Optimizing immunotherapy in locoregional and metastatic urothelial cancer

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Summarizing discussion and future perspectives

Preoperative cisplatin-based chemotherapy is the standard of care for locoregional UC, given the 5% absolute overall survival benefit (1). Yet, many patients do not respond and experience no clinical benefit from preoperative chemotherapy. Thus, novel treatment strategies are needed to improve the outcome in UC. In recent years, there have been major successes with immunotherapy throughout the UC landscape. This discussion is focused on immunotherapy in metastatic **(Thesis part I)** and locoregional **(Thesis part II)** urothelial cancer. In addition, the tumor-immune contexture and potential biomarkers that may inform prognosis and immunotherapy response are also discussed **(Thesis part III)**.

PART I. IMMUNE CHECKPOINT INHIBITORS IN METASTATIC UROTHELIAL CANCER PATIENTS

Immunotherapy was introduced to the first-line setting after previous successes in patients having platinum-refractory disease in UC. Based on single-arm phase II clinical trial data, atezolizumab and pembrolizumab were approved for first-line metastatic cisplatin-ineligible UC (2,3). However, the label was restricted by the EMA and FDA based on early preliminary data from the IMvigor130 (atezolizumab) and Keynote-361 (pembrolizumab) trial (4). In these randomized phase III trials, first-line atezolizumab and pembrolizumab were tested and compared to several treatment arms, including frontline chemotherapy. Early results from these trials indicated that clinical benefit from atezolizumab or pembrolizumab may be inferior to chemotherapy for platinum-eligible patients having PD-L1–low tumors (4). As a consequence, the use of first-line atezolizumab and pembrolizumab was restricted by the EMA and FDA to cisplatin-ineligible patients with PD-L1–positive tumors only (4).

In this thesis, we found that chemotherapy is still active after progressing to frontline ICIs (**chapter 2** ORR 64%; **chapter 3** ORR 58%) (5,6). However, despite these impressive responses, findings in **chapter 3** also indicated that a substantial number of patients (43%) who progress to frontline ICIs do not receive further systemic treatment and are at risk of early death (6). Recently, the FDA further restricted the label for first-line pembrolizumab to include only patients ineligible for all platinum-based chemotherapy irrespective of PD-L1 based on the mature keynote-361 results (7), whereas the label for first-line atezolizumab has not been changed to date and is still under review by the authorities. Thus, results from our retrospective study in **chapter 3** support the FDA/EMA decision to even further restrict the label for frontline ICI (6, 7).

To date, the use for first-line checkpoint inhibition in metastatic UC remains controversial due to the limited clinical benefit and rapid clinical deterioration that excludes patients from benefitting from chemotherapy. Ideally, response rates should double and have synergy in terms of efficacy to higher the probability of disease control at the start of immunotherapy. To increase the clinical benefit from first-line immunotherapy, combination strategies with different checkpoint inhibitors and chemotherapy have been investigated. Immunotherapies targeting PD-(L)1 plus CTLA-4 have been combined and compared to platinum-based chemotherapy alone in metastatic UC. Results from the CheckMate 901 trial (unpublished) showed that first-line ipilimumab plus nivolumab combination immunotherapy did not significantly improve OS over standard-of-care chemotherapy in metastatic UC patients having ≥1% PD-L1 expression (primary endpoint) (8). Likewise, no OS benefit was found for durvalumab monotherapy or tremelimumab plus durvalumab versus standard-of-care chemotherapy in first-line metastatic UC (DANUBE trial) irrespective of PD-L1 expression (9). Multiple trials are ongoing to test whether novel ICIs (e.g. anti-LAG-3) in combination with more common and approved immunotherapies may potentially enhance efficacy and survival outcome by antagonizing checkpoint resistance mechanisms (10,11).

