Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Scienze Chirurgiche, Oncologiche e Gastroenterologiche

CORSO DI DOTTORATO DI RICERCA IN: Oncologia clinica e sperimentale e Immunologia

CICLO: XXXVI

Clinical relevance of molecular biomarkers in urothelial carcinomas treated with chemotherapy, immunotherapy or targeted-therapies

Tesi redatta con il contributo finanziario dell'Istituto Oncologico Veneto IOV-IRCCS

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Summary

Abstract

Background: Advanced urothelial carcinoma (UC) is a neoplastic disease with a very poor prognosis. In the last few years novel treatments beyond platinum-based chemotherapy such as immunotherapy with checkpoint inhibitors (CPIs) and targeted therapy with Fibroblast-Growth-Factor-Receptor (FGFR) inhibitors have reached clinical practice. Nevertheless, the role of Programmed Death receptor Ligand 1 (PD-L1) evaluation on tumor tissue in predicting response to CPIs is still debated. On the other hand, while predictive for specific inhibitors, it is not clear if FGFR alterations are able to discern a different response to chemotherapy or CPIs.

Materials and Methods: To evaluate the clinical role of PD-L1 and FGFR aberrations two different approaches were applied. For PD-L1 a systematic review and meta-analysis was performed, selecting three measures of effect: the objective response ratio (ORR) and the Hazard Ratio (HR) for overall survival (OS) of patients treated with CPIs with elevated PD-L1 expression vs patients with low PD-L1 expression; the HR for OS of patients with elevated PD-L1 expression treated with CPIs vs chemotherapy. To evaluate the role of FGFR alterations all consecutive patients with advanced UC from 2018 to 2022 tested for FGFR and treated with at least one line of systemic therapy were enrolled in a retrospective, mono institutional study. Clinical data was collected from clinical charts, anonymized, and organized in an electronic database. Survivals were estimated with Kaplan-Meir method, and the association between the presence of FGFR alterations and OS, PFS was analyzed with the log-rank test.

Results: After literature research, 13 studies were deemed eligible, of which 8 for ORR and OS comparison between patients with high and low PD-L1 expression treated with CPIs, and 5 with OS comparison between patients with high PD-L1 treated with CPIs vs chemotherapy. The pooled ORR for tumor response was 2.9 with a 95% CI of 2.32 to 3.63 and a $p<0.0001$ in favor of the patients with an elevated expression of PD-L1. For the same subgroups, the pooled HR was 0.65 with a 95% CI 0.57 to 0.73 in favor of high PD-L1 expression. For patients with high PD-L1 expression treated with immunotherapy the pooled HR was 0.79 with a 95% CI 0.61 to 1.02; displaying only a trend for reduction in the risk of death when compared with patients treated with chemotherapy. In our institution from 2018 to 2022, 160 patients with UC were tested for FGFR alterations, of them 148 were eligible for this study. Of the 126 patients of the chemotherapy group 23 harbored FGFR mutations or translocations (18.3%). Median PFS was 7 months in the FGFR negative subgroup and 9 months in the positive one, HR = 1.32, p= 0.23. Median OS was 20 months in the former subgroup vs 15 months, HR= 0.98. Of 72 patients treated with CPIs, 13 were positive for FGFR (18.1%). Median PFS in this group was 2 vs 4 months in the FGFR negative one, HR= 1.22, p= 0.5. Patients with a FGFR alteration had a median OS of 5 versus 17 months, with an HR of 1.58, p= 0.2. Excluding the patients who received FGFR inhibitors after immunotherapy, median OS was 3.5 months with an HR of 3.5, p= 0.001.

Conclusions: PD-L1 has a prognostic role for metastatic UC, and an elevated expression is associated with a higher chance of response to CPIs. However, it does not seem to have enough predictive power to lead to the choice of a treatment with CPIs over chemotherapy, especially in the first line setting. On the other hand, FGFR alterations, seem to be associated with a lower OS in patients treated with immunotherapy, if they do not receive an FGFR inhibitor after the failure of CPIs. Conversely, no correlation with response to platinum-based chemotherapy was found.

Introduction

Urothelial carcinoma (UC) of the bladder and upper urinary tract is a quite common malignant disease, accounting for 4% of all cancers in the European population, interesting 12.4-21.8 per 100.000 males-years, and 2.7-4.3 per 100.000 females-years. Moreover, it is the second most prevalent urological tumor, after prostate cancer. The vast majority of urothelial carcinomas origin from the bladder, while the ones originating from the upper urinary tract represent a small, yet significant, portion of the total (around 5-10%) [1].

UC is a double-sided disease. The localized, non muscle-invasive disease (≤ pT1 according to the international Tumor-Node-Metastasis: TNM classification) could usually be treated with endoscopic approaches and, in the case of high-risk of recurrence, with intra-bladder instillations of Bacillus of Calmette-Guérin (BCG) or chemotherapeutic drugs, such as Mitomycin C or Gemcitabine [2]. These treatments are usually very effective, and the 5-year overall survival (OS) is excellent. On the other hand, invasive disease (pT2 or superior) requires neoadjuvant chemotherapy and invasive surgery, with radical cystectomy with lymphadenectomy being the standard of care [3]. Despite the most aggressive treatments, the prognosis is still suboptimal in the localized disease setting, and dismal in the advanced one. Consequently, most of the translational and pharmaceutical research is focused on the advanced and locally invasive setting.

The traditional treatment for metastatic or locally-advanced (not eligible for surgery) UC has long been systemic chemotherapy, in particular platinum-based regimens. The first chemotherapeutic combination widely used in clinical practice for UC was Cisplatin plus Methotrexate plus Doxorubicin plus Vinblastine (MVAC). The common and severe toxicity of this regimen is the main reason for its decline, in favor of the Cisplatin plus Gemcitabine (GC) combination. Indeed, these two regimens demonstrated similar OS rates of around 14 months in a phase 3 study, but GC showed far less hematologic and mucosal toxicity [4]. Even if GC is better tolerated than MVAC, there are still many contraindications for Cisplatin: a decreased renal function (usually considered as estimated glomerular filtration rate inferior to 60 mL/min), presence of peripheral neuropathy, acoustic impairment, and problems of cardiac insufficiency. Patients unfit for Cisplatin can be treated with Carboplatin plus Gemcitabine, a combination which was able to achieve a median OS of 9.3 months in a phase 2 study [5], and which can be administered safely in this particular group of patients. Even if not reimbursed in Italy, for patients unfit for Cisplatin and with an elevated expression of Programmed-Death receptor Ligand 1 (PD-L1) combined score (CPS), the European Medicines Agency (EMA) approved to use of an anti-PD1 antibody, Pembrolizumab [6].

Indeed, in the last few years, the use of checkpoint inhibitors antibodies (CPIs) has progressively expanded in many fields of medical oncology, and many studies have been conducted on UC with CPIs, in particular anti-PD1, anti-PD-L1 and anti-Cytotoxic-T-Lymphocyte-Associated protein 4 (CTLA4).

After the results of these studies, EMA approved many CPIs for the treatment of UC, in different disease settings. In particular, Pembrolizumab has been approved and it is currently used for the treatment of advanced UC in patients unfit for Cisplatin with a CPS score equal or greater than 10, as already mentioned, and for the treatment of UC in patients who experienced disease progression after a first line, platinum-based, chemotherapy, having showed a clear benefit in OS versus second line chemotherapy in a phase 3, randomized study [7]. More recently, Avelumab has been approved for the maintenance therapy in patients affected by UC, who showed response or disease stability after first line, platinum-based chemotherapy, based on a significant increase in OS (21.4 months vs 14.3 months of the standard of care) in a phase 3 trial [8]. Avelumab is an anti-PD-L1, and it was already approved by the American Food and Drug Administration (FDA) in the second line setting, similarly to Pembrolizumab (while in Europe Avelumab is not yet approved for this indication). These two drugs are the immunotherapeutics used in the clinical practice in Italy at the moment of this writing. PD-L1 evaluation is not mandatory for the most important indications, differently from what happens in non-small cell lung cancer (NSCLC), for instance.

After the CPIs "revolution" in the treatment of UC, other kinds of drugs are being developed. In 2019 the FDA granted accelerated approval for Erdafitinib, an inhibitor of Fibroblast-Growth-Factor-Receptor 2 and 3 (FGFR2-3). FGFR signaling pathway has a role in different pro-neoplastic functions such as proliferation, angiogenesis, and cellular mobility [9], and it can be classified as oncogene. The hyperactivation of FGFR3 is well documented in a fraction of UC [10], thus the idea of inhibiting this pathway as a therapeutic strategy. Indeed, Erdafitinib showed promising activity in a phase 2 trial [11], and this drug is currently being tested in a phase 3 trial, the THOR trial. Very recently, at the American Society of Medical Oncology (ASCO) 2023 annual meeting, the preliminary results of the THOR trial were presented, showing a benefit in OS for FGFR-positive patients, already treated with platinum based chemotherapy and a CPI, who received Erdafitinib, when compared to investigator's choice of chemotherapy. Moreover, at European Society of Medical Oncology (ESMO) 2023 annual meeting, the updated data of THOR trial cohort 1 were presented, reporting a median OS of 12.1 months for patients treated with Erdafitinib compared to 7.8 months for the ones who received sperimentator's choice of chemotherapy, with a hazard ratio of 0.64 (0.47-0.88). However, only a minority of UCs showed hyperactivation of FGFR3, and it is not clear if this molecular characteristic is able to discern a different biology of the disease, such as EGFR or ALK alterations in NSCLC. Indeed, taking EGFR mutated or ALK translocated NSLC as an example of oncogene-driven disease, many data suggest a different response to CPIs, when compared to EGFR, ALK wild type NSCLC. For instance, immunotherapy performed poorly in the CHECKMATE 057, a phase 3 trial comparing second line Nivolumab versus Docetaxel in advanced NSCLC [12]. Very similar results were obtained by phase 3 trials with other CPIs, such as Pembrolizumab and Atezolizumab. A meta-analysis pooling the results for survival of patients treated with immunotherapy versus chemotherapy in this setting including three phase 3 trials showed a pooled hazard ratio of 0.66 (95% CI, 0.58-0.76) for patients with EGFR wild type, while it

was 1.05 (95% CI, 0.7-1.55) for the ones harboring EGFR mutations [13]. While waiting for the final results of phase 3 trials on novel FGFR inhibiting drugs, which should prove the predictive role of FGFR aberrations for this kind of therapy, the behavior of FGFR mutated UC should be explored for therapies already used in everyday clinical practice.

Moving in this direction, a retrospective study by Santiago-Walker was presented as abstract at 2019 annual ASCO GU conference, showing a trend for worse survival for patients affected by advanced UC with FGFR alterations treated with CPIs [14]. A larger retrospective study by Rezazedeh was presented at ESMO 2020, still finding only a trend for lower OS in patients with FGFR mutation or translocations and treated with CPIs in any line [15]. Similarly, the role of FGFR alterations in the chemotherapy setting have not been largely explored. A retrospective study by Necchi was published in 2019, not evidencing any prognostic effect [16]. In conclusion, the role of FGFR alterations outside their possible power in predicting response to FGFR inhibitors is yet unclear.

While new promising drugs have approached the healthcare market in the last few years, there are some considerations to be done. While the survival benefits of those novel therapies are confirmed in phase 3 trials, the same studies and the clinical real-life experience showed that not all patients with UC are able to get that benefit. For instance, observing the Kaplan-Meier curve of OS in the pivotal Pembrolizumab trial, the KEYNOTE-045 [7], it is clear that in the first weeks of treatment, patients with metastatic UC treated with Pembrolizumab actually have a higher risk of death than the ones treated with chemotherapy. On the other hand, as time goes on, it becomes clear that patients who experienced a tumor response to Pembrolizumab have a longer OS. In the same trial PD-L1, the traditional biomarker associated with the use of CPIs, was tested with two different cut-offs, but it was not able to find out the patients who gained no effect (or a detrimental effect) from Pembrolizumab. At the moment, there is no way to select patients for the immunotherapy treatment, nor in the second line setting nor for the Avelumab maintenance therapy [8]. Moreover, with CPIs treatment together with their immune-related adverse events, another, subtler form of toxicity approached the healthcare systems: financial toxicity.

