serious events occurred beyond that time. At the time of data cutoff, objective response rate (ORR) was 39% for the IC2/3 group (including 16% complete response [CR] rate) and 12% for the IC0/1 group. Durable responses were observed, with a median duration of response (DOR) of 18.0 months in the IC2/3 group and 26.3 months in the IC0/1 group, with long-term responses seen even in a number of patients who discontinued therapy; 40% of responders had ongoing response at the time of data cutoff. In exploratory analysis, median progression-free survival (PFS) was 2.7 months and median OS was 10.1 months with a trend toward longer survival in patients with higher PD-L1 status.[17,18] Based on the results of this study, the US Food and Drug administration (FDA), in May 2014, granted breakthrough designation to atezolizumab for the treatment of patients with advanced UC previously treated with platinum-based chemotherapy.[19]

In early 2016, the results from cohort 2 of IMvigor 210 (NCT02108652), a phase II single-arm, two-cohort, multicenter trial of atezolizumab in locally advanced and metastatic UC, were reported.[20] This study included 2 cohorts: cohort 1 comprised patients ineligible for cisplatin-based chemotherapy as first-line treatment and cohort 2 comprised patients who had progressed during or following treatment with platinum-based chemotherapy. Overall, 310 patients in cohort 2 received atezolizumab and were available for analysis. Similar to the abovementioned study, PD-L1 expression on ICs was classified into 4 levels – IC0, IC1,

IC2 and IC3. ORR was 15% overall, including 26% in the IC2/3 group and 18% in the IC1/2/3 group, which compared favorably with a historical control ORR of 10% for cytotoxic chemotherapy in this setting. Most responses were durable and occurred even in subgroups with poor prognostic factors such as liver/visceral metastases. After a median follow-up of 11.7 months, the median DOR was not reached and ongoing response was reported in 84% of responders. Also, 17% of the 121 patients who were treated beyond radiographic progression subsequently showed target lesion reduction of at least 30% from baseline, suggesting possible "pseudoprogression" in the initial scans. Necchi et al later presented updated data from this study showing that almost a third of patients who continued atezolizumab beyond radiographic progression had subsequent tumor shrinkage, but only 4% has subsequent RECIST v1.1 response; selection bias may have been present in those patients.[21] Median time to response (TTR) was 2.1 months. Median PFS by immune-modified RECIST criteria was 2.7 months (4.0 months in the IC2/3 group). Median OS was 7.9 months for the whole cohort (11.4 months for IC2/3 group). In pre-specified exploratory analyses, response to atezolizumab was found to be independently associated with high mutational load and luminal cluster II subtype based on The Cancer Genome Atlas (TCGA) gene expression analysis. Compared to previously available second-line therapies for advanced UC, atezolizumab was well tolerated; TRAEs of any grade were reported in 69% of patients, with only 16% experiencing grade 3–4 TRAEs with no treatment-related deaths. The most frequent TRAEs in this cohort were fatigue (30%), nausea (14%) and decreased appetite (12%).[20]

Balar et al later published results from cohort 1 of IMvigor 210 (NCT02951767). This cohort included 119 patients with locally advanced/metastatic UC who were ineligible to receive cisplatin-based chemotherapy and received atezolizumab as the first-line therapy.[21] Cisplatin ineligibility was defined by one or more of the following: glomerular filtration rate $<$60 mL/min per 1.73 m^2, $\geq$ grade 2 hearing loss/neuropathy and ECOG Performance Status (PS) 2. Patients were grouped by PD-L1 expression on ICs as with cohort 2. After a median follow-up of 17.2 months, ORR was 23% for the whole cohort (28% for IC 2/3 group); the association between ORR and IC expression in cohort 1 was less pronounced than in cohort 2. Responses were durable with median DOR not reached, and 19 of 27 responses ongoing. Median PFS was 2.7 months, while median OS was 15.9 months. This observed median OS was notable when placed in context with first-line carboplatin-based (9.3 months) or cisplatin-based therapy (15.2–15.9 months) in eligible patients. Additionally, promising outcomes were reported in patients 80 years or older and those with upper tract disease, two groups traditionally associated with poor prognosis. TRAEs were reported in 66% of patients, 16% with grade 3–4 TRAEs, 8% leading to treatment discontinuation and one treatment-related death (due to sepsis). The most frequent TRAEs were fatigue (30%), diarrhea (12%) and pruritus (11%).[22]

Based on the results of cohort 2 of the IMvigor 210, the FDA, on May 18, 2016, granted accelerated approval to atezolizumab as second-line therapy in patients with locally advanced or metastatic UC who had disease progression during or after platinum-based therapy or within 12 months of neoadjuvant/adjuvant platinum-based therapy.[19] FDA later granted accelerated approval to atezolizumab as first-line agent in locally advanced or metastatic UC in cisplatin-ineligible patients, based on the data of the cohort 1 of the IMvigor 210 trial.[22] The recommended dose is 1,200 mg IV infusion every 3 weeks until disease progression or unacceptable toxicity.[23] Atezolizumab is being further evaluated in multiple ongoing trials in various treatment settings, including high-risk non-muscle-invasive bladder cancer (NMIBC) as well as in adjuvant, neoadjuvant and metastatic settings for muscle-invasive bladder cancer (MIBC), as listed in Table 1.

On May 9, 2017, Roche announced that IMvigor211, a phase III trial comparing atezolizumab to chemotherapy in patients with locally advanced or metastatic UC that progressed during or after treatment with platinum-based chemotherapy, did not meet its primary efficacy endpoint of OS in the patient subset with IC2/3 PD-L1 expression compared to chemotherapy, although the efficacy and safety profile of atezolizumab in that trial were consistent with what was previously observed. There was a hierarchical statistical design, in which the primary efficacy endpoint of OS was to be tested first in patients with the highest levels of PD-L1 expression (IC2/3), followed by those with PD-L1 expression (IC1/2/3) and ultimately in the overall study population; statistical significance needed to be demonstrated at each level for the next level to be evaluated. Interestingly, there was statistically significant OS benefit with atezolizumab over chemotherapy in the overall study population and there was discussion whether patients on vinflunine performed better than that previously expected. Results from this trial were presented in June 2017, and the publication is now available.[24,25] It is notable that the FDA approvals of atezolizumab in locally advanced or metastatic UC were maintained and similar approvals were also granted by the European Medicines Agency (EMA). The approvals based on single-arm phase II

Table 1 Examples of ongoing ICI clinical trials in UC (not an exhaustive list)

Disease setting	Study ID	Title	Phase	Allocation	Intervention	Primary outcome measures	N	Completion estimated time	Status
Non-muscle-invasive bladder cancer									
BCG-unresponsive	NCT02844816	Atezolizumab in treating patients with recurrent BCG-unresponsive non-muscle invasive bladder cancer (S1605)	II	Single group assignment	Atezolizumab	Event-free survival	148	February 2019	Recruiting
BCG-unresponsive	NCT02901548	Durvalumab for BCG-refractory urothelial carcinoma in situ of the bladder	II	Single group assignment	Durvalumab + cystoscopy	CRR at 6 months	34	December 2020	Recruiting
BCG-unresponsive	NCT02625961	Pembrolizumab in patients with high risk non-muscle invasive bladder cancer unresponsive to BCG (KEYNOTE-057)	II	Single group assignment	Pembrolizumab	CRR, DFS	260	December 2021	Recruiting
Recurrent	NCT02808143	Pembrolizumab and BCG solution in treating patients with recurrent non-muscle-invasive bladder cancer	I	Single group assignment	Pembrolizumab (intravesical) + BCG (intravesical)	DLTs, incidence of AEs	27	January 2019	Recruiting
Neoadjuvant (NMIBC and MIBC)	NCT02451423	A phase II study of atezolizumab in non-metastatic transitional cell bladder cancer	II	Single group assignment	Atezolizumab (3 dose levels)	Pathologic complete response rate; change in CD3 cell count/μm^2	42	December 2019	Recruiting
Muscle-invasive bladder cancer									
Adjuvant	NCT02450331	A study of atezolizumab versus observation as adjuvant therapy in participants with high-risk muscle-invasive urothelial carcinoma after surgical resection (IMvigor010)	III	Randomized	Atezolizumab vs observation	DFS	800	January 2019	Recruiting
Adjuvant	NCT02632409	An investigational immunotherapy study of adjuvant nivolumab versus placebo in subjects with high risk invasive UC (CheckMate 274)	III	Randomized	Nivolumab vs placebo	DFS	640	April 2020	Recruiting
Adjuvant	NCT03244384	Pembrolizumab versus observation in muscle invasive and locally advanced urothelial cancer (AMBASSADOR)	III	Randomized	Pembrolizumab vs observation	DFS, OS	739	February 2019	Recruiting
Neo-adjuvant	NCT02736266	Neoadjuvant pembrolizumab for muscle-invasive urothelial bladder carcinoma	II	Single group assignment	Pembrolizumab × 3 cycles before cystectomy	Pathologic complete response	90	January 2019	Recruiting

