frontiers | Research Topics

# Reviews in genitourinary oncology

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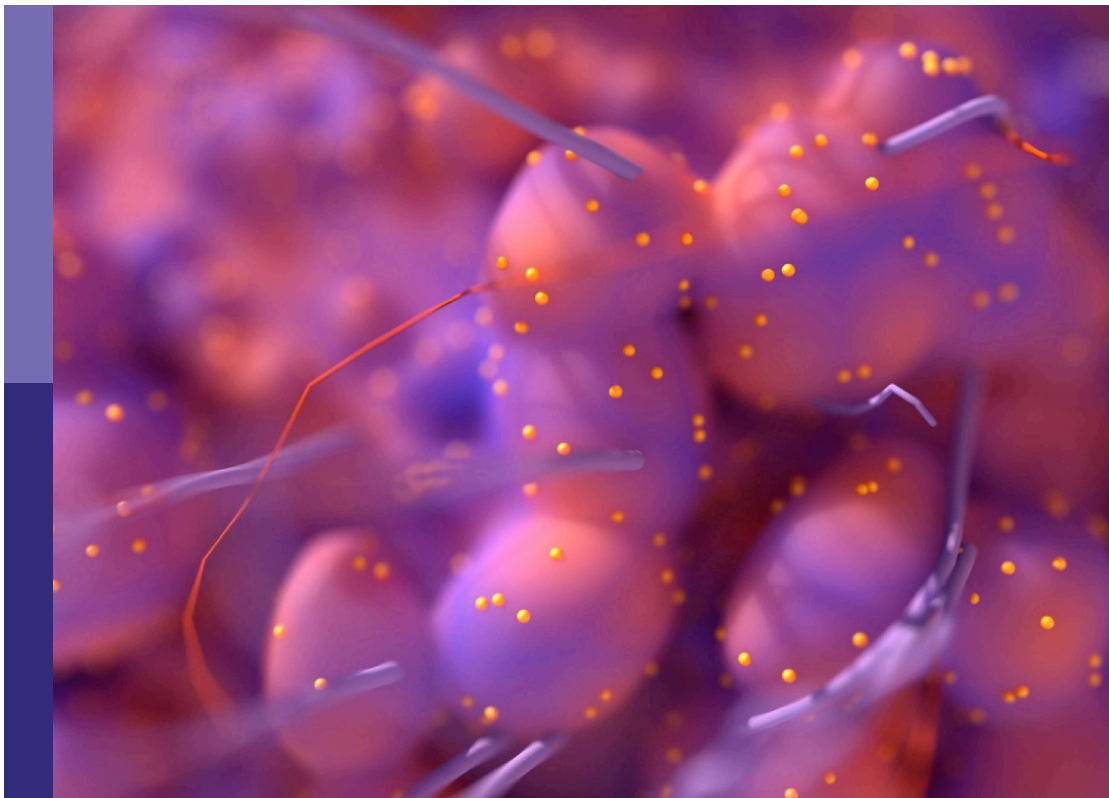

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# Reviews in genitourinary oncology

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# Editorial: Reviews in genitourinary oncology

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Editorial on the Research Topic
Reviews in genitourinary oncology

GU malignancies represent approximately one fourth of all solid tumors and over the past 20 years there have been huge improvements due to a rapid evolution in diagnostic modalities, with the emergence of novel biomarkers and clinical validation of new diagnostic tools, and to a broadening of therapeutic options. The understanding of the molecular interplay within the tumor microenvironment has resulted in an improved drug design and discovery, with the rise of novel agents, including second-generation anti-androgens, radioactive molecules, PARP inhibitors, checkpoint inhibitors and targeted therapy, each enabling to implement a precision medicine approach. In parallel, dramatic changes have also occurred in the field of radiation oncology, due to its ability to achieve a higher conformality around the target while reducing the dose to the surrounding healthy tissues, as well as in urological surgery, that has safely incorporated minimally invasive endoscopic techniques.

This Research Topic is aimed at widening the knowledges in various aspects of GU oncology, emphasizing interdisciplinary contributions. The issue currently includes 18 reviews/metanalyses mainly covering renal, bladder, and prostate cancers from different perspectives including basic science, genomic, clinical research, and translational research. All contributions to this Research Topic focus on one or more of the aforementioned research areas.

One of the hot topics deals with the relevant advances and main changes in the imaging in GU oncology. Liu et al. evaluated the detection rate of fluoro-prostate-specific membrane antigen (18F-PSMA-1007) PET/CT in patients with different serum PSA levels in the setting of both primary staging and biochemically recurrent prostate cancer. They found that the detection rate of 18F-PSMA-1007 PET/CT was slightly higher in primary prostate tumors than in biochemical recurrence, and that it is improved with increasing serum PSA levels. Tian et al. assessed a multiparametric MRI clear cell likelihood score algorithm for the classification of small renal masses: for all cT1 renal masses, the pooled sensitivity and specificity were 0.80 (95% CI 0.74–0.85) and 0.76 (95% CI 0.67–0.83), respectively, showing a moderate to high accuracy for identifying clear cell RCC from other RCC subtypes, and with a moderate inter-reader agreement.

Biomarkers have become a significant focus of research, and namely on how they can help predict response to systemic therapy, identify treatment resistance, and tumors' immunogenity. Xie et al. investigated the diagnostic, clinicopathological, and prognostic utility of circRNAs in prostate cancer: the aggregated data from their metanalysis revealed an AUC of 0.81, with a sensitivity of 0.82 and a specificity of 0.62 for diagnostic value, indicating that circRNAs could be employed as diagnostic biomarkers for prostate cancer, and that aberrant circRNA expression was strongly linked to poor overall survival in terms of prognostic value. Rizzo et al. demonstrated a robust rationale for the use of IL-8 as a potential prognostic and predictive biomarker in the use of ICIs and TKI in mRCC, which has the potential to customize the treatment for each individual patient, although confirmatory studies are needed. Jiang et al. focused on the role of tumor-derived exosomes in clear cell RCC metastasis, drug resistance and diagnosis, and highlighted the potential to act as biological markers and meaningful targets for early diagnosis and monitoring of disease at once. Burley et al. explored the basics of cancer-associated fibroblasts (CAFs) biology in bladder cancer and identified key therapeutic challenges associated with CAFs, such as the lack of specific CAF markers, the paucity of research into bladder-specific CAFs and their relationship with inferior responses to radical radiotherapy, and also the opportunities of being employed as single agents and in combination with existing therapies. Wu et al. reported on the recent findings on lactate dehydrogenase C4 (LDH-C4) and highlighted that not only it can be employed as an important parameter in evaluating semen quality and male reproductive function but has the potential to provide new clues for the early diagnosis of testicular tumors.

Immunotherapy, that has historically been centered on systemic cytokines for the treatment of metastatic kidney cancer, or the Bacillus Calmette–Guérin vaccine for non-metastatic bladder cancer, has enormously evolved in the past decade, especially in the field of immune checkpoint inhibition, to a point that immune checkpoint inhibitors (ICIs) are now used extensively in the treatment of kidney and bladder cancers. Wang et al. summarized the recent studies that looked at the interaction networks of B cells with other cells, discussed the role of B cells in RCC development and progression, and assessed their impact on RCC immunotherapy, while Raghubar et al. examined the mechanisms behind the transition of proximal tubular epithelial cells (PTEC) in clear cell RCC development, and the interactions that may limit the response to targeted immune therapy, finally concluding that stromal cells are key drivers in recurrent and locally invasive clear cell RCC.

The uro-oncologic treatment's landscape has seen a tremendous growth with major advancements in prostate, bladder, and urothelial cancers due to a better understanding of tumour biology and the underlying genetic and molecular alterations. Zhu et al. summarized the current data and addressed whether neoadjuvant (NAC) or adiuvant (AC) chemotherapy is effective for variant histologies bladder cancers. In general, they found that favorable OS and CSS observed in patients with frequent histologies who receive NAC or AC are confirmed in those with variant histologies. Interestingly however, a subgroup analysis revealed that NAC independently improved OS in sarcomatoid and neuroendocrine tumors but not in squamous histology. With the development of molecular research, a variety of biomarkers are expected to predict the response to cisplatin-based chemotherapy, thus driving the use of NAC/AC in the future, as also pointed out by Roviello et al., who scrutinized the role of neoadjuvant therapy in muscle invasive bladder cancer (MIBC), highlighting recent advances that can change the clinical practice, and concluded that molecular signatures have the potential for reshaping the selection for tailored treatment and disease monitoring. Zheng et al. show that erdafitinib, a pan-FGFR inhibitor, resulted in a higher objective response rate (0.38 versus 0.10) and lower progressive disease rate (0.26 versus 0.68) in urothelial carcinoma patients compared to those with other solid tumor patients, and that the drug was more effective in presence of fibroblast growth factor receptor (FGFR) alteration, particularly when a specific FGFR alteration (FGFR3-TACC3) was observed. Kloskowski et al. shed light on the potential therapeutic activity of Quinolones, a broad-spectrum antibiotics frequently prescribed by urologists due to their higher accumulation in urine and prostate tissue than in serum, and speculated that the use of modified quinolones in combination with other chemotherapeutics can enable toxic effects at lower drug doses in bladder cancer treatment.

A significant progress has also occurred in the surgical treatment of bladder cancer, mainly due to the implementation of robotic surgery, as emphasized by Long et al. who analyzed the difference in efficacy between robotic-assisted radical cystectomy (RARC) and laparoscopic radical cystectomy (LRC) in bladder cancer and found that the former one is a safe and effective treatment with reduced surgical blood loss and postoperative complications compared to LRC.

Remarkable achievements in the recent years have revolutionized the management of advanced prostate cancer with the opportunity of customizing treatment to the specific cancer and the individual patient. Wolf et al. have discussed the emerging knowledge about the role of Prostate Cancer Stem Cells (PCSCs) as a potential therapeutic target, although a selective and effective targeting of PCSCs remains challenging at this stage, and efforts are needed to improve the characterization of PCSCs using (single-cell) genomics and proteomics. Li et al. explored the current landscape in the management of metastatic hormone-sensitive prostate cancer (mHSPC) following the development of several novel agents and the combination of different therapeutic strategies. Chen et al. provided an overview of the research on bone metastases in prostate cancer based on a bibliometric analysis covering the past 22 years, and in particular showed that the latest research focused on the tumor microenvironment and biomarkers with the aim of exploring the mechanism and the therapeutic targets of bone metastases.

Finally, Camero et al. elucidated the mechanisms of the radioresistance of rhabdomyosarcoma (RMS), the most common soft tissue sarcoma in children, frequently accounting the genitourinary tract. This knowledge paves the way to combined

therapeutic approaches that can radiosensitize cancer cells to finally ameliorate the overall survival of patients with RMS, especially for the most aggressive subtypes.

In conclusion, this Research Topic displayed exciting developments in the diagnosis and treatment of GU malignancies incorporating novel mechanisms, biomarkers for selection of targeted therapy, and innovative treatment approaches. Advances in the management of patients with bladder cancer, prostate cancer, and renal cell carcinoma can be expected from these efforts.

## Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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# Quinolones as a Potential Drug in Genitourinary Cancer Treatment—A Literature Review

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Quinolones, broad-spectrum antibiotics, are frequently prescribed by urologists for many urological disorders. The mechanism of their bactericidal activity is based on the inhibition of topoisomerase II or IV complex with DNA, which consequently leads to cell death. It has been observed that these antibiotics also act against the analogous enzymes present in eukaryotic cells. Due to their higher accumulation in urine and prostate tissue than in serum, these drugs seem to be ideal candidates for application in genitourinary cancer treatment. In this study, an extensive literature review has been performed to collect information about concentrations achievable in urine and prostate tissue together with information about anticancer properties of 15 quinolones. Special attention was paid to the application of cytotoxic properties of quinolones for bladder and prostate cancer cell lines. Data available in the literature showed promising properties of quinolones, especially in the case of urinary bladder cancer treatment. In the case of prostate cancer, due to low concentrations of quinolones achievable in prostate tissue, combination therapy with other chemotherapeutics or another method of drug administration is necessary.

**Keywords: quinolones, fluoroquinolones, cancer, bladder, prostate, urine**

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## INTRODUCTION

Quinolones are chemotherapeutics discovered in 1962 by Lescher et al. who have synthetized nalidixic acid (1). The application of this drug is limited due to the short time of action and fast-occurring bacterial resistance. Insufficient properties of nalidixic acid led to the formation of fluoroquinolones characterized by a broader antibacterial spectrum and improved pharmacokinetic properties (2). The quinolones and their derivatives are synthetic antibiotics that show antibacterial activity against Gram (+) and Gram (–) bacteria. The mechanism of their bactericidal activity is based on the inhibition of topoisomerase II or IV complex with DNA, which consequently leads to cell death (3). It has been observed that these antibiotics also act against the analogous enzymes present in eukaryotic cells. Due to the high concentrations of these antibiotics achievable in the urine and prostate, they are widely used in the treatment of genitourinary tract infections.

Various antibiotics are used in cancer treatment. Their antiproliferative and proapoptotic properties and influence on epithelial to mesenchymal transition are used for tumor growth

inhibition (4). Also, quinolones, especially ciprofloxacin, were tested on many cell lines *in vitro*, indicating their potential usage for cancer patients. Induction of apoptosis, cell cycle arrest, and disruption of mitochondrial membrane potential are examples of quinolones' mechanism of action against cancer cells (5). Despite potential anticancer properties of different antibiotics, it should be noticed that these types of drugs can also negatively influence cancer development. Antibiotics, as well as chemotherapeutics, besides removing pathogenic bacteria, can also affect natural microbiota. Especially important is gut microbiota, whose disruption can lead to cancer generation by promotion of chronic inflammation, alteration of normal metabolism, genotoxicity, and weakening of the immune response (4). The microbiome is also present in the urinary tract, which for a long time was considered sterile (6). Recent studies have shown that modulation of the microbiome can improve therapeutic response to immune checkpoint inhibitors, which can influence the effectiveness of immunotherapy in cancer treatment (7, 8). Antibiotic therapy can reduce immunotherapy effectiveness, by damaging the microflora. In order to eliminate the influence of antibiotics on intestine microflora, drugs can be administrated parenterally. However, experiments performed on animal models showed that antibiotics, both after oral and intravenous administration, can cause gut dysbiosis. The advantage of intravenous administration is that the richness and diversity of interstitial microbiota return to the pretreatment level more quickly (9). Intravesical infusion can disrupt the urinary microbiome, which can also lead to a decrease in immunotherapy (Bacillus Calmette-Guerin) effectiveness in bladder cancer treatment (6). Combined therapy, using fecal microbiota transplantation after the end of antibiotic therapy or chemotherapy, can reduce the negative influence of these drugs on the immune system and cancer treatment effectiveness (10). Antibiotics show an ambivalent role in tumor growth and progression; these properties should be considered before applying them in cancer patients.

Quinolones are administrated mostly intravenously or orally; some of these drugs are available as eye drops. These drugs are classified into four generations with different antibacterial spectra (**Figure 1**) (11).

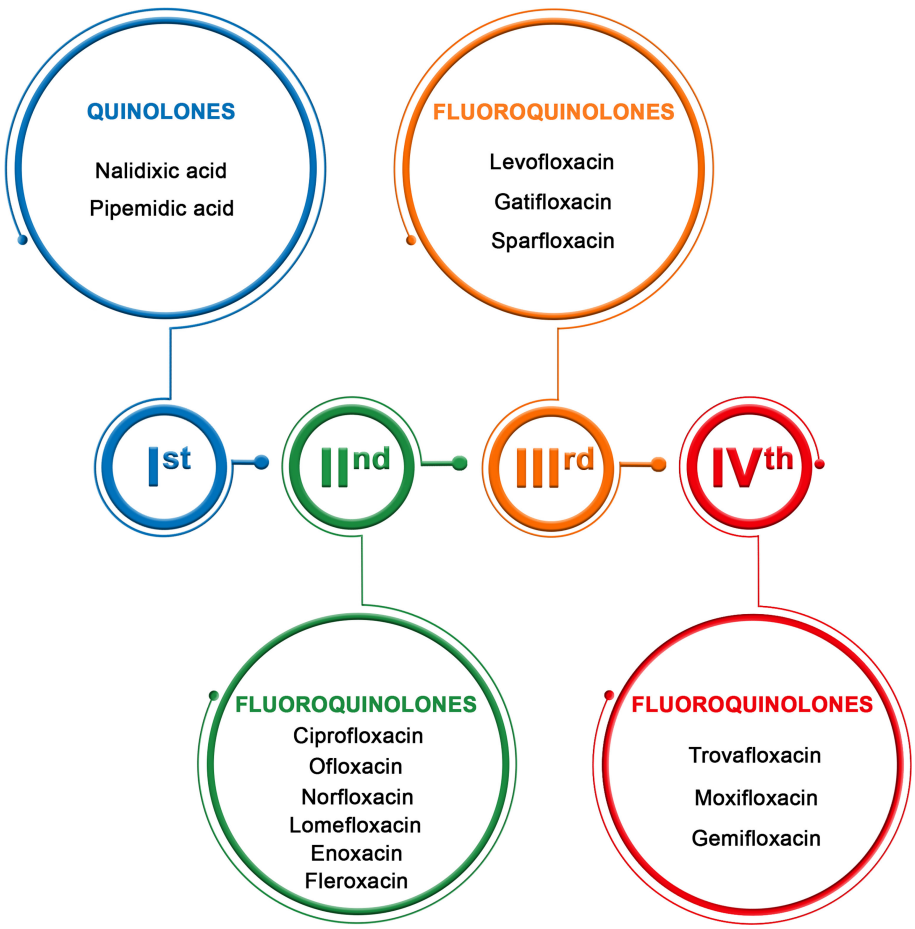


**FIGURE 1** | Generation of quinolones (11).

## BLADDER AND PROSTATE CANCER EPIDEMIOLOGY

Prostate cancer (PCa) is the most frequent type of cancer among men in Europe and second around the globe. According to the recent incidences in 2020, almost 1,400,000 new PCa patients with 375,000 deaths were noticed (12). Over the last few years, the stabilization of incidence rates in Western and Northern regions of Europe has been observed. As for the Eastern and Southern regions, the continuous rise of incidence was determined (13). High PCa occurrence is a global problem; it is the most frequently diagnosed cancer in men in over one-half of countries globally (12).

Bladder cancer (BCa) is a much less common type of cancer than PCa. Worldwide, it is the 10th most common type of cancer, with 573,000 new cases and 213,000 deaths. It is more common in men than in women (4-times) (12). The incidence rates are much higher for Europeans, especially in Southern and Northern parts, and men in the USA than in other parts of the world. This pattern is believed to be a reflection of the tobacco smoking prevalence 20–30 years ago. The highest mortality was observed in Eastern Europe, where the smoking prevalence just started to decrease recently, and the results will be observed after a few decades (14).

## QUINOLONES IN UROLOGY

Since the discovery of nalidixic acid in 1960, quinolones have become one of the most commonly prescribed antibiotics in urology (15). In the USA, 31,500,000 fluoroquinolone prescriptions were registered in 2014. A similar trend was reported in Canada, where 3.1 million prescriptions for fluoroquinolones were reported in 2018 (16). Currently available fluoroquinolones approved by the US Food and Drug Administration (FDA) for the treatment include ciprofloxacin, levofloxacin, norfloxacin, and ofloxacin, while ciprofloxacin and levofloxacin are the most commonly prescribed in urological practice worldwide (17). The excellent activity of fluoroquinolones against gram-negative bacilli and their exceptional penetration to urine positioned them as the major antibiotics applied in urological departments and outpatient clinics. The higher genitourinary drug concentrations that occur with renally cleared quinolones promote their effectiveness in the treatment of genitourinary infections (18). Indeed, fluoroquinolones are effective against genitourinary infections, but increases in the use of fluoroquinolones in recent years have resulted in the gradual development of fluoroquinolone resistance among gram-negative bacilli. In particular, resistance in *E. coli* has dramatically emerged due to fluoroquinolone overuse and has become a challenge in the medical therapy of patients with urinary tract infection (UTI) (19). For instance, Mean et al. found that 51% of fluoroquinolone regimens used in French teaching hospitals were reevaluated as inappropriate based on local microbiological guidelines (20). Fluoroquinolone resistance is mediated by multiple mechanisms including chromosomal point mutations in the genes encoding DNA gyrase and/or topoisomerase IV, mutations that cause the decreased expression of outer membrane proteins (OMPs), changes in the lipopolysaccharide (LPS) component of the cell envelope, and enhanced fluoroquinolone efflux by efflux pumps such as AcrAB. In particular, the use of ciprofloxacin should be strictly avoided in urologic patients with suspicion of extended-spectrum β-lactamase-producing *Escherichia coli* (ESBL-EC) (21).