The elevated costs of CPIs is well known, and the cost-effectiveness of these treatments from an economic point of view have been explored in different studies. A review from Walia [17] evidenced that to improve cost-effectiveness of CPIs one of the key-points is the presence of better selection criteria, possibly biomarker driven. In the same way, Pichler [18] commented on a cost-effective study about Pembrolizumab in the second line setting by Sarfaty [19], underlining the importance of a better patient selection, to reduce the financial burden of CPIs on the healthcare systems. Therefore, it can be stated that research and validation of prognostic and predictive biomarkers in advanced UC is a priority in the field of medical oncology.

In this work, different methodologies have been applied to better explore the role of important molecules such as PD-L1 and FGFR, according to existing literature and data. PD-L1 has been measured in a large number of randomized clinical trials (RCTs); even if many of these trials were not

designed with a biomarker-based approach. Consequently, it has been decided to approach PD-L1 with a critical, systematic review and meta-analysis of the existing data, extrapolating from phase 2 and 3 trials the results concerning the PD-L1 positive subgroups. FGFR molecular alterations on the other hand have not been as widely studied, especially outside trials concerning FGFR inhibitors. Therefore, we collected all consecutive patients affected by advanced UC treated at our Institution and whose tumor tissue was tested for FGFR alterations, designing an observational trial to explore the correlation between FGFR mutational status and oncological outcomes.

Programmed Death receptor Ligand 1 (PD-L1) as a biomarker for UC, a meta-analysis

Background

The main innovation in the field of oncological treatment of advanced stage UC has consisted in the introduction of CPIs, in particular anti-PD1 and anti-PD-L1 antibodies.

These drugs have been extensively experimented in a large variety of solid neoplasms, especially NSCLC [20]. To prescribe CPIs for the treatment of NSCLC an assessment of the PD-L1 expression on tumor tissue is mandatory [21]. On the contrary, for the treatment of UC there is only one indication approved by EMA (two by FDA): platinum-ineligible patients in the first-line setting need to have a CPS of 10 or more to receive Pembrolizumab [6].

The reason for this difference lies in the fact that the phase III randomized trials which allowed the registration for use of CPIs such as Pembrolizumab for pre-treated metastatic UC, and Avelumab as maintenance therapy after platinum-based chemotherapy, showed an OS benefit in the intention to treat (ITT) population, regardless of PD-L1 status [7] [8]. Nevertheless, in the majority of prospective trials with CPIs PD-L1 is usually assessed on tumor tissue [22]. Unfortunately, every different CPIs seem to be connected with a different PD-L1 staining assay, which the FDA defines as "companion diagnostics". For instance, the already mentioned Pembrolizumab, is linked to the CPS score, which consists in an immunohistochemical (IHC) assay for PD-L1 with the Dako 22c3 antibody, calculating the combined score of positive PD-L1 cells both on tumor and the infiltrating immune-cells. On the other hand, Atezolizumab is linked to another algorithm, using the Ventana SP142 antibody, and the score is based on the portion of positive infiltrating immune cells alone, without considering tumor cells. Moreover, positivity cut-offs are different with different assays. In the previous example, the latter is considered positive if 10 or greater, while the former if 5% or more of immune cells stains for PD-L1. Other CPIs such as Nivolumab and Durvalumab ha still different companion diagnostics, using Dako 28-8 and Ventana SP263 antibodies for IHC, respectively, and they consider the sum of positive tumor and infiltrating immune cells (with a cut-off of 25% or greater) or the portion of tumor cells staining for PD-L1 (with a cut-off of 5%, TPS score), respectively [23]. The different tests are not always interchangeable. A large study on 251 different urothelial carcinoma tissues, showed that while Dako 28-8, 22c3 and SP263 showed similar performances, SP142 was not as interchangeable [24]. On the other hand, it should be considered that in other settings, such as the treatment of NSCLC, different scoring methods and antibodies, seem to be equally predictive of response to CPIs [25].

Considering the importance of establishing a predictive biomarker for the treatment with CPIs, many reviews and meta-analyses in the last few years focused on different aspects of PD-L1 expressions.

However, the results have been inconclusive, and sometimes contradictory, based on the inclusion criteria of the different analyses. For instance, an elevated PD-L1 expression seemed to be linked to poorer prognosis in patients with localized UC in a recent meta-analysis performed by Wen et al [26], where a correlation with a higher T stage was also detected. Indeed, a higher expression of PD-L1 showed to be a negative prognostic factor in many solid tumors [27], especially in the localized and locally advanced stage. On the contrary, when considered the advanced stage, PD-L1 expression seems to be correlated with a greater median OS, if patients are treated with CPIs in solid tumors in general [28] and in UC in particular [29]. Pooling three important phase 3 studies for advanced UC of CPIs versus chemotherapy in the first line: DANUBE [30], KEYNOTE-361 [6] and IMVIGOR-130 [31], Rizzo et al. concluded for an OS benefit for CPIs in the group of patients with high PD-L1 expression. On the other hand, another meta-analysis conducted with a different methodology by Martini et al., pooling the same three studies, demonstrated that no particular benefit in OS could be detected in patients treated with CPIs [32]. To establish the prognostic and predictive role of a biomarker, different steps should be taken, following a roadmap [33]. In particular, after the discovery in the preclinical phase, the biomarker should be prospectively qualified, exploring the correlation between it and the desired clinical outcome. After that, clinical trials for novel, targeted therapies, with randomization defined by the biomarker under evaluation are necessary to establish its predictive value. As already explained, this is not the case for advanced UC, where all the most important trials were not randomized according to PD-L1 expression. Our meta-analysis has the objective to pool together the results from the phase 2 and phase 3 trials with a population of patients with UC treated with CPIs, and in whom PD-L1 expression was measured. The aim is to get the most solid data on the prognostic and predictive role of PD-L1 expression in this population and to generate more hypotheses for future randomized clinical trials.

Materials and Methods

Measure of effect

This study is structured as a systematic review and meta-analysis. Considering the two objectives of evaluating the prognostic and predictive role of PD-L1 expression on tumoral tissue, we decided to evaluate three measures of effect:

1) The objective response ratio (ORR) of patients treated with CPIs with elevated PD-L1 expression vs patients with low PD-L1 expression.

2) The Hazard Ratio (HR) for OS of patients treated with CPIs with elevated PD-L1 expression vs patients with low PD-L1 expression.

3) The Hazard Ratio (HR) for OS of patients with elevated PD-L1 expression treated with CPIs vs chemotherapy.

With the first two parameters, the difference of response in patients treated with CPIs could be evaluated in the two subgroups of elevated and low PD-L1. ORR is usually a secondary endpoint in phase 2 and phase 3 studies and it could help in understanding the different activity of the experimental intervention on the neoplastic disease. In this study ORR is defined as the fraction of patients with a complete or partial radiological response out of the total, according to the standardized RECIST 1.1 criteria [34]. The difference in OS in clinical studies is usually evaluated with HR, as long as the assumption of proportional hazards is respected. Improving OS is the final endpoint of any oncological treatment, and necessary to test the prognostic value of any biomarker. On the other hand, to be considered predictive, any biomarker should be tested in a population selected by the biomarker and randomized to receive the experimental therapy (in our case immunotherapy) or the standard as the control arm.

Search strategy

On April 2023, a systematic review was conducted on the PubMed, Web of Science and Scopus databases with the following keywords: [mesh] urothelial carcinoma OR bladder cancer OR urothelial cancer AND metastatic OR advanced AND [mesh] PDL-1 OR PD-1 OR CTLA-4 AND immunotherapy OR [mesh] checkpoint inhibitors OR antiPDL-1 OR antiPD-1 OR anti-CTLA-4. Papers were checked by titles and abstracts for initial screening. Full text review followed in the initially screened manuscripts. References from selected studies were hand searched and cross-referenced.

Inclusion and exclusion criteria

The following inclusion criteria were determined for the first two parameters: prospective clinical trial of patients affected by stage IV urothelial carcinoma treated with CPIs, PD-L1 tested and reported, comparison of ORR and OS between patients with high and low PD-L1 expression.

For the third measure of effect the inclusion criteria comprehended: randomized controlled study of CPIs vs chemotherapy, PD-L1 tested and reported, comparison of OS between patients with high and low PD-L1 expression. For all the objectives: observational or retrospective studies, letters to the editor, comments, etc. were excluded. Other reviews or meta-analyses were excluded. Analysis of single arms or subgroups of the selected study were included if informative. If hazard ratios with confidence intervals were not available, Kaplan-Meier curves with risk tables for our endpoints have to be published or accessible.

Data extraction

Differentiating between the first two and the last measure of effect, two investigators independently extracted the following information: number of patients in the low PD-L1 arm, number of patients in the high PD-L1 arm, number of partial and complete responses in the low and high PD-L1 arm, median OS in the low and high PD-L1 arm, and median OS in the chemotherapy and immunotherapy arm in

the high PD-L1 subgroup, respectively. HRs with 95% confidence intervals were retrieved where available. If not available, logHR and standard error (SE), where calculated from individual patient data (IPD), extracted from published Kaplan-Meier curves [35], a summary of the accuracy estimation of the process will be provided in the results.

Risk of bias assessment

The Cochrane Collaboration risk of bias tool was used to assess the risk of bias for the selected studies.

Statistical analysis

To assess the summary ORR we calculated the composite Odds Ratio (OR) of the ratio of partial plus complete responses in the two arms (low and high PD-L1 expression). Form published HR for survival and 95% confidence interval, logHR and SE were calculated and pooled together. Forest plots were designed to summarize the relation between the expression of PD-L1 and OR for response and HR for survival. Another forest plot was designed to summarize the difference in survival between patients with high PD-L1 expression treated with chemotherapy or immunotherapy. Heterogeneity among outcomes of the selected studies was assessed with Cochrane Q-test and the I^2 statistic with significance at $p<0.05$ or I^2 greater than 50%. A random effect model was used to calculate pooled HRs for all the results, independently of heterogeneity, considering the differences in the dosing of PD-L1 and the probable different true effect size in different studies. Publication bias was evaluated using funnel plots. All statistical analyses were performed with R v.4.2.3. IPD from Kaplan Meir curves were extracted with ScanIt(R) software and then analyzed with R v.4.2.3.

Results

Study selection and Characteristics

After literature research, 13 studies were deemed eligible, of which 8 for the first two endpoints and 5 for the last one. Figure 1 reports the search steps. All the selected studies were prospective trials including patients with advanced urothelial carcinoma, treated with systemic therapy, with an immunotherapy containing arm. In all the studies PD-L1 was assessed and quantified, even with different methods. A summary of the selected trials with drugs involved and different PD-L1 assessments is reported in Table 1. For studies with more arms, the comparison was between the arm with CPI monotherapy versus chemotherapy, excluding the chemo plus immunotherapy arm, which was considered a possible confounding factor. If more than one PD-L1 positive group was identified, according to different percentages of the biomarker expression, it was always considered as PD-L1 positive the group with the highest expression for this meta-analysis.