(*Continued*)

Table 1 (*Continued*)

Disease setting	Study ID	Title	Phase	Allocation	Intervention	Primary outcome measures	N	Completion estimated time	Status
Neo-adjuvant	NCT02365766	Neoadjuvant pembrolizumab in combination with gemcitabine therapy in cisplatin-eligible/ineligible UC subjects	I/II	Non-randomized	Pembrolizumab + gemcitabine + cisplatin (in eligible patients) before cystectomy	RFS, OS, radical cystectomy rate	81	July 2018	Recruiting
Neo-adjuvant	NCT02812420	Durvalumab + tremelimumab in patients with muscle-invasive, high-risk UC who are ineligible for cisplatin-based neoadjuvant chemotherapy	I	Single group assignment	Durvalumab + tremelimumab followed by cystectomy	Toxicity	15	March 2019	Recruiting
Neo-adjuvant	NCT02845323	Neoadjuvant nivolumab with and without urelumab in patients with cisplatin-ineligible muscle-invasive urothelial carcinoma of the bladder	II	Randomized	Nivolumab vs nivolumab + urelumab	Tumor infiltrating $CD8^+$ T-cell density at cystectomy	44	September 2018	Recruiting
Bladder-sparing	NCT02621151	Pembrolizumab, gemcitabine and concurrent hypofractionated RT for muscle-invasive UBC	II	Single group assignment	Pembrolizumab + gemcitabine + RT	2-year bladder-intact DFS	54	May 2021	Recruiting
Advanced/metastatic UC									
First-line	NCT02807636	Study of atezolizumab as monotherapy and in combination with platinum-based chemotherapy in participants with untreated locally advanced or metastatic urothelial carcinoma (IMVigor130)	III	Randomized	Atezolizumab + gemcitabine + carbo/cisplatin vs placebo + gemcitabine + carbo/cisplatin vs atezolizumab	PFS, OS, % patients with AEs	1,200	December 2018	Recruiting
First-line	NCT02989584	A pilot safety study of atezolizumab combination with cisplatin + gemcitabine in patients with metastatic bladder cancer	I/II	Single group assignment	Atezolizumab + gemcitabine + cisplatin	Dose-limiting toxicity rates	30	December 2018	Recruiting
First-line	NCT03093922	A study of two dosing schedules of atezolizumab in combination with gemcitabine and cisplatin as first-line treatment for metastatic bladder cancer	II	Randomized	2 dosing schedules of atezolizumab + gemcitabine + cisplatin	Best confirmed ORR	31	March 2019	Recruiting

(*Continued*)

Table 1 (*Continued*)

Disease setting	Study ID	Title	Phase	Allocation	Intervention	Primary outcome measures	N	Completion estimated time	Status
First-line	NCT03133390	Atezolizumab with or without bevacizumab in cisplatin-ineligible patients	II	Randomized	Atezolizumab + bevacizumab vs atezolizumab	OS	118	May 2018	Not yet recruiting
First-line	NCT03115801	A phase II randomized trial of immunotherapy plus radiotherapy in metastatic genitourinary cancers	II	Randomized	Atezolizumab vs atezolizumab + RT	Best ORR	112	December 2020	Recruiting
First-line	NCT02853305	Study of pembrolizumab with or without platinum-based combination chemotherapy versus chemotherapy alone in UC (MK-3475-361/KEYNOTE-361)	III	Randomized	Pembrolizumab vs pembrolizumab + gemcitabine + cis/carboplatin vs gemcitabine + cis/carboplatin	PFS, OS	990	January 2019	Recruiting
First-line	NCT03036098	Nivolumab in combination with ipilimumab compared to the standard of care chemotherapy in patients with untreated inoperable or mUC (CheckMate 901)	III	Randomized	Nivolumab + ipilimumab vs gemcitabine + cis/carboplatin	PFS, OS	690	April 2020	Recruiting
First-line	NCT02603432	A study of avelumab in patients with locally advanced or metastatic urothelial cancer (JAVELIN bladder 100)	III	Randomized	Avelumab + best supportive care vs best supportive care	OS	668	July 2019	Recruiting
First-line	NCT02516241	Durvalumab with or without tremelimumab versus standard of care chemotherapy in patients with unresectable stage IV UC (DANUBE)	III	Randomized	Durvalumab + tremelimumab vs durvalumab vs standard of care chemotherapy	PFS	1,005	April 2018	Close to accrual
First-line	NCT03150836	Radiation therapy and durvalumab, with or without tremelimumab, in patients with unresectable, muscle-invasive or metastatic UBC that are ineligible or refusing chemotherapy	II	Randomized	Durvalumab + RT vs durvalumab + tremelimumab + RT	Toxicity, PFS	74	September 2021	Not yet recruiting
First-line	NCT02527434	Study of tremelimumab in patients with advanced solid tumors	II	Single group assignment	Tremelimumab vs durvalumab vs tremelimumab + durvalumab	ORR	64	March 2018	Active, but not recruiting

(*Continued*)

Table 1 (*Continued*)

Disease setting	Study ID	Title	Phase	Allocation	Intervention	Primary outcome measures	N	Completion estimated time	Status
First/second-line	NCT02891161	Durvalumab and radiation therapy followed by adjuvant durvalumab in patients with locally advanced UBC (unresectable, medically unfit for surgery, or cisplatin ineligible) (DUART)	Ib/II	Non-randomized	Durvalumab + RT followed by durvalumab	DLT rate, PFS, disease control rate	42	November 2018	Recruiting
Second-line	NCT02928406	A study of atezolizumab in locally advanced or metastatic urothelial or non-urothelial carcinoma of the urinary tract	III	Single group assignment	Atezolizumab	% patients with AEs	1,000	May 2022	Recruiting
Second-line	NCT02437370	Pembrolizumab and docetaxel or gemcitabine hydrochloride in treating patients with urothelial cancer	I	Non-randomized	Pembrolizumab + docetaxel/ gemcitabine	Maximum tolerated dose of pembrolizumab	38	May 2018	Recruiting
Second-line	NCT02581982	Paclitaxel and pembrolizumab in treating patients with platinum-refractory mUC	II	Single group assignment	Pembrolizumab + paclitaxel	ORR	27	March 2019	Recruiting
Second-line	NCT02351739	Combination of ACP-196 and pembrolizumab in subjects with platinum resistant UBC (KEYNOTE143)	II	Randomized	Pembrolizumab vs pembrolizumab + ACP-196	ORR	75	December 2017	Closed to accrual
Second-line	NCT02880345	Combined pembrolizumab and hypofractionated radiation in patients with advanced UC who have progressed on anti-PD-1/PD-L1 monotherapy (RADVAX)	–	–	Pembrolizumab + RT in 2 different fractionation schedules	No. of AEs	14	August 2018	Not yet recruiting
Second-line	NCT02496208	Cabozantinib-s-malate and nivolumab with or without ipilimumab in treating patients with metastatic genitourinary tumors	I	Non-randomized	Cabozantinib + nivolumab + ipilimumab	AEs, recommended phase 2 dose	135	December 2017	Recruiting
Maintenance	NCT02500121	PD-1 inhibitor pembrolizumab as maintenance therapy after initial chemotherapy in metastatic bladder cancer	II	Randomized	Pembrolizumab vs placebo	PFS	200	November 2018	Recruiting

Abbreviations: AEs, adverse events; CRR, complete response rate; DFS, disease-free survival; DLT, dose limiting toxicities; ICI, immune checkpoint inhibitor; MIBC, muscle-invasive bladder cancer; mUC, metastatic urothelial carcinoma; NMIBC, non-muscle-invasive bladder cancer; ORR, objective response rate; OS, overall survival; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand-1; PFS, progression-free survival; RFS, relapse-free survival; UC, urothelial cancer.

trial raised interest; however, it can probably be explained by the recently unmet need in those disease settings as well as the totality of available data, including depth and durability of response, safety and tolerability, and ease of use in a challenging patient population. Confirmatory studies are being conducted; IMvigor211 was one of them and while it did not meet its primary endpoint based on the design, it did demonstrate efficacy and safety data consistent with IMvigor210 trial.[25] Moreover, the IMvigor130 trial is a large randomized phase III multicenter trial comparing atezolizumab alone or in combination with platinum-based chemotherapy to platinum-based chemotherapy alone in patients with advanced UC as first-line therapy; study is actively accruing patients (NCT02807636).[26]