Potential treatment synergy may be obtained from combining chemotherapy and immunotherapy, given the high response rates to chemotherapy and durable response rates to ICI. In the keynote-361, pembrolizumab plus chemotherapy was also explored as treatment arm and compared to pembrolizumab monotherapy and chemotherapy alone (cis/gem or carbo/gem). Surprisingly, the study did not meet its primary endpoints (OS and PFS) compared to platinum-based chemotherapy (12), suggesting no additional survival benefit for chemo-immunotherapy. Chemo-immunotherapy as combination treatment was also tested and compared to single-agent atezolizumab and standard-of-care chemotherapy alone in the IMvigor 130. While OS data is not yet mature, atezolizumab plus platinum-based chemotherapy was associated with a significantly higher progression-free-survival when compared to atezolizumab alone or platinum-based chemotherapy (13). The findings in IMvigor 130 are surprising, as atezolizumab in general appears to induce lower response rates in urothelial cancer when compared to pembrolizumab. It is currently unknown whether the addition of anti-CTLA-4 to platinum-based chemotherapy plus anti-PD-1/anti-PD-L1 such as pembrolizumab or atezolizumab may enhance response and long-term clinical outcome in the first-line setting in UC.

Optimizing the timing of immunotherapy administration (e.g. sequencing) may potentially enhance rates and contribute to improved long-term clinical outcome. A sequential chemo-immunotherapy approach has been tested in metastatic UC in the randomized

clinical trial named JAVELIN (14). In this trial, a total of 700 patients with locally advanced or metastatic UC and an ongoing response or stable disease upon platinum-based chemotherapy were switched to maintenance avelumab (anti-PD-L1) plus best supportive care or best supportive care alone (14). The treatment arm that sequenced immediately to avelumab following chemotherapy showed an absolute survival benefit of 13% at 1-year when compared to best supportive care. The survival benefit was irrespective of PD-L1 expression levels, whereas the outcome in the PD-L1 positive group appeared slightly better (14). These results indicate that earlier immunotherapy administration following chemotherapy is better, given that patients are less likely to lose the opportunity to benefit from immunotherapy.

Beyond conventional chemo-immunotherapy combination strategies, promising results are found when ICI are combined with antibody-drug conjugates such as enfortumab vedotin (15). This drug binds nectin-4 on tumors cells and subsequently delivers chemotherapy (Monomethyl auristatin E) to these nectin-4 positive cells. In a phase III clinical trial, enfortumab vedotin significantly prolonged overall survival when compared to standard of care chemotherapy in patients with advanced UC that progressed to platinum-based chemotherapy and anti-PD-1 or anti-PD-L1 (16). In addition, remarkable response rates were found in a phase II trial testing enfortumab vedotin plus pembrolizumab in cisplatin-ineligble UC (17). This chemo-immunotherapy combination is also tested in a randomized phase III trial against standard-of-care chemotherapy and results from these trials are awaited with high interest (18).

Altogether, it is currently unclear how first-line checkpoint inhibition will evolve in the treatment landscape of stage IV UC given the uncertainties surrounding it. Upcoming results from ongoing clinical trials and further refinement of the timing and appropriate therapy combinations are needed to exploit the potential of immunotherapy in the first-line setting in metastatic UC.

PART II. PREOPERATIVE CHECKPOINT IMMUNOTHERAPY IN UROTHELIAL CANCER

Although the role of checkpoint inhibitors in first line metastatic UC is still under debate, immunotherapy has been introduced to the preoperative setting. Reasons to introduce ICI to the preoperative setting include response durability and the higher response rates observed when treating patients in first-line rather than second line in the metastatic setting (2). Additionally, patients having only lymph node metastases tend to have higher response rates to immunotherapy than patients with visceral