Given in 3- to 10-day courses, fluoroquinolones effectively treat uncomplicated UTIs caused by susceptible *Escherichia coli*. However, in light of rising resistance, current prescribing guidelines recommend fluoroquinolones as second-line drugs (22). In general, efforts are being made to change therapeutic strategies and limit the use of fluoroquinolones in urology. European Urology Association (EUA) guidelines advocate the use of fluoroquinolones in strictly defined conditions, including uncomplicated pyelonephritis, prostatitis, and epididymitis. Correspondingly, due to the progressive increase in fluoroquinolone resistance, alternative antibiotics such as fosfomycin or targeted agents are recommended as routine prophylaxis before urological procedures (23). Despite justified concerns about the resistance to fluoroquinolones, they are still an important part of antimicrobial management in urology. For bacterial prostatitis and uncomplicated pyelonephritis, they are the most appropriate agents for treatment because of their unique pharmacological characteristics. In this situation, it is critical to strictly follow guidelines when prescribing fluoroquinolones to prevent further resistance increases and deprive the urologists of this effective antibiotic class.

## AIM OF THE STUDY

Quinolones' anticancer properties were analyzed alone or combined with standard chemotherapeutics in many *in vitro* studies. Quinolones, after oral and intravenous administration, accumulate in higher concentrations in urine and prostate tissue than in serum, which is why more attention was paid to the potential use of these drugs in genitourinary cancer treatment. Additionally, these drugs are well tolerated by patients and can be administrated for a long time in high doses, enabling them to maintain high concentrations for many weeks.

The aim of this study was to analyze the anticancer properties of quinolones with particular emphasis on prostate and bladder cancers.

## MATERIALS AND METHODS

### Data Sources

An extensive literature review was performed using the PubMed database in order to identify studies involving the anticancer properties of quinolones and their derivatives. The PubMed database was searched using the following terms: anticancer activity of quinolones, bladder cancer, prostate cancer, cancers of the genitourinary system, and treatment of genitourinary system cancers. Only original full-text publications written in

English have been analyzed. Studies involving treatment with quinolones (alone or in combination with standard chemotherapeutic agents) for normal and cancer cells other than those originating from the genitourinary system were also included. Additionally, the PubMed database was searched for information about concentrations achievable in urine and prostate tissue after oral or intravenous administration of different quinolones. The databases were reviewed until the end of June 2021.

## Data Extraction

Extracted data included the following elements: type of quinolones and their derivatives, quinolone concentrations, type of tested cells, incubation time, influence on cell viability, changes induced in cells, measurement results, the way of assessing results, and the effectiveness of tested compounds. Among the 17 quinolones described, antitumor activity was examined for 15 of them, and no cytotoxic effects of grepafloxacin and finafloxacin have been analyzed so far in the literature. For other compounds, 54 full-text articles were included for the analysis of anticancer properties of quinolones. Detailed data extraction for each quinolone is presented in **Figure 2**. In order to search for information on quinolone concentrations achievable in prostate tissue or urine, combination of quinolone name and "prostate" or "urine" was used. Articles containing necessary information were chosen for **Tables 1**, **2** preparation.

# RESULTS

## Concentration in Urine and Potential Action Against Urinary Bladder Cancer Cells

Most of the analyzed quinolones reach high concentrations in the urine (**Table 1**). In the case of nalidixic acid and pipemidic acid, the antitumor properties against bladder and prostate tumor cell lines have not yet been tested. These compounds, although exhibiting promising properties, confirmed in leukemia, osteosarcoma, and ovarian, breast, and pancreas cancers, belong to the old generation and are currently less

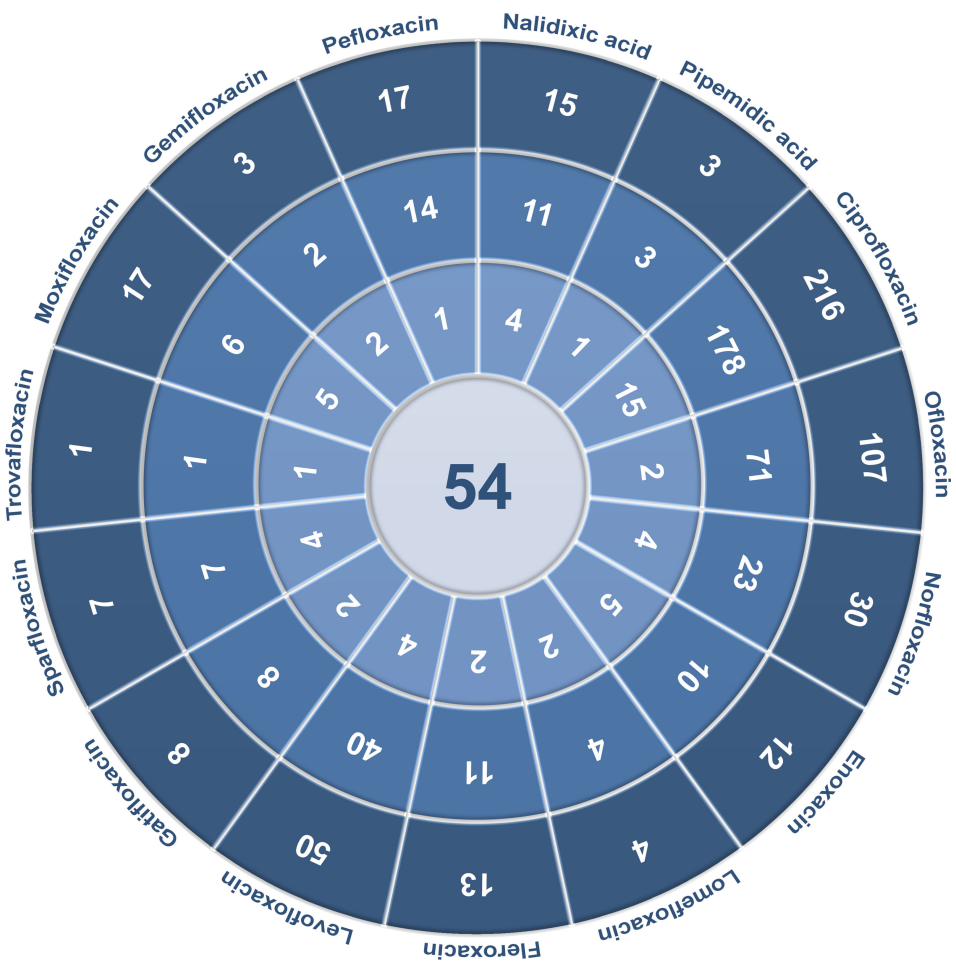


**FIGURE 2** | Detailed analysis of data extraction.

**TABLE 1** | Concentrations achieved in urine and serum after a specific dose application for individual quinolones.

| Quinolone | Dose [mg] | Concentrations achieved in the urine [μg/ml] | Concentrations achieved in serum [μg/ml] | Reference |
|---|---|---|---|---|
| **Nalidixic acid** | 1,000 | 100 | 27.3 | (24) |
| **Pipemidic acid** | 100 | 400 | 1.23 | (25) |
| | 400 | —— | 3.64 | (26) |
| | 500 | 900 | 3.38 | (25) |
| **Ciprofloxacin** | 250 | 205–261 | 1.35–1.42 | (27) |
| | 500 | 255–518 | 2.60–2.89 | |
| | 750 | 243–846 | 3.41–4.21 | |
| **Ofloxacin** | 400 | 119 | 5.5 | (28) |
| **Fleroxacin** | 200 | >200 | —— | (29) |
| | 100 | 68 | 2.9 | (30) |
| | 400 | 224 | 4.2 | |
| | 800 | 490 | 9.4 | |
| | 1,200 | 715 | 11.9 | |
| **Norfloxacin** | 200 | 200 | 0.75 | (31) |
| | 400 | 478 | 1.58 | |
| | 800 | 697 | 2.41 | |
| | 1,200 | 992 | 3.15 | |
| | 1,600 | 1045 | 3.87 | |
| **Enoxacin** | 200 | 119–685 | 1.83 | (32) |
| | 800 | 379–1287 | 6.58 | |
| **Lomefloxacin** | 100 | | 1.11 | (33) |
| | 200 | | 2.46 | |
| | 400 | 104–713* | 3.02 | |
| | 600 | | 4.79 | |
| | 800 | | 7.46 | |
| **Pefloxacin** | 400 | —— | 3.66–9.06 | (34) |
| | 800 | 219 | —— | (35) |
| **Levofloxacin** | 500 | 406 | 5.61 | (36) |
| **Grepafloxacin** | 400 | 15.4 | 1.5 | (37) |
| **Gatifloxacin** | 400 | 111–649 | 1.53–2.46 | (38) |
| **Sparfloxacin** | 400 | 5.4–19.9 | 1.2–1.9 | (39) |
| **Trovafloxacin** | 200 | —— | 3.2 | (40) |
| | 300 | 7.3 | 3.0 | |
| **Moxifloxacin** | 400 | 127 | 6.80 | (41) |
| **Gemifloxacin** | 160 | —— | 0.92 | (42) |
| | 320 | 55 | 2.01 | |
| | 480 | —— | 2.0 | |
| | 640 | —— | 3.03 | |
| **Finafloxacin** | 25 | —— | 0.24 | (43) |
| | 50 | —— | 0.44 | |
| | 100 | 0.1–101 | 1.32 | |
| | 200 | 0.5–166 | 1.90 | |
| | 400 | 1.4–328 | 5.06 | |
| | 800 | 0.7–257 | 11.1 | |

*These values are range of concentrations achieved in urine for all tested doses of lomefloxacin.*

frequently used. Similar to the first generation of quinolones, lomefloxacin reaches high concentrations in the urine. However, due to phototoxicity and the lack of effective action of this drug on cancer cells, its use is limited. A solution to this problem may be the modification of lomefloxacin, which could increase its effectiveness against cancer cells and reduce its toxic effects. Moxifloxacin and pefloxacin have also not been yet tested against genitourinary tract cancer cells. The analyzed databases contain many publications about the cytotoxic properties of these chemotherapeutic agents against other cancer cells, like leukemia and colon, pancreas, and breast cancers. Considering the urinary accumulation at 130 μg/ml, this compound could potentially be used in the treatment of bladder cancer. Finafloxacin also reaches high concentrations in the urine; however, its activity has not been tested against cancer cells so far. Marginal concentrations in the urine are reached by compounds such as grepafloxacin, sparfloxacin, trovafloxacin, and gemifloxacin, which precludes them for use in the treatment of genitourinary system cancers (**Table 1**).

Ciprofloxacin, ofloxacin, fleroxacin, enoxacin, norfloxacin, and levofloxacin were the only quinolones tested on bladder cancer cell lines (EJ, T24, J82, BOY, HTB9, HT1197, HT1376, TCCSUP, MBT-2, and BC-5867); however, the majority of the compounds mentioned above (except enoxacin and norfloxacin) were tested only on the T24 cell line (**Table 3**). Most of the research was carried out using ciprofloxacin. $IC_{50}$ presented in **Table 3** was, in most cases, due to lack of specific values, calculated on the basis of available data in analyzed manuscripts. Data from the study of Ebisuno et al. were not included in the analysis due to high differences in cell viability

calculated with two different methods (29). Based on the collected data, it can be concluded that enoxacin has a comparable activity like ciprofloxacin and norfloxacin on the EJ cell line due to a similar $IC_{50}$ value after 72 h of incubation. Ciprofloxacin has a stronger activity than levofloxacin, ofloxacin, and fleroxacin due to the lower $IC_{50}$ values obtained for three (T24, J82, and TCCSUP) or one (T24) tested cell line, respectively. Levofloxacin has better cytotoxic properties against the T24 cell line and similar to the BOY cell line compared to ofloxacin. The calculated $IC_{50}$ values of ciprofloxacin, norfloxacin, enoxacin, and levofloxacin are achievable in the urine (**Table 1**), which makes these compounds possible in the treatment of bladder cancer. The $IC_{50}$ values calculated for ofloxacin after the 400-mg dose in most cases (**Table 3**) are not achievable in urine, limiting this compound's use. In the case of fleroxacin, the $IC_{50}$ values obtained after 12 h of incubation with T24 and MBT-2 cell lines are not achievable in urine. The solution to this problem may be the use of a higher dose of those quinolones and their long-term use. Long-term therapy using these quinolones is safe and does not cause side effects. In addition, ciprofloxacin and ofloxacin (no data for levofloxacin) show a stronger cytotoxic effect in acidic pH, and such conditions are present in urine. The results discussed above indicate that all quinolones presented in **Table 3** are promising candidates for use in the treatment of bladder cancer. According to collected data, ciprofloxacin seems to be the most effective due to the lowest $IC_{50}$ values calculated for all tested cell lines. Low $IC_{50}$ values were also calculated for norfloxacin, levofloxacin, and enoxacin, but compared to ciprofloxacin, a small number of data available in literature do not allow to draw reliable conclusions about their effectiveness. In our recent study, we additionally, for the first time, analyzed the influence of ciprofloxacin and levofloxacin on bladder cancer cells in 3D culture (spheroids). We showed that $IC_{50}$ values calculated after drug incubation with cells in standard 2D culture were not effective in 3D culture. Only the concentration reducing 90% of cell viability was effective in cell viability reduction and caspase 3/7 activation in 3D culture. These concentrations were 1,500 and 245 μg/ml for ciprofloxacin and 4,670 and 730 μg/ml for levofloxacin, respectively, after 24 and 48 h of incubation with drugs. The concentration obtained after 48 h of incubation with ciprofloxacin is achievable in urine, which suggests the potential of this drug in clinical application. Additionally, we tested both drugs on a non-malignant uroepithelial cell line. Our results showed that both tested drugs were more effective against cancer cell lines which were more pronounced in 3D culture (62).

## Concentration in Prostate Tissue and Potential Action Against Prostate Cancer Cells

There is a lack of data in the literature about the concentration of some quinolones in prostate tissue. This concentration has not been determined for nalidixic and pipemidic acids, grepafloxacin, sparfloxacin, gemifloxacin, and finafloxacin. The use of other quinolones, in most cases, allows reaching a higher concentration in the prostate tissue than in the serum (**Table 2**). The highest concentration was obtained after the use of levofloxacin; high concentrations were also obtained for lomefloxacin and moxifloxacin. In the case of other quinolones, good penetration of the drug into the prostate gland was observed, which allowed achieving over two times higher concentrations in this organ than in the serum. In comparison to the concentrations obtained in urine, the concentrations obtained in the prostate gland are much lower, which limits the use of quinolones alone in the treatment of prostate cancer. In the case of gatifloxacin, the prostate tissue dose was not determined; however, its concentration in the prostatic secretion, seminal fluid, and ejaculate was tested, reaching the highest concentration at 3.10 μg/ml.

Only five quinolones (ciprofloxacin, levofloxacin, enoxacin, norfloxacin, and gatifloxacin) have been so far studied on prostate cancer cell lines (LNCaP, 22Rv1, VCaP, PC3, and DU-145). In six studies, the use of ciprofloxacin was especially focused on prostate cancer, and the other two studies analyzed modified quinolones on the panel of various cell lines; therefore, information about prostate cancer is very limited (**Table 3**). The PC3 cell line was analyzed in almost all studies. In the study of El-Rayes et al. and Sousa et al., only one concentration of ciprofloxacin was tested, which is why it is not possible to calculate $IC_{50}$ values (75, 76). In another study, values presented on the graph do not allow for $IC_{50}$ calculation; only information showing that ciprofloxacin is less toxic for normal prostate epithelium (MLC88991) can be obtained (77). Ninety percent of inhibition of prostate cancer cell growth was observed at a concentration of 50 μg/ml; this concentration is difficult to obtain in the prostate gland (**Table 2**). After application of enoxacin at a concentration of 40 μg/ml for 5 days, inhibition of cell growth of all five prostate cancer lines was observed in the range of 17%–59%; the most sensitive cell line was LNCaP. In the study of Pinto et al., the $IC_{50}$ values for ciprofloxacin were 254 and 172 μg/ml for PC3 and LNCap, respectively; after a 24-h exposure, this value decreased to 70 μg/ml for both cell lines after a 72-h exposition to this drug (71, 72). In another study, the $IC_{50}$ value calculated for this same cell line after the 24-h exposition was below 16 μg/ml, which is a significantly lower dose than that calculated by Pinto et al. (73). Levofloxacin, compared to ciprofloxacin, was more effective against the DU-145 cell line after a 24-h incubation, while after 48 h the situation was opposite. In this study, the advantage of both drugs over cancer cells was demonstrated (62). In the case of norfloxacin, it was shown that prostate cancer cells were the most resistant to this drug among all tested cancer cell lines. The $IC_{50}$ value calculated for norfloxacin was approximately 11.2 μg/ml, similarly in the case of ciprofloxacin; these concentrations are over two times higher than the maximum values obtained in prostate tissue (74). In the case of gatifloxacin, only its modified forms were examined; the experiment was performed on a panel of 58 cancer cell lines, including two prostate cancer cell lines (PC3 and DU145). Etoposide was used as a control (78). The $IC_{50}$ value obtained for prostate cancer was approximately 6 μg/ml, which is a relatively low concentration. However, it is almost twice as high as that obtained in prostatic secretion. Moreover, a

**TABLE 2 |** Concentrations achieved in the prostate tissue after a specific dose application for individual quinolones.

| Quinolone | Dose [mg] | Concentrations achieved in the prostate tissue [μg/g] | Concentration in tissue/concentration in serum | Citation |
|---|---|---|---|---|
| **Ciprofloxacin** | 200 | 1.02–5.81 | 2.45 | (44) |
| **Ofloxacin** | 200 | 1.70–6.37 | 2.11 | (45) |
| | 300 | 1.94–4.55 | ~1 | (46) |
| | 400 | 2.40–5.58 | ~0.95 | (28) |
| **Fleroxacin** | 400 | 0.58–6.80 | 1.12 | (47) |
| **Norfloxacin** | 200 | 0.63–4.35 | 5.71 | (45) |
| | 400 | 0.30–1.73 | 0.87 | (48) |
| | 400 | <0.25–2.55 | 1.68 | (49) |
| **Enoxacin** | 200 | 4.5–1.2 | 1.3 | (50) |
| | 400 | 5.1 | 2.2 | (51) |
| | 400 | 5.15 | 2.54 | (52) |
| **Lomefloxacin** | 400 | 1.1–10.1 | 2.2 | (53) |
| | 400 | 4.02 | 1.8 | (54) |
| | 400 | 2.5–10.0 | 1.53 | (55) |
| **Pefloxacin** | 800 | 4.39 | ~1 | (56) |
| **Levofloxacin** | 500 | 17 | 2.96 | (57) |
| | 500 | 1.23–20.8 | —— | (58) |
| **Trovafloxacin** | 200 | 4.94 | —— | (59) |
| **Moxifloxacin** | 400 | 9.54 | ~2 | (60) |

modified compound of gatifloxacin was less effective than that of etoposide. Although quinolones accumulate at a higher concentration in prostate tissue than in serum, reachable concentrations are much lower than these $IC_{50}$ values calculated for ciprofloxacin, norfloxacin, and modified gatifloxacin. This problem may be resolved by the use of modified quinolones like nanocomposites. The use of such compounds significantly improves anticancer activity by reducing the toxic dose; however, it is not known whether the modification of quinolones will affect its accumulation in prostate tissue. Another solution is the improvement of quinolone accumulation in prostate tissue using direct injection to prostate tissue or use modification of drug delivery systems like lipid-based complexes. The obtained results indicate that quinolones cannot be used as independent anticancer drugs in the treatment of prostate cancer; the solution may be their use in combination therapy with standard-use chemotherapeutics.