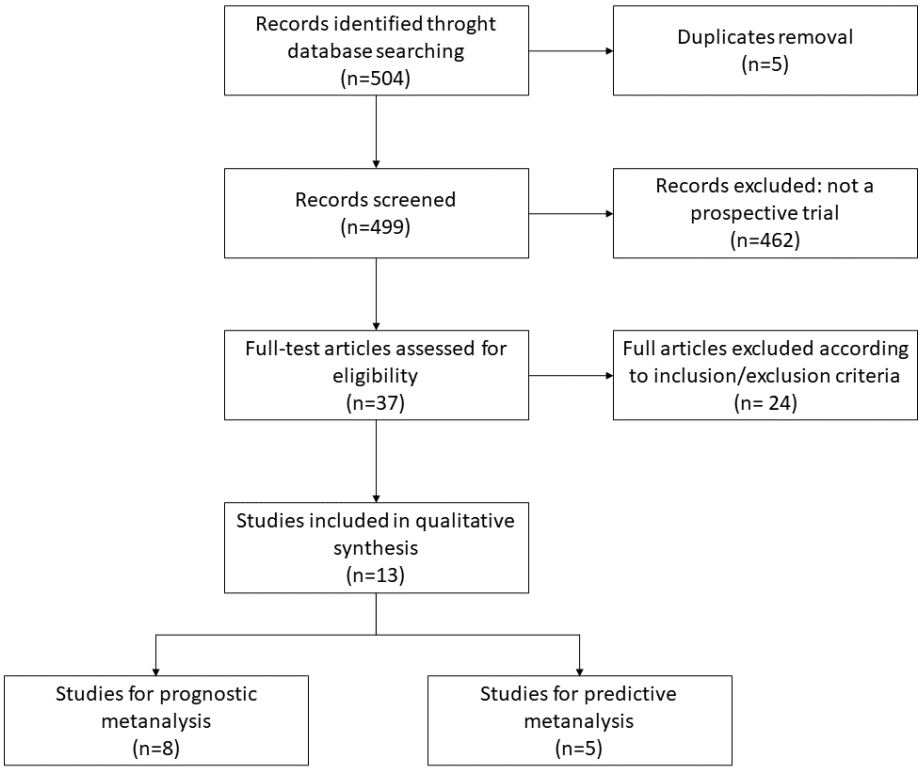


Figure 1: Flow chart of paper screening by inclusion and exclusion criteria.

Table 1: studies included in the meta-analysis

Study	Phase	ICI involved	Randomized	PD-L1 assessment	Citation
IMVIGOR-210	II	Atezolizumab	No	SP142 antibody % on immuno-infiltrating cells (positive > 5%)	Rosenberg et al., Lancet., 2016 [36]
MEDI4736	I/II	Durvalumab	No	SP263 antibody % on TC or immuno-infiltrating cells (positive > 25%)	Powles et al., JAMA Oncol., 2017 [37]
ARIES	II	Avelumab	No	CPS score	Iacovelli et al., Ann Oncol., 2022 [38]
CHECKMATE-275	II	Nivolumab	No	Dako % on TC (positive > 5%)	Galsky et al., Int J Clin Oncol., 2019 [39]
KEYNOTE-052	II	Pembrolizumab	No	CPS score	Balar et al., Lancet Oncol., 2017 [40]
POLARIS-3	II	Toripalimab	No	SP263 % on TC (positive > 1%)	Sheng et al., Clin Cancer Res., 2022 [41]
SAUL	IV	Atezolizumab	No	SP142 antibody % on immuno-infiltrating cells (positive > 5%)	Sternberg et al., Eur Urol., 2019 [42]
STRONG	IV	Durvalumab	No	SP263 antibody % on TC or immuno-infiltrating cells (positive > 25%)	Sonpavde et al., Eur J Cancer., 2022 [43]
KEYNOTE-361	III	Pembrolizumab	vs chemotherapy (1st line)	CPS score	Powles et al., Lancet Oncol., 2021 [6]
IMVIGOR-211	III	Atezolizumab	vs chemotherapy (pre-treated)	SP142 antibody % on immuno-infiltrating cells (positive > 5%)	Powles et al., Lancet., 2018 [44]
DANUBE	III	Durvalumb + Tremelimumab	vs chemotherapy (1st line)	SP263 antibody % on TC or immuno-infiltrating cells (positive > 25%)	Powles et al. Lancet Oncol., 2020 [30]
KEYNOTE-045	III	Pembrolizumab	vs chemotherapy (pre-treated)	CPS score	Bellmunt et al., New ENg J Med., 2017 [7]
IMVIGOR-130	III	Atezolizumab	vs chemotherapy (1st line)	SP142 antibody % on immuno-infiltrating cells (positive > 5%)	Galsky et al., Lancet., 2020 [31]

Association between response and PD-L1 expression

A total of 8 studies including 2703 patients and 499 events (complete plus partial responses) were identified for the first measure of effect meta-analysis. The pooled OR for tumor response was 2.9 with a 95% CI of 2.32 to 3.63 and a p<0.0001 in favor of the patients with an elevated expression of PD-L1 on their tumor tissue. The meta-analysis did not show significant heterogeneity with a Q=5.47; p=0.6, and I^2 < 50%.

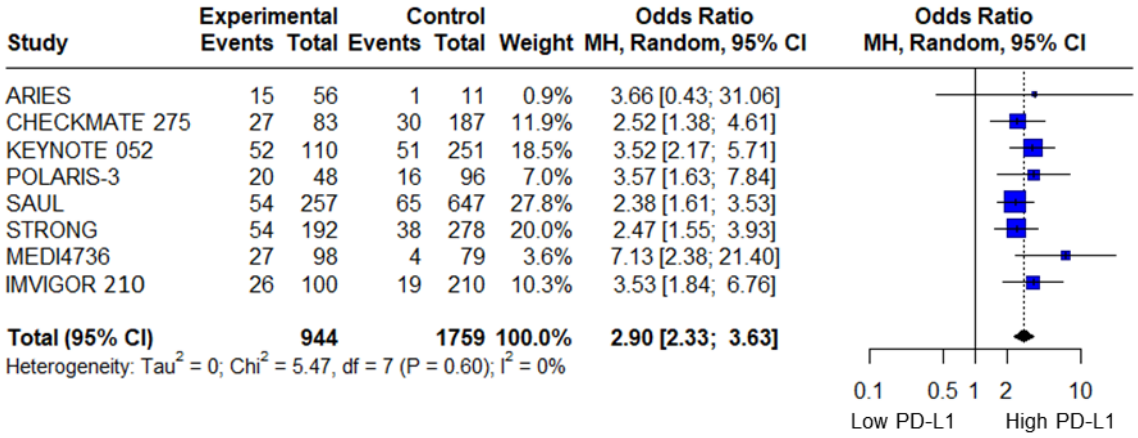


Figure 2: meta-analysis of probability of tumor response in patients treated with CPIs according to high or low PD-L1 expression.

Association between OS and PD-L1 expression in patients treated with CPIs

A total of 8 studies including 2703 patients were considered. The pooled HR was 0.65 with a 95% CI 0.57 to 0.73; displaying a reduction in the risk of death for patients whose tumors expressed high PD-L1 levels. The meta-analysis did not show significant heterogeneity with a Q=6.47; p=0.49, and I^2 < 50%.

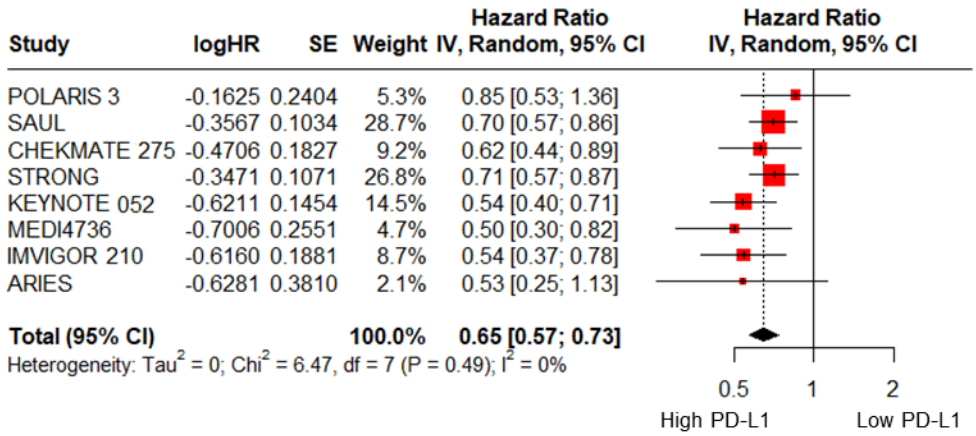


Figure 3: meta-analysis of pooled HR of reconstructed IPD of patients treated with CPIs according to high or low PD-L1 expression.

A summary of the estimated accuracy of reconstructed IPD is reported in figure 9. The greatest variability was found in the ARIES study, which had the lowest sample size and number of events.

Association between OS and treatment in patients with high PD-L1 expression

A total of 5 studies including 2058 patients were considered. The pooled HR was 0.79 with a 95% CI 0.61 to 1.02; displaying a trend for reduction in the risk of death for patients with high PD-L1 expression treated with immunotherapy. The meta-analysis did not show significant heterogeneity with a Q=6.45; p=0.17, and $I^2 < 50\%$.

Considering the ITT population of the same studies, unselected for PD-L1, 3797 patients were randomized to receive chemotherapy or CPIs. The pooled HR was 0.86 (95% CI, 0.72 - 1.02). The heterogeneity was moderate with $I^2 > 50\%$ (53%).

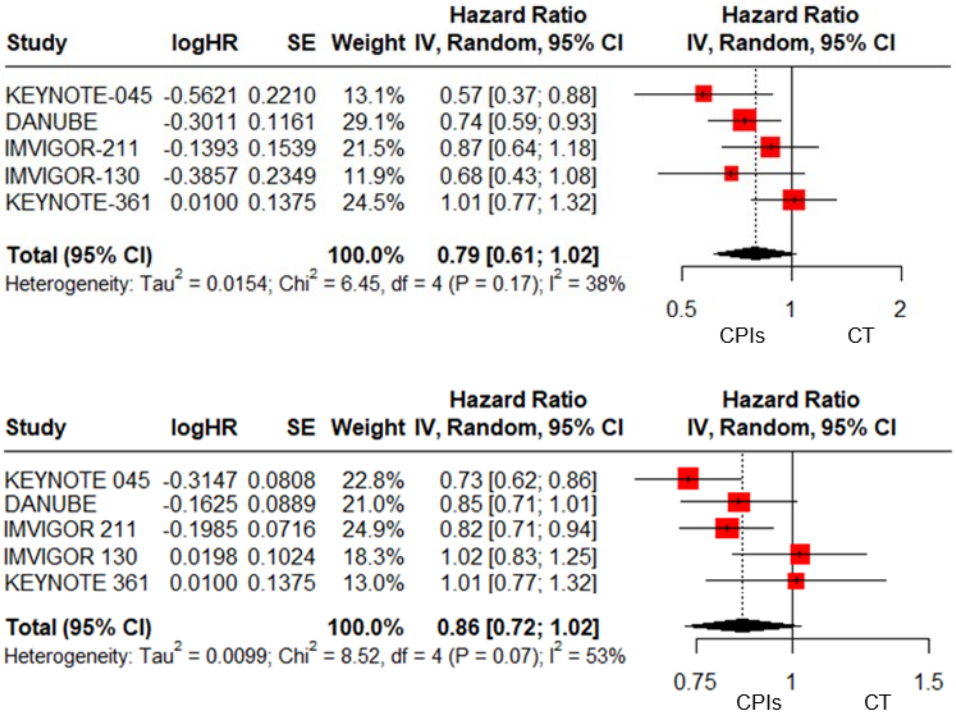


Figure 4: meta-analysis of pooled HR of patients with high PD-L1 treated with CPIs or chemotherapy, followed by the same analysis in the ITT population.

Considering the low number of involved studies, no funnel plot for assessing publication bias was produced. A subgroup meta-analysis was then performed dividing the studies involving the first or subsequent line of therapy.