disease (2). The introduction of immunotherapy to the perioperative setting may improve clinical outcome, as has been shown for melanoma (19). In the randomized CheckMate-274 trial, patients were allocated to adjuvant nivolumab or placebo after previously having cystectomy with or without prior cisplatin-based chemotherapy. This study found a significantly longer disease-free survival (DFS) in patients receiving adjuvant nivolumab when compared to the placebo group (20.8 versus 10.8 mo) (20). A higher DFS with nivolumab was observed irrespective of PD-L1 positivity or previous neoadjuvant cisplatin-based chemotherapy status (20). In contrast, no DFS difference was found between adjuvant atezolizumab and observation groups in the IMvigor010 trial (21). There is growing interest in preoperative checkpoint immunotherapy in UC. In the PURE-01 (pembrolizumab) and ABACUS (atezolizumab) trial, a pCR rate of 37% and 31% was found (22,23), respectively. However, pathologic complete responses were primarily found in less advanced (cT2N0) tumors (23), while more advanced tumors (cT3-4N0) showed only limited to no response. These results emphasize the need for more effective treatment strategies in patients with more extensive disease (cT3-4N0) or loco-regional lymph node involvement (T2-4N+). In this thesis, a promising pathological response rate (58% had pCR or non-invasive disease) was demonstrated in patients having locoregionally-advanced (stage III) UC treated with sequenced ipilimumab (3 mg/kg) plus nivolumab (1 mg/kg) (24). The pCR rate and survival outcome to ipilimumab plus nivolumab in NABUCCO appeared to be better when compared to a similar patient cohort treated with neo-adjuvant/induction chemotherapy in a retrospective analysis (25). Thus, combination immunotherapy may be a potent treatment strategy to treat a patient population that in general has a high risk of recurrence. In NABUCCO, a high occurrence of grade 3-4 immune-related adverse events was also observed (24). This excluded a subset of patients from receiving the third and last treatment cycle (nivolumab monotherapy) in NABUCCO cohort 1. In various malignancies (including melanoma), a lower toxicity rate with preserved efficacy was observed when using ipilimumab 1 mg/kg instead of ipilimumab 3 mg/kg) in addition to nivolumab preoperatively (26).

In an attempt to optimize tolerability and efficacy of preoperative ipilimumab and nivolumab in UC, the extension study of NABUCCO (cohort 2) tested two cycles of ipilimumab 3 mg/kg plus nivolumab 1 mg/kg (cohort 2A) versus two cycles of ipilimumab 1 mg/kg plus nivolumab 3 mg/kg (cohort 2B), followed by a third cycle of nivolumab 3 mg/kg in both cohorts involving cis-ineligible/refusal patients (27). Thus, compared to NABUCCO cohort 1, patients in cohort 2 received nivolumab in addition to ipilimumab in the first treatment cycle. The pCR rate in cohort 2A (43% pCR) was consistent with the pCR rate in cohort 1 (46% pCR), whereas cohort 2B (ipi 1 mg/kg + nivo 3 mg/kg) showed a substantially lower pCR rate (7% pCR) (27). The study is not yet mature enough to assess whether the survival outcome of cohort 2A is in line with cohort 1 and whether

survival differences can be found between cohort 2A and 2B. Results from NABUCCO cohort 2 are in contrast to findings in other cancers such as melanoma, where comparable pathological response rates were observed for high and low-dose ipilimumab (26). Reasons for these outcome differences may involve more limited tumor foreignness in UC when compared to other cancers such as melanoma (28,29). Yet, the exact mechanisms driving the need for more CTLA-4 blockage in UC remain to be elucidated.