## Anticancer Properties of Quinolones

The mechanism of action of quinolones is mainly based on the inhibition of bacterial topoisomerase II; however, they also show activity against analogous enzymes in eukaryotic cells. Other changes in eukaryotic cells associated with quinolones' mechanism of action are shown in **Supplementary Table 1**. One of the mechanisms of action is the dysfunction of the mitochondria, which, according to Lawrence et al., can be linked with the theory of endosymbiosis and similarity in the structure of mitochondria to bacterial cells to which quinolones are active (79). The addition of

**TABLE 3 |** $IC_{50}$ index (μg/mL) of individual quinolones for bladder and prostate cancer cell lines depending on incubation time (hours).

| Cell line | Ciprofloxacin | | | | | Ofloxacin | | | | Fleroxacin | | | Norfloxacin | | Levofloxacin | | | Enoxacin | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Hours** | **24** | **48** | **72** | **96** | **120** | **24** | **72** | **96** | **120** | **12** | **24** | **48** | **24** | **72** | **24** | **48** | **96** | **72** | |
| **Bladder cancer** | | | | | | | | | | | | | | | | | | | |
| EJ | —— | —— | ~67 | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | ~64 | —— | —— | —— | ~57 | (61) |
| T24 | ~160 ~100 ~87 | ~50 ~40 | ~40 ~5 | ~60 ~85 | ~30 | ~730 | ~120 | ~150 | ~100 | ~1,150 | ~550 | ~350 | —— | —— | ~500 | ~180 | ~150 | —— | (62–68) |
| J82 | ~140 | —— | ~50 | —— | ~25 | ~1,400 | ~240 | —— | ~55 | —— | —— | —— | —— | —— | —— | —— | —— | —— | (67) |
| BOY | —— | —— | —— | —— | —— | —— | —— | ~60 | —— | —— | —— | —— | —— | —— | —— | —— | ~65 | —— | (65) |
| TCCSUP | ~250 | —— | ~70 | ~50 ~70 | ~10 | ~2,130 | ~160 | ~270 | ~140 | —— | —— | —— | —— | —— | —— | —— | —— | —— | (63, 67, 68) |
| HTB9 | ~38 ~200 | ~77 | ~41 ~100 | ~15 | —— | —— | —— | ~50 | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | (63, 66, 68) |
| HT1197 | —— | —— | ~34 | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | (69) |
| HT1376 | —— | —— | ~17 | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | (69) |
| MBT-2 | —— | —— | —— | —— | —— | —— | —— | —— | —— | ~990 | ~625 | ~350 | —— | —— | —— | —— | —— | —— | (64) |
| BC-5867 | ~40 | ~40 | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | (70) |
| **Prostate cancer** | | | | | | | | | | | | | | | | | | | |
| PC3 | 254 >16 | 79 | 70 | 64 | —— | —— | —— | —— | —— | —— | —— | —— | 11 | —— | —— | —— | —— | —— | (71–74) |
| LNCaP | 172 | 80 | 70 | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | (71, 72) |
| DU-145 | ~165 | ~80 | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | —— | ~330 | ~150 | —— | —— | (62) |

quinolones to cell culture led to cell cycle arrest, mainly in the S and G2/M phases. In the case of moxifloxacin, levofloxacin, and sparfloxacin complexes with gold (III), a higher number of cells in the G0/G1 and subG1 phases were observed compared to control. Inhibition of topoisomerase led to the inhibition of DNA synthesis and its fragmentation. Downregulation of *TOP2A* and *TOP2B* genes, two isomers of topoisomerase II, were observed after ciprofloxacin and levofloxacin treatment (62). Quinolones induced cell death mainly through apoptosis, rarely necrosis (62, 75, 77, 80–82). Apoptosis was not observed after the use of gatifloxacin and moxifloxacin. Also, studies of the level of apoptosis indicators such as caspase-3, Bax, and Bcl-2 protein or ROS were carried out. Bax and Bcl-2 are intracellular proteins that are known to regulate apoptosis. The Bax protein inactivates the Bcl-2 protein that protects cells against apoptosis, thereby increasing the Bax/Bcl-2 ratio. An increase in the level of this factor may be responsible for the induction of apoptosis. In addition, Bax translocation into the mitochondria is thought to accelerate the onset of apoptosis. Mitochondrial membrane permeability disorders were also observed, which led to the activation of caspases and DNA fragmentation (77). Caspase-3 is an important enzyme that is required for the final phase of apoptosis. ROS has also been shown to stimulate apoptotic pathway signaling by activation of caspase-3 (83). The effect of quinolones on metastasis treatment was also analyzed; gatifloxacin showed a reduction in the migration and invasion of cancer cells by influencing epithelial to mesenchymal transition (EMT) (84, 85). Also, changes in *TP53* and *CDKN1* gene expression after treatment with ciprofloxacin or levofloxacin were observed (62, 86). Analysis of differentially expressed genes (DEGs) using the STRING database version 11.5 allows for detection of three pathways, which could play a potential role in fluoroquinolones' action. The highest strength (above 1.9) was noticed for apoptosis, platinum drug resistance, and p53 signaling pathway. Based on analyzed publications, the potential mechanism of quinolones' action was summarized as shown in **Figure 3**. Many studies indicate that quinolones in combination with other commonly used anticancer drugs (etoposide, cisplatin, doxorubicin, epirubicin, imatinib, 5-fluorouracil, irinotecan, docetaxel, camptothecin, tamoxifen, etc.) and as complexes with metals such as copper, platinum, ruthenium, zinc, and gold have better cytotoxic effects (5). Metallic derivatives of antibacterial drugs are gaining more and more interest because the coordination of metal by a synergistic effect leads to various pharmacological activities, including antiproliferative, antimicrobial, antifungal, and antiviral activities (87). Quinolones like etoposide, doxorubicin, and mitoxantrone have the same cellular target—topoisomerase II—but distinct mode and sites of action within this enzyme (72). Etoposide works by suppressing the ability of topoisomerase II to ligate DNA molecules, whereas quinolones have little effect on ligation but stimulate the rate of DNA cleavage by topoisomerase II (88). Additionally, etoposides' action leads to the release of pro-inflammatory cytokines, such as IL-8, TNF-α, and IL-1β, which, depending on the tumor cell line, is inhibited by quinolones. The release of proangiogenic IL-8 is undesirable and considered as a side effect of the use of etoposide (89). Doxorubicin, similar to ciprofloxacin, helps to stabilize double-stranded DNA complexes

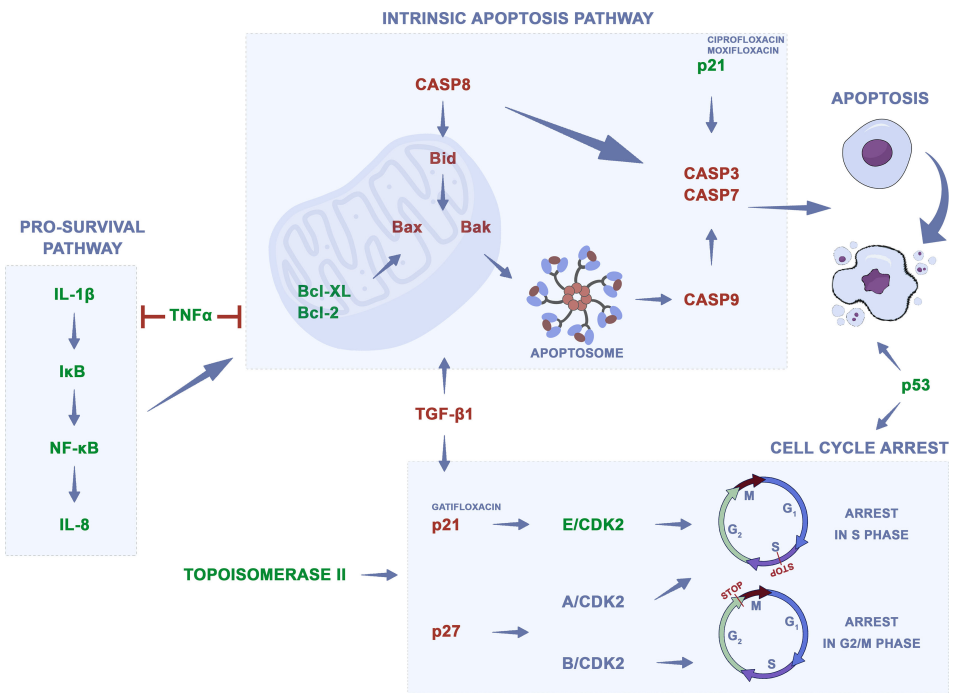


**FIGURE 3** | Potential mechanism of quinolones action on cancer cells. Changes in gene regulation after quinolones treatment, which lead to apoptosis and cell cycle arrest, were presented on the basis of analyzed data. Green—downregulation, red—upregulation.

with topoisomerase II; the difference is that ciprofloxacin is a non-intercalating drug (71). In such cases, when the mechanism of action is similar, the treatment with two drugs should be sequential, not simultaneous (71). Different mechanisms of action show drugs like cisplatin, imatinib, vinblastine, 5-fluorouracil (5-FU), and docetaxel. Cisplatin poses the ability to bind with purine bases and interfere with DNA repair mechanisms, causing damage to genetic material, and the consequent induction of apoptosis enhances the activity of quinolones (90). Vinblastine and docetaxel interact with mitotic spindle, imatinib prevents platelet-derived growth factor receptor activation, and 5-FU is classified as a pyrimidine anti-metabolite leading to inhibition of DNA synthesis (64, 71, 72). Although these drugs have different metabolic pathways and generally act synergistically, they can affect the same molecular process like G2/M arrest or apoptosis induction by Bcl-2 downregulation. That is why in order to obtain the best therapeutic effect, it is important to choose the right drug combination and method of its administration (71, 72). Not all studies compared the properties of quinolones against cancer and normal cells; however, if such analyses were performed, quinolones did not affect or showed little activity on normal cells, which means that they show selective action against cancer cells (74, 77, 91–96). Modification of quinolones' structure can strengthen their properties (97–105). However, in such cases introduction of a new drug on the market is difficult due to expensive and long-term procedures including all stages of clinical trial conduction. The advantage of unmodified drugs is the possibility of their repositioning without such complicated procedures. Quinolones can also be used in combined therapy as a supportive drug, which can lead to improvement in treatment efficacy (106–110).

It should be also mentioned that, beside direct action, through topoisomerase II inhibition, quinolones can also affect the neoplastic process through indirect effects, by modulation of the immune response (111). The immunological system plays an essential role in cancer development and progression, and it can detect and destroy abnormal cells preventing or slowing down cancer growth. However, cancer cells develop different mechanisms, which allows avoiding their destruction by the immune system (112). Enhancing the immune response can lead to the stronger elimination of cancer cells. Fluoroquinolones can induce an immunomodulatory effect by activation or inhibition of specific cytokines. It was shown that fluoroquinolones, like ciprofloxacin, are able to upregulate interleukin 2 (IL-2) production, which is the most important growth factor for lymphocyte T and NK cells. This effect, together with activation of IL-3 and GM-CSF synthesis, which stimulates bone marrow generation, could be significant in immune-compromised cancer patients (113). Additionally, fluoroquinolones inhibit pro-inflammatory cytokines, like IL-1β, IL-6, IL-8, and TNF-α, which together with stimulation of IL-2 can enhance immunomodulatory effects. Inhibition of IL-8 by moxifloxacin was observed in colon cancer and IL-1β and TNF-α in leukemia cells (88, 89, 109). IL-1β and IL-8 are involved in the NFκβ signaling pathway leading to cell survival, and their downregulation could have an impact on cell death induction. The TNF signaling pathway can activate antiapoptotic and pro-survival signals and can lead to apoptosis induction. Its downregulation can indicate that apoptosis of cancer cells could be induced not directly but indirectly by the inhibition of pro-survival signals (**Figure 3**). On the other hand, fluoroquinolones can increase the level of anti-inflammatory cytokines, like IL-10, which inhibit the production of IL-2, IL-3, or GM-CFS (111). That is why, in order to learn more about the mechanism of action of quinolones on regulation of the immune system in cancer cells, more experiments have to be performed.

## DISCUSSION

Urinary bladder and prostate cancers are a serious problem in modern urology. Current treatment methods are based mainly on surgical excision of the affected organ. Quinolones are commonly used by urologists; additionally, these drugs can be used in high doses and can be administrated for a long time without severe side effects which enable to obtain its high concentration, especially in urine (114). Together with their anticancer properties confirmed on bladder cancer cell lines, these drugs have enormous potential as supporting drugs in bladder cancer treatment. An ideal therapeutic goal can be the state after transurethral resection of bladder tumor (TURBT). This procedure is characterized by a high percentage of relapses probably by a small amount of cancer cells that remain in the bladder, and after reimplantation in the bladder wall can induce tumor growth (115, 116). Use of quinolones directly after the procedure as an intravesical therapy and their intravenous or oral administration for another couple of weeks can lead to the rid of residual cancer cells and thus reduction in the risk of bladder cancer recurrence.

Although many *in vitro* studies confirming the anticancer properties of quinolones, including bladder and prostate cancer, have been conducted, more preclinical and clinical studies are necessary. One clinical study evaluating the effectiveness of ciprofloxacin in bladder cancer treatment terminated due to poor accrual is registered on clinicaltrial.gov (NCT00003824). Three quinolones have been tested on an animal model so far. After oral administration of fleroxacin, a reduction in chemically induced bladder cancer was observed only when tested fluoroquinolone was used together with 5-FU (64). Trovafloxacin and ciprofloxacin were tested against murine leukemic cells accompanied by bacterial lung infection. Results of this study showed that both drugs were effective in the treatment of lung infection, but only trovafloxacin was effective in preventing metastasis of leukemia cells (117). This indicates that in order to confirm promising *in vitro* properties of quinolones, more *in vivo* studies have to be performed. According to the analyzed literature, a better candidate for *in vivo* testing is ciprofloxacin. This fluoroquinolone reaches a higher concentration in urine than fleroxacin; additionally, calculated $IC_{50}$ values, reducing bladder cancer cell viability, are significantly lower for ciprofloxacin. During the new study construction, it is very important to use the appropriate way of

drug administration in order to achieve its optimal concentration in urine and prostate tissue. Intravenous administration can lead to a higher accumulation of these quinolones compared to an oral one.

Quinolones accumulate in higher concentrations also in other organs like the lung, that is why the use of these drugs is not limited only to bladder and prostate cancers (118). Data collected in **Supplementary Table 1** indicate that quinolones were considered as chemotherapeutic agents in many cancer models and, besides bladder and prostate cancers, were analyzed on colon, pancreatic, breast, liver, and lung cancer, including melanoma, leukemia, and sarcoma cell lines. In our opinion, urinary bladder cancer is the most promising target due to the definitely higher concentration of quinolones achievable in urine. Such concentration is not possible to achieve in tissue, that is why in other cancers, including prostate, modified quinolones or a different method of drug administration should be developed.

Quinolones are extensively investigated, which is why it can be expected that more preclinical and first clinical studies utilizing these drugs will be completed in the near future. In the next few years, the development of new quinolone derivates can lead to an increase in their efficiency; additionally, new quinolones like finafloxacin or delafloxacin, which was not tested on cancer cells so far, may have better anticancer properties than their older representatives. Currently, these drugs are used in urology as anti-inflammatory agents, but according to their properties these drugs in the near future could be reprofiled into anticancer agents (5). In our opinion, quinolones in the next 5 to 10 years, will be applied as supportive drugs in genitourinary cancer treatment.

## CONCLUSIONS

Taken together, quinolones are very promising agents for genitourinary cancer therapy. A more promising application of quinolones is urinary bladder cancer treatment because most of the drugs analyzed in this study (except grepafloxacin, gemifloxacin, sparfloxacin, and trovafloxacin) reach very high concentrations in urine both after oral or intravenous administration. Additionally, long-time administration of quinolones in high doses is safe, and if the risk of drug resistance is not taken into account, such therapy doses do not cause side effects. That is why quinolones are frequently used by urologists (16). Another argument for quinolone application is a low cost of such therapy (5). Although all quinolones tested in this study reach a higher concentration in prostate tissue than in serum, in all cases obtained, values are too small for inhibition of cancer growth. The solution to this problem can be use of another way of drug administration like intraprostatic injections, which allow to receive higher doses in prostate. The use of modified quinolones or in combination with other chemotherapeutics can enable toxic effects at lower drug doses. A growing amount of evidence shows that bacterial infection can induce chronic inflammation, which, in the future, can lead to prostate cancer development. Quinolones can be used in the prophylaxis of prostate inflammation and prostate cancer because of its dual action against bacterial and cancer cells (so called two-hit hypothesis) (119).

## AUTHOR CONTRIBUTIONS

TK: study design, data analysis, data collection, writing; SF: data collection, writing; JA: writing; KS: writing; MR: writing; TD: review and editing, supervision; MP: review and editing, supervision. All authors contributed to the article and approved the submitted version.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.890337/full#supplementary-material

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# Prostate Cancer Stem Cells: Clinical Aspects and Targeted Therapies

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Despite decades of research and successful improvements in diagnosis and therapy, prostate cancer (PC) remains a major challenge. In recent years, it has become clear that PC stem cells (PCSCs) are the driving force in tumorigenesis, relapse, metastasis, and therapeutic resistance of PC. In this minireview, we discuss the impact of PCSCs in the clinical practice. Moreover, new therapeutic approaches to combat PCSCs are presented with the aim to achieve an improved outcome for patients with PC.

**Keywords: prostate cancer, prostate cancer stem cells, prostate cancer stem cell hypothesis, prostate cancer stem cell antigens, prostate cancer stem cell therapy**

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## INTRODUCTION

Prostate cancer (PC) is the most common cancer and the second leading cause of cancer death in men from industrial countries. More than 1.41 million new cases and more than 375,000 deaths by this tumor are expected worldwide every year (1).

If the tumor is limited to the prostate, a good chance of cure is the surgical resection [radical prostatectomy (RP)] or radiation of the organ. Both treatment options are associated with adverse effects, such as incontinence and sexual dysfunction, which negatively affect the quality of life. The prerequisite for cure is the complete removal of the tumor. If residual tumor cells persist, the tumor may soon relapse and begin to metastasize. Overall, biochemical failure after RP in node-negative patients occurs in approximately 15%–40% of cases within 5 years and is independent of the surgical approach [reviewed in (2)]. The only potentially curative treatment for patients with local recurrence at the earliest sign of biochemical failure is salvage radiation therapy, preferably for PSA levels <0.2 ng/ml (3). In case of metastasized tumors, treatment options include androgen deprivation therapy (ADT) and chemotherapy (4, 5). However, chemo- and castration-resistant PC commonly develop and mainly contribute to therapy failure and mortality (6, 7).

One model that explains heterogeneity, tumor-initiating capability, and therapeutic resistance of tumors is the cancer stem cell (CSC) hypothesis. The CSC hypothesis postulates that cancer cells are hierarchically organized and form different heterogeneous subpopulations within a tumor. CSCs are on top of the hierarchy and represent cancer cells with stem cell-like properties, such as self-renewal, pluripotency, and plasticity, that evolve during the lifetime of a tumor (8, 9). Different factors are discussed that might foster the emergence of CSCs, like de-differentiation through genetic

**Abbreviations:** ADT, androgen deprivation therapy; AR, androgen receptor; ABCG2, ATP-binding cassette sub-family G member 2 transporter; CSC, cancer stem cell; CRPC, castration-resistant prostate cancer; DPCC, differentiated prostate cancer cells; EMT, epithelial–mesenchymal transition; NEPCC, neuroendocrine prostate cancer cell; PAP, prostatic acid phosphatase; PCPC, prostate cancer progenitor cells; PCSC, prostate cancer stem cell; RP, radical prostatectomy; PSA, prostate-specific antigen.

and epigenetic alterations, clonal expansion, and adaptation through epithelial–mesenchymal transition (EMT) as well as transdifferentiation under the influence of the tumor microenvironment or under therapeutic pressure [reviewed in (10)]. CSCs were found to be the driving force in tumor progression, metastasis, and therapeutic resistance, and new strategies are being developed to identify and treat them (11). Our minireview describes clinical aspects of prostate CSCs (PCSCs) and new therapeutic options, aiming to achieve a cure for hitherto incurable stages of the disease.

## PROSTATE CANCER STEM CELLS

PC cells with stem cell characteristics, such as self-renewal, pluripotency, and plasticity, were isolated from patients undergoing RP for the first time in 2005 (12). PCSCs can also be obtained from established PC cell lines, especially of metastatic origin (12–14) and are characterized by sphere formation under non-adherent culture conditions, high clonogenicity, high rate of self-renewal, and the ability to form phenotypically mixed populations of non-clonogenic cells (12, 15). Different antigens, which are involved in various signaling pathways of tumorigenesis, metastasis, and therapeutic resistance of PC, were identified to characterize PCSCs (**Table 1**) (58).