Avelumab

Avelumab (MSB0010718C) is a fully human anti-PD-L1 IgG1 antibody that selectively inhibits PD-1/PD-L1 interactions without interfering with the PD-1/PD-L2 pathway.[27] By retaining a native Fc region, avelumab has been found to induce TC lysis in vitro via ADCC, suggesting a possible additional mechanism of anticancer activity.[28] However, it has been reported not to have a significant effect on native ICs allaying concerns regarding deleterious effects on tumor immunity.[29]

JAVELIN Solid Tumor (NCT01772004) is a phase I, open-label, multi-cohort trial aimed at assessing the safety, pharmacokinetics and activity of avelumab in patients with metastatic/locally advanced solid tumors. Based on the results of the dose-escalation phase, 10 mg/kg IV every 2 weeks was chosen as the study dose in phase Ib dose expansion cohorts for various tumor types including advanced UC.[30] Interim results from the UC dose expansion cohort of the JAVELIN trial were published in April 2017 and subsequently, updated safety and efficacy results from pooled analysis of two UC cohorts within the trial were presented at the 2017 ASCO Genitourinary Cancers Symposium (n=153), 2017 ASCO Annual Meeting (n=161) and the 2017 European Society of Medical Oncology (ESMO) meeting (n=249). This study included patients with advanced UC who had progressed/recurred after platinum-based therapy or were cisplatin-ineligible, but not pre-selected based on PD-L1 expression. Based on the pooled analysis, patients received avelumab for a median duration of 12 weeks; 23.3% had upper tract disease and only 2.8% were platinum-naïve. Among the patients who had ≥6 months of follow-up (median 13.6 months), confirmed ORR was 17.3% (25.6% with PD-L1 expression ≥5% and 13.7% with PD-L1 <5%), with a CR rate of 4.4%. Responses were ongoing in 79% patients at data cutoff; median DOR was 20.1 months. Median PFS was 1.6 months, median OS was 8.2 months and the Kaplan–Meier OS rate at 12 months was 41.9%. Avelumab was well tolerated; 68.3% of patients had any grade TRAEs (10.4% grade ≥3), while 17.3% had immune-related adverse events (IRAEs) (3.6% grade ≥3). Most common TRAEs were infusion-related reactions (23.3%) and fatigue (17.3). One treatment-related death was reported (pneumonitis).[31–34]

On May 9, 2017, based on the results of the above-mentioned trial, FDA granted accelerated approval to avelumab as second-line therapy for patients with locally advanced or metastatic UC with disease progression during or following platinum-containing chemotherapy or within 12 months of neoadjuvant or adjuvant platinum-containing chemotherapy.[35] The recommended dose is 10 mg/kg IV infusion every 2 weeks until disease progression or unacceptable toxicity.[36] Moreover, the JAVELIN Bladder 100 phase III randomized trial is evaluating whether switch maintenance with avelumab plus best supportive care vs best supportive care alone can benefit patients who had received platinum-based chemotherapy as first line without progression (NCT02603432).[37]

Durvalumab

Durvalumab (MEDI4736) is a high-affinity human IgG1 κ monoclonal antibody against PD-L1 that blocks its interaction with PD-1 and CD80 (B7.1) but does not induce tumor or IC lysis via ADCC.[38] Interim results from the UC dose expansion cohort of Study 1,108 (NCT01693562), a phase I/II, open-label, multicenter trial of durvalumab in locally advanced or metastatic solid tumors including UC, were first published in June 2016 (n=61).[39] Updated efficacy and safety results were presented at the 2017 ASCO Genitourinary Cancers Symposium (n=103) and the 2017 ASCO Annual Meeting (n=191).[40,41] Responses were rapid and durable; median TTR was 1.4 months, median DOR was not reached at data cutoff and 45% of responders had ongoing responses beyond 6 months. Confirmed ORR was 17.8% (27.6% in the PD-L1 high [≥25% expression on TC or IC] group [n=98] vs 5.1% in the PD-L1 low/negative [<25% on TC and IC] group [n=79]) with a CR rate of 3.7%. Median PFS and OS were 1.5 months and 18.2 months, respectively with a 1-year OS rate of 55.0%. Durvalumab was well tolerated; 63.9% of patients had TRAEs (6.8% grade ≥3). Most common TRAEs were fatigue (13.1%), diarrhea (9.8%) and anorexia (8.2%). Two patients had to discontinue therapy due to adverse events (AEs). No treatment-related deaths

were reported.[39–42] Jin et al presented results of exposure efficacy and safety analysis for the UC cohort at the 2017 ASCO Annual Meeting; overall, the distribution of pharmacokinetic metrics, on a 10 mg/kg IV every 2-week regimen, was similar between responders and non-responders, with no obvious trends connecting pharmacokinetic exposures to change in tumor size, ORR or risk of grade ≥3 AEs.[43] Powles et al reported that tumor shrinkage and OS correlated with increased albumin, decreased neutrophil/lymphocyte ratio and decreased durvalumab clearance, supporting the hypothesis that durvalumab may be associated with a reduction in protein catabolism, inflammation and cancer cachexia among responders.[44]

Based on the abovementioned results, FDA, on May 1, 2017, granted accelerated approval to durvalumab as second-line therapy for patients with locally advanced or metastatic UC who had disease progression during or following platinum-containing chemotherapy or within 12 months of neoadjuvant or adjuvant platinum-containing chemotherapy.[45] The recommended dose is 10 mg/kg IV infusion every 2 weeks until disease progression or unacceptable toxicity.[46] DANUBE (NCT02516241) is a phase III, randomized, multicenter study comparing durvalumab monotherapy vs durvalumab/tremelimumab vs standard of care chemotherapy as first-line therapy in patients with advanced UC; the study is close to accrual and results are anticipated within 2018.[47]

PD-1 inhibitors

Pembrolizumab

Pembrolizumab (MK-3475) is a humanized monoclonal antibody of the IgG4 κ isotype directed against PD-1 that acts by blocking its interaction with PD-L1, thereby impeding inhibitory signaling in T cells and enhancing antitumor immunity.[48] KEYNOTE-012 (NCT01848834), a non-randomized multi-cohort phase Ib study, first provided evidence for safety and activity of pembrolizumab in locally advanced/metastatic UC.[49] This study enrolled 33 patients with PD-L1-positive (≥1% PD-L1 expression on TC and/or tumor stroma by IHC assay different than assays used in other ICI trials) advanced UC. All enrolled patients were included in safety analyses, while 27 were evaluable for response. Overall, 76% were pretreated with at least one regimen for advanced disease, while the remaining 24% had received platinum-based therapy in the adjuvant or neoadjuvant setting but had progressed within 12 months. In total, 30 patients had pure urothelial, 2 had mixed and 1 had small-cell histology, 32 had distant metastases and 1 patient biopsy-proven N3 stage. Among the 27 response evaluable patients, ORR was 26% with 11% CR rate after a median follow-up of 13 months. Median TTR was 2 months, while median DOR 10 months, with 2 patients with ongoing CR at data cutoff. Median PFS and OS were between 2 and 13 months, while the PFS and OS at 12 months were 15% and 50%, respectively. Four patients who were PD-L1 positive on screening were subsequently found to be PD-L1 negative using the clinical trial assay and did not respond; PD-L1 expression correlated with response. Pembrolizumab was overall well tolerated: 61% had any grade TRAEs (15% grade ≥3) TRAEs, 2 patients discontinued treatment due to TRAEs (grade 3 rhabdomyolysis; grade 3 hypercalcemia) but no treatment-related death was noted. Most common TRAEs were fatigue (18%) and peripheral edema (12%).[49]

Balar et al presented results from interim analysis on the first 100 patients of KEYNOTE-052 (NCT02335424), a phase II, multicenter trial of pembrolizumab as first-line therapy for locally advanced/metastatic UC in cisplatin-ineligible patients. Two different cutoff points were used for PD-L1 expression: combined positive score (CPS) (TC + IC PD-L1 expression) ≥1 and ≥10. ORR for the first 100 patients after a median follow-up of 8 months was 24%, with a CR rate of 6% (ORR for the CPS ≥10 group was 36.7% with an impressive CR rate of 13.3%). Median DOR was not reached; 67% experienced TRAE (16% ≥ grade 3) and 5% had to discontinue therapy due to TRAEs.[50] Updated results and biomarker analyses were presented at the 2017 ASCO annual meeting.[51] Overall, 370 patients were available for safety and efficacy analyses, 85% had visceral metastases, 10% had prior adjuvant/neoadjuvant platinum-based chemotherapy and 19% had upper tract disease. Confirmed ORR was 29%, with a CR rate of 7% (increased ORR with longer follow-up). ORR was comparable across subgroups based on age, ECOG PS and disease location. Median TTR was 2 months and the response was durable; median DOR was not reached; and 82% of responses lasted ≥6 months and 67% of responses were ongoing at data cutoff. For PD-L1 expression analyses, patients were divided into two groups: training set, which included the first 100 patients (used to identify CPS cutoff point), and validation set, which included all evaluable patients except the training set (used to validate CPS cutoff); CPS ≥10 was determined to be the optimal cutoff point, with an ORR of 51% in the CPS ≥10 group vs 23% in the CPS <10 group. Overall, 66% of patients reported TRAEs of any grade, with 19% reporting grade ≥3 TRAEs. Most common TRAEs were fatigue (18%) and pruritus (17%), while most common IRAE was hypothyroidism (10%); 7% discontinued therapy due to TRAEs and one treatment-related

death was noted (myositis).[50,51] Updated results by Grivas et al showed that pembrolizumab was well tolerated with notable anti-tumor activity in elderly and poor performance status patients, comparable to the entire study population, suggesting that it could be used even in advanced UC patients who cannot tolerate chemotherapy.[52]