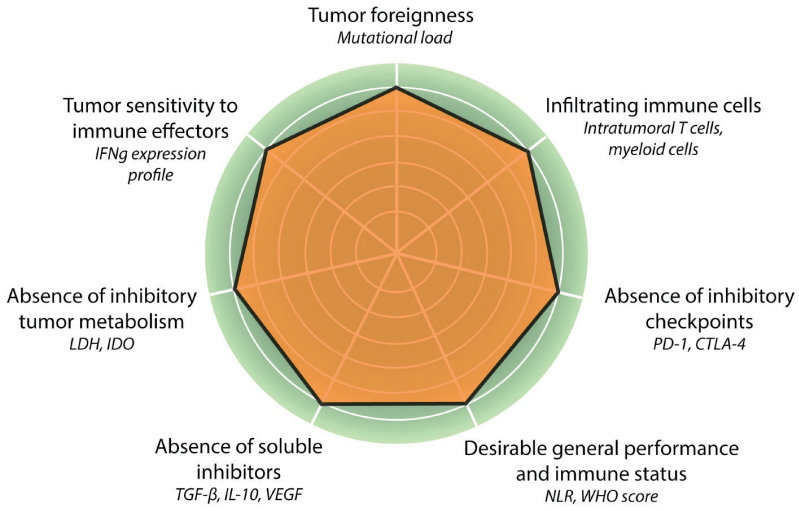
Given the recent successes with preoperative combination immunotherapy in UC, bladder sparing treatment strategies are also being explored. In the phase II Indi-Blade study, induction ipilimumab plus nivolumab is tested in 50 patients having cT2-4aN0-2 urothelial cancer, who are amenable for chemoradiation (30). Although this treatment approach might be beneficial in terms of efficacy and morbidity, no results are published on induction combination checkpoint Inhibition and subsequent chemoradiotherapy to date. As in metastatic UC, the combination of checkpoint inhibition and platinum-based chemotherapy has also been tested and compared to single-agent treatments in the preoperative setting, aiming to benefit from best of both worlds. Various single-arm trials showed a high response rate to preoperative chemo-immunotherapy in UC. In the single-arm phase II BLASST-1 trial, 41 patients (cT2-T4aN0-1M0) were treated with preoperative nivolumab plus cisplatin/gemcitabine prior to cystectomy (31). In total, 66% showed pathological response, including 49% pCR, showing the potential of this combination therapy (31). This is slightly higher then results from studies testing neo-adjuvant cisplatin-based chemotherapy alone (22-40% pCR) (1,32). However, outcome comparisons across small single-arm trials testing single/combination-immunotherapy or chemo-immunotherapy is limited due to varying study populations and different treatment cycles and dosing schedules. Thus, results from ongoing randomized trials will be crucial to better understand the efficacy and adverse events of each treatment strategy. Larger randomized clinical trials are currently ongoing to test whether chemo-immunotherapy as a preoperative treatment strategy may further enhance pCR rates and survival when compared to the appropriate platinum-based chemotherapy alone. These studies include KEYNOTE-866 (pembrolizumb plus cisplatin/gemcitabine), NIAGARA (durvalumab plus cisplatin/gemcitabine) and ENERGIZE (nivolumab or nivolumab plus IDO1-inhibitor linrodostat combined with cisplatin/gemcitabine), whom all provide adjuvant therapy in the combined chemo-immunotherapy arms. Given the recent successes in the metastatic setting, antibody-drug conjugates such as enfortumab vedotin are also extensively being tested in the peri-operative treatment setting in UC, both in cisplatin-ineligible (e.g. KEYNOTE-905) and cisplatin-eligible (e.g. KEYNOTE-B15) patient populations.

In conclusion, while neo-adjuvant cisplatin-based chemotherapy is currently the standard of care in UC, the neo-adjuvant treatment landscape may look very different within five years, potentially paving the way for bladder-sparing treatment strategies and multimodal therapies that include immunotherapy. This should be accompanied by appropriate adjuvant treatment strategies in patients having insufficient benefit from preoperative treatment modalities.

PART III. BIOMARKERS FOR IMMUNOTHERAPY RESPONSE PREDICTION IN UROTHELIAL CANCER.