PCSCs might originate from normal prostate stem cells, normal prostate progenitor cells, or differentiated normal prostate cells after genetic and epigenetic alterations or changes in the tumor microenvironment (15). It was found that activation of the proto-oncogene MYC, loss of the tumor suppressor PTEN, or mutations in the repair genes *BRCA2, ATM*, and *CHEK2*, induce genomic instability and drive progression and heterogeneity of PC (10, 59, 60). Polson and colleagues showed a high frequency of the TMPRSS2:ERG gene fusion not only in differentiated PC cells but also in PCSC (61). When the transcription factor ERG comes under the control of the prostate-specific, androgen-regulated TMPRSS2 gene promoter, enhanced ERG expression is found. Since enhanced EGR expression can influence differentiation, self-renewal, and maintenance of SCs, it is discussed that the TMPRSS2: ERG fusion might also play a decisive role in the emergence and maintenance of PCSCs (62).

PCSCs can evolve from basal or luminal epithelial cells after oncogenic transformation (11). Recent findings from a single-cell sequencing study in mice suggest that differentiated luminal cells that survived castration can contribute to prostate regeneration by acquisition of stem cell-like regenerative properties (63). There is also evidence that PCSCs might come from the basal cell layer, because tissue-derived tumor-initiating cells in immunocompromised mice expressed basal markers (such as p63), but did not express the androgen receptor (AR) or markers of luminal differentiation (PSA and PAP) (64). Moreover, there might be a loss of basal cells and expansion of luminal cells during PC tumorigenesis (65). For example, Choi and colleagues demonstrated that inactivation of the tumor suppressor PTEN induced the differentiation of basal cells to transformation-competent luminal cells (66).

PCSCs can differentiate into PC progenitor cells (PCPCs) or differentiated PC cells (DPCCs), which leads to the typical formation of heterogeneous prostate tumors with increasing grading that is determined by the Gleason score. Interestingly, Castellon et al. found highest expression of the stem cell markers CD133, CD44, and ABCG2 in medium-grade Gleason biopsies compared to lower- or higher-grade biopsies or lymph-node and bone metastases (67). This suggests that PCSCs reach a significant number at stages, in which the tumor seems to be confined to the gland and in which surgical treatment or radiation is usually with curative intention. However, many PC patients develop biochemical relapse despite local therapy (68, 69). It is therefore assumed that PCSCs remain in the surgical or radiation area or have already entered blood circulation and colonized lymph nodes or other organs due to their ability to migrate and persist in extra-prostatic tissues (67). Number and signatures of PCSCs in local tumors are therefore discussed as prognostic factors for PC recurrence (70). For example, significantly enhanced expression of the stem cell markers SOX2, OCT4, KLF4, and ABCG2 in recurrent PC tissues in comparison to non-recurrent PC tissues was found after RP (71).

Radiation is a therapeutic option for local disease, recurrence, or advanced PC. However, radioresistance of PC cells is an obstacle for successful radiation therapy. A subpopulation of PC cells with CSC characteristics was found after radiation that was marked by enhanced PI3K/Akt/mTOR signaling (72). PCSCs therefore appear to contribute to the formation of radioresistant tumors.

PC cells metastasize preferentially in lymph nodes, liver, and bones (73). The spine, pelvis, and ribs are the most frequently observed sites of bone metastasis in PC. This distribution is often multifocal, and preferable involvement of the axial skeleton suggests an affinity to the red bone marrow. It seems that the bone marrow is particularly suitable as a metastatic site for PC cells, because it is strongly supplied with a low blood flow rate. In addition, it seems that the bone marrow, which harbors the hematopoietic stem cells, forms a suitable niche for disseminated PCSCs. About 10% of patients already harbor bone metastases at the time of diagnosis and 70%–80% of patients, who relapse after RP, fatally progress to advanced disease with bone metastases. This confirms that there are already subpopulations of PC cells in early-diagnosed, local prostate tumors with stem-cell like properties that are able to disseminate and colonize distant organs. Metastatic PC cells are marked by a high expression of integrins that promote their adherence to a broad spectrum of proteins of the bone extracellular matrix, and release factors (FGFs, IGFs, VEGF, or Wnt pathway-related factors, originally involved in bone formation and maintenance) for persistence [reviewed in (74)]. Castellon and colleagues found only low expression of PCSC markers in metastases from lymph nodes and bone, but explained this phenomenon with prevalence of PCPCs and DPCCs (67).

PCSCs are marked by low or lack of androgen receptor (AR) expression (75) and, as a result, by a missing or reduced PSA release. Therefore, they might escape a PSA screening and

**TABLE 1 |** Antigens associated with PCSCs.

| Target antigen | Structure | Function | Role in PC | Ref. |
|---|---|---|---|---|
| $\alpha2\beta1$ integrin | Type I collagen receptor | Cell adhesion, signaling | Self-renewal, proliferation, differentiation, migration, invasion, metastasis | (12, 16, 17) |
| $\alpha_6$ integrin/ D49f | Transmembrane glycoprotein | Cell signaling | Sphere formation, differentiation, tumor progression, invasion | (17–19) |
| ABCB1 | Transmembrane protein | Transporter | Chemoresistance | (20–22) |
| ABCG2 | Transmembrane protein | Transporter | Stem cell maintenance, chemoresistance | (23, 24) |
| ALDH | Cytosolic enzyme | Aldehyde dehydrogenase | Tumorigenicity, clonogenicity, tumor progression, self-renewal, migration, metastasis, radioresistance | (25) (26–28) |
| | | | ALDH1A1 expression positively correlated with Gleason score and pathologic stage | (26) |
| AR variant 7 | Androgen receptor splice variant | Transcription factor | Acquisition of stem cell characteristics, EMT | (29) |
| | | | Associated with enhanced progression to mCRPC and shorter survival | (30) |
| CD117/c-kit | Type III tyrosine kinase receptor | Cell signaling, survival, metabolism, growth, proliferation, apoptosis, migration, differentiation | CSC maintenance, sphere formation, proliferation, migration, invasion, tumor progression, bone metastasis, therapeutic resistance | (31) |
| | | | Increased expression during clinical progress | (32) |
| CD133/Prominin-1 | Glycosylated pentaspan transmembrane protein | Precise physiological function unknown | Tumorigenicity, self-renewal, sphere formation, proliferation, differentiation, invasion, chemo/radioresistance | (12, 33–36) |
| CD166/ activated leukocyte cell adhesion molecule (ALCAM) | Transmembrane glycoprotein | Cell adhesion | Sphere formation, bone metastasis | (37, 38) |
| | | | Upregulated in CRPC | (37) |
| CD44 | Transmembrane protein, hyaluronic acid receptor | Cell adhesion, signaling | Self-renewal, proliferation, differentiation, invasion, metastasis | (12, 39) |
| CXCR4 | Chemokine receptor | Chemotaxis, hematopoietic stem cell maintenance | CSC maintenance, clonogenicity, differentiation, migration, metastasis, chemoresistance | (40–42) |
| | | | Overexpressed in metastatic disease | (43) |
| E-cadherin/ ECAD | Transmembrane glycoprotein | Cell adhesion, regulation of epithelial morphogenesis, and differentiation | Sphere formation | (44) |
| | | | Expression correlates with recurrence after RP and metastasis | (45, 46) |
| EpCAM/ CD326 | Transmembrane glycoprotein | Cell adhesion, signaling, migration, proliferation, differentiation | CSC maintenance, proliferation, invasion, metastasis, chemo-/ radioresistance | (47, 48) |
| | | | Overexpressed in local and metastatic disease | (47) |
| | | | Overexpressed in chemo-/radioresistant stages | (49, 50) |
| EZH2 (enhancer of zeste homolog 2) | Cytosolic enzyme | Histone-lysine N-methyltransferase | PCSC maintenance and growth | (51–53) |
| | | | Coactivator for transcription factors in CRPC, including AR | (53) |
| | | | Positive EZH2:ECAD status strongly associated with recurrence after RP | (53) |
| TG2 (tissue trans-glutaminase) | Cytosolic enzyme | Protein-glutamine γ-glutamyltransferase | Invasion, chemoresistance, EMT | (54) |
| Trop2 (trophoblast cell-surface antigen 2)/ tumor-associated calcium signal transducer 2 (TACSTD2)/ epithelial glycoprotein-1 (EGP-1) | Transmembrane protein | Calcium signal transducer | Self-renewal, sphere formation, proliferation, migration, invasion, metastasis | (55, 56) |
| | | | Upregulated in invasive stages | (57) |

androgen receptor expression and measurable PSA values might only be detected when the metastatic PCSCs have already differentiated and expanded. This could be an explanation for the often observed discrepancy between detection of biochemical recurrence (defined by a rising PSA profile) and already existing progressive disease (76).

First-line treatment of advanced PC is ADT. In general, tumors initially respond well to ADT; however, the therapeutic effect of ADT only lasts for a limited period of 12–33 months. At that point, ADT-resistant PC cells emerge and form castration-resistant tumor lesions (77). There is growing evidence that PCSCs contribute to this phenomenon. Since PCSCs were found to be AR-negative and have the ability to grow androgen independently, they might have a survival benefit when treated with ADT (78). Indeed, whereas $AR^{+}/PSA^{+}$ tumor cells form the main cell population in untreated androgen-sensitive tumors, enrichment of $AR^{-/lo}$ and $PSA^{-/lo}$ cells was found in untreated higher grades of the disease and in CRPC (79). Moreover, genes associated with CSCs, like *OCT4, SOX2, NANOG, BMI1, BMP2, CD44, SOX9*, and *ALDH1*, were found to be upregulated in enzalutamide-resistant cells (80). There is evidence that truncated AR variants, which lack the ligand binding domain, but retained transcriptional activity, play a decisive role. In particular, the variant AR-V7 might be involved in EMT and promotes the emergence of PCSCs (81).

Re-programming of PC cells to stem-like cells during ADT was demonstrated in a recent study. After androgen depletion over 90 days, a re-differentiation to a stem-like phenotype was observed in LNCaP cells, which was marked by growth as floating spheres and enhanced expression of CD133, ALDH1A1, and the multidrug resistance protein transporter ABCB1A. Interestingly, ABCB1A expression in the re-differentiated stem-like cells was associated with enhanced resistance against docetaxel and 2-hydroxyflutamide (20). This provides a rationale that chemoresistance may already be induced in prostate tumors during ADT and reinforces the medical guidelines recommending chemotherapy in hormone-naïve PC (82).

An example for transdifferentiation is the emergence of neuroendocrine PC cells (NEPCCs) in about 25% of patients after treatment with ADT (83). NEPCCs are hormone-refractory and secrete peptide hormones and growth factors for paracrine stimulation of surrounding cells in the microenvironment (83). It was found that loss of PTEN concurrently with inactivation of the tumor suppressors TP53 and Rb1 caused plasticity of PC cells with enhanced metastatic potential and conversion from adenocarcinoma to neuroendocrine PC [reviewed in (84)].

PCSCs also contribute to chemoresistance of PC. Docetaxel (DTX)-resistant cells showed enhanced expression of CD44 and CD133, leading to enhanced migration and invasion (85–87). Moreover, enhanced activity of Notch signaling was found, which promoted DTX resistance by upregulation of ABCC1 (88).

## TARGETING PCSCs

Current treatments against PC, like ADT, chemotherapy, and radiotherapy, aim to remove bulk tumors, but do not seem to affect resistant PCSCs effectively. Therefore, research is increasingly being conducted into new therapeutic approaches against PCSCs. Such approaches comprise the targeting of PCSC-associated pathways, the targeting of the PCSC microenvironment, and immunotherapies.

### Targeting PCSC-Associated Signaling Pathways

The Hedgehog (Hh), Wnt, Notch, and NF-kB pathways, which regulate proliferation, survival, metastasis, apoptosis, recurrence, and therapeutic resistance, were identified to be associated with PCSCs (11, 89–91). Different strategies have therefore been developed to target these pathways by inhibitors or RNA silencing (90, 92–94). Specific inhibitors against the Hh pathway (Sonidegib, GANT-61, and GDC-0449), the Wnt pathway (3289-8625, LGK974, Foxy-5, and OMP-54F28), the Notch pathway (RO4929097), and the NFkB pathway [bortezomib, PS1145, BMS345541, Aspyrin, 17-(allylamino)-17-demethoxygeldanamycin, and BKM120] are tested in preclinical and clinical trials with the intention of attacking PCSCs for an improved therapeutic outcome (90).

The PI3K/AKT/mTOR pathway is associated with PC progression and ADT resistance (95). Suppression of this pathway is therefore discussed to restore sensitivity against ADT, chemotherapy, and radiation (96, 97). In a recent study, an enhanced sensitivity of LNCaP cells against paclitaxel was determined after siRNA knockdown of the stem cell marker CD133. Mechanistically, an induction of the tumor suppressor PTEN accompanied by a decrease of AKT and c-myc oncogenes was found (33). Chang and colleagues were able to restore radiosensitivity and to induce apoptosis in radioresistant PCSCs by the use of the dual PI3K/mTOR inhibitor BEZ235 (72). Marhold and colleagues found elevated HIF1α levels and an enhanced HIF target gene expression in PCSCs under hypoxic conditions. This was accompanied by drug resistance to selective mTOR inhibitors. The authors therefore proposed a deregulation of the PI3K/AKT/mTOR pathway through HIF1α for quiescence and maintenance of PCSCs by attenuating CSC metabolism and growth *via* mTOR signaling and promoting survival by AKT signaling (98). Since hypoxia often prevails in the tumor microenvironment, targeting the HIF1α pathway might damage PCSCs while sparing normal stem cells.

ABC transporters were found to contribute to drug resistance of PSCs (99) and PCSCs (67). Liu and colleagues examined that the intracellular domain of NOTCH1, called ICN1, directly binds to the promoter region of ABCC1 and that inhibition of NOTCH1 with shRNA decreased ABCC1 expression and restored chemosensitivity of PCSCs (88). ABCG2 was found to play a decisive role in ADT-resistant PSCs by efflux of intracellular androgens. When ABCG2 was blocked by the inhibitor Ko143, an increasing nuclear AR level was observed followed by enhanced AR regulated gene expression and increased differentiation with ADT-sensitive luminal phenotype (23). Future experiments have to prove whether targeted differentiation is a new strategy to sensitize PCSCs to conventional therapies.

## Targeting the PCSC Microenvironment

Multiple signaling pathways exist between epithelial cells, stromal cells, and the extracellular matrix of the prostate tumor microenvironment to support tumor progression from the primary site to regional lymph nodes and distant metastases. For example, the CSC niche was found to induce Hh, Wnt, NF-κB, Notch, or PI3K/AKT/mTOR signaling in CSCs (100). Therefore, targeting of these pathways aims to disrupt the interaction between the microenvironment and the tumor cells in order to stop tumor spread [reviewed in (100, 101)].

Since PCSCs are also dependent on a microenvironment, called the PCSC niche, for the maintenance of their stemness properties, research is ongoing to investigate whether targeting of the tumor microenvironment might also lead to damage of PCSCs (11). The monoclonal antibody bevacizumab can be used to target the vascular endothelial growth factor (VEGF) and to reduce the tumor neovasculature for disruption of CSC niches. Bevacizumab-resistant PCSCs were found to have Rac1-mediated ERK activation, and Rac1 inhibition or P-Rex1 downregulation increased their sensitivity to bevacizumab (102). The CXCL12/CXCR4 chemokine pathway was also found to be activated in CD44/CD133-positive PCSCs and to affect cell adhesion, clonal growth, and tumorigenicity. The use of the CXCR4 antagonist AMD3100 inhibited sphere formation and restored the chemosensitivity of PCSCs (103). Since CD44 associates with the extracellular matrix hyaluronic acid (HA) (104), HA-coated liposomes containing cabazitaxel were generated for the inhibition of migration and the triggering of apoptosis in CD44-positive PC cells (105).

## Immunotherapy of PCSCs

PCSCs show enhanced expression of cell surface markers that can serve as target antigens for new immunotherapeutic approaches (**Table 1**). Recently, chimeric antigen receptor (CAR)-modified T-cell therapy targeting CSC-associated tumor antigens emerged as a new therapeutic approach for the treatment of CSCs (106). Zhu et al. demonstrated that anti-C133 CAR-T therapy leads to toxicity of patient-derived glioblastoma CSCs *in vitro* and in an orthotropic tumor model *in vivo* (107). Another study by Deng et al. showed that CAR T cells targeting the CSC marker EpCAM reduced PC progression in preclinical models (49). Currently, there is only one completed phase I/II clinical study of CD133-directed CAR T cells for the treatment of relapsed and/or chemotherapy refractory advanced hepatocellular carcinoma (NCT04427449) (108). However, several clinical trials using CAR-T cells targeting CSC surface markers are in the recruiting stage, representing a promising therapeutic option for the treatment of PCSCs in the future (106).

A further immunotherapeutic approach includes the use of dendritic cells preloaded with the PCSC-associated antigens CD44 and EpCAM for the activation of cytokine-induced killer T cells. This led to high cytotoxicity against PCSCs *in vitro* and antitumor activity *in vivo* in PCSC-derived xenograft models (109). Ma and colleagues generated aptamer-based liposomes loaded with curcumin to target CD133-positive PC cells and found antitumor activity in a PC mouse xenograft model (110). Interestingly, CD133 is expressed on both CSCs and differentiated tumor cells, but seems to be differentially folded and glycosylated, and therefore presents different target epitopes (111). Since different antibodies recognize different glycosylated CD133 epitopes (112), evaluation of glycosylation patterns of markers in PCSCs and differentiated PC cells could lead to the development of antibodies specifically directed against PCSCs in the future. Other strategies comprise the targeting of multiple antigens to enhance PCSC specificity or the targeting of potentially relevant splice variants.

# CONCLUSIONS

PCSCs were identified as the driving force in PC. There is emerging knowledge about the role of PCSCs, and new therapeutic approaches aim to achieve an improved therapeutic outcome. Selective and effective targeting of PCSCs, however, remains challenging, since cellular plasticity and intra- as well as inter-tumoral heterogeneity drive tumor progression and therapeutic resistance against conventional therapies (113, 114). From a clinical perspective, the understanding of the interactions between PCSCs, differentiated PC cells, and the TME is of utmost importance, but these interactions are very difficult to reproduce *in vitro*. Furthermore, some CSC markers (e.g., CD133 and ALDH) are expressed not only on malignant cells but also on healthy stem cells causing on-target/off-tumor toxicity (115, 116). Therefore, treatment side effects can be hindrances for a successful therapy of PCSCs. In the future, the characterization of PCSCs using (single-cell) genomics and proteomics could lead to improved prognosis and more individualized therapy for patients with PC to probably achieve complete cure of advanced hitherto incurable stages of the disease.

# AUTHOR CONTRIBUTIONS

PW drafted the manuscript and designed the figure. IW provided the data for the table. IW, CG, and PW wrote, discussed and reviewed the final manuscript. All authors contributed to the article and approved the submitted version.

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SYSTEMATIC REVIEW
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# Neoadjuvant and Adjuvant Chemotherapy for Variant Histology Bladder Cancers: A Systematic Review and Meta-Analysis

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**Context:** To improve the prognosis of variant histology (VH) bladder cancers, clinicians have used neoadjuvant chemotherapy (NAC) or adjuvant chemotherapy (AC) on the basis of radical cystectomy (RC). Despite some new data, the evidence remains mixed on their efficacy.

**Objective:** To update the current evidence on the role of NAC and AC for VH bladder cancers.

**Evidence Acquisition:** We searched for all studies investigating NAC or AC for bladder cancer patients with variant histology in PubMed, Embase, and the Cochrane Central Register of Controlled Trials up to December 2021. The primary end points were recurrence-free survival (RFS), cancer-specific survival (CSS), and overall survival (OS).