KEYNOTE-045 (NCT02256436), a phase III, open-label, international trial, randomized 542 patients with advanced UC that recurred or progressed after platinum-based therapy to receive either pembrolizumab or investigator's choice of paclitaxel, docetaxel or vinflunine.[53] Patients were selected irrespective of PD-L1 expression status and randomization was stratified according to ECOG PS (0/1 vs 2), liver metastases (yes vs no), hemoglobin level (<10 vs ≥10 g/dL) and time since last dose of prior chemotherapy (<3 vs ≥3 months). Co-primary endpoints were OS and PFS in all patients and in those with PD-L1 CPS ≥10. Initial results after a median follow-up of 14.1 months were published, while updated results of survival analysis after median follow-up of 18.5 months were presented at the 2017 ASCO Annual Meeting.[53,54] Median OS was 10.3 months (pembrolizumab) vs 7.4 months (chemotherapy) (P=0.0004), and the survival benefit was maintained with longer follow-up. The reported hazard ratio for death with pembrolizumab was 0.73 (95% CI: 0.59–0.91). OS rates were 44.4% and 30.2% at 12 months and 36.1% and 20.5% at 18 months with pembrolizumab and chemotherapy, respectively. OS appeared longer in the overall patient population than in the CPS ≥10 patient subset, suggesting that higher PD-L1 protein expression may be a negative prognostic biomarker based on this assay. ORR was significantly higher with pembrolizumab than with chemotherapy, both in the overall study population (21.1% vs 11.0%) and within the subset with CPS ≥10 (20.3% vs 6.7%; P=0.0034). Median TTR was 2.1 months in each arm. There was no significant difference in PFS in the total population (median 2.1 months with pembrolizumab vs 3.3 months with chemotherapy; hazard ratio 0.98; 95% CI 0.81–1.19; P=0.42) or in patients with CPS ≥10 (hazard ratio 0.89; 95% CI 0.61–1.28; P=0.24). Median DOR was 4.4 months with chemotherapy and not reached with pembrolizumab at the time of data cutoff. Continued responses at ≥12 months were seen in 69% (pembrolizumab) vs 36% (chemotherapy) of patients. Any grade TRAEs were fewer with pembrolizumab vs chemotherapy (61.3% vs 90.2%), as were grade ≥3 TRAEs (16.5% vs 49.8%) and TRAE-related discontinuation of therapy (5.6% vs 11.0%). Most frequent TRAEs of any grade with pembrolizumab were pruritus (19.5%), fatigue (13.9%) and nausea (10.9%), and the most common TRAE grade ≥3 were pneumonitis (2.3%), colitis (1.1%) and nephritis (0.8%). One patient died from treatment-related pneumonitis.[53,54] De Wit et al reported that health-related quality of life (HRQoL) was substantially better for longer duration in patients on pembrolizumab than on chemotherapy, both among patients with continued response as well as those with progression by week 15.[55]

Petrylak et al, in subgroup analyses from KEYNOTE-045, showed that pembrolizumab was associated with OS benefit over each individual chemotherapy agent in the trial (hazard ratio [95% CI]: paclitaxel, 0.77 [0.57–1.06]; docetaxel, 0.78 [0.56–1.08]; vinflunine, 0.71 [0.52–0.96]), though PFS rates were similar.[56]

Based on the results of KEYNOTE-045 trial, the FDA, on May 18, 2017, granted regular approval to pembrolizumab as second-line therapy for patients with locally advanced or metastatic UC with disease progression during or following platinum-based therapy or within 12 months of neoadjuvant or adjuvant therapy with a platinum-containing regimen. FDA also granted accelerated approval to pembrolizumab as first-line agent in locally advanced or metastatic UC in cisplatin-ineligible patients, at the same dose of 200 mg as IV infusion every 3 weeks until disease progression or unacceptable toxicity, based on the KEYNOTE-052 trial.[49,57] Similar approvals have been granted by the EMA in Europe.

It is worth noting that pembrolizumab is the only ICI with proven OS benefit over chemotherapy in a large randomized phase III trial and thus has the highest level of evidence in the platinum-refractory setting in advanced UC. Moreover, the Keynote 361 trial is a large randomized phase III multicenter trial comparing pembrolizumab alone or in combination with platinum-based chemotherapy to platinum-based chemotherapy alone in patients with advanced UC as first-line therapy; study is actively accruing patients (NCT02853305).[58]

Nivolumab

Nivolumab is a fully human monoclonal IgG4 antibody that binds PD-1 with high affinity, blocking its interaction with both PD-L1 and PD-L2, but does not activate ADCC or CDC.[59] CheckMate 032 (NCT01928394) was a phase I/II, two-stage, multi-arm, multicenter trial for multiple advanced solid tumors including UC patients with progression after at least one previous platinum-based therapy for locally advanced/metastatic UC, or previously refused standard chemotherapy for advanced disease, or had recurrence within 12 months of platinum-based adjuvant/neoadjuvant therapy. The UC cohort of the trial involved three treatment arms: 1) N3 – nivolumab 3 mg/kg every 2 weeks (monotherapy);

2) N1I3 – nivolumab 1 mg/kg + ipilimumab 3 mg/kg every 3 weeks for 4 cycles, followed by nivolumab 3 mg/kg every 2 weeks; and 3) N3I1 – nivolumab 3 mg/kg + ipilimumab 1 mg/kg every 3 weeks for 4 cycles, followed by nivolumab 3 mg/kg every 2 weeks. The results of 78 patients randomized to nivolumab monotherapy arm (N3) were reported first.[60] These patients were heavily pretreated (67% had received ≥2 previous regimens), 78% had visceral metastases and 51% had <1% PD-L1 expression. At the time of data cutoff, the median duration of follow-up was 15.2 months and patients had received a median of 8.5 doses of nivolumab. Among the 74 response-evaluable patients, ORR was 24.4% with a CR rate of 6%. ORR was comparable in patients with PD-L1 expression <1% and ≥1% (26% vs 24%). At the time of data cutoff, median DOR was 9.4 months and median TTR was 1.5 months. Median OS was 9.7 months and median PFS 2.8 months in the whole cohort, while median OS was 16.2 months and median PFS 5.5 months in patients with PD-L1 expression ≥1%. Overall, 59% patients reported grade 1–2 TRAEs, while 22% reported grade 3–4 AEs. Most common TRAEs were fatigue (33%), pruritus (29%) and rash (15%). Serious IRAEs included colitis, thrombocytopenia, AKI and pneumonitis. Two patients discontinued therapy due to TRAEs (grade 4 pneumonitis; grade 4 thrombocytopenia) and subsequently died.[60]

Results from the nivolumab/ipilimumab arms (N1I3 [n=26] and N3I1 [n=104]) of the abovementioned trial were presented at the Society for Immunotherapy of Cancer (SITC) Meeting in 2016.[61] Median follow-up was 7.8 months in the N1I3 group and 16.7 months in the N3I1 group. ORRs were 38.5% and 26%, while CR rates were 4% and 3% in the N1I3 group and the N3I1 group, respectively. Mean DOR had not been reached in either group. The frequencies of grade 1–2 TRAEs were 46.1% (N1I3) and 52.9% (N3I1), while that of grade 3–4 TRAEs were 30.8% (N1I3) and 31.7% (N3I1). One treatment-related death was reported with N3I1 (pneumonitis) and none with N1I3. Authors suggested that the N1I3 dosing schedule may provide the most favorable risk-benefit ratio, although data with longer follow-up are anticipated.[61] These results supported the design of CheckMate 901, a large confirmatory randomized phase III trial comparing N1I3 to platinum-based chemotherapy in patients with advanced UC as first-line therapy (NCT03036098).[62]