Immune checkpoint immunotherapy has had a major impact on the treatment landscape in UC, both as first-line and second-line therapy in the metastatic setting and as pre-operative treatment in locally advanced UC. Yet, a substantial subset of patients do not respond and may suffer serious adverse events without experiencing clinical benefit or a durable response. Biomarkers may help to discriminate responders from non-responders to guide patient selection in the immunotherapy landscape. Biomarkers such as PD-L1 expression and a high tumor mutational burden (TMB) have been associated with response in metastatic UC. Yet, these biomarkers lack sufficient predictive power for clinical utility (33). The preoperative setting may be more suited for biomarkers exploration, given that operable patients have limited disease heterogeneity (restricted to bladder or LNs) without exposure to prior systemic therapies, as well as availability of paired tissue biopsies (TUR vs cystectomy). Trials (e.g.. PURE-01 and ABACUS) testing neo-adjuvant ICI monotherapy in UC showed that response was significantly higher in tumors having baseline pre-existing $CD8^+$ T-cell immunity based on high CD8 presence and interferon-γ signaling (23,34), whereas cold tumors (immune-desert) were unresponsive to atezolizumab (23). In this thesis, we showed that pCR to ipilimumab plus nivolumab in NABUCCO was independent of baseline $CD8^+$ T-cell density by multiplex immunofluorescence and inflammatory signatures such as interferon-gamma, tumor inflammation and T-cell effector signatures (24). Thus, suggesting that the addition of anti-CTLA4 can induce responses in immunologically "cold" tumors. While baseline TLS and B-cell abundance did not differ between responders and non-responders in NABUCCO (24), other trials revealed higher baseline TLS and B-cell abundance in pCR tumors compared to non-pCR tumors, as in a trial testing preoperative durvalumab tremelimumab in UC (35). Thus, conflicting results on baseline candidate biomarkers for immunotherapy response were found between comparable studies and between studies testing ICI monotherapy vs combination immunotherapy. This is both true for the metastatic setting and non-metastatic setting.

While a high-TMB, PD-L1 expression and CD8 T-cell infiltration show predictive value for ICI response in various cancers (36,37), none of them can perfectly predict response, particularly in isolation. Biomarker interpretation is limited by variations in biomarker assays and thresholds, whereas tumor heterogeneity within a tumor lesion also plays a role. In addition, biomarkers show a complex interplay between one another that affects anti-tumor immunity and tumor biology (29,38). For example, IFN-γ secreting CD8 T-cells can induce PD-L1 expression on tumor cells and immune cell subsets (38). Thus, resulting in mechanism that contribute to immune suppression. Additionally, MMR-deficient tumors can harbor mutations that translate into immunogenic tumor neoantigens that may be recognized by the immune system. Recognition of these neoantigens through T-cell receptor (TCR) ligation is subject to factors such as HLA heterogeneity and the host microbiome (e.g. molecular mimicry) (29), whereas tumor elimination by tumor-specific T-cells is dependent on the level of tumor-infiltrating lymphocytes and the level of antigen presentation within the tumor. All these cells and processes show complex interactions that facilitate anti-tumor immunity. The nature and complex interplay of these biomarkers demonstrate the limitation of single-biomarkers approaches and underline the need for composite biomarker approaches that account for the balance between anti-tumor immunity and immune suppression. In this thesis, we proposed the UC immunogram (39), a theoretical framework that integrates candidate biomarkers to ultimately inform individualized treatment based on multiple biomarkers. Incorporating multiparametric biomarkers into predictive quantitative models will be a major challenge to implement the immunogram into clinical practice.



To fully exploit the UC immunogram and to assess the full spectrum of biomarkers, multiple biopsies within and across tumors may be needed for robust analysis, also accounting for tumor heterogeneity. For thorough assessment, blood and stool samples should be obtained to assess peripheral biomarkers, host germline DNA and the host microbiome from genetic and non-genetic biomarker analysis (39). Furthermore, circulating tumor DNA and circulating tumor cells in blood can expand the field of response biomarkers, also in terms of dynamics. Future studies are needed to explore low concentration candidate biomarkers in liquid blood biopsies. As discussed in this thesis, characterizing the tumor contexture and immune cell phenotypes in a systematic manner should be a major aim for biomarker discovery (40). This should be organ (primary tumor vs metastasis) and site specific (primary tumor vs TLS vs lymph node), as well as stratified for various tissue types (tumor vs stroma). Nevertheless, tumor immune biology is complex and the resolution achieved by biomarkers may be limited, particularly when using single-markers and only one tumor lesion in case of metastatic disease. Analysis of more advanced biomarkers may be challenging in terms of practicality and could be challenging to deploy in clinic.