Considering the first line of therapy, for patients with high PD-L1 expression, the pooled HR was 0.82, with a 95% CI of 0.49 to 1.35 with a heterogeneity < 50% (46%). On the other hand, in the ITT population, the pooled HR was 0.94 (95% CI 0.72 - 1.23) with a lower heterogeneity of 9%.

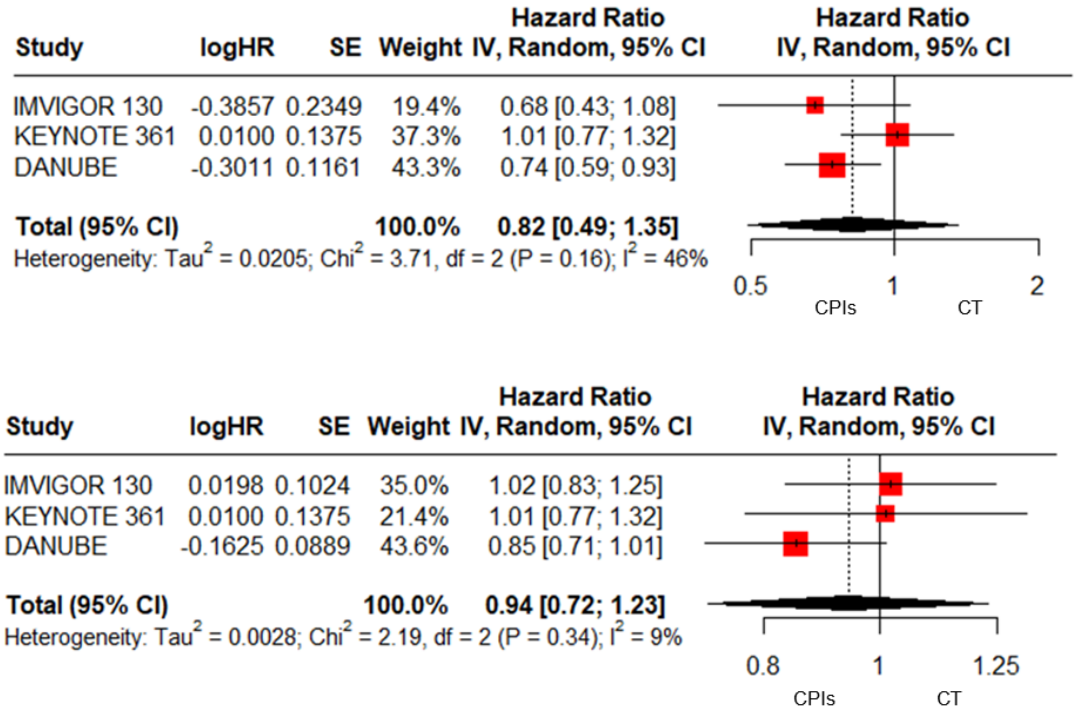


Figure 5: meta-analysis of pooled HR for patients with high PD-L1 treated with CPIs or chemotherapy in the first line setting, followed by ITT meta-analysis.

Considering the studies which involved patients in subsequent lines of therapy (after the platinum-based first-line), the ones with high PD-L1 expressions showed a pooled HR of 0.73 (95% CI, 0.05 to 10.37) and a I^2 of 59%. Similarly, the pooled HR for the ITT population was 0.78 (95% CI, 0.37-1.62) with a heterogeneity I^2 = 14%. Figure 6 shows the forest plots for this analysis.

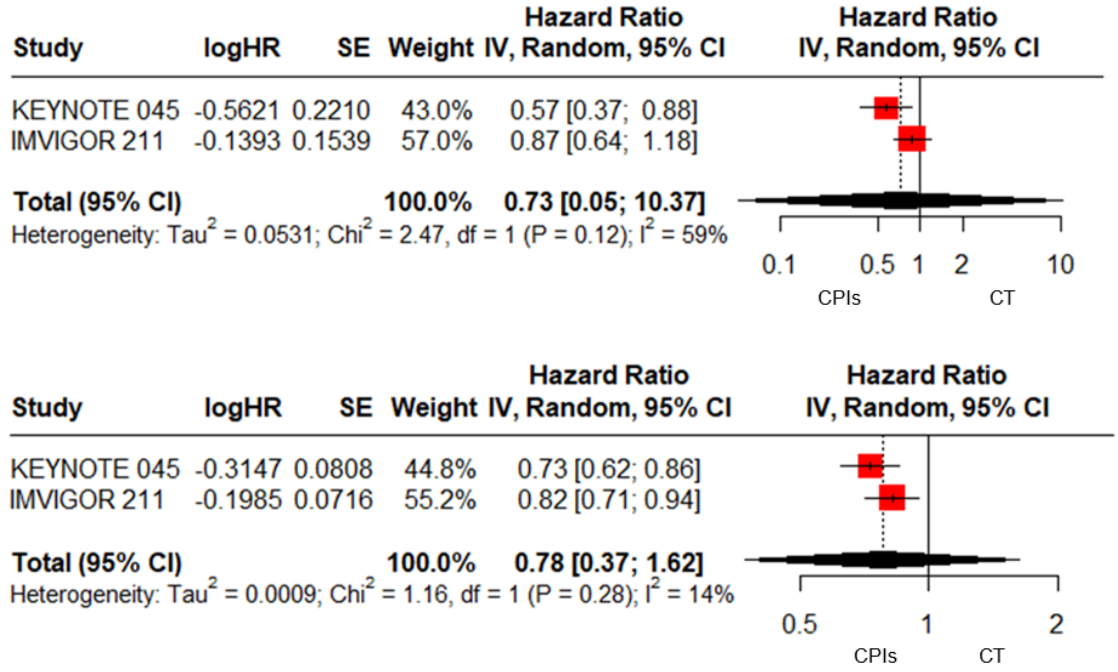


Figure 6: meta-analysis of pooled HR for patients with high PD-L1 treated with CPIs or chemotherapy in the second or advance line setting, followed by ITT meta-analysis.

Risk of bias assessment

The funnel plot for assessing publication bias considering the association between OS and PD-L1 expression did not show greatly asymmetrical patterns (Figure 7). As per Cochrane recommendations, funnel plots were not designed for meta-analyses with fewer studies.

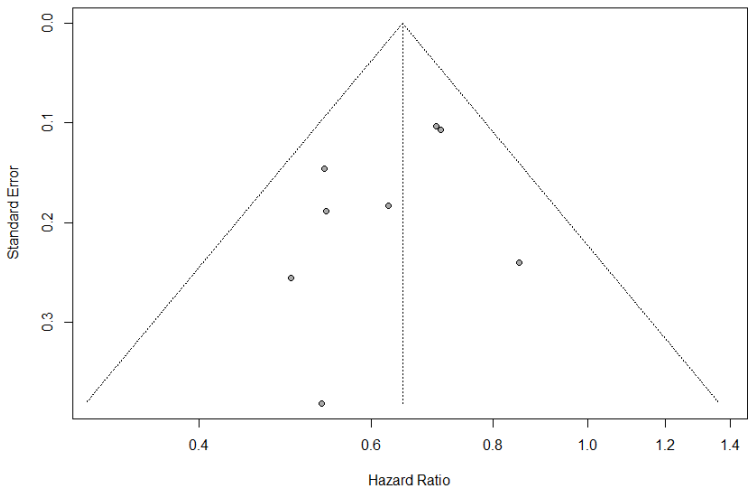
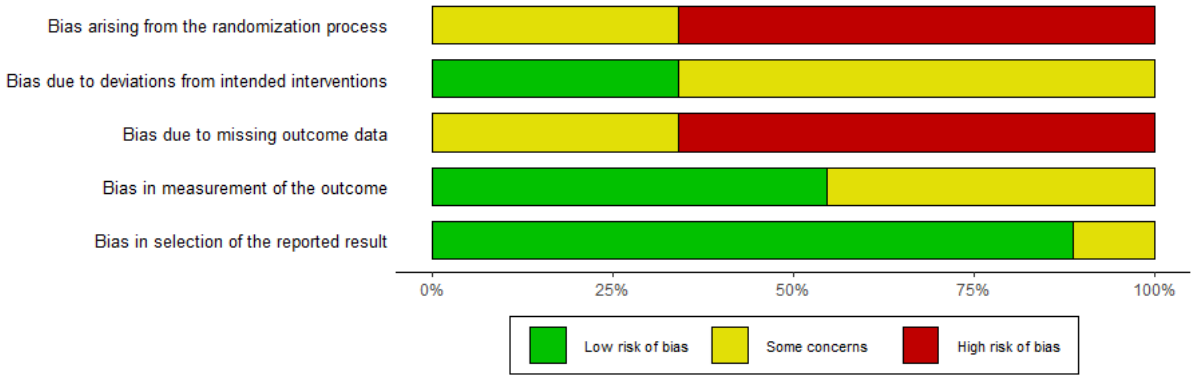
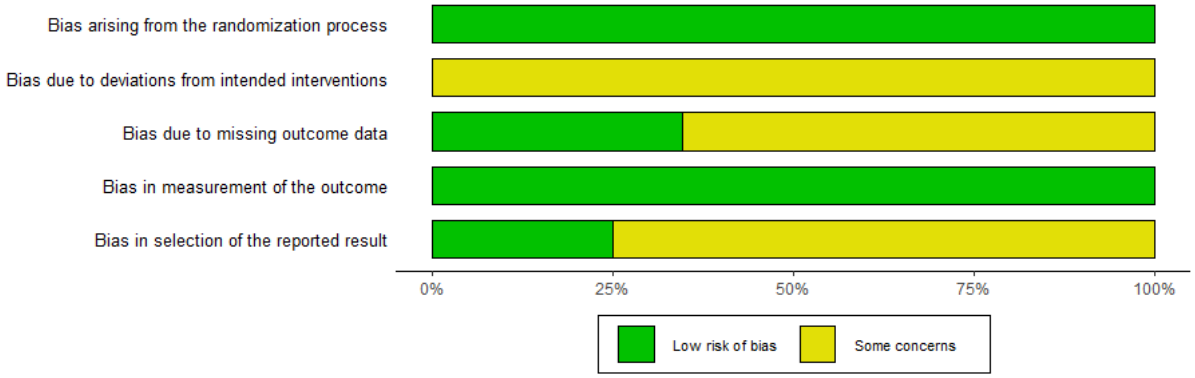


Figure 7: funnel plot for publication bias for studies selected for association between OS and PD-L1 expression.

Risk of bias was assessed independently for the two different sets of study and summarized in Figure 8. For each element an evaluation was given between low risk of bias, some concern about risk of bias, or high risk of bias. Five elements were considered: bias in the randomization process, deviations from intended interventions, missing outcome data, bias in the measurement of the outcome and bias in the selection of reported results.



(A)



(B)

Figure 8: risk of bias assessment for the first two measures of effect (A): response ratio and OS for patients treated with CPIs with high or low PD-L1. (B): OS for patients with high PD-L1 treated with CPIs or CT.