**Evidence Synthesis:** We identified 18 reports comprising a total of 10,192 patients in the NAC studies. In patients with VH, the use of NAC did improve CSS (hazard ratio [HR] 0.74, 95% confidence interval [CI] 0.55–0.99, p = 0.044), and OS (HR 0.74, 95% CI 0.66–0.84, p = 0.000), but not RFS (HR 1.15, 95% CI 0.56–2.33, p = 0.706). Subgroup analyses demonstrated that receiving NAC was associated with better OS in sarcomatoid VH (HR 0.67, 95% CI 0.54–0.83, p = 0.000) and neuroendocrine VH (HR 0.54, 95% CI 0.43–0.68, p = 0.000). For AC, we identified eight reports comprising a total of 3254 patients. There was a benefit in CSS (HR 0.61, 95% CI 0.43–0.87, p = 0.006) and OS (HR 0.76, 95% CI 0.60–0.98, p = 0.032). Subgroup analyses demonstrated that only neuroendocrine VH had better CSS (HR 0.29, 95% CI 0.13–0.67, p = 0.174) when receiving AC.

**Conclusions:** NAC or AC for VH bladder cancers confers an OS and CSS benefit compared with RC alone. For NAC, the benefit was independently observed in the sarcomatoid and neuroendocrine subgroups. As for AC, only neuroendocrine subgroups improved CSS.

**Systematic Review Registration:** https://www.crd.york.ac.uk/prospero/, identifier CRD42021289487.

**Keywords: urinary bladder neoplasms, meta-analysis, variant histology, neoadjuvant chemotherapy, adjuvant chemotherapy**

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# 1 INTRODUCTION

Bladder cancer is the 10th most commonly diagnosed cancer worldwide, with approximately 573,000 new cases and 213,000 deaths in 2020 (1). In patients with bladder cancer, about 75% of instances are classified as pure urothelial carcinoma, while the remaining 25% harbor variant histology (VH) (2–4). The presence of VH has been regarded as a poor prognostic factor in several studies (4–6). Compared with pure urothelial carcinoma, VH in patients with urothelial carcinoma of the bladder is associated with increased risks of disease recurrence as well as cancer-specific and overall mortality (7). However, there is a relative gap in knowledge of the treatment in these patients.

At present, whether neoadjuvant chemotherapy (NAC) or adjuvant chemotherapy (AC) is effective for VH bladder cancers is still uncertain. For example, while a secondary analysis of the Southwest Oncology Group directed intergroup randomized trial S8710 suggests NAC was an independent predictor of improved overall survival and cancer-specific survival on multivariate analysis in bladder cancer patients with squamous and glandular differentiation (8), some single-center retrospective studies concluded that the use of NAC did not improve recurrence-free, cancer-specific or overall survival (9–11). In the use of AC, there is a small case series found that no administration of AC was independently associated with poor overall survival in cases with glandular differentiation. Conversely, another study reported the administration of AC did not significantly improve survival outcomes in any histological variant (12).

Evidence concerning NAC or AC in the treatment of patients with histological variants is scarce and quite divergent. Thus, we performed this systematic review and meta-analysis to summarize the current data and to determine whether NAC or AC is effective for VH bladder cancers.

# 2 EVIDENCE ACQUISITION

## 2.1. Search Strategy

This systematic review was performed according to the Preferred Reported Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines, and registered with the International Prospective Register of Systematic Reviews (CRD42021289487) (13). A systematic literature search of PubMed, Embase, and the Cochrane Central Register of Controlled Trials was performed to identify studies regarding the role of chemotherapy in VH performed prior to December 2021. The following terms were used: "urinary bladder neoplasms/transitional cell carcinoma/bladder cancer," "variant/difference/mix," "adjuvant chemotherapy/neoadjuvant chemotherapy," "cystectomy," "multivariable/adjusted," and relevant variants of these search terms. The full search term algorithms are shown in **Appendix 1**. The literature search was unrestricted concerning publication date, region, and language. Studies that were performed at the same centers with overlapping time periods were excluded. Full articles were retrieved for further review.

## 2.2. Inclusion Criteria and Study Eligibility

The eligibility of each study was evaluated taking into account participants, interventions, comparators, outcomes, and study design approach (PICOS): Participants, bladder cancer patients with VH who intended to undergo radical cystectomy; Interventions, bladder cancer patients with VH who underwent radical cystectomy with systemic NAC or AC; Comparators, bladder cancer patients with VH who only underwent radical cystectomy; Outcomes, comparison of overall survival (OS), cancer-specific survival (CSS), and recurrence-free survival (RFS); and Study design, no restrictions on research design, but only studies with multivariate analyses were considered for meta-analysis. We considered randomized controlled trials (RCTs) and nonrandomized observational studies, as well as population-based cohorts (Surveillance, Epidemiology, and End Results [SEER], National Cancer Data Base [NCDB]) for inclusion into the systematic review and meta-analysis.

Reviews, letters, editorials, and case reports were excluded. In case of multiple reports of the same cohort, the most complete data aggregated with the longest follow-up duration were selected. In case different outcomes were examined, both articles were included to gather comprehensive data.

## 2.3. Data Extraction

Data extraction was performed by two authors (Zw Zhu and Yy Xiao) with any discrepancy resolved by a third author (Zs Zhu). Data on the paper (first author name, publication year, country, center, period of patient recruitment, and study type), participant demographics and oncologic characteristics (VH type, clinical T stage, and pathological TN stage), treatment characteristics (type of chemotherapy regimen and follow-up duration), outcomes (OS, CSS, RFS, pCR, and pDS), and results (numbers of events, hazard ratios [HRs], 95% confidence intervals [CIs], and p-values) were extracted.

## 2.4. Risk of Bias Assessment

The Cochrane Handbook for Systematic Reviews of Interventions was used to assess the risk of bias. Due to only nonrandomized comparative studies, RoB was determined by examining the risk of preassigned confounders. The confounding factors were identified as the most important prognostic factors at the time of treatment. The articles were therefore reviewed based on the adjustment for the effects of age, gender, tumor staging and grading, positive surgical margins, and receipt of NAC/AC. The RoB of each study was assessed independently by two authors (Zw Zhu and Yy xiao). The overall RoB level was judged as "low," "intermediate," or "high".

## 2.5. Statistical Analyses

VH was defined as nonpure urothelial carcinoma, including urothelial carcinoma with VH or pure VH in this analysis. The effects of NAC/AC on OS, RFS, and CSS were measured using hazard ratios (HRs). In studies with only HRs and p-values we

calculated the corresponding 95% CIs (14, 15). Forest plots were used to assess HRs to describe the relationships between NAC (or AC) and OS, RFS, and CSS. Subgroup analyses of "micropapillary," "squamous," "glandular," "sarcomatoid," and "small cell" VH were performed.

Between-study heterogeneity was assessed using $\chi^2$ and $I^2$ tests. A Cochran Q statistic p-value < 0.05 and $I^2$ statistic >50% indicate statistically significant heterogeneity between trials (16). When no significant heterogeneity was observed, fixed-effect models through the inverse-variance method were used for calculation. In the event that at least 10 studies were included, funnel plots were to be used to assess publication bias.

Sensitivity analyses were conducted, where we removed each study one at a time, to determine the impact on the overall pooled result.

All statistical analyses were performed using Review Manager version 5.4.1(The Cochrane Collaboration, 2020.) and STATA/MP 14.0 (Stata-Corporation, 2014.).

# 3 RESULTS

## 3.1. Study Selection and Characteristics

The initial search identified 1598 publications. (425 in PubMed, 1111 in EMBASE, and 62 in Cochran library). Of these, 1217 studies remained for review after removing duplicates. A total of 1156 articles were excluded after screening the titles and abstracts, and a full text review was performed for 61 articles. After applying the selection criteria, we included 22 studies in the final analysis (**Figure 1**). All included studies were non-randomized and observational. The RoB assessment indicated an intermediate to high level of bias across the studies (**Supplementary Figure S1**).

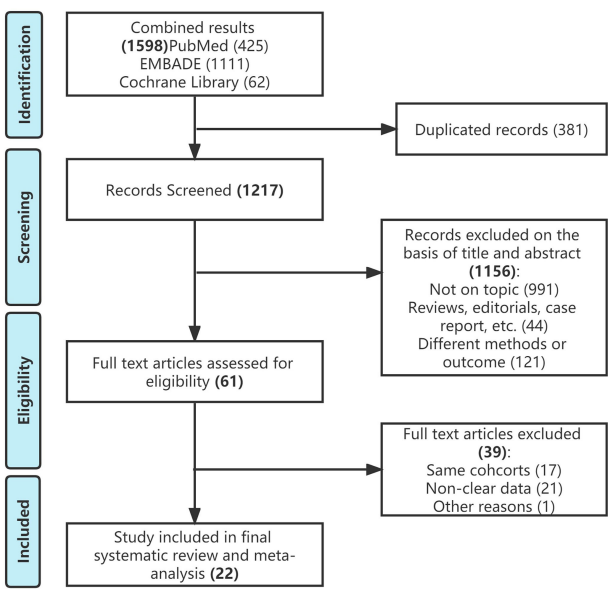


**FIGURE 1** | Study selection flowchart according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis guidelines.

There were 14 studies evaluating the role of NAC for VH (8, 10, 11, 17–27). Six were populations-based studies, seven studies came from different centers, and one study was based on both center and SEER. There were four studies evaluating the role of AC for VH (12, 28–30). Two were populations-based studies, while the remaining were based on different center's database. Four studies assessed both NAC and AC in VH, three of which were based on NCDB (9, 31–33). There were a total of 18 reports comprising 10,192 participants in the NAC studies, 978 (9.6%) of which had received NAC. With the exception of six studies that ignored patients' clinical stage, most of the studies included MIBC patients (**Supplementary Table S1**). A total of eight reports comprising 3254 people was included in the AC studies, of whom 665 received AC. Three of them included MIBC patients, the remaining five included all VH patients regardless of their stage (**Supplementary Table S2**).

## 3.2. Meta-Analysis

### 3.2.1. Neoadjuvant and Adjuvant Chemotherapy in VH

A total of 18 studies reported on survival outcomes in patients who had NAC prior to RC. Forest plots of HR and 95% CI for RFS, CSS, and OS are illustrated in **Figure 2**. The Cochrane Q-

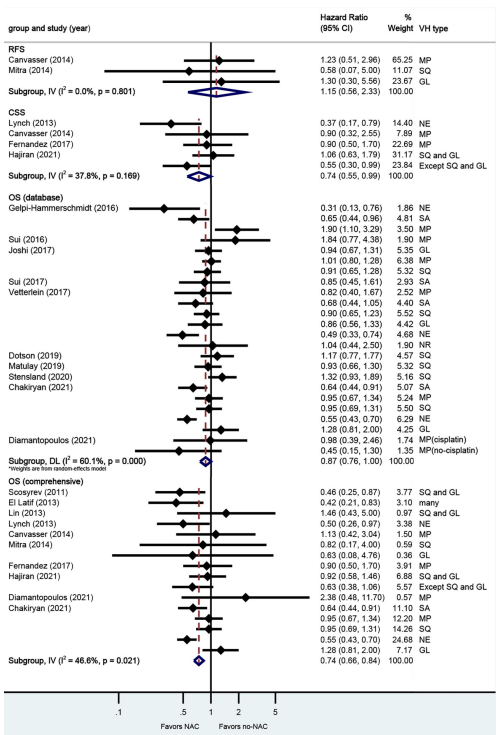


**FIGURE 2** | Forest plots of studies investigating the association of neoadjuvant chemotherapy with survival outcomes in variant histology. MP, micropapillary; SQ, squamous; GL, glandular; NE, neuroendocrine; SA, sarcomatoid; NR, not reported.

test (chi-square 57.60, p [0.000]) and I2 test (60.1%) revealed significant heterogeneity in OS (data base), with the funnel plot identifying five studies over the pseudo-95% CI (**Supplementary Figure S2**.). No significant heterogeneity in the Cochrane Q or I2 test was detected for other end points. Ten studies reported on the data base with a pooled HR of 0.87 (95% CI 0.76–1.00, p = 0.052) on OS. We selected the study by Chakiryan et al. for final analysis because it had high weight and stable RoB performance. Receiving NAC was not associated with RFS (HR 1.15, 95% CI 0.56–2.33, p = 0.706), but associated with better CSS (HR 0.74, 95% CI 0.55–0.99, p = 0.044) and better OS (HR 0.74, 95% CI 0.66–0.84, p = 0.000) in this pooled analysis.

A total of eight studies reported on survival outcomes in patients who had AC after RC. Forest plots of HR and 95% CI for RFS, CSS, and OS are illustrated in **Figure 3**. The Cochrane Q-test (chi-square 20.95, p [0.004]) and I2 test (66.6%) revealed significant heterogeneity in OS (comprehensive), with the funnel plot identifying two studies over the pseudo-95% CI (**Supplementary Figure S3**). No significant heterogeneity in the Cochrane Q or I2 test was detected for other end points. Four studies reported on the data base with a pooled HR of 0.92 (95% CI 0.82–1.05, p = 0.209) on OS. We selected the study by Berg et al. for final analysis because it had high weight and stable RoB performance. Receiving AC was not associated with RFS (HR 0.82, 95% CI 0.55–1.20, p = 0.304), but associated with better CSS (HR 0.61, 95% CI 0.43–0.87, p = 0.006), and better OS (HR 0.76, 95% CI 0.60–0.98, p = 0.032) in this pooled analysis.

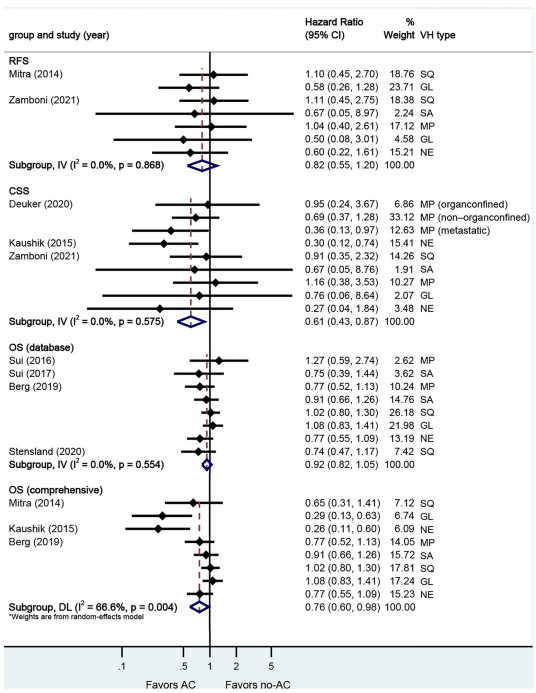


**FIGURE 3** | Forest plots of studies investigating the association of adjuvant chemotherapy with survival outcomes in variant histology. MP, micropapillary; SQ, squamous; GL, glandular; NE, neuroendocrine; SA, sarcomatoid; NR, not reported.

### 3.2.2. Neoadjuvant and Adjuvant Chemotherapy in Micropapillary VH

A total of eight studies reported on survival outcomes in patients who had NAC prior to RC. Forest plots of HR and 95% CI for RFS, CSS, and OS are illustrated in **Figure 4A**. No significant heterogeneity in the Cochrane Q or I2 test was detected for all end points. Six studies reported on the data base with a pooled HR of 1.05 (95% CI 0.88–1.24, p = 0.597) on OS. We selected the study by Joshi et al. for final analysis because it had high weight and stable RoB performance. Receiving NAC was not associated with RFS (HR 1.23, 95% CI 0.51–2.96, p = 0.644), CSS (HR 0.90, 95% CI 0.53–1.52, p = 0.695), or OS (HR 1.02, 95% CI 0.82–1.26, p = 0.880) in this pooled analysis.

A total of four studies reported on survival outcomes in patients who had AC after RC. Forest plots of HR and 95% CI for RFS, CSS, and OS are illustrated in **Figure 4B**. No significant heterogeneity in the Cochrane Q or I2 test was detected for all end points. Two studies reported on the data base with a pooled HR of 0.85 (95% CI 0.60–1.21, p = 0.367) on OS. Receiving AC was not associated with RFS (HR 1.04, 95% CI 0.40–2.61, p = 0.935), CSS (HR 0.68, 95% CI 0.44–1.07, p = 0.096), or OS in this pooled analysis.

### 3.2.3. Neoadjuvant and Adjuvant Chemotherapy in Squamous VH

A total of seven studies reported on survival outcomes in patients who had NAC prior to RC. Forest plots of HR and 95% CI for CSS and OS are illustrated in **Figure 5A**. No significant heterogeneity in the Cochrane Q or I2 test was detected for all end points. Six studies reported on the data base with a pooled HR of 1.00 (95% CI 0.87–1.15, p = 0.972) on OS. We selected the study by Vetterlein et al. for final analysis because it had high weight and stable RoB performance. Receiving NAC was not associated with CSS (HR 0.58, 95% CI 0.07–5.00, p = 0.617) or OS (HR 0.90, 95% CI 0.66–1.23, p = 0.494) in this pooled analysis.

A total of four studies reported on survival outcomes in patients who had AC after RC. Forest plots of HR and 95% CI for RFS, CSS, and OS are illustrated in **Figure 5B**. No significant heterogeneity in the Cochrane Q or I2 test was detected for all end points. Two studies reported on the data base with a pooled HR of 0.95 (95% CI 0.77–1.18, p = 0.640) on OS. We selected the study by Berg et al. for final analysis because it had high weight and stable RoB performance. Receiving AC was not associated with RFS (HR 1.10, 95% CI 0.58–2.09, p = 0.759), CSS (HR 0.91, 95% CI 0.35–2.32, p = 0.845), or OS (HR 0.98, 95% CI 0.78–1.23, p = 0.851) in this pooled analysis.

### 3.2.4. Neoadjuvant and Adjuvant Chemotherapy in Glandular VH

A total of four studies reported on survival outcomes in patients who had NAC prior to RC. Forest plots of HR and 95% CI for CSS and OS are illustrated in **Figure 6A**. No significant heterogeneity in the Cochrane Q or I2 test was detected for all end points. Three studies reported on the data base with a pooled HR of 0.99 (95% CI 0.79–1.25, p = 0.972) on OS. We selected the

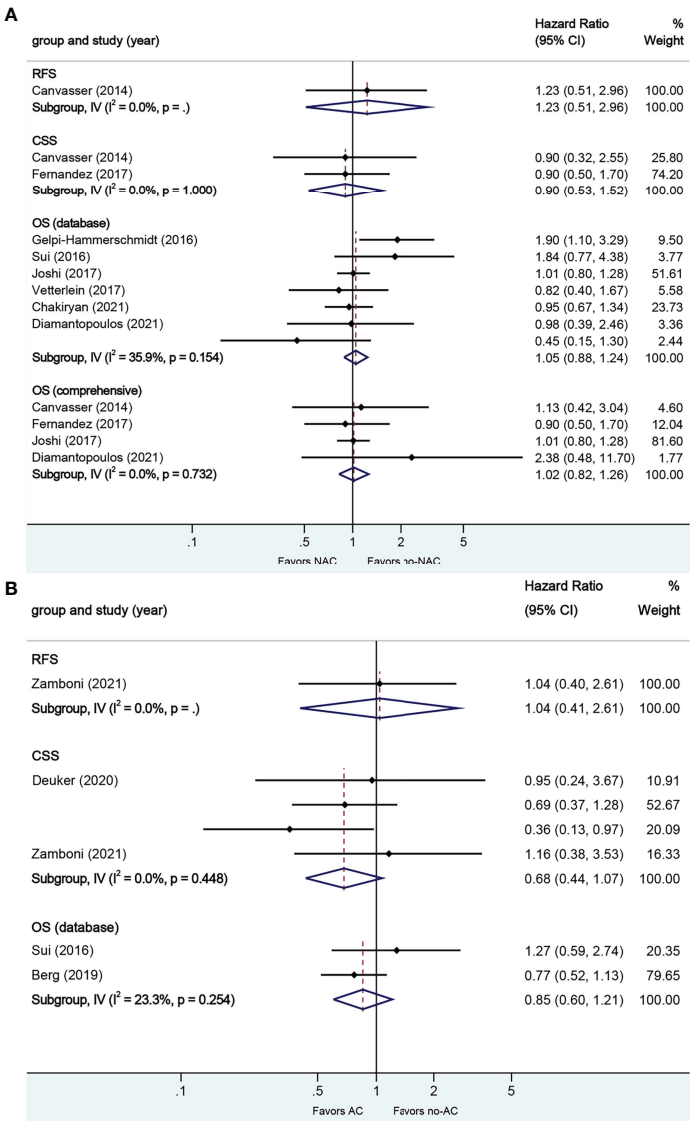


**FIGURE 4** | Forest plots of studies investigating the association of chemotherapy with survival outcomes in micropapillary variant histology. **(A)** neoadjuvant chemotherapy; **(B)** adjuvant chemotherapy.

study by Joshi et al. for final analysis because it had high weight and stable RoB performance. Receiving NAC was not associated with CSS (HR 1.30, 95% CI 0.30–5.56, p = 0.725) or OS (HR 0.93, 95% CI 0.67–1.29, p = 0.665) in this pooled analysis.