In conclusion, checkpoint immunotherapy shows a great promise and its use evolves throughout the UC landscape, including the perioperative setting. Beyond a better understanding of tumor immune biology, a further refinement of patient study populations, distinctive and composite biomarker use, and promising diagnostics such as circulating tumor DNA to discriminate responders from non-responders is warranted.

FUTURE CONSIDERATIONS

Over the last decade, there has been a remarkable increase in systemic therapies due to the introduction of therapies that specifically target the immune system. Not only for UC, but extending over various cancers (e.g. melanoma and lung cancer). In UC, immune checkpoint inhibitors (ICIs) have shown promising activity as monotherapy in the first-line and preoperative setting and prolonged survival in the platinum-refractory patients. Still, many patients progress upon immunotherapy, underpinning the need for strategies that enhance benefit from immunotherapy. Optimizing the right combinations, the appropriate timing, and sequencing for cancer immunotherapy may ultimately improve outcome to cancer immunotherapy. While conventional cancer therapies such as chemotherapy, irradiation, targeted therapy and surgery directly target cancer, immunotherapy targets immune cells. All of these treatments have a different mechanism of action and work differently. Only when we understand the therapeutic mechanisms, we can utilize the anti-tumor potential of these established therapies and find the ap-

propriate combinations and timing for these treatments in the metastatic and perioperative setting.

Although T cells have always been the main focus in cancer immunotherapy, other immune cells such as natural killer cells, macrophages and dendritic cells also play vital roles in adaptive immunity. Thus, it will be important to understand their role in tumor-immunity and how to effectively target them. Each of these immune cells have unique immune regulatory features and therapeutic potential. Beyond immune cells, the tumor-microenvironment consists of other important cells and immune features that impact tumor-immunity, such as fibroblasts and tertiary lymphoid structures. We need to understand their role in tumors and across cancers to identify distinctive response biomarkers. Finding the right combination treatment strategies for individual tumors can specifically target these components, ultimately improving clinical outcome of UC patients. Unfortunately, translational research and biomarkers are limited by our current biomarker approaches. Biomarker findings across trials are complicated by variability in biomarker assays (i.e. PD-L1 assessment) and heterogeneity in tumor tissue used to assess biomarkers. Thus, clouding the interpretation of these findings. Biomarkers analysis should be done on more homogeneous collections of tissue and biopsy acquisition from multiple tumor and organ sites is warranted. Like patients, all tumors are unique. This also includes tumors at different organ sites within the same patient. Unfortunately, biopsies are mainly obtained from a single metastatic lesion for ethical and practical reasons. Tumor heterogeneity, accompanied by mixed responses, warrants the need for multiple fresh biopsies from distinct tumor sites in a single patient at various timepoints. This would allow analysis of the tumor-immune contexture at different disease sites, as well as single-cell analysis. Non-invasive biomarkers (e.g. liquid biopsies; ctDNA) can further expand this comprehensive biomarker approach. Yet, it's naïve to underestimate cancer and to think that liquid biomarkers will compensate for our lack of understanding of tumor and biomarker complexity. I personally believe that single-biopsy approaches and liquid biopsies do simply not suffice. It is highly likely that biopsies from multiple tumor and organ sites are needed to ultimately understand cancer, biomarker complexity and response to various treatment combinations.

In conclusion, future studies should focus on understanding distinct cancer therapies and immune-cells that orchestrate anti-tumor immunity. This will ultimately improve immunotherapy timing, sequencing and ideal immunotherapy combinations to enhance cancer immunotherapy. In addition, biomarker analysis on tumor tissue obtained from distinct tumor sites may be needed to facilitate this.

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