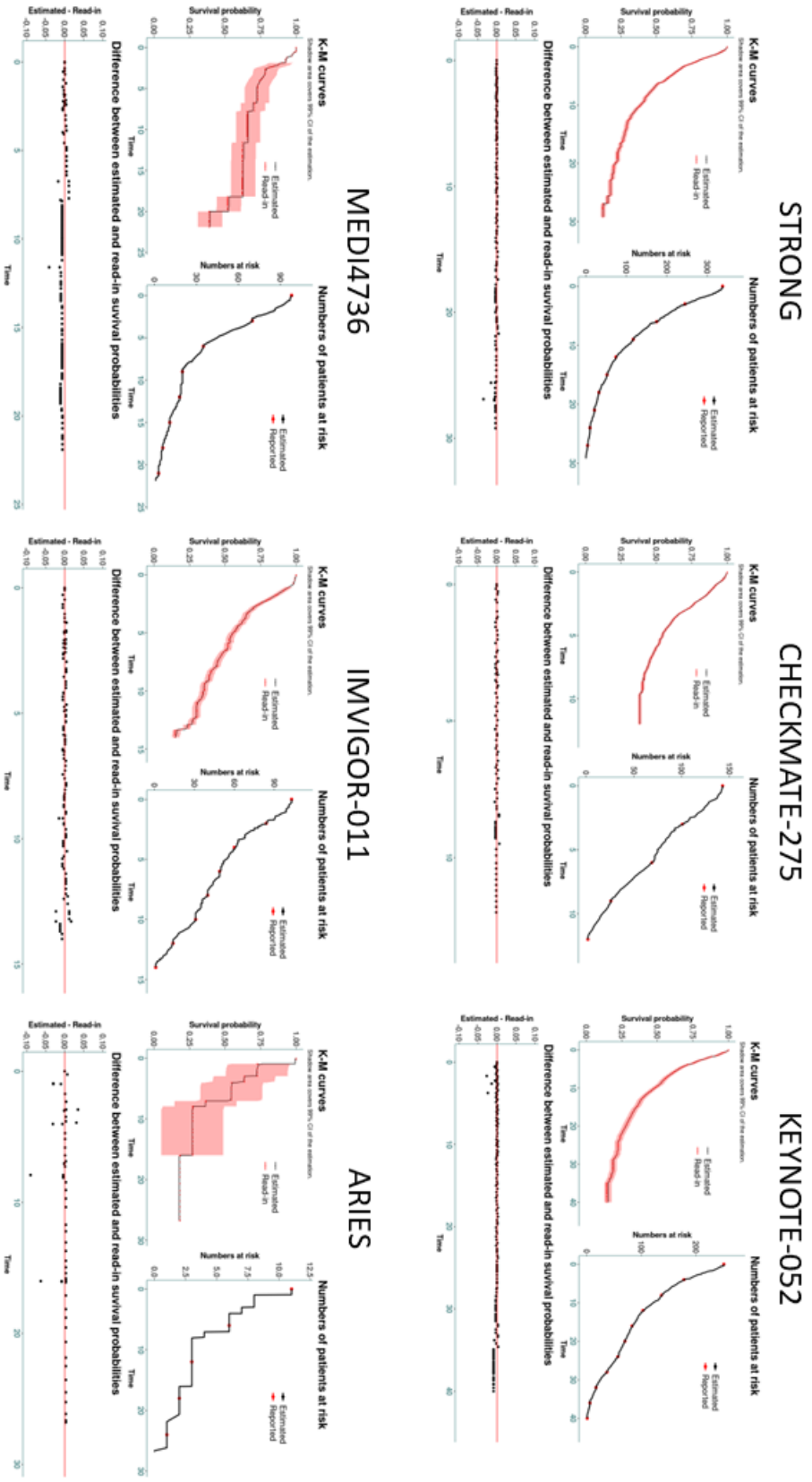


Figure 9: Summary of the estimated accuracy of reconstructed IPD and HR for the Kaplan-Meir curves of the selected studies for OS meta analysis in patients with high vs low PD-L1 expression.

Discussion

The role of PD-L1 as a predictive biomarker in the context of immunotherapy for the treatment of UC is still discussed. While it is mandatory for very specific indications, it is still not widely used in clinical practice. The aim of this meta-analysis was to establish if PD-L1 expression on tumor tissue could be prognostic and/or predictive in patients with UC treated with CPIs. As older meta-analyses with the first studies on CPIs already demonstrated, it seems that patients with elevated PD-L1 expression in their tumor tissue, even if measured with different methods, have a higher chance of a partial or complete remission (according to RECIST 1.1 criteria) when treated with CPIs, when compared with patients in the same setting, but with a low PD-L1 expression. Indeed, our analysis showed an OR of 2.9 (95% IC, 2.33- 3.63) for response in the former subgroup of patients. Moreover, it seems that the greater ORR may translate into an OS benefit. As many of the studies included in this first analysis were phase 2 with only one arm (immunotherapy), they did not report the HR for survival with CI for the high and low PD-L1 subgroups, as it was not the main endpoint. As described in the methods section, IPD were retrieved from the Kaplan-Meier curves, and logHR and SE estimated from them separately for each study. Pooling the estimated logHR together, in our analysis patients with high PD-L1 expression had a higher median OS than their pairs with low PD-L1, with a HR for death of 0.65 (95% CI, 0.57- 0.73). From these results, it could be concluded that high PD-L1 expression seems to be prognostic in patients affected by UC treated with CPIs, as it is able to predict better response to treatment and better survival.

These findings are in line with other reviews and meta-analyses [45] [46], even if the OS results should be approached with caution, as the results are based on estimations, and not on direct IPDs. The heterogeneity between the 8 included studies for the first two measures of effect was low. Moreover, examining the forest plots, it can be seen as there is a concordance of results between all trials. Looking at the funnel plot no particular publication bias is reasonably suspected.

However, the most important role of a biomarker in this setting is its predictive power. The positivity or negativity of a biomarker should be able to guide the treatment decisions, as already seen in HER-2 positive breast cancer [47] or EGFR mutated/ALK translocated NSCLC [48] [49]. From our meta-analysis, it seems that PD-L1 could not be considered predictive. Patients with high PD-L1 expression showed no significantly different median OS if treated with CPIs or traditional chemotherapy, with an HR of 0.79 and a 95% CI of 0.61 to 1.02. As can be seen in the forest plot, 3 out of 5 studies considered did not show a clear OS benefit for the high PD-L1 subgroup. Considering the different approaches among the different trials in measuring PD-L1, we decided to be more conservative, applying a random effect model, even if the heterogeneity tested with Cochrane Q and I^2 were lower than the pre-specified parameters. Our conservative approach could explain the lack of statistical significance, and the presence of only a trend for better survival. Indeed, it is interesting to notice how even studies with the same drug (e.g., Pembrolizumab in the two KEYNOTE studies) and the same PD-L1 test (CPS score

in this case) showed completely different results concerning differences in OS based on PD-L1 expression, with the difference being the first or advanced line of therapy.

The benefit in OS for the ITT population in second or further lines is confirmed by the KEYNOTE-045 and IMVIGOR-211 trials. Curiously, comparing the PD-L1 high populations in these two trials the benefit seems to be similar or even lower than in the ITT. Of course, it is worth considering that the sample size for PD-L1 high population is far lower than in the ITT, inevitably increasing the standard error and the uncertainty of the meta-analysis. However, observing the pooled HR and the confidence interval in the forest plot of the 3 studies in first line (DANUBE, KEYNOTE-361 and IMVIGOR-130) even in this setting it does not seem that the effect of a higher PD-L1 is much different from the ITT population. Moreover, in the first line setting, the therapy with CPIs is not beneficial in terms of increasing OS, when compared to platinum-based chemotherapy, regardless of PD-L1.

Thus, the results of this meta-analysis confirm that, at the moment, there is not enough evidence to prefer to treat a patient with immunotherapy, rather than chemotherapy, based on PD-L1 expression. Up today, the best choice in first line treatment for advanced UC was platinum-based chemotherapy followed by maintenance Avelumab in the case of response or stability [8]. It should be noted that even in this phase 3, randomized trial, the OS benefit of the treatment was both in the intention-to-treat population and in the high PD-L1 subgroup. Very recently, at the ESMO 2023 annual meeting, the results of the KEYNOTE-A39 and CheckMate 901 trials were presented at the plenary oral session. The latter is a phase 3 study comparing the combination of Enfortumab Vedotin plus Pembrolizumab and traditional platinum-based chemotherapy while the former compared Cisplatin plus Gemcitabine plus Nivolumab versus Cisplatin plus Gemcitabine. Both studies show very promising results in improving OS in an unselected population of patients with advanced UC. While the complete results with subgroup analyses are not yet available, these two regimens are probably going to become a new standard for first-line treatment for all patients, regardless of PD-L1 expression.

Probably, the expression of PD-L1, when elevated, is a positive prognostic factor in these advanced patients, differently from the one with localized UC. To gain predictive power, PD-L1 could be associated with other biomarkers, both molecular and clinical, to try to find a combination of elements that could be combined in a predictive score able to predict response to CPIs or chemotherapy in different settings. For UC many prognostic models are available, such as the Bellmunt or the Sonpavde score, pooling clinical elements such as the presence of liver metastases, or the presence of anemia and the patient performance status to get a prognostic score [50]. Perspective studies combining these items with PD-L1 expression could give more answers on the role of this biomarker for UC.

Conclusions

PD-L1 evaluation could be useful as a prognostic biomarker in patients with advanced UC receiving immunotherapy. However, the results did not show enough predictive power to help in the choice between CPI therapy and first-line Cisplatin-based chemotherapy or other options in later lines of treatment.

Fibroblast Growth Factor Receptor (FGFR) as a biomarker for UC, a monocentric, retrospective, real life experience

Background

FGFR is a family of tyrosine kinase receptors which consists of four members: FGFR 1 to 4. Each one of them is formed by an extracellular binding domain, a transmembrane domain and, lastly, an intracellular tyrosine kinase domain. When bound by their ligands, the intracellular domain activates different signaling pathways, including mitogen-activated protein kinase (MAPK) which increase cell proliferation, the development of angiogenesis, and promote cell survival [51]. Consequently, the activation of FGFR by mutation, such as single-nucleotide variants or translocations, could lead to the development of neoplastic diseases. Indeed, this family of receptors is found to be altered in around 5-10% of all cancer types, but with greater frequency in intrahepatic cholangiocarcinoma and UC [52]. In UC, the most important member of the family is FGFR3. For this gene, the most frequent alterations are single base mutations, especially S249C which affect the extracellular domain, stabilizing the receptor dimerization [53]. Other commonly found mutations are R248C, Y375C, G372C and N542S. Fusions and translocations could also be found, especially the FGFR3-TACC3 fusion [52]. Most of the alterations have been identified and studied by sequencing UC tumor tissue with next generation sequencing (NGS) on tumor DNA. However, up to today there is also an FDA approved test on tumor RNA, the QIAGEN therascreen®, which is the companion diagnostic of the Erdafitinib drug [11]. Unfortunately, this test is not yet available worldwide for patients outside of clinical trials or academic research. Other kind of genomic testing, such as FoundationOne® CDx which uses a highly sensitive hybrid capture-based NGS to examine a large number of genes related to cancer, have been validated for a large number of alterations, including FGFR2 for treatment of cholangiocarcinoma [54] and it will probably be for FGFR3 in the near future. Other approaches exist, such as the measurement of FGFR mRNA within situ hybridization, which has been used in the FORT-2 trail, a phase Ib/II testing a combination of Rogaratinib (a FGFR inhibitor) and Atezolizumab. In this trial only patients with FGFR mRNA overexpression could be enrolled. The FORT-2 showed promising results, with an ORR of 54% and a disease control rate of 83% (Rosenberg et al., 2021).

Despite most trials focusing on advanced disease, FGFR3 mutations seem to be more frequent in low grade, non muscle invasive UC, in particular in papillary pTa (up to 87% of cases), while they are less commonly found in carcinoma in situ (CIS) or in high grade muscle invasive tumors [56]. Moreover, in a large study by van Rhijin et al examining 260 primary UC, it was found that FGFR3 mutations were almost always mutually exclusive with TP53 alterations, which is usually associated with high grade, muscle invasive disease [57]. These findings lead to the concept that UC of the bladder may follow two distinct evolution pathways: the non-invasive or papillary pathway, characterized by FGFR3

alterations, and the CIS, high-grade, muscle invasive pathway, enriched with TP53 mutations. However, FGFR3 mutations or translocations are found in around 18% of advanced UC [58], which develops almost exclusively from invasive, high grade disease.