A total of three studies reported on survival outcomes in patients who had AC after RC. Forest plots of HR and 95% CI for RFS, CSS, and OS are illustrated in **Figure 6B**. The Cochrane Q-test (chi-square 9.58, p [0.002]) and I2 test (89.6%) revealed significant heterogeneity in OS (comprehensive); no significant heterogeneity in the Cochrane Q or I2 test was detected for other end points. One study based on the data base (HR 1.08, 95% CI 0.83–1.41, p = 0.569) was included in final analysis. Receiving AC was not associated with RFS (HR 0.57, 95% CI 0.27–1.17, p = 0.127), CSS (HR 0.76, 95% CI 0.06–8.64, p = 0.829), or OS (HR 0.59, 95% CI 0.16–2.14, p = 0.422) in this pooled analysis.

### 3.2.5. Neoadjuvant and Adjuvant Chemotherapy in Sarcomatoid VH

A total of four studies reported on survival outcomes in patients who had NAC prior to RC. All of the studies reported on the data base on OS. Forest plot of HR and 95% CI for OS is illustrated in **Figure 7A**. No significant heterogeneity in the Cochrane Q or I2 test was detected for end points. Receiving NAC was associated with better OS (HR 0.67, 95% CI 0.54–0.83, p = 0.000) in this pooled analysis.

A total of three studies reported on survival outcomes in patients who had AC after RC. Forest plots of HR and 95% CI for

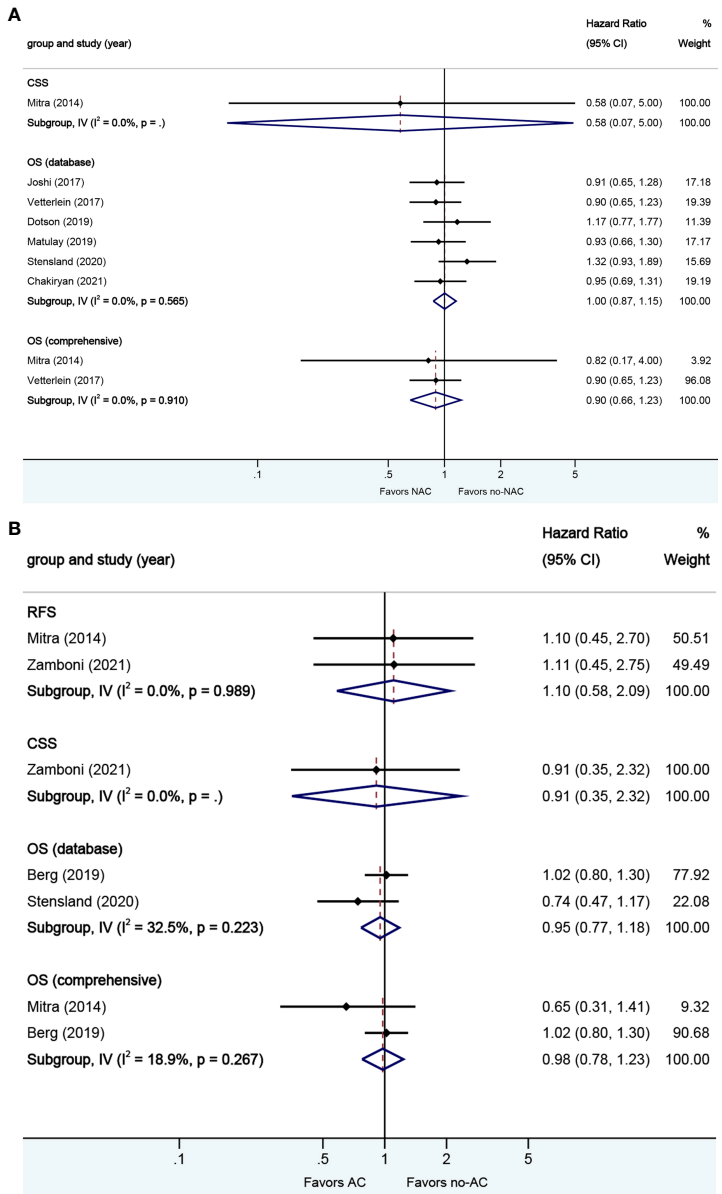


**FIGURE 5** | Forest plots of studies investigating the association of chemotherapy with survival outcomes in squamous variant histology. **(A)** neoadjuvant chemotherapy; **(B)** adjuvant chemotherapy.

RFS, CSS, and OS are illustrated in **Figure 7B**. No significant heterogeneity in the Cochrane Q or I2 test was detected for all end points. Two studies reported on the data base with a pooled HR of 0.88 (95% CI 0.66–1.17, p = 0.371) on OS. Receiving AC was not associated with RFS (HR 0.67, 95% CI 0.05–8.97, p = 0.762), CSS (HR 0.67, 95% CI 0.05–8.76, p = 0.761), or OS in this pooled analysis.

### 3.2.6. Neoadjuvant and Adjuvant Chemotherapy in Neuroendocrine VH

A total of four studies reported on survival outcomes in patients who had NAC prior to RC. Forest plots of HR and 95% CI for CSS and OS are illustrated in **Figure 8A**. No significant heterogeneity in the Cochrane Q or I2 test was detected for all end points. Three studies reported on the data base with a pooled HR of 0.52 (95% CI 0.42–0.63, p = 0.000) on OS. We selected the study by Chakiryan et al. for final analysis because it had high weight and stable RoB performance. Receiving NAC was associated with better CSS (HR 0.37, 95% CI 0.17–0.79, p = 0.011) and better OS (HR 0.54, 95% CI 0.43–0.68, p = 0.000) in this pooled analysis.

A total of three studies reported on survival outcomes in patients who had AC after RC. Forest plots of HR and 95% CI for RFS, CSS, and OS are illustrated in **Figure 8B**. The

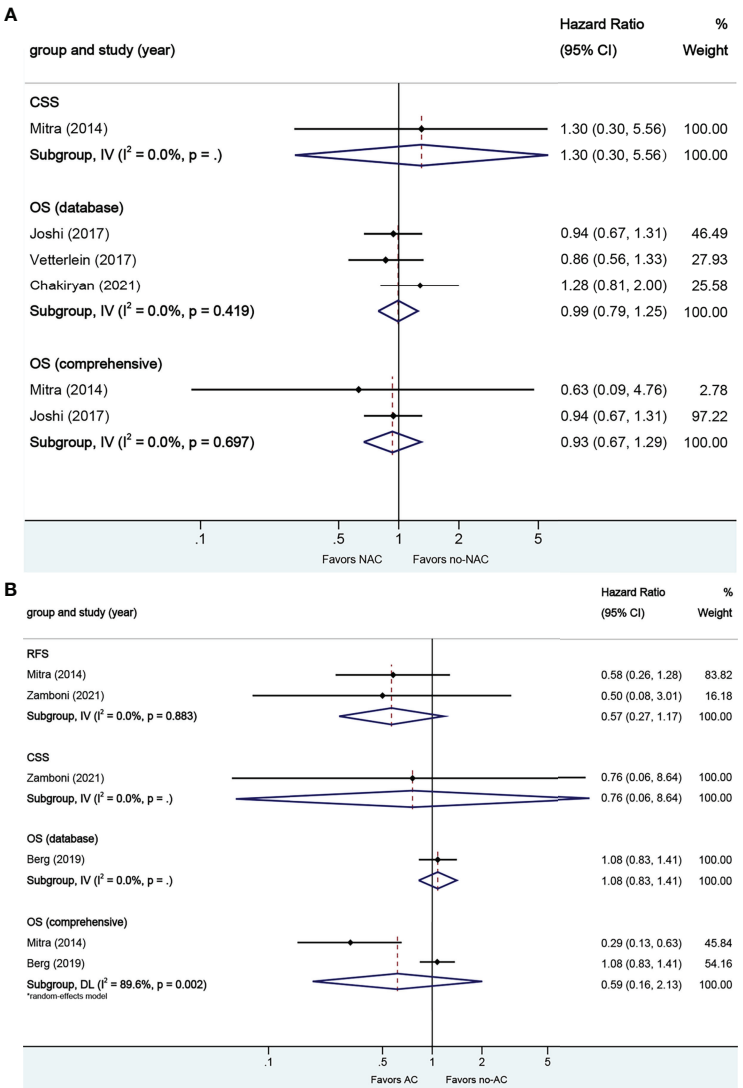


**FIGURE 6** | Forest plots of studies investigating the association of chemotherapy with survival outcomes in glandular variant histology. **(A)** neoadjuvant chemotherapy; **(B)** adjuvant chemotherapy.

Cochrane Q-test (chi-square 5.41, p [0.020]) and I2 test (81.5%) revealed significant heterogeneity in OS (comprehensive); no significant heterogeneity in the Cochrane Q or I2 test was detected for other end points. One study based on the data base (HR 0.77, 95% CI 0.55–1.09, p = 0.134) was included in final analysis. Receiving AC was not associated with RFS (HR 0.60, 95% CI 0.22–1.61, p = 0.314) or OS (HR 0.48, 95% CI 0.17–1.38, p = 0.004), but was associated with better CSS (HR 0.29, 95% CI 0.13–0.67, p = 0.174) in this pooled analysis.

## 3.3. Sensitivity Analysis

Sensitivity analyses were performed through sequential deletion of any individual study to measure the effects of each study. Overall HRs were not significantly influenced by any individual study, suggesting the robustness and reliability of the results in our meta-analysis.

# 4 DISCUSSION

## 4.1. Role of NAC

This meta-analysis investigated the role of NAC and AC in VH bladder cancers. VH is usually associated with advanced stage, lymphovascular invasion, and lymph node metastasis (34). Although many studies have reported that patients with VH had a poor response to NAC compared to patients with pure urothelial carcinoma (35–37),the study on whether NAC is effective for VH is rare and draws different conclusions. In this respect, the present study helped identify the effect of NAC and

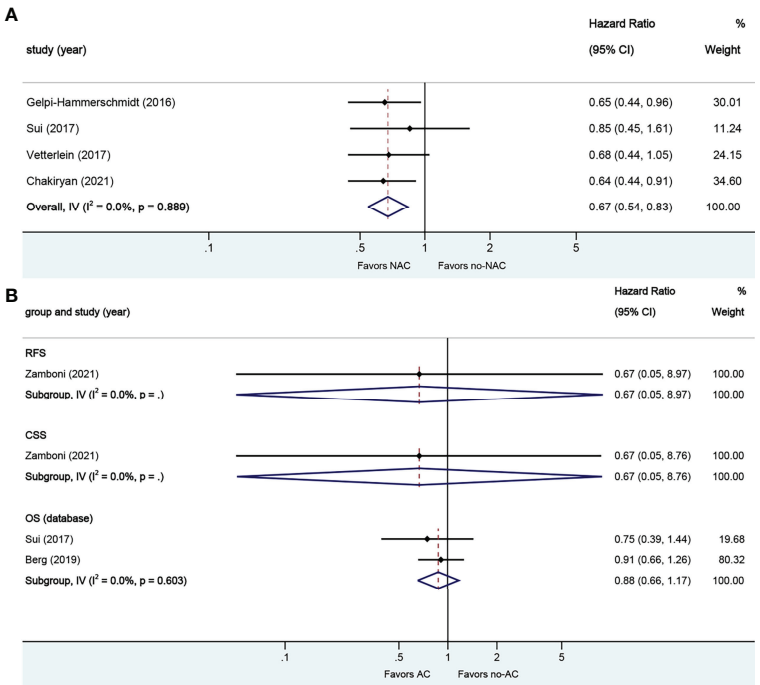


**FIGURE 7** | Forest plots of studies investigating the association of chemotherapy with survival outcomes in sarcomatoid variant histology. **(A)** neoadjuvant chemotherapy; **(B)** adjuvant chemotherapy.

AC in VH. Our meta-analysis demonstrated that patients with sarcomatoid and neuroendocrine VH would benefit from NAC. For patients with sarcomatoid differentiation, our findings do not align with what was envisioned. Because it is known that this rare VH is generally more aggressive and is at a more advanced stage at the time of diagnosis (38), and almost half agree immediate RC in EAU-ESMO Consensus Statements (no consensus achieved) (39).Given that the result was drawn from four populations-based studies, it should be treated with caution. In our study, no benefit was found in patients with micropapillary, squamous, or glandular VH, but we have reservations about the use of neoadjuvant chemotherapy in these patients. Although our study found no significant benefit in OS in these patients, the complete response rate (down-staging to T0) and/or partial response rate (pathological down-staging) of patients receiving NAC were obvious in some of the studies (40, 41). For example, MEEKS et al. collected data on patients with bladder cancer treated at the Memorial Sloan-Kettering Cancer Center (MSKCC) (40). Of the 44 patients with muscle-invasive micropapillary carcinoma, 29 received NAC. Down-staging to pT0 occurred in 13 (45%) of those who received neoadjuvant chemotherapy compared with two (13%) of those who did not (P = 0.049). They concluded that patients with the micropapillary variant of urothelial carcinoma should not be excluded from consideration for neoadjuvant chemotherapy. Considering the disadvantages of NAC, such as delaying RC leading to inadvertent disease progression and toxicities related to chemotherapy, administration of NAC should be used with caution.

## 4.2. Role of AC

The major downside of post-operative chemotherapy is that patients often suffer a decline in their physical performance after RC, which leads to patients being unable to tolerate chemotherapy. After RC, clinicians obtain the most accurate pathological staging from specimens. They can judge the next treatment measures *via* RC specimens. In this systematic review and meta-analysis, we found that only patients with neuroendocrine VH who received AC have a CSS benefit compared with those who underwent RC alone.

## 4.3. VH in the Future

With the development of molecular medical research, a variety of potential biomarkers have been evaluated to predict response to cisplatin-based chemotherapy (42). However, although the use of NAC/AC may be guided by tumor molecular characteristics in the future, the VH will continue to influence clinicians' decision-making for a long time.

Based on the current study, further clinical risk stratification in patients with VH may better guide the treatment. On the basis of VH classification, Rosiello et al. further divided patients with squamous VH into three groups according to TNM stages (1:T3–4aN0M0, 2:TanyN1–3M0, 3:T4bN0–3 or M1) (29). They found that chemotherapy benefited patients in T4bN0–3 or M1, while no significant benefit was found in the 1 and 2 groups. In our meta-analysis, receiving NAC was not associated with CSS or OS in squamous VH. Deuker et al. have stratified micropapillary patients into three groups: T1-2 N0M0, T3–4 N0M0/TanyN1–3 M0, Tany Nany M1 (43). They found that chemotherapy for

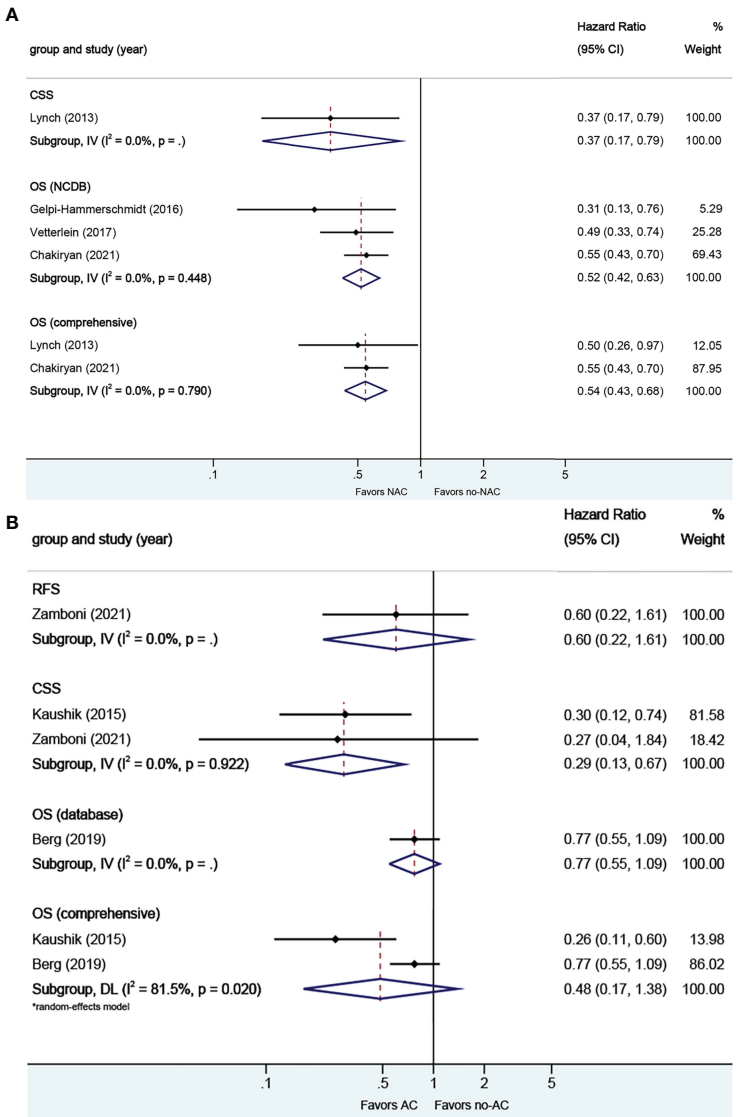


**FIGURE 8** | Forest plots of studies investigating the association of chemotherapy with survival outcomes in neuroendocrine variant histology. **(A)** neoadjuvant chemotherapy; **(B)** adjuvant chemotherapy.

micropapillary VH is effective in Tany Nany M1 stages, but of no beneficial effect in the T1-2 N0M0 stage. In our meta-analysis, receiving NAC was not associated with RFS, CSS, or OS in micropapillary VH. In addition, Speir et al. divided patients with squamous VH into two cohorts based on the percentage of squamous VH in the TUR specimen: <50% or ≥50% squamous VH (44). They found favorable results in patients with <50% involvement by squamous VH who received NAC.

## 4.4. Study Strengths and Limitations

The treatment of histological variants of bladder neoplasm is strongly debated, and the role of chemotherapy has not yet been properly assessed. We performed a precise evaluation of the available studies. This review can certainly stimulate the production of randomized studies. In addition, during the search, we found a systematic review of similar topic (45). Their research focused on the systematic review and extensively introduced the study in the use of NAC for variant histologies. Compared to their study, our study focused on the meta-analysis, and the retrieval database was relatively broad, but only the studies of multivariate analysis were considered. Therefore, some of the articles included in the study are different from theirs. We believe that our conclusions are based on meta-analysis and are more reliable.

Our study is not devoid of limitations. All studies included in this meta-analysis were retrospective and might show selection bias. Furthermore, multiple series with negative results may be unpublished, and the published studies contain some small

sample size research which may impact the overall quality of data. Lots of studies depended on database entries and might have suffered from a lack of secondary pathology reviews. Some studies included for analysis did not contain follow-up data or contained follow-up of less than 2 years, thus some conclusions regarding survival outcomes are unreliable.

In addition, heterogeneity was detected in the OS (AC, comprehensive) analysis, limiting the value of these results. The analyses conducted without regard to their particular VH type, and the inclusion of the populations-based studies which always account for high weight, may have contributed in large measure to significant heterogeneity in this meta-analysis. While this may be mitigated with a random-effect model, conclusions should be interpreted with caution.

Furthermore, our study investigates NAC and AC for all of VH, which may be oversimplified because not all VH types have similar biological behavior. For example, squamous VH has been considered a chemoresistant tumor, while neuroendocrine VH has been seen as sensitive to chemotherapy.

## 5 CONCLUSIONS

In conclusion, our meta-analysis found favorable OS and CSS in patients with VH who received NAC or AC. In the subgroup analyses, NAC independently improved OS in sarcomatoid and neuroendocrine subgroups. The results in AC report a significant CSS benefit in patients with neuroendocrine VH. However, this finding should be interpreted with caution because of the limitations of this studies which include the heterogeneity of the population of interest and the retrospective nature of the primary data evaluated.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

ZWZ, ZW, and ZSZ put forward the concept of the study and designed the study. SH and YX contributed to the data acquisition. ZWZ and ZW contributed to prepare the manuscript and the statistical analysis. ZSZ reviewed the manuscript. All authors read and approved the final manuscript.