CheckMate 275 (NCT02387996), a phase II multicenter single-arm trial, was aimed at assessing the safety and efficacy of nivolumab monotherapy (3 mg/kg every 2 weeks until disease progression or unacceptable toxicity) in patients with metastatic or surgically unresectable UC with disease progression or recurrence despite at least one platinum-based regimen.[63] Of the 265 patients who were evaluable for response, 29% had previously received ≥2 lines of chemotherapy in the metastatic setting, 46% had ECOG PS ≥1 and 84% had visceral metastases at baseline. Median follow-up was 7.0 months. Confirmed ORR was 19.6% in the overall population (28.4% with PD-L1 expression ≥5%, 23.8% with PD-L1 ≥1% and 16.1% with PD-L1 <1%) with overall CR rate of 2%. Median DOR was not reached. Median OS was 8.7 months (11.3 months with PD-L1 ≥1% and 5.9 months with PD-L1 <1%), and median PFS 2.0 months. The toxicity profile of nivolumab was comparable to that described in multiple trials in various solid tumors, and clearly favorable compared to cytotoxic chemotherapy in this setting. TRAEs occurred in 64% of patients available for safety analyses, with 18% grade 3–4 TRAEs. Most common TRAEs were fatigue (17%), diarrhea (9%) and pruritus (9%). Most common IRAEs were cutaneous (17%) and endocrine (14%). Three treatment-related deaths occurred: one from pneumonitis, one from acute respiratory failure and one from cardiovascular failure.[63] Necchi et al recently reported that HRQoL in 168 trial patients who had assessments at baseline and at least once later remained stable or improved on nivolumab.[64]

Based on the results of the CheckMate 275 trial, the FDA, on February 2, 2017, granted accelerated approval to nivolumab as second-line therapy for patients with locally advanced or metastatic UC who had disease progression during or following platinum-containing chemotherapy or within 12 months of neoadjuvant or adjuvant treatment with a platinum-containing regimen.[65] The recommended dose is 240 mg IV every 2 weeks until disease progression or unacceptable toxicity, while more recently the dose of 480 mg every 4 weeks was approved by FDA across several nivolumab indications.[66]

CTLA-4 inhibitors

Ipilimumab

Ipilimumab is a fully human IgG1 anti-CTLA-4 antibody that has been approved by the FDA for treatment of melanoma.[67] In 2010, Carthon et al reported the results from a phase I trial of ipilimumab as neoadjuvant therapy in a small cohort of 12 patients with localized (cT1–cT2) muscle-invasive bladder UC. Primary end points were safety and immune monitoring. The drug was well tolerated in both 3 mg/kg (n=6) and 10 mg/kg (n=6) doses. Most patients had grade 1–2 TRAEs. Four patients developed grade 3 TRAEs and 2 patients had treatment-related delay in surgery (all in the 10 mg/kg arm). Eight patients had downstaging in their

surgical specimens after ipilimumab, when compared to pre-treatment transurethral resection of bladder tumor specimens. Also, 4 patients converted from positive to negative in urine cytology and/or FISH analysis after ipilimumab. Analysis of tumor and peripheral blood samples revealed posttreatment increase in a subset of effector T cells ($CD4^{+}ICOS^{hi}$ T cells), which had previously been shown to be immune biomarker of increased clinical benefit with anti-CTLA-4 therapy in metastatic melanoma.[68]

In a phase II trial (NCT01524991), patients with metastatic UC received gemcitabine + cisplatin (GC) for 2 cycles, followed by 4 planned cycles of GC + ipilimumab, as first-line therapy. Overall, 36 patients were enrolled with median age of 60 years; 75% had Karnofsky Performance Status ≤90% and 58% had visceral metastases. Patients received a median of 5 cycles of GC and 3 doses of ipilimumab. ORR was 64%, with a CR rate of 14%. Median PFS was 8.0 months after median follow-up of 10.4 months. The primary end point, 1-year OS, had not yet been reported. The most common grade ≥3 TRAEs were neutropenia (36%), hyponatremia (31%), anemia (25%) and thrombocytopenia (19%), while the most frequent grade ≥3 IRAEs were colitis (6%), hypophysitis (3%), hyperthyroidism (1%) and rash (1%).[69]

Tremelimumab

Tremelimumab (formerly known as CP-675,206 and ticilimumab) is a fully human monoclonal IgG2 antibody directed against CTLA-4.[70] Though it showed promise in phase I and II melanoma trials, results from interim analysis of a phase III trial were negative, leading to its termination. Results were also negative in a phase II mesothelioma trial.[71]

Results of a planned safety and efficacy analysis from the UC cohort of NCT02527434, a phase II multicenter open label trial evaluating tremelimumab in advanced solid tumors, were recently presented at the 2017 SITC meeting. Eligible patients had locally advanced or metastatic UC who had progressed on, were ineligible for or refused prior chemotherapy. The primary endpoints were safety and ORR. As of April 2017, 32 patients were eligible for efficacy and safety analyses. All of them had stage IV tumor, and most had received prior platinum-based therapy. ORR was 18.8%, with a CR rate 6.2%. Median TTR was 3.3 months and responses were durable (median DOR not reached). Median PFS and OS were 3.7 months and 9.6 months, respectively. TRAEs occurred in 53.1% patients with grade ≥3 events reported in 18.8% patients. No treatment-related deaths were reported.[72]

Tremelimumab is being evaluated in ongoing trials as neoadjuvant therapy in MIBC and in advanced/metastatic UC. Results from the large randomized DANUBE phase III trial (NCT02516241) are pending[47] (Table 1).

Putative biomarkers

Despite promising results from ICI trials in UC, not all patients respond to these therapies. Biomarkers that can accurately predict response to these agents can help patient selection and thus maximize therapeutic benefit while limiting unnecessary immune-related toxicity. However, as of yet, there is no molecular biomarker with proven clinical utility in UC, but intensive research is ongoing.

PD-L1 expression

PD-L1 expression on TC and/or ICs, measured by IHC, is the most extensively studied biomarker with conflicting results among trials regarding its potential prognostic and/or predictive value based on the assay used. The distinction between prognostic vs predictive value is important and very hard to derive from single arm phase II trials. Various commercially available in vitro diagnosis (IVD) assays have been used in clinical trials for detecting PD-L1 expression in UC, each in combination with one of the PD-1/PD-L1 inhibitors described earlier (Table 2). The Ventana PD-L1 (SP142) assay (uses rabbit monoclonal anti-PD-L1 clone SP142) has been FDA approved as a complementary IVD with atezolizumab for use in UC, based on results from IMVigor 210 trial.[73] The SP263 PD-L1 assay from Ventana (uses rabbit monoclonal antibody clone SP263) has been approved by the FDA as a complementary IVD for use with durvalumab in UC.[45] The IHC 22C3 pharmDx PD-L1 assay from Dako (uses mouse anti-PD-L1 clone 22C3), which was used in the KEYNOTE-012, KEYNOTE-045 and KEYNOTE-052 trials, has been FDA approved as a companion assay with pembrolizumab in non-small cell lung cancer (NSCLC), and is currently being validated in UC in clinical studies.[74] The Dako IHC 28-8 pharmDx PD-L1 assay (uses rabbit monoclonal antibody clone 28-8), used in the CheckMate 032 and CheckMate 275 trials, has been FDA approved for use with nivolumab in NSCLC and melanoma, and has been validated in UC based on clinical studies.[60] The proprietary IHC 73-10 PD-L1 assay from Dako was used in combination with avelumab in the JAVELIN Solid Tumor trial, but has not yet been validated for use in UC.[33] All these assays are performed using formalin-fixed paraffin-embedded tissues.[75] However, none of those assays are approved as companion diagnostic test in UC and are not used in routine clinical practice.