It is hypothesized that pT2 UC with FGFR3 alteration as a major feature could develop from NMIBC that have progressed, gaining mutation in other key genes such as CDKN2A. [59]. From this biological background, a different behavior of the FGFR3 "addicted" disease could be expected at the patient's bedside. However, few studies explored this possibility, most of the ongoing trials are focused on the new FGFR inhibitors. In this observational study, patients' characteristics, response to traditional chemotherapy and to immunotherapy with CPIs have been compared in the group harboring FGFR alterations and in the wild-type one, with the aim to explore the presence of significant differences in a real world population.

Materials and Methods

We retrospectively evaluated all consecutive patients treated at our Institution affected by advanced UC from 2018 up to 2022, tested for FGFR alterations for clinical trial or clinical practice, with at least 6 months of follow-up. The test was considered acceptable if performed with the TFGFR or QIAGEN therascreen®, which is FDA approved, or with NGS with an exon coverage in FGFR 2 and 3 genes of at least 100X. The alterations considered as pathologic to define a patient as FGFR "positive" were G370C, R248C, S249C, Y373C point mutations, and TACC3v1 and TACC3v3 fusions. The test had to be performed on tumor tissue of the primitive tumor or a metastasis, with histological confirmation of urothelial carcinoma.

All patients have to be treated with at least one line of systemic therapy for metastatic disease: chemo or immunotherapy (if Cisplatin-ineligible). Adjuvant and neoadjuvant platinum-based chemotherapy could have been administered previously for these patients. In the case of a relapse later than 12 months after the treatment, a new platinum-based first-line therapy was started, otherwise the patients started a second-line therapy. The treatment was administered as per standard clinical practice, according to national and international medical oncology guidelines and drugs labels. On the basis of the treatment received, other medications could have also been administered to patients, such as anti-nausea drug (especially Aprepitant and Ondasentron), steroid premedication with dexamethasone, and granulocyte colony stimulating factors (G-CSF) as appropriate.

Radiological evaluation was performed at the baseline, before the first therapy dose, and then every three months. Response to therapy was evaluated with RECIST 1.1 criteria. Contrast enhanced CT scan of thorax, abdomen and pelvis or CT scan of the thorax with abdomen and pelvis MRI were considered acceptable radiological examinations for evaluating response and disease progression. Unequivocal

clinical progression or death were considered as a progression event even in the absence of a radiological confirmation.

Clinical data was collected from clinical charts, anonymized, and collected in an electronic database. The association between the presence of FGFR alterations and OS and PFS for patients treated with platinum-based chemotherapy and/or immunotherapy, was calculated with a log-rank test, and illustrated with Kaplan-Meier curves. After checking the adherence to proportional hazards with Schoenfield method, differences of survival or progression-free survival were calculated as hazard ratios with Cox proportional hazards method. The OS analysis was conducted both with patients receiving FGFR inhibitors included and excluded. Association between nominal variables were studied with Chi squared test or Fisher's exact test, as appropriate for the sample size. Variables significant at $p< 0.1$ at univariate analysis were analyzed in multivariate. Results were considered as statistically significant at $p < 0.05$.

All statistical analyses were performed with R v.4.2.3.

Results

Patients' characteristics

In our institution from 2018 to 2022, excluding the most recent patients with an inadequate follow-up, 160 patients were tested from FGFR alterations, both as part of a clinical trial or clinical practice. From each patient one or more samples from the primitive neoplasm or a metastasis were analyzed. Twelve patients (7.5%) were excluded from the final analysis, as the result of the test (or tests) was sample failure. At the end, 148 patients with a FGFR assessment were eligible for this study. Median follow-up was 21 months (95% CI; 17-26 months).

The majority of the 148 included patients were male (n=113, 76.4%). The smoker or non-smoker status was available for 129 patients: there was a prevalence of smokers and ex-smokers (n=63, 48.8% and n= 38, 29.5%), while non-smokers were only 28 (21.7%).

All 148 patients were affected with histologically confirmed urothelial carcinoma. Of them, 35 (23.6%) developed UC in the upper urinary tract (UTUC), while 113 (76.4%) in the bladder (BC). Some of the analyzed patients presented a UC with a variant histology such as squamous, plasmacytoid, or neuroendocrine (n=39, 26.4%).

A minority of patients had a precedent history of non-invasive bladder cancer (NMIBC) treated with operative cystoscopy and intra-bladder installations of chemotherapy or BCG (n=29, 19.6%). Most of the patients during their treatments received surgery on the bladder or upper urinary tract (n= 129, 87.2%), but only 15 of them (11.6%) received neoadjuvant chemotherapy, while 39 (30.3%) received adjuvant platinum-based treatment.

All 148 presented at diagnosis with advanced stage disease or had a disease relapse after surgery, as per inclusion criteria. Of them, 16 (10.8%) had evidence of bone metastases during first-line treatment,

while 19 (12.8%) had liver metastases. At the beginning of the first-line therapy, most of our population showed a good performance status, with 106 patients with an ECOG of 0 (71.6%), 35 with an ECOG of 1 (23.7%) and 7 with an ECOG of 2 (4.7%).

Of the selected 148 patients, 144 received platinum-based chemotherapy with either Cisplatin (n=94, 63.3%) or Carboplatin (n= 50, 36.7%). The two regimens used were Cisplatin plus Gemcitabine or Carboplatin plus Gemcitabine; no patient was treated with MVAC or dose dense MVAC. Eighteen patients also received Avelumab as maintenance therapy after chemotherapy. Avelumab has been available in Italy within an expanded access program since 2022. On the other hand, 4 patients were considered not eligible for chemotherapy, and received first line Pembrolizumab, after PD-L1 evaluation with a CPS > 10. Additional 68 patients received anti-PD1 or anti-PD-L1 (mainly Pembrolizumab and Atezolizumab) as second or third line therapy. In total, 72 patients received immunotherapy, not including Avelumab maintenance. Patient characteristics are summarized in Table 2.

Median OS from the first diagnosis of metastatic disease to death for all the cohort was 19 months (95% CI, 16 - 27 months). Factors analyzed in the OS analysis in the whole group were:

- Presence of a histologic variant: 18 (95% CI, 15- 34) months vs 20 (95% CI, 17 - 28) months, p= 0.4.
- Carboplatin instead of Cisplatin chemotherapy: 21 (95% CI, 13- 37) months vs 19 (95% CI, 17 - 29) months, p= 0.6.
- PS at baseline: ECOG 0 median of 21 (95% CI, 17- 31) months, ECOG 1 median of 18 (95% CI, 14 - NA) months, ECOG 2 median of 12 (95% CI, 8 - NA), p= 0.7.
- Presence of bone metastases: 15 (95% CI, 10- NA) months vs 20 (95% CI, 17- 28) months, p= 0.1.
- Presence of liver metastases: 11 (95% CI, 7- NA) months vs 20 (95% CI, 19- 281) months, p= 0.02.

Mutational status

Out of 148 with a FGFR alteration status result, 30 (20.3%) presented a mutation or translocation of FGFR2-3. There were 11 patients with S249C FGFR3 mutations (36.7%), 9 Y373C FGFR3 mutations (30%), 5 TACC3v1 translocations 16.7%), 3 R238C FGFR3 mutations (10%) and 1 G37C0C deletions (3.3%). Two of the patients with S249C mutation also presented the Y373 mutation in their tumor tissue and were included in both the analysis for S249C and Y373. No other association between other mutations or translocations was detected in our sample.

Table 2: Patients' characteristics.

Characteristics	**n. of patients**	***%***
Male	76	*76.4%*
Smokers	63	*42.6%*
Non-smokers	38	*25.7%*
Ex-smokers	28	*18.9%*
Smoker status unknown	9	*6.1%*
Primary tumor in the bladder	113	*76.4%*
Primary tumor in the upper tract	35	*23.6%*
Patients with a variant histology	39	*26.4%*
Precedent history of non-invasive BC	29	*19.6%*
Previous surgery of the primary tumor	129	*87.2%*
ECOG PS 0	106	*71.6%*
ECOG PS 1	35	*23.7%*
ECOG PS 2	7	*4.7%*
Presence of bone metastases at first line	16	*10.8%*
Presence of liver metastases at first line	19	*12.8%*

Association between mutational status and patient characteristics

The presence of FGFR alterations did not seem to be correlated with gender in our mono-institutional experience: 9 out of 35 female patients presented the alterations (25.7%), while 21 out of 113 males resulted positive to the FGFR test (18.6%). The difference was not statistically significant with p = 0.5. In the same way, smoker status did not seem to influence the prevalence of FGFR alterations. Indeed 8 patients in the non-smoker group (28.6%) presented with FGFR mutations or translocations, versus 13 in the ex-smoker group (20.6%) and 7 in the active smoker group (18.4%), p= 0.59.

The distribution of FGFR alterations was different between UTUC and BC: 11 out of 35 patients (31.4%) and 19 out of 113 (16.8%), respectively, without statistical significance (p= 0.06). A previous diagnosis of NMIBC did not seem to be correlated to FGFR alterations: of 29 patients with previous pTa or pT1 UC 7 showed positive FGFR test (24.1%), versus 23 out of 119 (19.3%), p=0.75. In the same way, the distribution of FGFR alterations was not significantly different in pure UC histology (22/109, 18.5%) and in UC with a component of squamous, neuroendocrine or plasmacytoid differentiation (8/39, 20.5%), p= 0.99.

Table 3: Different distribution of characteristics based on FGFR status.

Characteristics	**n. of patients FGFR***	***%***	**n. of patients FGFR wt**	***%***
Male	21	*18.6%*	92	*81.4%*
Smokers	7	*11.1%*	56	*88.9%*
Non-smokers	8	*21.1%*	30	*78.9%*
Ex-smokers	13	*46.4%*	15	*53.6%*
Smoker status unknown	-	-	-	-
Primary tumor in the bladder	19	*16.8%*	94	*83.2%*
Primary tumor in the upper tract	11	*31.4%*	24	*68.6%*
Patients with a variant histology	8	*20.5%*	31	*79.5%*
Precedent history of non-invasive BC	7	*24.1%*	22	*75.9%*

Response to platinum-based chemotherapy

As already mentioned, 144 patients were treated with platinum based chemotherapy. Of them, 54 had a complete or partial response, with an ORR= 37.5%. On the other hand, 39 patients experienced disease progression at the first radiological evaluation (27.1%). Eighteen patients treated with Avelumab maintenance were excluded from survival analysis. Therefore, the following analyses on PFS and OS will be on 126 patients. In the overall chemotherapy group, median PFS was 7 months (95% CI, 6 - 8 months) with 120 progression events registered at the end of the follow-up period. In the same group, median OS was 19 months (95% CI, 15 - 28 months) with 82 events.

Median PFS and OS for the overall chemotherapy group are reported in Figure 10.