## SUPPLEMENTARY MATERIAL

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# Detection rate of fluorine-18 prostate-specific membrane antigen-1007 PET/CT for prostate cancer in primary staging and biochemical recurrence with different serum PSA levels: A systematic review and meta-analysis

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**Background:** We performed a systematic review and meta-analysis to evaluate the detection rate (DR) of fluoro-prostate-specific membrane antigen ($^{18}$F-PSMA-1007) PET/CT in patients with different serum prostate-specific antigen (PSA) levels in the setting of primary staging of prostate cancer (PCa) or biochemically recurring PCa.

**Methods:** A comprehensive electronic literature search of the PubMed, Embase, and Cochrane Library databases was conducted in accordance with the PRISMA statement. This study was registered in the PROSPERO database (registration number: CRD42022331595). We calculated the DR of $^{18}$F-PSMA-1007 PET/CT in PCa.

**Results:** The final analysis included 15 studies that described 1,022 patients and 2,034 lesions with $^{18}$F-PSMA-1007 PET/CT in PCa. The DR of $^{18}$F-PSMA-1007 PET/CT in patients with PCa in primary staging ranged from 90% to 100%, with a pooled estimate of 94% (95% CI: 92%–96%). The DR of $^{18}$F-PSMA-1007 PET/CT in patients with PCa in BCR ranged from 47% to 100%, with a pooled estimate of 86% (95% CI: 76%–95%). The DRs of PSA levels >2.0, 1.1–2.0, 0.51–1.0, and ≤0.5 ng/ml detected by $^{18}$F-PSMA-1007 PET/CT in a patient-based analysis were 97% (95% CI: 93%–99%), 95% (95% CI: 88%–99%), 79% (95% CI: 68%–88%), and 68% (95% CI: 58%–78%), respectively.

**Conclusion:** This meta-analysis concluded that $^{18}$F-PSMA-1007 PET/CT had a high application value for prostate cancer, including primary tumors and biochemical recurrence. The DR of $^{18}$F-PSMA-1007 PET/CT was slightly higher in primary prostate tumors than in biochemical recurrence.

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**Systematic Review Registration:** https://www.crd.york.ac.uk/prospero/, identifier CRD42022331595.

KEYWORDS

prostate cancer, biochemical recurrence, meta-analysis, $^{18}$F-PSMA-1007, PET/CT, prostate-specific antigen

# 1 Introduction

Prostate cancer (PCa) is the second most common malignancy in men, excluding non-melanoma skin cancers such as basal and squamous cell carcinomas (1, 2). Typically, PCa patients do not exhibit characteristic clinical symptoms during the early stages of the disease; therefore, by the time PCa is diagnosed, many patients are already advanced in the disease and the tumor cannot be removed (3). Therefore, early diagnosis and treatment are important for PCa. Between 27% and 53% of all patients undergoing radical prostatectomy or radiation therapy develop a rising prostate-specific antigen (PSA) level (PSA recurrence) (4). Importantly, patients with PSA recurrence after radical prostatectomy or primary radiation therapy have different risks of subsequent PCa-specific mortality (4). A recent study investigated the impact of biochemical recurrence (BCR) on hard endpoints and concluded that patients experiencing BCR are at an increased risk of developing distant metastases and PCa-specific and overall mortality (5).

The precise staging of PCa by imaging methods is essential for proper disease management, as treatment options differ for localized PCa, locally advanced PCa, or metastatic disease (6). Prostate-specific membrane antigen (PSMA) is a transmembrane glycoprotein with glutamate carboxypeptidase activity (7). Prostate-specific membrane antigen expression is highly upregulated in advanced, metastatic, and poorly differentiated prostate cancers and increases with tumor aggressiveness; on the other hand, the overexpression of PSMA has not been found in benign prostatic diseases (8). Fluorine-18-PSMA-1007 ($^{18}$F-PSMA-1007) positron emission tomography/computed tomography (PET/CT) is an advanced imaging modality used to assess PCa (9). PET/CT images of the salivary glands, liver, gallbladder, prostate, kidney, and small intestine have a physiological uptake of $^{18}$F-PSMA-1007; a positive result can also be found in areas with localized abnormal radioactivity uptake, such as in lymph nodes and bones, which can be an indication of metastases (10). Compared with $^{68}$Gallium-PSMA-11 ($^{68}$Ga-PSMA-11), the most used PSMA imaging agent, $^{18}$F-PSMA-1007 has many advantages (6, 11, 12). First, $^{18}$F is produced by a cyclotron, which ensures that $^{18}$F-PSMA-1007 can be synthesized stably and in large quantities. However, the utility of [$^{68}$Ga] Ga circumvents the need for an on-site cyclotron since it is produced from a $^{68}$Ge/$^{68}$Ga generator (13). Second, the $^{18}$F-PSMA-1007 has a longer half-life (110 min) than the $^{68}$Ga-PSMA-11 (68 min), which facilitates distribution to other regions (11). Third, the deficiency of $^{68}$GA-PSMA-11 is that it is excreted mainly through the urinary system. If the tracer accumulates in the urinary tract, it may affect the diagnosis of local recurrence after radiotherapy (12). However, $^{18}$F-PSMA-1007 mainly focuses on hepatobiliary excretion, and the low urine activity can avoid this effect, which is conducive to the display of recurrence and metastasis. Finally, the low positron energy, long half-life, and rapid clearance *in vivo* of $^{18}$F-PSMA-1007 are convenient for a delayed scan. It can obtain higher tumor-to-background images and is more sensitive in the detection of recurrence than $^{68}$Ga-PSMA-11 (14, 15).

According to several publications (15–17), $^{18}$F-PSMA-1007 PET/CT tests are highly valuable for detecting prostate cancer primary lesions and biochemical recurrences. One study (4) involving an intraindividual comparison of prostate cancer patients with $^{18}$F-PSMA-1007 and $^{18}$F-fluorodeoxyglucose found that the former had a higher detection rate for primary lesions than the latter [100% (21/21) vs. 67% (14/21)]. For extra-prostatic lesions, the former showed a true positive rate of 60% and the latter 79%. Based on the $^{18}$F-PSMA-1007 PET/CT results of Giesel et al. (16), 204 (71.3%) of PCa patients showed evidence of recurrence. The percentages of PSA levels greater than or equal to 2, 1 to less than 2, 0.5 to less than 1, and 0.2 to less than 0.5 ng/ml detected by PET/CT were 94.0%, 90.9%, 74.5%, and 61.5%, respectively. Using $^{18}$F-PSMA-1007 PET/CT, German researchers (15) analyzed 100 cases of pathologically confirmed biochemically recurrent prostate cancer. Among patients with ≤0.5, 0.51–1.0, 1.1–2.0, and >2.0 ng/ml PSA levels, the pathological scanning rates were 86%, 89%, 100%, and 100%, respectively. As a result of the small sample sizes, regional differences, and differing PSA levels, the results of these studies were highly heterogeneous. For this reason, to evaluate the value of $^{18}$F-PSMA-1007 PET/CT in prostate cancer, it is important to carry out a meta-analysis or systematic review of the previous studies. Despite several published meta-analyses (18–20) assessing the rate of detecting BCR using $^{18}$F-PSMA-1007 PET/CT, no studies evaluated the efficacy of $^{18}$F-PSMA-1007 PET/CT for both primary staging and biochemical recurrence in PCa patients with different serum PSA levels.

Therefore, the aim of this meta-analysis and systematic review was to evaluate the application value of $^{18}$F-PSMA-1007 PET/CT in patients with different serum PSA levels in the setting of primary staging of PCa or biochemically recurring PCa.

## 2 Materials and methods

This meta-analysis was in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (see **Supplementary Material** for the PRISMA 2020 Checklist). This study was registered in the PROSPERO database (registration number: CRD42022331595).

### 2.1 Data sources and search strategy

We performed electronic literature searches of the PubMed, Embase, and Cochrane Library databases for English-language articles from the earliest available date of indexing through 30 September 2021. We also manually searched the reference lists of the identified publications to identify additional studies. The following keywords were used for the selection of studies: PSMA, prostate-specific membrane antigen, prostate cancer, prostate recurrence, positron imaging, PET, and $^{18}$F-PSMA-1007.

### 2.2 Study selection

The inclusion criteria for the relevant studies were as follows: a) $^{18}$F-PSMA-1007 PET/CT was used to identify and characterize PCa; b) subjects were diagnosed with PCa by histopathology, imaging examinations, or clinical follow-up; c) sufficient data to calculate detection rate (DR) of $^{18}$F-PSMA-1007 PET/CT in PCa were reported; and d) analyses were performed on a per-patient or per-lesion basis.

The exclusion criteria were as follows: a) overlapping papers; b) review articles, animal experiments, editorials or letters, comments, and conference proceedings; c) a lack of access to the full text; d) insufficient data to assess detection rate from individual studies; and e) a sample size of fewer than 10 patients or lesions.

### 2.3 Data extraction

In this study, the lesion-based analyses included local recurrence, lymph node, and bone and soft tissue lesions. In patient-based studies, the presence of lesions can be used as a covariate analysis. During data extraction, a positive $^{18}$F-PSMA-1007 scan was defined as follows: intraprostatic lesions were defined as positive if the tracer uptake was focal and higher than the surrounding prostate tissue (15, 21). Other soft tissue and bone metastases were judged as positive when there were obvious morphological changes; meanwhile, the corresponding lesions showed increased radiotracer uptake above normal surroundings (4, 22).

A data abstraction sheet was developed. Two researchers (XL and TJ) independently assessed the collected data that included basic information (authors, publication year, and country), study design (prospective or retrospective), patient characteristics, imaging purpose (initial stage or BCR), sample size (patients or lesions), imaging agent ($^{68}$Ga-PSMA-11 or $^{18}$F-PSMA-1007), administered activity, level of PSA, and Gleason score for characterizing PCa. In cases of disagreement, a consensus was reached on inclusion or exclusion by discussion, and if necessary, a third researcher (CG) was consulted.

### 2.4 Quality assessment

The methodological quality of the included studies was critically appraised based on the modified Quality Assessment of Diagnostic Accuracy Studies version 2 (QUADAS-2) (23), as recommended by the Cochrane Collaboration. Each item was evaluated as "high," "low," or "unclear." Each paper was scored independently by two evaluators (XL and TJ), and any discrepancies were resolved. The Review Manager software (The Cochrane Collaboration, version 5.3.5, London, United Kingdom) was used to assess the quality.

### 2.5 Statistical analysis and data synthesis

In this study, the data of every eligible study were collected. Descriptive statistics (such as mean, standard deviation, and count) were used to summarize continuous variables, while percentage and count were used for categorical variables. The primary objective was to estimate the DR with a 95% confidence interval (95% CI). Detection rate was defined as the ratio between the number of patients or lesions with at least one suspected lesion detected by imaging facility and the total number of PCa patients who underwent the scan. A bivariate normal random-effects model for measures was used to analyze and pool the diagnostic performance of previous studies (24). Heterogeneity was analyzed using the $\chi^2$ test, with a $P$-value of less than 0.05 suggesting heterogeneity. In addition, the $I^2$ statistic was adopted to evaluate the degree of heterogeneity (25). Based on Cochrane's handbook, a rough classification of the $I^2$ index is as follows: low (0%–40%), moderate (30%–60%), substantial (50%–90%), and considerable variability (75%–100%). The value of $P$ <0.05 or $I^2$ >50% indicated that there was greater heterogeneity in the specimens (26). Based on the results, the random-effects model was used for further analysis; otherwise, a fixed-effect model was performed. Meanwhile, when there was substantial statistical heterogeneity,

we performed subgroup analysis to identify potential sources of bias (27). As described by Deeks and colleagues (28), we examined the possibility of publication bias by using an effective sample size funnel plot and a regression test of asymmetry. Tests for significance were two-tailed, with a statistically significant *P*-value threshold of 0.05. All statistical analyses were carried out using Stata version 16.0 software (StataCorp LP, College Station, TX, USA).

# 3 Results

## 3.1 Literature search and study selection

After a comprehensive computerized search was performed and the reference lists were extensively cross-checked, our study identified 255 records. After reviewing titles and abstracts, 128 records were excluded because they were non-human studies, duplicated reports, reviews, editorials, conference abstracts, or small case series. Additionally, 104 unrelated abstracts were removed. By reading the full texts, seven articles were eliminated because of a lack of sufficient information to calculate the detection rate. Two literature studies (22, 29) were published by the same institution, and the data were duplication. Therefore, only data from the latest article (22) were extracted for meta-analysis. Finally, 15 studies met the inclusion and exclusion criteria, all of which were subjected to a systematic review and meta-analysis. No other articles were found after screening the references of these articles. The detailed process of literature screening is shown in **Figure 1**.

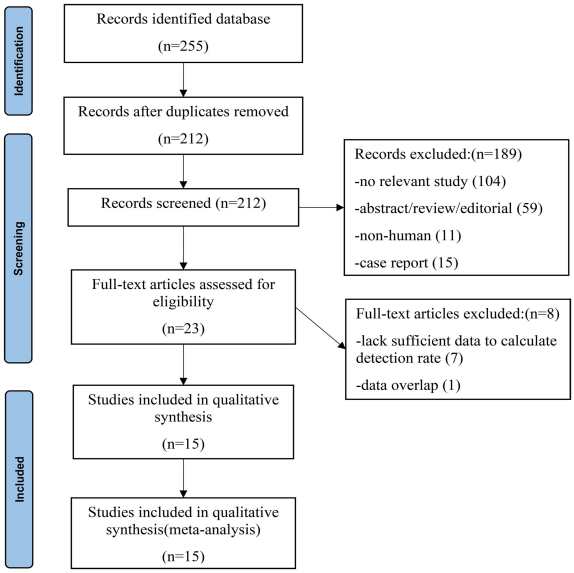


FIGURE 1
Flowchart of the search for eligible studies on $^{18}$F-PSMA-1007 PET/CT in patients of prostate cancer.

## 3.2 Characteristics of the included studies

The major characteristics of the 15 studies (4, 11, 15–17, 21, 22, 30–37) included in the meta-analysis are described in **Tables 1**, **2**. The 15 articles were published between 2017 and 2021, consisting of 12 retrospective studies (75%) and three prospective studies (25%) (21, 22, 33).

Seven studies (4, 17, 21, 22, 30–32) assessed the primary staging of prostate cancer. Nine studies (11, 15, 16, 32–37) assessed the biochemical recurrence of prostate cancer. One study (32) evaluated both the primary staging of prostate cancer and the biochemical recurrence.

All studies used PET/CT as an imaging modality. Three studies (17, 22, 30) simultaneously evaluated $^{18}$F-PSMA-1007 PET/CT and magnetic resonance imaging (MRI). The imaging agents $^{18}$F-PSMA-1007 and $^{68}$Ga-PSMA-11 were compared simultaneously in three studies (11, 21, 37). Nearly half of the studies were from Germany (46.7%), and the other studies were from the Netherlands (30, 32), Israel (21), Belgium, Finland (29), Sweden (31), and China (4), respectively.

In total, there were 1,022 PCa patients and 2,034 PCa lesions in the included studies, and the ages of the patients ranged from 48 to 86 years. The number of cases in each study ranged from 10 to 251. The serum PSA levels ranged from 0.01 to 2,000 ng/ml. We conducted all analyses based on per-patient and/or per-lesion data. Unfortunately, only three (15, 16, 33) eligible studies have evaluated the serum PSA grouping.

## 3.3 Risk of bias and applicability

The risk of bias and applicability concerns for the included studies were assessed using QUADAS-2, as shown in **Figure 2** and **Supplementary Table 1**. All the included studies were of moderate to high quality.

## 3.4 Quantitative analysis (meta-analysis)

### 3.4.1 18F-PSMA-1007 PET/CT for prostate cancer in primary staging

Seven included studies assessed the $^{18}$F-PSMA-1007 PET/CT in the setting of primary staging. The DR of $^{18}$F-PSMA-1007 PET/CT in patients with PCa ranged from 90% to 100%, with a pooled estimate of 94% (95% CI: 92%–96%) (**Figure 3A**). There was no heterogeneity between studies ($I^2$: 0.00%).

The funnel plot for publication bias assessment is shown in **Figure 3B**. Egger's regression intercept for DR pooling was 0.16 (95% CI: −0.36 to 0.69, $P$ = 0.460), also indicating that publication bias was absent.

TABLE 1 Basic study and patient characteristics.

| Author | Year | Country | Study design | No. of patients/ lesions | Age (years) | Imaging purpose | Type of patients evaluated | Median (range)PSA values at PET/ CT (ng/ml) | Gleason score |
|---|---|---|---|---|---|---|---|---|---|
| Zhou et al. (4) | 2021 | China | R | 21/124 | Median:66 | Initial stage | Patients with BCRPCa previously treated with ADT (81%) or RP (52%) | 41.20 (5.00–200.00) | ≤6: 0%, 7: 42%, ≥8: 58% |
| Rauscher et al. (11) | 2020 | Germany | R | 102/371 | Mean: 71 ± 8 | Biochemical recurrence | BCRPCa | 0.87 (0.20–13.59) | 6–7: 61.8%, 8–10: 38.2% |
| Rahbar et al. (15) | 2018 | Germany | R | 100/NR | Mean: 68.75 ± 7.6 | Biochemical recurrence | Patients with BCRPCa previously treated with RP (92%) or RT (45%) or ADT (27%) | 1.34 (0.04–41.3) | ≤6: 8%, 7: 56%, ≥8: 36% |
| Kesch et al. (17) | 2017 | Germany | R | 10/372 | Median: 67 (62–77) | Initial stage | Patients with PPCa | 13.1 (5.8–40.0) | ≤6: 0%, 7: 30%, ≥8: 70% |
| Trägårdh et al. (31) | 2021 | Sweden | R | 39/118 | Mean: 65 ± 5.6 | Initial stage | Patients with PPCa | NR | NR |
| Kuten et al. (21) | 2019 | Israel | P | 16/145 | Median: 68.5 | Initial stage | Patients with PPCa | 6.35 (5.1–10.9) | ≤6: 0%, 7: 81%, ≥8: 19% |
| Malaspina et al. (22) | 2021 | Finland | P | 79/218 | Median: 72 | Initial stage | Patients with PPCa | Median 12 (3–2,000) | ≤6: 100%, 7: 0%, ≥8: 0% |
| Privé et al. (30) | 2020 | Netherlands | R | 53/46 | NR | Initial stage | Patients with PPCa | 12 (7.7–20) | ≤6: 9%, 7: 36%, ≥8: 55% |
| Wondergem et al. (32)[a] | 2021 | Netherlands | R | 69/NR | NR | Initial stage | Patients with PPCa | 14.7 (2.4–577) | ≤6: 0%, 7: 94%, ≥8: 0%, unknown: 6% |
| | | | | | | Biochemical recurrence | Patients with BCRPCa previously treated with RP (33.3%) or RT (66.7%) | 2.4 (0.4–7.8) | NR |
| Giesel et al. (16) | 2019 | Germany | R | 251/NR | Median: 70 (48–86) | Biochemical recurrence | Patients with BCRPCa previously treated with RT after RP (43.8%) or ADT (53.4%%) | 10.9 (0.6–250) | ≤6: 5.2%, 7: 49.8%, ≥8: 33.1%, unknown: 11.2% |
| Witkowska-Patena et al. (33) | 2019 | Poland | P | 40/NR | Mean: 69 ± 7 | Biochemical recurrence | Patients with BCRPCa previously treated with RP (80%) or RT (20%) | 0.7 (0.01–2.0) | Mean 7.1 ± 1, median 7 (5–9) |
| Sachpekidis et al. (34) | 2019 | Germany | R | 17/NR | Median: 66 | Biochemical recurrence | Patients with BCRPCa previously treated with RP or RT (100%) | 1.2 (0.2–237.3) | ≤6: 4%, 7: 44%, ≥8: 24%, unknown: 28% |
| Dietlein et al. (35) | 2020 | Germany | R | 27/NR | Mean: 67.2 ± 7.8 | Biochemical recurrence | Patients with BCRPCa previously treated with RP (93%) or RT (7%) | 0.3–27.7 | NR |
| Ahmadi Bidakhvid et al. (36) | 2021 | Belgium | R | 175/580 | Mean: 69 ± 8.8 | Biochemical recurrence | Patients with BCRPCa previously treated with RP (78%) or RT (35.9%) or high-intensity focused ultrasound (0.7%) or ADT (93.3%) | Median 1.6 (0.07–429) | ≤6: 8%, 7: 49%, ≥8: 43% |
| Morawitz et al. (37) | 2021 | Germany | R | 23/60 | Mean: 71 ± 8.5 | Biochemical recurrence | BCR after RP (100%) | 1.5 (0.2–7.0) | NR |

[a]This study evaluated both the primary staging of prostate cancer and the biochemical recurrence.
P, prospective; R, retrospective; NR, not reported; PPCa, primary prostate cancer; BCR, biochemical recurrence; ADT, androgen-deprivation therapy; RP, radical prostatectomy; RT, radiation therapy.