Studies in multiple cancers, including UC, have shown correlation between PD-L1 expression and rates of response to ICI.[20,22,64,76,77] A recent meta-analysis comparing outcomes

Table 2 Characteristics and performance of PD-L1 assays used in ICI UC trials

Drug	Trial	IHC assay	TC scoring	IC scoring	TC + IC scoring	Outcome measures
Atezolizumab	IMvigor 210 (phase II); cisplatin-treated[20]	SP142 (Ventana)	TC0 <1% TC1 ≥1% but <5% TC2 ≥5% but <50% TC3 ≥50%	IC0 <1% IC1 ≥1% but <5% IC2 ≥5% but <10% IC3 ≥10%	N/A	IC staining: ORR 27% (IC2/3), 10% (IC1), 9% (IC0) ($P<0.001$) TC staining: no correlation with response
	IMvigor 210 (phase II); cisplatin-ineligible[22]	SP142 (Ventana)	TC0 <1% TC1 ≥1% but <5% TC2 ≥5% but <50% TC3 ≥50%	IC0 <1% IC1 ≥1% but <5% IC2 ≥5% but <10% IC3 ≥10%	N/A	No correlation (TC or IC) with response
Pembrolizumab	KEYNOTE-012 (phase I)[49]	IHC 22C3 (Dako)	PD-L1 expression <1% vs ≥1%	N/A	PD-L1 CPS <1% vs ≥1%	CPS: ORR 0% (<1%) vs 24% (≥1%) TC staining: ORR 27% (<1%) vs 14% (≥1%)
	KEYNOTE-045 (phase III) pembrolizumab vs chemotherapy[53]	IHC 22C3 (Dako)	N/A	N/A	PD-L1 CPS ≥10% vs <10%	HR for death 0.57 (0.37N/A0.88) in CPS ≥10%; 0.80 (0.61N/A1.05) in CPS <10%
	KEYNOTE-052 (phase II)[50]	IHC 22C3 (Dako)	N/A	N/A	PD-L1 CPS ≥10% vs <10% PD-L1 CPS ≥1% vs <1%	ORR 36.7% (CPS ≥10%), 25.4% (CPS ≥1%), 24.0% (all patients)
Nivolumab	CheckMate 032 (phase I/II)[60]	IHC 28-8 (Dako)	PD-L1 expression <1% vs ≥1%	N/A	N/A	No correlation with ORR OS 16.2 months (≥1%) vs 9.9 months (<1%)
	CheckMate 275 (phase II)[63]	IHC 28-8 (Dako)	PD-L1 expression ≥5%, ≥1%, and <1%			ORR 28.4% (≥5%), 23.8% (≥1%), 16.1% (<1%) OS 11.3 months (≥1%) vs 5.9 months (<1%)
Durvalumab	NCT01693562 (phase I/II)[39]	SP263 (Ventana)	PD-L1 expression <25% vs ≥25%	PD-L1 expression <25% vs ≥25%	PD-L1 positive (≥25% on TC or IC) PD-L1 negative (<25% on TC and IC)	ORR 46.4% (PD-L1 positive) vs 0% (PD-L1 negative) TC only: ORR 46.7% (≥25%) vs 22.2% (<25%) IC only: ORR 55.6% (≥25%) vs 12.5% (<25%)
Avelumab	NCT01772004 (phase Ib)[31]	IHC 73-10 (Dako)	PD-L1 expression ≥5% vs <5%	N/A	N/A	ORR 53.8% (≥5%) vs 4.2% (<5%) PFS 48.1 weeks (≥5%) vs 7.1 weeks (<5%) 12-month OS 75.5% (≥5%) vs 56.3% (<5%)

Abbreviations: CPS, combined positive score; HR, hazard ratio; IC, immune cell; ICI, immune checkpoint inhibitor; IHC, immunohistochemistry; ORR, objective response rate; OS, overall survival; PD-L1, programmed cell death ligand-1; PFS, progression-free survival; TC, tumor cells; UC, urothelial cancer; N/A, not available.

of anti-PD-1/PD-L1 therapy vs chemotherapy as salvage regimens in phase II and III clinical trials of metastatic UC revealed that ICIs were associated with significantly higher median OS and ORR in PD-L1-positive therapy compared to single agent or doublet chemotherapy, while the differences were not significant in PD-L1 unselected patients.[78] However, clinical trials in UC have employed varying IHC assays and thresholds/cutoff points for PD-L1 positivity, making the results very difficult to generalize and adopt in practice (Table 2). Objective responses occur in patients with PD-L1 negative tumors in most studies, suggesting that its negative predictive value for clinical response is clearly suboptimal. There is also evidence that PD-L1 expression may be transient and dynamic, leading to significant inter-tumor and intra-tumor heterogeneity.[79] PD-L1 IHC might potentially be informative, particularly in combination with other potential biomarkers. This is being investigated in several clinical trials; however, PD-L1 IHC score should not be currently used to select advanced UC patients for ICI in routine clinical practice.

Mutational and neoantigen burden

Neoantigens produced by somatic or germline mutations in TC are important drivers of endogenous anti-tumor immunity.[80] Tumors with high mutational load and neoantigen burden have been demonstrated to have microenvironment rich in ICs and Th1-associated cytokines.[81] This is counterbalanced by upregulated expression of immune checkpoint ligands, such as PD-1, PD-L1, CTLA-4, LAG-3 and TIM3, IDO, facilitating immune escape and in turn making these tumors more responsive to immunotherapy strategies.[82] Higher tumor mutational load, measured by

whole exome or targeted next-generation sequencing, was shown to correlate with response to immunotherapy in melanoma and NSCLC.[83,84] In both the cohorts of IMvigor210 UC trial, higher mutational load correlated with greater response to and longer OS with atezolizumab, and this association was independent of PD-L1 expression or TCGA profiling cluster.[20,22] However, often responders had tumors with low mutational load while non-responders displayed high mutational load, calling into question the predictive accuracy of this biomarker. The combination of high mutational load and low intra-tumor neoantigen heterogeneity (<1%) may be a better predictor of durable clinical benefit than mutational load alone, as shown by McGranahan et al in NSCLC.[85] Similar data correlating higher mutational load with better response to and longer survival with nivolumab were shown in updates from the CheckMate 275 trial in UC.[63]

Mismatch repair, DNA damage response gene mutations and microsatellite instability

MMR genes are responsible for recognizing and repairing nucleotide base mispairing that arise during DNA replication and recombination, as well as correcting other forms of DNA damage. Microsatellites are repetitive sequences of bases scattered throughout coding and non-coding regions of the genome, where DNA polymerases are particularly error prone. Defects in the MMR machinery lead to 100–1,000-fold increase in rates of frame-shift and missense mutations (microsatellite instability [MSI]). MSI due to germline mutations in *MMR* genes results in Lynch (hereditary non-polyposis colorectal cancer) syndrome, which is also associated with UC among other cancers.[86]

MMR-deficient (dMMR) tumors exhibit high neoantigen burden and thus more likely to respond to ICI blockade. Le et al, in a proof-of-concept study of pembrolizumab in patients with progressive metastatic cancer, observed that dMMR tumors had significantly higher mutational load and higher ORR and PFS rates when compared to MMR-proficient tumors.[87] Subsequently, the study was expanded to include 86 patients with dMMR tumors of 12 different types, demonstrating robust and durable responses across all tumor types (ORR 53%, CR rate 21%, PFS and OS not reached at data cutoff), supporting the hypothesis that dMMR tumors are more sensitive to ICI.[88] Teo et al recently reported, based on an analysis of 52 patients with advanced UC, that the presence of alterations in DNA damage response genes correlated positively with response to PD-1/PD-L1 blockade, and this effect was independent of mutational load.[89] Similarly, Iyer et al in a study of UC patients found that dMMR tumors, though rare (3%), were characterized by high mutational load, MSI-high status, strong association with Lynch syndrome and durable response to ICI.[90]

The FDA, on May 23rd 2017, contingent on the results of a confirmatory trial, granted accelerated approval to pembrolizumab for the treatment of patients with unresectable or metastatic, MSI-high/dMMR solid tumors that progressed following prior treatment. This was a first-of-its-kind approval of a cancer therapy based on a biomarker, regardless of tumor type. This approval was based on data from 149 patients with dMMR/MSI-high tumors across five single-arm clinical trials (90 patients had colorectal cancer and 59 had any of 14 other cancer types). ORR for pembrolizumab was 39.6%, with a clinical response rate of 7.4%. Responses were durable (lasting ≥6 months in 78% of responders) and included non-colorectal cancers.[91]

Gene expression (mRNA) subtypes

TCGA had initially identified four subtypes (clusters I–IV) of UC based on RNA-sequencing analysis of 129 tumors: cluster I with papillary-like morphology and dysregulation of FGFR3, clusters I and II with features similar to luminal A breast cancer and presence of urothelial markers, cluster III with expression of stem cell markers and features similar to basal-like breast and squamous cell carcinomas and cluster IV, which is similar to cluster III but with features of surrounding stroma and muscle.[92,93] Results from clinical trials have been discordant regarding the correlation between mRNA subtypes and response to ICI. Exploratory analysis in both the platinum-treated and cisplatin-unfit cohorts of the IMvigor210 trial had revealed that mRNA cluster II was associated with the highest ORR.[20,22] However, in the CheckMate 275 nivolumab trial, cluster III had the highest ORR.[94] Hence, further studies are needed to clarify the role of mRNA clusters as putative predictive biomarkers. Also, lack of a standardized assay and insufficient negative predictive value for clinical response are limiting factors. Moreover, updated results of the TCGA analysis as well as other datasets are very intriguing and merit further evaluation in regard to ICI therapy.[95–97]

Transforming growth factor-β signaling

Utilizing tumor samples from participants in the IMvigor210 study, Mariathasan et al demonstrated that increased transforming growth factor-β (TGFβ) signaling signature in fibroblasts within the peritumoral stroma was associated with a lack of tumor response to atezolizumab, particularly in patients with an immune-excluded phenotype (ie, $CD8^+$ T cells excluded from tumor parenchyma and found mostly

in the peritumoral stroma). Further, using a mouse model that recapitulates this immune phenotype, they showed that co-administration of a TGFβ blocking antibody with anti-PD-L1 led to increased penetration of $CD8^+$ T cells into the tumor, thereby provoking vigorous anti-tumor immune response and tumor regression.[98] Also, dual blockage of PD-L1 and TGFβ using a novel bifunctional protein (M7824), derived from the fusion of avelumab and a TGFβ receptor 2 domain, was shown in a recent study to render UC cell lines more susceptible to antigen-specific $CD8^+$ T-cell and tumor necrosis factor-related apoptosis-inducing ligand-mediated lysis, when compared to avelumab alone.[99] These findings suggest that TGFβ signaling in the stroma may be a negative predictor of response to anti-PD-L1, particularly in immune-excluded tumors, a common phenotype in advanced UC.