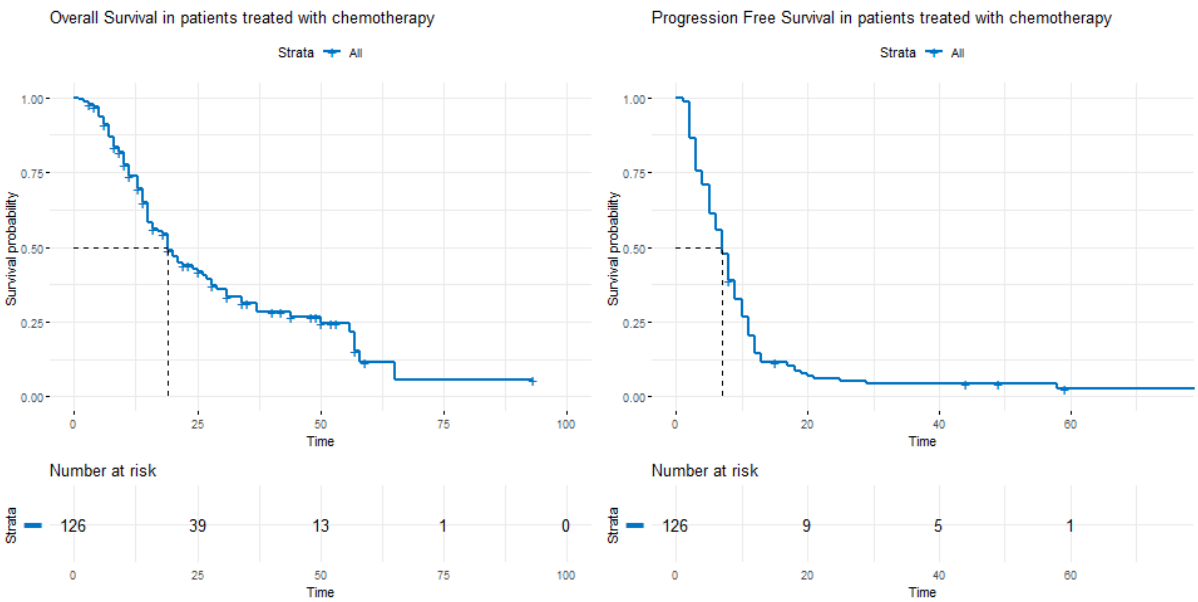


Figure 10: Kaplan-Meir curves of OS and PFS of patients treated with platinum-based chemotherapy.

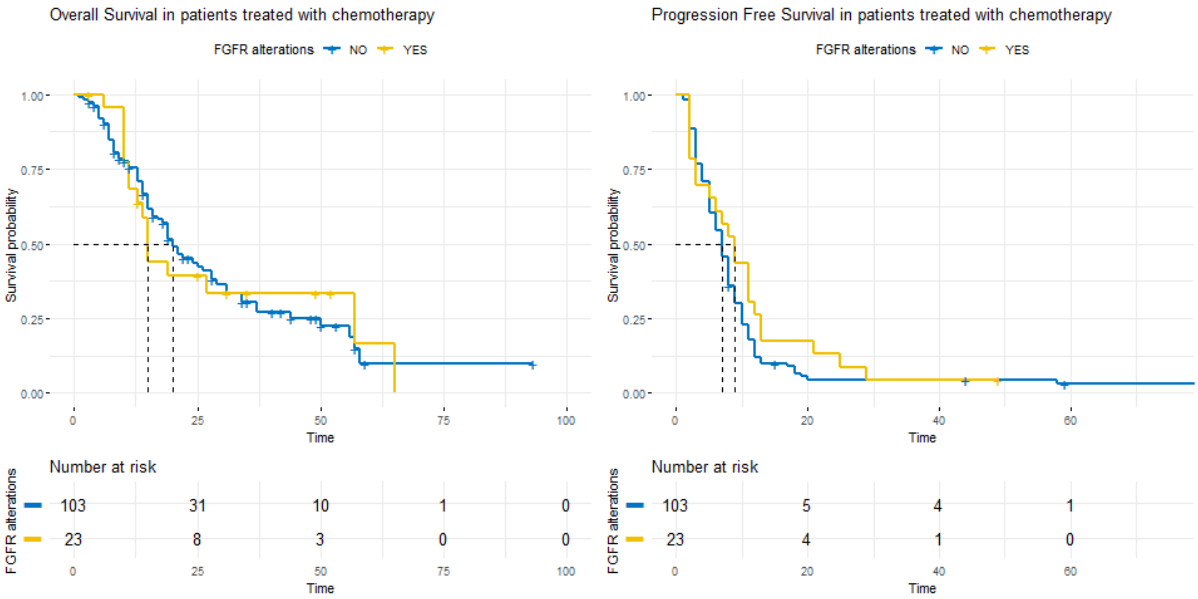


Figure 11: Kaplan-Meier curves of OS and PFS in patients treated with platinum-based chemotherapy according to FGFR status.

Considering the presence or absence of FGFR alterations, in the 126 patients of the chemotherapy group 23 harbored FGFR mutations or translocations (18.3%). In the FGFR positive subgroup ORR was 26.1% versus 33% of the FGFR negative one (p= 0.71). In the same way, the ratio of patients with progression as better response to chemotherapy was similar: 30.4% of FGFR positive patients and 31.1% of FGFR negative ones (p= 0.99). Median PFS was 7 months (95% CI, 6 - 8 months) in the FGFR negative subgroup and 9 months (95% CI, 5-13 months) in the positive one with an HR = 1.32 (95% CI, 0.47 -1.21), p= 0.23. Median OS was 20 months in the former subgroup (95% CI, 16 - 29 months) vs 15 months (95% CI, 13 - NR) with an HR= 0.98 (95% CI, 0.58 - 1.75). Curves of median PFS and OS are reported in Figure 11. Excluding patients who received an FGFR inhibitor after chemotherapy, median OS in the FGFR positive subgroup was 15 months (95% CI, 13- NR), with an almost identical HR= 0.98.

Response to immunotherapy with anti PD-1 or anti PD-L1

Excluding the patients receiving Avelumab maintenance, 72 patients were treated with a "pure" therapy line with anti PD-1 or PD-L1. The ORR for the group was 30.6%, while 56.9% of patients experienced disease progression as the best response. In the overall immunotherapy group, median PFS calculated from the first dose of anti-PD 1/PD-L1 drug and radiological or clinical disease progression was 3.5 months (95% CI, 3 - 6 months) with 70 events. Median OS calculated from the beginning of immunotherapy to death for any cause was 15 months (95% CI, 7-18 months) with 47 events at the end of the follow-up period. Median PFS and OS are reported in Figure 12.

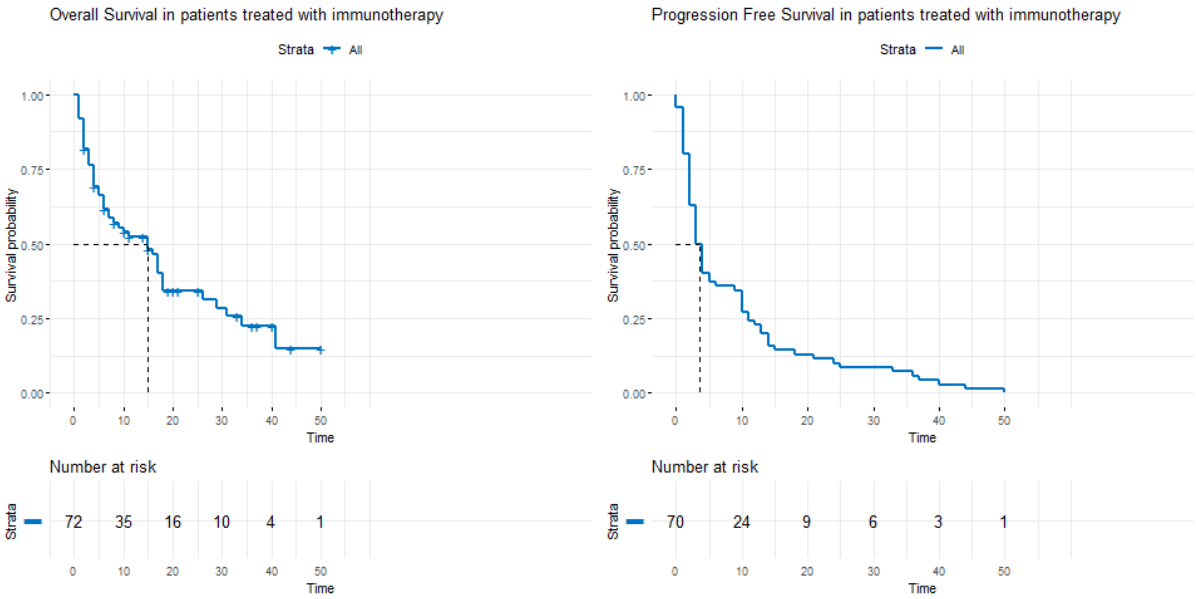


Figure 12: Kaplan- Meier curves of OS and PFS in patients treated with CPIs.

In this group of patients, 13 were positive for FGFR alterations (18.1%). Positive patients had an ORR of 23.1%, while the FGFR negative subgroup showed an ORR of 27.5%. The difference was not statistically significant, with a p= 0.74 (Fisher's exact test). Similarly, 69.2% of patients harboring FGFR alterations experienced disease progression as best response to immunotherapy, versus 46.4% (p= 0.54). Differentiating PFS for the FGFR positive and negative subgroups, median PFS in the latter was 2 months (95% CI, 1 - NA) while in the former was 4 months (95% CI, 3 - 9 months), the HR was 1.22 with p= 0.5. Patients with a FGFR alteration had a median OS of 5 months (95% CI, 2 - NR), versus 17 months (95% CI, 9- 26 months), with an HR of 1.58, p= 0.2.

Excluding the patients who received FGFR inhibitors after immunotherapy, median OS was 3.5 months (95% CI, 2- NR) with an HR of 3.5, p= 0.001. Difference in OS in this subgroup was reported in Figure 13. Multivariate Cox regression analysis for the presence of FGFR alterations, presence of bone and presence of liver metastases showed that FGFR alterations is an independent prognostic factor for OS (altered vs not altered HR= 4.3, 95% CI, 1.93-9.7, p< 0.001).

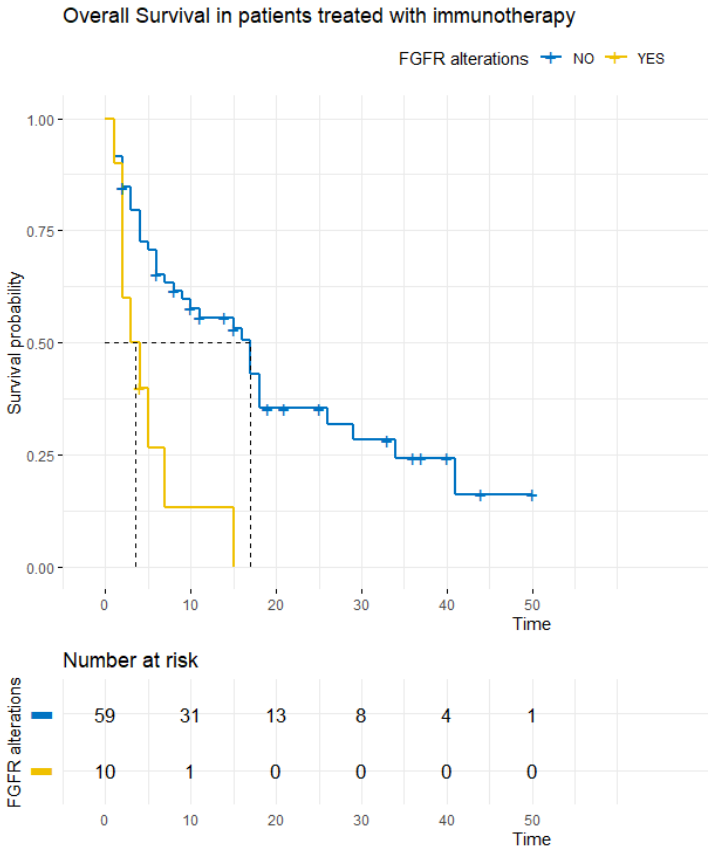


Figure 13: Kaplan Meier curves of patients treated with CPIs according to FGFR status excluding the ones who received a subsequent line of therapy with FGFR inhibitors.