TCR gene signatures

Next-generation sequencing of the CDR3 regions of TCR provides insight into the clonality of tumor-infiltrating T lymphocytes: low TCR diversity suggests clonal expansion of tumor antigen-specific T cells, while high diversity suggests either lack of clonal expansion of $CD8^+$ T cells or expansion of immunosuppressive regulatory T cells.[100] The former phenotype has been found to correlate with longer recurrence-free survival and the latter with higher rates of tumor recurrence post-cystectomy.[101,102] Four *TCR* gene signatures: interferon-γ (IFNγ), expanded immune, TCR signaling and de novo were explored in the UC cohort of KEYNOTE-012 pembrolizumab trial, and TCR signaling was associated with the longest PFS and clinical benefit.[74]

Among a subgroup of metastatic UC patients from a single center (n=24) treated with atezolizumab as part of the IMVigor 210 trial, high baseline tumor T-cell infiltration and clonality as well as increased on-treatment peripheral expansion of TCR clonality correlated with greater response. Also, patients with lower baseline peripheral TCR clonality had improved OS and PFS.[103,104] Althammer et al, in a biomarker analysis of baseline tumor samples from 43 patients with UC treated with durvalumab in a phase I/II trial (NCT01693562), showed that tumors with high densities of both CD8(+) and PD-L1(+) cells had longer survival compared to those with CD8/PD-L1(−) profiles ($P<0.06$).[105] TCR sequencing may be a useful biomarker to predict response to ICI, although it needs to be studied further.

IFN-γ-related gene signature

Ayers et al using Nanostring platform on mRNA isolated from pretreatment formalin-fixed paraffin-embedded tumor samples derived and refined an 18-gene T-cell inflamed gene expression profile (GEP), which correlated well with response to anti-PD-1 therapy. Furthermore, the potential predictive role of the T-cell GEP score was validated across multiple solid tumors (including UC), and there were signals that it may perform favorably when compared to PD-L1 IHC in PD-L1 unselected patients.[106] This 18-gene T-cell inflamed GEP score was shown in a biomarker analysis from the KEYNOTE-052 trial to have significant association with response to pembrolizumab. Also, a sizable number of additional responders (31/81) were captured using this signature beyond that predicted by PD-L1 IHC.[51] These results suggest that such signatures may be more robust in capturing the biology of tumor immune microenvironment than PD-L1 IHC alone. Bais et al through biomarker analysis of the 1,108 trial UC cohort showed that patients treated with durvalumab in the top tertile for expression of IFN-γ-related mRNA in their tumors had better ORR, PFS and OS than those in the lower two tertiles.[107] Also from the 1,108 UC cohort, using multiplex immunoassay on serum samples from 158 patients prior to durvalumab therapy, Guo et al demonstrated that high serum concentrations of IFN-γ-inducible proteins, CXCL9 and TNF-related weak inducer of apoptosis, were associated with longer OS, while high levels of myeloid cell-associated proteins, including interleukin-8, C-reactive protein, interleukin-6 and macrophage colony-stimulating factor, were associated with shorter OS and disease progression.[108]

Peroxisome proliferator-activated receptors-γ/retinoid X receptor-α pathway activation

Studies have revealed a negative correlation between peroxisome proliferator-activated receptors-γ (PPARγ) expression and T-cell inflamed phenotypes.[109] Recently, Korpal et al, using a syngeneic mouse bladder tumor model, showed that S427F/Y hotspot mutations of retinoid X receptor-α (RXRα) and overexpression of PPARγ cause ligand-independent activation of PPARγ/RXRα heterodimer in bladder cancer cell lines. This leads to downregulation of several pro-inflammatory cytokines, thereby allowing the tumors to reprogram their immune microenvironment to be less inflamed, thus making them more resistant to immunotherapies.[110]

Treatment-related adverse events

Reported rates of TRAEs of any grade approximately range from 57% to 81% across clinical trials of PD-1/PD-L1 inhibitors in UC, with only 4.9%–22% of patients reporting grade 3–4 TRAEs and 4%–8% requiring treatment discontinuation.[17,39,60]

Most commonly reported TRAE was fatigue (13%–36%) and the most common (but still rare) cause of treatment-related mortality was immune-mediated pneumonitis.[39,60] Incidence of grade 3–4 TRAE was significantly higher with the combination of nivolumab/ipilimumab, which is consistent with data from other cancer types.[61] We provide a brief but not exhaustive review of IRAE in the following text. There are several recently developed and dynamically updated guidelines for the management of IRAEs by different organizations, eg, ASCO, ESMO, National Comprehensive Cancer Network, SITC, etc., while registries, databases, care paths and multi-disciplinary tumor boards can contribute to better data capture and sharing, as well as multi-expert and more standardized IRAE management. Early recognition and optimal IRAE management are really critical.[111–113]

Systemic

Fatigue is the most commonly observed TRAE in UC trials of PD-1/PD-L1 inhibitors, affecting up to 36% of patients. It is usually mild with only <3% reporting grade ≥3 fatigue or requiring treatment delay or discontinuation.[60] Fatigue should prompt evaluation for cancer progression or IRAEs such as thyroid disorders, hypophysitis, adrenal insufficiency or hepatitis, among others.

Infusion reactions, which may include chills, pyrexia, flushing, wheezing, urticaria, abdominal pain and hypotension, are more common with CTLA-4 inhibitors. Among the PD-1 pathway inhibitors, reported rates have varied from 0.8% to 20.5%, with up to 1.6% grade 3–4 symptoms.[31,39,53] Mild to moderate symptoms can be treated by slowing or interrupting the infusion and administering non-steroidal anti-inflammatory drugs or acetaminophen. Grade 4 symptoms require permanent discontinuation and administration of corticosteroids and antihistamine agents.[114,115]

Dermatological

These are the most common immune-related events reported with both anti-CTLA-4 (47%–68%) and anti-PD-1 (30%–40%) therapies in clinical trials involving solid tumors.[116] Most common cutaneous toxicities with PD-1 blockade in UC trials were pruritus (3.3%–29%) and rash (0.8%–1.8%).[39,53,60] Other reported toxicities with ICI include lichenoid reactions, vitiligo (more common with anti-CTLA-4 therapy), urticaria, eczema, alopecia and oral mucositis/dryness (may mimic oral candidiasis and is more frequent with anti-PD-1). Flares of underlying dermatological disorders, such as psoriasis, may also occur.[114] Potentially serious IRAEs, such as Stevens-Johnson syndrome/toxic epidermal necrolysis, exfoliative dermatitis, bullous pemphigoid and erythema multiforme, have also been observed.[117] Grade 1–2 events can be treated with topical corticosteroids and symptomatic measures including oral antihistamines. While therapy can be continued for grade 1 events (involving <10% body surface area), grade 2 events (10%–30% body surface area) will need interruption of therapy and consideration of skin biopsy. Grade 3–4 symptoms will require systemic corticosteroids, possible skin biopsy, and interruption (grade 3) or permanent discontinuation (grade 4) of therapy.[114,116]

Gastrointestinal

Gastrointestinal AEs range from diarrhea (only increased frequency of stool) to immune-mediated colitis (abdominal pain ± diarrhea, with imaging and/or endoscopic evidence of colonic inflammation). These are more common with anti-CTLA-4 than anti-PD-1 therapies.[116] Reported rates of immune-mediated colitis with anti-PD-1 agents in UC trials are 0.8%–2.3%, with 0.3%–1.1% grade 3–4 events.[23,46,53] Other causes of diarrhea such as *Clostridium difficile* colitis, parasitic infestations, viral or bacterial gastroenteritis and other non-infectious causes should be ruled out, if appropriate.[118,119] Patients with severe symptoms need interruption (grade 2–3) or permanent discontinuation (grade 4 or recurrent grade 3) of ICI, with administration of systemic corticosteroids and consideration of infliximab or mycophenolate mofetil if symptoms remain steroid refractory, eg, after 3 days.[114,116]

Hepatic

Hepatic toxicity may manifest as asymptomatic elevation of transaminases ± bilirubin, or less often as symptomatic hepatitis, eg, with fever, fatigue and jaundice. Rates of immune-mediated hepatitis with anti-PD-1 therapy in UC trials have ranged from 1.0% to 3.0%, with 1.0%–2.0% patients reporting grade ≥3 events.[20,22,63] Hepatotoxicity was much more common with combined PD-1 + CTLA-4 blockade (>10%).[116] Other causes of hepatitis, including reactivation of viruses, eg, hepatitis B or C, other medications/toxins, etc., should be ruled out. Liver function tests should be measured at baseline and periodically during treatment.[23] Patients with grade ≥2 hepatitis need interruption or discontinuation (grade ≥3) of therapy, with initiation of systemic corticosteroids. If no improvement is noted, eg, within 7 days, alternative immunosuppression (tacrolimus, mycophenolate mofetil or cyclophosphamide) should be considered. Infliximab is contraindicated in this setting due to its potential hepatotoxicity.[114,116]

Pulmonary

Immune-mediated pneumonitis is a rare but potentially severe and life-threatening complication of ICI, though mild symptoms or asymptomatic pulmonary infiltrates are more common occurrences. This IRAE has been found to be more common with anti-PD-1 than anti-CTLA-4 therapy.[120] Reported rates of pneumonitis in UC trials with anti-PD-1 agents were 1.1%–4.1%, with 0.2%–2.3% of patients experiencing grade ≥3 pulmonary toxicity.[23,53] However, there are concerns that rates of pneumonitis in clinical practice may be higher than observed in trials, and a multicenter post-marketing analysis involving various tumors revealed pneumonitis rates >5% with anti-PD-1 monotherapy, though grade ≥3 events remained infrequent at <1%.[121] Most common symptoms are cough and shortness of breath, and radiographic presentations are variable, including organizing pneumonia (most common pattern in clinical trials), interstitial pneumonitis-like, hypersensitivity-like, sarcoidosis-like or non-specific ground glass patterns.[121,122] Opportunistic infections, such as *Pneumocystis jirovecii*, viral and atypical bacterial pneumonia should be ruled out, and bronchoscopy with bronchoalveolar lavage or lung biopsy may be required.[119] Therapy should be held for grade 2 pneumonitis, and permanently discontinued for persistent or recurrent grade 2 or any grade ≥3 symptoms. Patients with grade ≥2 pneumonitis require systemic corticosteroids, with consideration of cyclophosphamide or infliximab if symptoms do not improve, eg, in 48 hours.[114,116,120]

Endocrine

The most commonly reported immune-related endocrinopathies in ICI trials are thyroid disorders, hypophysitis, adrenal insufficiency and type 1 diabetes mellitus. ICI can cause hypothyroidism, hyperthyroidism or thyroiditis. Hypothyroidism is the most common endocrine IRAE noted in anti-PD-1 trials with rates of 2.5%–10.0%, followed by hyperthyroidism in 0.6%–4.9% of patients.[23,46,51,63] Thyroid function should be checked at baseline, periodically thereafter while on therapy and also as indicated by signs and symptoms.[23] Thyroiditis can cause initial hyperthyroidism, but eventually transitions to a state of hypothyroidism. Since most immune-related thyroid disorders can be managed effectively with hormone supplementation or anti-thyroid medications, interruption of therapy is seldom required.[114,116]

Hypophysitis, an uncommon IRAE (<1% in ICI trials), may present with non-specific symptoms related to pituitary hormone deficiencies (thyroid stimulating hormone [TSH], adrenocorticotropic hormone [ACTH], growth hormone, luteinizing hormone and follicular stimulating hormone) and less frequently with symptoms secondary to mass effect, such as headaches and visual disturbances. TSH is the most frequently deficient hormone in this setting.[123] Brain magnetic resonance imaging may reveal pituitary enlargement and heterogeneous enhancement.[123,124] Treatment involves replacement of deficient hormones, interruption (grade 2) or permanent discontinuation (grade ≥3) of therapy, and systemic corticosteroids (grade ≥2 toxicity). Some patients may need lifelong hormone replacement.[114,116]

Immune-mediated adrenalitis has been reported in <1% of patients in ICI trials in UC.[23,46,53] Patients may present with non-specific symptoms such as asthenia, fatigue, anorexia, nausea, vomiting, etc., or in adrenal crisis with hypotension and electrolyte imbalances. Testing will reveal low cortisol and high ACTH levels.[116] Treatment of patients with grade ≥2 symptoms involves systemic corticosteroids ± mineralocorticoid supplementation and interruption or discontinuation of therapy.[23]

Type 1 diabetes mellitus is a rare IRAE (reported rates <0.5%) and is managed with insulin administration and interruption of therapy.[23] Corticosteroids are not recommended due to the potential to worsen hyperglycemia.[114,119]

Neurologic

Neurological IRAEs are rare, including isolated reports of aseptic meningoencephalitis, transverse myelitis, Guillain-Barré syndrome, posterior reversible encephalopathy syndrome, myasthenia gravis and peripheral neuropathy.[125–128] Those were mostly reported in patients on anti-CTLA-4 therapy but can also occur with anti-PD-1 therapy.[129] Any grade ≥2 neurological toxicity requires systemic corticosteroids and interruption or permanent discontinuation of therapy.[23,114]

Renal

Though renal dysfunction may occur secondary to various insults, biopsy-proven acute interstitial nephritis has been a rare occurrence in ICI trials (0.5%–1.0%).[46,63] Immune-related nephritis may require systemic immunosuppression including corticosteroids and interruption of therapy.[23,114]

Opportunistic infections

Infection rates as high as 38% have been reported in UC trials.[23,46] This may be due to immunosuppression resulting from a combination of advanced malignancy, ICI therapy and prolonged treatment of IRAEs with corticosteroids and/or other immunomodulatory agents. The urinary tract was the most commonly reported site of infection in these trials. Patients with severe infections need treatment with antimicrobials and interruption or discontinuation of ICI.[23]

Other immune-related adverse events

Other significant IRAEs reported in <1% of patients in ICI trials include myopericarditis, myositis, rhabdomyolysis, inflammatory arthritis, vasculitis, sicca syndrome, immune thrombocytopenia, hemolytic anemia, acquired hemophilia A, uveitis, scleritis, episcleritis and pancreatitis.[36,46,57,66,130] Treatment involves immunosuppression and interruption of therapy depending on severity of symptoms.

Conclusion

After an almost three-decade-long stalemate in advances in the treatment of advanced UC, ICIs with their favorable tolerability and efficacy profiles have ushered in new treatment paradigms. Comprehensive tables that summarize key data with anti-PD-1 and anti-PD-L1 inhibitors were presented at the last day of the most recent (2017) Annual ASCO Meeting as part of highlights of genitourinary (non-prostate) cancers and are available online (https://meetinglibrary.asco.org/), while updated data are also summarized in a recent review.[131] There has been a spate of clinical trials evaluating the role of ICIs in UC, including NMIBC and neoadjuvant, adjuvant and advanced/metastatic settings in MIBC. As these agents get tested in earlier stages of the disease, long-term remissions and higher cure rates are being tested. The discovery and validation of putative biomarkers may contribute to better patient selection in the future. The optimal combinations with several treatment modalities and sequences of active agents are being assessed in various clinical trials. There is a very steep learning curve regarding recognition and management of IRAEs, while registries, databases, care paths, multi-disciplinary tumor boards and guidelines are being developed to support early recognition and uniform management approaches and help the dissemination of practical clinical information that can aid optimal patient care. At this current era of molecular medicine, methods in precision and personalized care are being evaluated with the aim to effectively incorporate a growing spectrum of ICI therapies into the therapeutic arsenal.[131]

Author contributions

Dr Gopalakrishnan was involved in manuscript preparation, critical revision and approval; Drs Koshkin, Ornstein and Papatsoris were responsible for critical revision and approval; and Dr Grivas was responsible for the concept, critical revision and approval. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

Dr Ornstein has done consulting with Pfizer. Dr Grivas has done consulting with Genentech, Merck & Co., Bristol-Myers Squibb, Exelixis, AstraZeneca, Clovis Oncology, EMD Serono, Biocept, Foundation Medicine, Seattle Genetics, Driver. The other authors report no conflicts of interest in this work.

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