Discussion

FGFR inhibitors promise to change the treatment of FGFR mutated UC. However, little data is known about this particular subset of the urothelial disease. FGFR mutations are indeed more common in the NMIBC, while they are rarer in advanced UC. In our observational study a fairly large number of patients (160) affected by metastatic or locally advanced UC and tested on tumor tissue for FGFR alterations have been enrolled. First of all, a sample failure of 7.5% should be underlined, which is lower than the one of the BLC2001 trial, the phase 2 trial of Erdafitinib, where 2214 patients were tested worldwide with 227 invalid test result (10.3%) (Loriot et al. N Eng J Med, 2019). As expected, the majority of patients were male (76%) confirming the 2:1 ratio which is usually reported in the literature for this disease [60]. In our population, fewer patients received a neoadjuvant treatment, compared to the adjuvant one, as many of the patients enrolled were not evaluated at our institution before surgery but underwent surgery in smaller hospitals, confirming the still low rate of neoadjuvant treatments for UC in low volume centers. The vast majority of the patients received first line platinum-based chemotherapy as per international standard, while around half of them received a second line therapy with CPIs, mainly Pembrolizumab. Patients treated with Avelumab maintenance were excluded from

the main analyses about PFS and OS for chemotherapy and CPI, because Avelumab can be prescribed only in case of response or stability of disease after chemotherapy, introducing a selection bias. In our population, the median OS from the diagnosis of metastatic disease to death was 19 months, which is far better than the 13.8 months of the first phase 3 trial of Cisplatin plus Gemcitabine versus MVAC [4]. Of course, this improvement is explained using more effective second line treatments, like CPIs, and ad Avelumab maintenance in a fraction of patients. On the other hand, median PFS for Cisplatin and Gemcitabine was 7 months in our study, almost identical to the median PFS of the clinical trial (7.3 months). Many possible prognostic factors have been considered in our analysis, with a possible correlation between bone and liver metastasis and a worse prognosis. Indeed, the negative prognostic meaning of bone metastases have already been explored in the Meet-URO 01 trial [61] while liver localizations have been associated with a worse outcome in many retrospective reports, even for patients treated with CPIs [62] [63].

In our group of patients 20.3% had FGFR3 alterations in their tumor tissue, very similar to the 20.9% of patients detected in the BLC2001 trial. The distribution of frequency of mutations and translocations were as expected by the literature, with a prevalence of the S249C point mutation (a third of the alterations detected). Many patients in our report were tested in the context of a clinical trial, the majority with the FDA approved QIAGEN therascreen®. FGFR3 alterations were detected by targeted exome NGS in a minority of patient, many with the Foundation One® test, which in the last years has been largely used in many clinical trials (especially basket trials) and even if not reimbursed by the Italian Healthcare System, it is commercially available to patients. While the two methods have some differences it should be noted that there is still not a "gold standard" and each new FGFR inhibitor is probably going to have a companion diagnostic test. The concordance of our data about the incidence of FGFR3 mutations with the literature and large international trials can lead to the conclusion that both methods are at the moment acceptable for identifying FGFR3 alterations.

We analyzed the correlation between FGFR3 status and different patient and tumor characteristics. From our results, the distribution of FGFR3 alterations was not significantly different between men and women. In the same way, no particular differences were detected in smokers, ex-smokers and not smokers. A greater portion of FGFR3 aberrations was found in UTUC rather than in bladder cancer with 31.4% vs 16.8% of cases, respectively. This result was at the limit of statistical significance with $p=0.06$, probably due to the smaller sample size of the UTUC subgroup. On the other hand, there was not a greater incidence of FGFR3 alterations in histological variants, when compared to pure transitional cell histology, or in patients with a previous history of NMIBC treated with local therapies. Concerning the response to platinum based chemotherapy, from our results there seem to be no significant differences in PFS, OS, or even ORR between the FGFR aberrant and wild type groups, similarly to another Italian published report by Necchi et al, which found no particular differences in OS or PFS (Necchi et al, 2019). This result was confirmed even excluding the few patients who received a FGFR inhibitor (in clinical trials or as a compassionate use program).

When considering patients treated with CPIs, even in this setting no particular difference in PFS or ORR emerged in our series of patients. However, when considering OS, there was a striking difference in median OS, with 5 months for patients with FGFR alterations and 17 months for patients with FGFR wild-type. However, probably due to a low sample size in the FGFR mutated subgroup, there was only a trend for statistical significance. Excluding the very few patients who received a FGFR inhibitor, a pre-planned analysis, the difference was even more evident with a median OS of 3.5 months for the FGFR aberrant subgroup. In this case the difference was significant with a p=0.001. Including this result in a multivariate analysis with the presence of bone or liver metastasis confirmed the negative prognostic role of FGFR alterations for patients with UC treated with CPIs. At the ESMO 2023meeting the results of cohort 2 of the THOR trial were presented, showing no benefit in OS for Erdafitinib compared to Pembrolizumab in the second line setting in a population of patients with FGFR alterations. Moreover, the positive results of first line combinations of Enfortumab Vedotin plus Pembrolizumab and Cisplatin plus Gemcitabine plus Nivolumab showed at the same event need to be considered: the therapeutic algorithm for metastatic UC is probably going to change, including CPIs as standard first line therapy. In this changing scenario FGFR could still be a prognostic biomarker and be decisive in the choice of a second line treatment.

It should be underlined that our study has some limitations: its retrospective nature makes it prone to selection bias, even if in our clinical practice the research of FGFR alterations was proposed to all consecutive patients deemed eligible for systemic treatment. Almost all patients had the FGFR test on tumor tissue of the primitive tumor rather than on a metastasis, both from radical cystectomy surgical specimen or diagnostic material from endoscopic procedures. This fact could be interpreted as a limitation but, on the other hand, it would be difficult to expose patients to the risks of a biopsy with the tumor tissue already available, outside of a clinical trial specifically designed to study the different mutation profile of primitive UC and its metastases. Moreover, all ongoing phase 3 trials with FGFR testing allow the use of the tumor tissue from the T. The sample size is limited, but it should be considered that only patients with advanced UC were included, excluding the ones not fit to receive systemic therapy.

Conclusions

FGFR aberrations were found in about 20% of a real life series of patients with advanced UC. They were slightly more frequent in UTUC rather than bladder. No significant difference was found between patients harboring FGFR alterations treated with chemotherapy and wild type concerning ORR, OS and PFS. On the other hand, OS was inferior for patients with FGFR mutations treated with CPIs. Prospective studies are needed to confirm these results.

Final remarks and future research

Despite extensive research, metastatic UC remains a deadly disease with a poor prognosis: only 8% of patients are alive after 5 years from diagnosis (NCI statistics 2023). In the first years of 2000s the median OS expected for these patients were about 15.8 months, a result from phase III clinical trials comparing Cisplatin and Gemcitabine to other chemotherapeutic regimens [4]. After the introduction of CPIs in clinical practice the median OS increased to about 23.8 months, especially with Avelumab maintenance, which proved to improve outcome in patients responding to chemotherapy [64]. It should be underlined that these results are from randomized clinical trials, with a selected population of patients without important comorbidities, eligible for chemotherapy and who achieved a response or at least disease stability after platinum-salts, in the case of Avelumab data. Indeed, in our study about the role of FGFR the 148 patients analyzed had a median OS of about 19 months, in line with the expectations, considering that data collection started well before the use of Avelumab became common clinical practice. However, response to chemotherapy and CPIs greatly vary between patients. There are occasional reports of long lasting responses, especially to CPIs, with long term survival [65] [66] while a considerable portion of patients experience progression at the first evaluation or even an hyperprogression [67]. Other novel, promising drugs, such as FGFR inhibitors, and antibody-drug conjugates Enfortumab Vedotin [68] and Sacituzumab Govitecan [69] promise to further improve outcomes for patients with advanced UC who progressed after chemotherapy and immunotherapy. All these innovations in the therapeutic field are much needed in a field with great clinical needs, but no advance has yet been made in patient selection. As therapeutic options expand there is a need for therapy personalization, which could be achieved mainly with the use of clinical or molecular biomarkers. In the treatment of UC, many clinical prognostic biomarkers have been explored such as the presence of bone metastases [61], the role of thyroid hormones [70] and the presence of circulating inflammation markers [71]. However, none of these could be used at the moment to make a choice between a therapeutic approach or another. Many studies are ongoing on molecular biomarkers in UC, but the only two ones who could approach the patient bedside up today are PD-L1 and FGFR. Both the biomarkers should be predictive, the first of response to CPIs, the second to FGFR inhibitors. In this study a critical review approach has been conducted on PD-L1, examining many clinical trials and pooling a large number of patients in a meta-analysis. The results are somewhat ambiguous, confirming that the measurement of PD-L1 as a biomarker in UC is still problematic. Indeed, a high expression of PD-L1 was very consistently linked to a greater chance of tumor response when treated with CPIs (according to RECIST 1.1 criteria) in all the studies pooled in the meta-analysis. However, median OS was not significantly different in patients with high PD-L1 treated with chemotherapy or CPIs. These results could lead to the conclusion that PD-L1 is more of a prognostic than a predictive biomarker, at least in the settings considered for the clinical trial included in our analysis. Still different methods of assessing

PD-L1 exist with immunohistochemistry, ignoring other, more refined analyses [72] which are more difficult to apply in a clinical setting. Every registration trial for each CPI used a companion diagnostic which is not interchangeable. It is important to underline that this fact could have a role in explaining the different results obtained in different trials, concerning the predictive role of PD-L1. Moreover, the heterogeneity could also rise from the differences between di primary tumor and the metastatic sites, which could have a different micro-environment, regulating the expression of PD-L1. As already discussed, the use of invasive biopsy to monitor the biomarker status on metastases is unrealistic in a real world scenario. Non-invasive methods are also being developed such as liquid biopsy, or even specific tracers for nuclear medicine diagnostic [73].

On the other hand, FGFR aberrations seem to be a predictive biomarker when considering FGFR inhibitors [74]. In this study a cohort of real life patients were analyzed and interestingly a trend for lower OS was detected for patients with UC harboring FGFR alterations treated with CPIs, when compared to ones with FGFR wild type. When excluding the few patients who received FGFR inhibitors after failure of CPI therapy, the difference was even greater, and statistically significant. A similar trend is observed also in "oncogene addicted" NSCLC [75], with combination strategies being currently developed with the aim of gaining the benefits of targeted and immunotherapies [76]. Our results contribute to this biological hypothesis also for UC, even if prospective trials are needed to confirm these findings. The research on this subject will proceed with a prospective trial called DIFFRACTION, with the first patients already enrolled in our Institute. With this trial, patients treated for advance UC at our Institute with FGFR alterations on tumor tissue are followed prospectively, and ctDNA from peripheral blood is collected before the start of a line of systemic therapy and at the first radiological evaluation. The aim of the study is to test the feasibility of liquid biopsy for the diagnosis and monitoring of FGFR altered, metastatic UC. The data collected will also be correlated with the therapy administered, in particular chemotherapy plus Avelumab maintenance or Pembrolizumab monotherapy. In our real world population, there was a higher fraction of FGFR3 mutations in UTUC, rather than in bladder cancer, even if the difference was not statistically significant ($p=0.06$). This data is in line with the existing literature [77] [78], but it should be noticed that most studies about molecular characterization of UTUC or BC were conducted on surgical samples of localized disease, and do not report any clinical outcome. In our report only metastatic patients were included, with a complete analysis of treatments and outcomes. Other differences in the expression of biomarkers between UTUC and BC will be explored in the AURORA trial, an observational study with the aim to analyze with immunohistochemistry some key biomarkers in UTUC, in particular Nectin-4, PD-L1 and proteins of the mismatch repair mechanism (MLH-1, MSH-2; MSH-6 and PMS-2).

Even more biomarkers are currently studied and more candidate biomarkers will probably be identified in the near future. There are reasons to be optimistic about the chances of a more precise, personalized treatment of UC, giving patients the most effective treatment and avoiding unnecessary adverse events from ineffective therapies. However, even with the most effective treatment in the best conditions,

advanced UC remains lethal, and for this reason great effort should be made in exploring novel therapies in the neoadjuvant and adjuvant setting, with the aim of reducing the number of patients who will develop metastatic UC and in improving biomarker detection for early diagnosis.

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