#### 3.4.1.1 Per patient-based or per lesion-based analysis

Four studies (4, 22, 31, 32) assessed the DR of $^{18}$F-PSMA-1007 PET/CT in a patient-based analysis, with a range of 91% to 100% and a combined estimate of 96% (95% CI: 91%–99%) (**Supplementary Figure 1**). There was no heterogeneity between studies ($I^2$: 22.13%).

Six studies (4, 17, 21, 22, 30, 31) assessed the DR of $^{18}$F-PSMA-1007 PET/CT in a lesion-based analysis, with a range of 53% to 94% and a combined estimate of 81% (95% CI: 66%–92%) (**Supplementary Figure 1**). The included studies were statistically heterogeneous in their estimate of DR ($I^2$: 96.47%).

The DR of $^{18}$F-PSMA-1007 PET/CT for PCa in primary staging was significantly different between patient-based and lesion-based analysis ($P$ = 0.02).

TABLE 2 Technical aspects of $^{18}$F-PSMA-1007 in the included studies.

| Author | Modality | Radiotracer | Radiotracer injection activity[a] (mean) | Time interval between radiotracer injection and image acquisition (mean) | Modality manufacturer | Scanning scope | Other imaging performed for comparison |
|---|---|---|---|---|---|---|---|
| Zhou et al. (4) | PET/CT | $^{18}$F-PSMA-1007 | 348 ± 52 MBq | 180 min | Biograph mCT-64 PET/CT scanner (Siemens) | From the vertex to the mid-thigh | $^{18}$F-FDG PET/CT |
| Rauscher et al. (11) | PET/CT | $^{18}$F-PSMA-1007 | 325 ± 40 MBq | 94 ± 22 min | Biograph mCT scanner (Siemens Medical Solutions) | NR | $^{68}$Ga-PSMA-11 PET/CT |
| Rahbar et al. (15) | PET-CT | $^{18}$F-PSMA-1007 | 338.02 ± 33.31 MBq | Median 120 min | Siemens mCT Scanner (Siemens Healthcare, Knoxville, TN, USA) | From the lower limbs to the skull | – |
| Kesch et al. (17) | PET/CT | $^{18}$F-PSMA-1007 | NR | 60 min, delay 180 min | Biograph mCT Flow Scanner (Siemens) | NR | mpMRI |
| Trägårdh et al. (31) | PET/CT | $^{18}$F-PSMA-1007 | 4.0 ± 0.4 MBq/kg | 120 ± 6 min | Discovery MI (GE Healthcare, Milwaukee, WI, USA) | From the skull base to the mid-thigh | – |
| Kuten et al. (21) | PET/CT | $^{18}$F-PSMA-1007 | 4 MBq/kg | 60 min | Discovery 690 PET/CT system (GE Healthcare) | From the tip of the skull to the mid-thigh | $^{68}$Ga-PSMA-11 PET/CT |
| Malaspina et al. (22) | PET/CT | $^{18}$F-PSMA-1007 | 250 MBq | 60 min | Discovery MI digital PET/CT system (GE Healthcare, Milwaukee, WI, USA) | From the vertex to the mid-thigh | MRI |
| Privé et al. (30) | PET/CT | $^{18}$F-PSMA-1007 | 250 MBq | 90 ± 10 min | Biograph mCT 4-ring, 40-slice TOF PET/CT Scanner (Siemens) | NR | MRI |
| Wondergem et al. (32) | PET/CT | $^{18}$F-PSMA-1007 | 324 MBq | 90 min | Biograph-16 TruePoint PET/CT (Siemens Healthcare, Knoxville, USA) | From the skull base to the inguinal region | $^{18}$F-DCFPyL PET/CT |
| Giesel et al. (16) | PET-CT | $^{18}$F-PSMA-1007 | 301 ± 6.46 MBq | 92 ± 26 min | Biograph mCT Flow Scanner (Siemens Medical Solutions) | NR | – |
| Witkowska-Patena et al. (33) | PET-CT | $^{18}$F-PSMA-1007 | 296 ± 14 MBq | 95 ± 12 min | Dedicated hybrid PET/CT system (Discovery 710; GE Healthcare, Chicago, IL, USA) | From the top of the head to the mid-thigh | $^{18}$F-FCH PET/CT |
| Sachpekidis et al. (34) | PET-CT | $^{18}$F-PSMA-1007 | Median 237 MBq | 60 min | Dedicated PET/CT system (Biograph mCT, 128S, Siemens, Erlangen, Germany) | From the skull to the feet | – |
| Dietlein et al. (35) | PET/CT | $^{18}$F-PSMA-1007 | 159 ± 31 MBq | NR | NR | NR | – |
| Ahmadi Bidakhvid et al. (36) | PET/CT | $^{18}$F-PSMA-1007 | 3 MBq/kg | 81 ± 16 min | Discovery MI-4 PET/CT (GE) | From the vertex to the upper thigh | – |
| Morawitz et al. (37) | PET/CT | $^{18}$F-PSMA-1007 | 229 ± 27 MBq | NR | Biograph mCT 128 (Siemens Healthineers, Erlangen, Germany) | From the skull base to the mid-thigh | $^{68}$Ga-PSMA-11 PET/CT |

NR, not reported; mpMRI, multiparameter magnetic resonance imaging; $^{68}$Ga-PSMA, Gallium-68; $^{18}$F, fluorine-18; PET/CT, positron emission tomography/computed tomography; $^{18}$F-FCH, fluorine-18-fluorocholine; PSMA, prostate-specific membrane antigen; FDG, fluorodeoxyglucose; DCFPyL, 2-(3-{1-carboxy-5-[(6-[(18)F]fluoro-pyridine-3-carbonyl)-amino]-pentyl}-ureido)-pentanedioic acid.

[a]Activity (mean activity of the radiotracer applied in MBq; NR, not recorded; reported target dose in MBq/kg).

#### 3.4.1.2 18F-PSMA-1007 PET/CT vs. MRI

Three studies (17, 22, 30) simultaneously compared the DR of $^{18}$F-PSMA-1007 PET/CT with MRI for PCa in primary staging in a lesion-based analysis. The pooled DR of $^{18}$F-PSMA-1007 PET/CT vs. MRI was 88% (95% CI: 79%–95%) vs. 81% (95% CI: 65%–94%), respectively (**Supplementary Figure 2**). There was no significant difference between the two groups ($P$ = 0.409).


FIGURE 2
Risk of bias and applicability concerns the summary **(A)** and graph **(B)** of the studies included in the systematic review according to the QUADAS-2 tool.

### 3.4.2 $^{18}$F-PSMA-1007 PET/CT for prostate cancer in BCR

Nine studies assessed the DR of $^{18}$F-PSMA-1007 PET/CT for prostate cancer in BCR in this group. The DR of $^{18}$F-PSMA-1007 PET/CT in patients with PCa ranged from 47% to 100%, with a pooled estimate of 86% (95% CI: 76%–95%) (Figure 4A). The included studies were statistically heterogeneous in their estimate of DR ($I^2$: 93.91%).

The funnel plot for publication bias assessment is shown in Figure 4B. Egger's regression intercept for DR pooling was −2.70 (95% CI: −5.81 to 0.41, $P = 0.079$), also indicating that publication bias was absent.

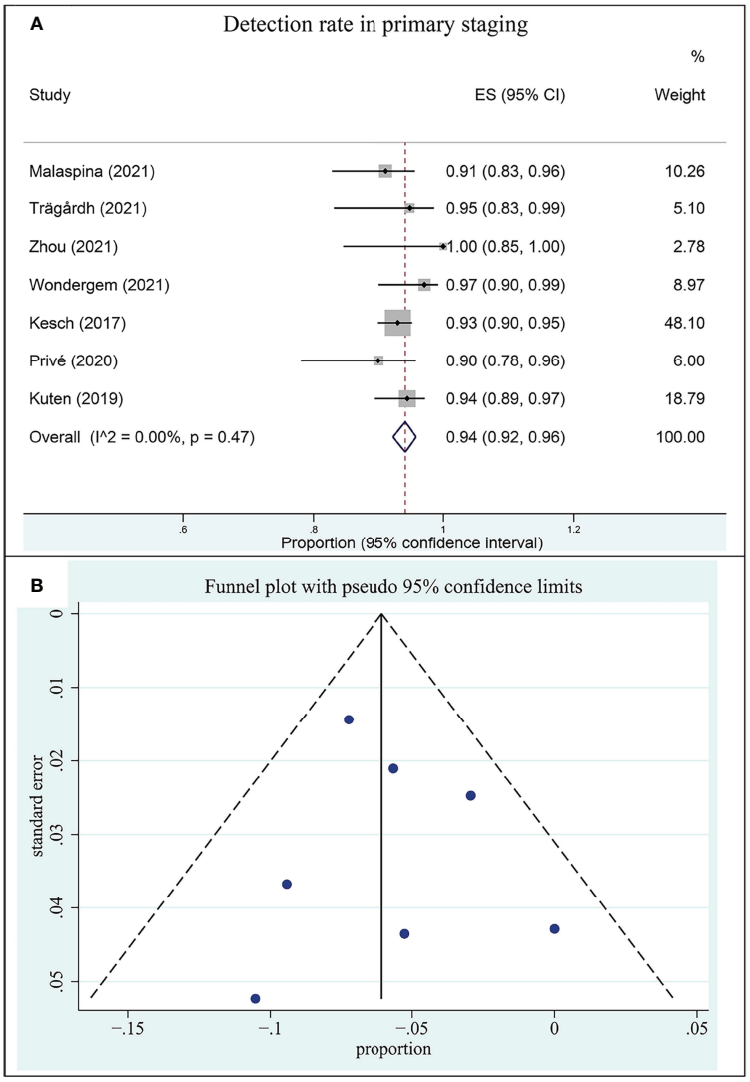


FIGURE 3
Plot of the pooled detection rate of $^{18}$F-PSMA-1007 PET/CT for prostate cancer in primary staging **(A)** and related funnel plot for publication bias assessment (**B**).

### 3.4.2.1 Without serum PSA grouping based on patient or lesion analysis

Nine studies (11, 15, 16, 32–37) assessed the DR of $^{18}$F-PSMA-1007 PET/CT for prostate cancer in BCR based on patient analysis without serum PSA grouping, with a range of 47% to 95% and a pooled estimate of 82% (95% CI: 74% to 88%) (**Supplementary Figure 3**). The included studies were statistically heterogeneous in their estimate of DR ($I^2$: 76.92%).

Four studies (11, 16, 36, 37) assessed the DR of $^{18}$F-PSMA-1007 PET/CT in a lesion-based without serum PSA grouping, with a range of 33% to 100% and a combined estimate of 78% (95% CI: 33%–100%) (**Supplementary Figure 3**). The included studies were statistically heterogeneous in their estimate of DR ($I^2$: 99.61%).

There was no significant difference in the DR of $^{18}$F-PSMA-1007 PET/CT for PCa in BCR between patient-based and lesion-based analyses ($P$ = 0.863).

### 3.4.2.2 Serum PSA subgroup based on patient analysis

Due to limited information, the pooled analysis was performed only for patient-based studies in the subgroup analysis performed with serum PSA.

Two studies (15, 16) assessed the pooled DR of $^{18}$F-PSMA-1007 PET/CT for PCa in BCR based on patient analysis. The DRs of PSA levels >2.0, 1.1–2.0, 0.51–1.0, and ≤0.5 ng/ml detected by $^{18}$F-PSMA-1007 PET/CT were 97% (95% CI: 93%–99%), 95% (95% CI: 88%–99%), 79% (95% CI: 68%–

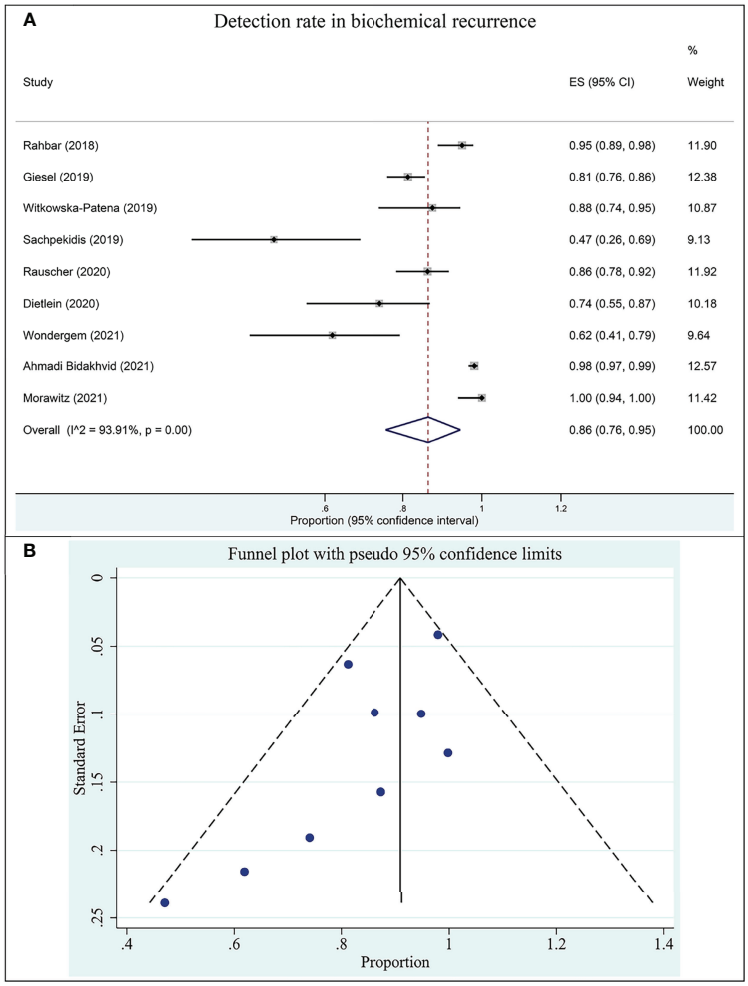


FIGURE 4
Plot of the pooled detection rate of $^{18}$F-PSMA-1007 PET/CT for prostate cancer in biochemical recurrence **(A)** and related funnel plot for publication bias assessment (**B**).

88%), and 68% (95% CI: 58%–78%), respectively (**Figure 5**). There was a significant difference between the four groups ($P$ = 0.00).

#### 3.4.2.3 $^{18}$F-PSMA-1007 PET/CT vs. 68Ga-PSMA-11 PET/CT

Two studies (11, 37) simultaneously compared $^{18}$F-PSMA-1007 with $^{68}$Ga-PSMA-11 PET/CT for PCa in biochemical recurrence. The pooled DRs of $^{18}$F-PSMA-1007 vs. $^{68}$Ga-PSMA-11 PET/CT in PCa were 87% (95% CI: 80%–92%) vs. 47% (95% CI: 38%–55%) in a patient-based analysis and 46% (95% CI: 41%–50%) vs. 89% (95% CI: 86%–92%) in a lesion-based analysis, respectively (**Supplementary Figure 4**). The pooled results should be interpreted carefully, given the fact that the results were only based on two studies.

## 4 Discussion

In the previously published meta-analyses (19, 20, 38, 39), Treglia et al. (38) analyzed the DR of $^{18}$F-labeled PSMA PET/CT for the biochemical recurrence of PCa. In their meta-analysis, four studies were included assessing the application value of 18F-PSMA-1007, with a pooled DR of 89%. However, Treglia et al. (38) did not perform subgroup analyses for each radiotracer at different serum PSA levels. Alberts et al. (40) performed a network meta-analysis on the diagnostic performance of different radiotracers in recurrent prostate cancer and believed that $^{18}$F-PSMA-1007 had a good advantage in the detection of prostate cancer lesions. However, their study has the following shortcomings: the literature after 2020 was not included, as this was the year when many new studies on $^{18}$F-PSMA-1007 were published; no grouping of

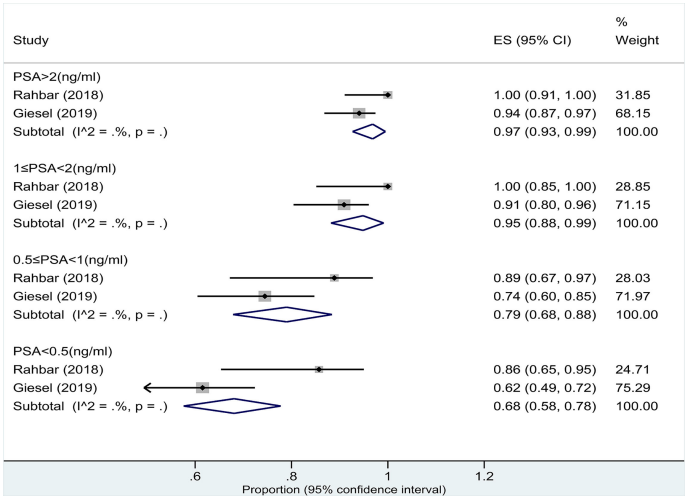


FIGURE 5
Plot of the pooled detection rate of $^{18}$F-PSMA-1007 PET/CT for prostate cancer with biochemical recurrence based on patient analysis with PSA levels >2.0, 1.1–2.0, 0.51–1.0, and ≤0.5 ng/ml.

lesions and patients was performed; and the DRs of different serum PSA levels were not analyzed. Therefore, it is necessary to re-conduct a meta-analysis on this background. Our meta-analysis suggested that $^{18}$F-PSMA-1007 PET/CT has a good DR in patients with different serum PSA levels in the setting of primary staging or BCR of PCa.

In our meta-analysis, the serum PSA was higher than 2 ng/ml in all primary staging patients, and the combined DR of $^{18}$F-PSMA-1007 PET/CT was 94% (95% CI: 92%–96%). In addition, we performed patient- and lesion-based subgroup analyses, and the pooled DRs of $^{18}$F-PSMA-1007 PET/CT were 96% (95% CI: 91%–99%) and 81% (95% CI: 66%–92%), respectively. Our study also found that the difference between the two groups was statistically significant ($P$ = 0.02). In other words, the DR of the former was significantly higher than that of the latter. Possible reasons for the difference in the results between the two subgroups include the following: first, in a patient-based analysis, a prostate cancer patient who has only one lesion is considered positive. However, in the lesion-based analysis, the number of lesions was large and there were many false-positive lesions, so the true positive rate decreased. Second, the subjects analyzed were not derived from the same study, and not all subjects were head-to-head comparisons. Third, there was significant heterogeneity between the studies in the lesion-based analysis ($I^2$: 96.47%). However, in the patient-based analysis, there was no heterogeneity ($I^2$: 22.13%). Grünig et al. (41) concluded that $^{18}$F-PSMA-1007 PET/CT detected specific uptake foci in bone in 51.4% of the patients with prostate cancer. In a recent original study, the overall positive detection rate of $^{18}$F-PSMA-1007 PET/CT was 91% in the BCR of prostate cancer (42). However, the study also found a significantly lower positive predictive value for $^{18}$F-PSMA in bone lesions compared to local recurrence and pelvic lymph nodes, which are a potential diagnostic weakness when using this tracer (42). Therefore, it can be concluded that the DR of the lesion-based analysis in this study was lower than that of the patient-based analysis.

Three studies simultaneously compared the DR of $^{18}$F-PSMA-1007 PET/CT with MRI for PCa in primary staging in a lesion-based analysis. However, our pooled results showed no significant difference in the DR of the two imaging modalities ($P$ = 0.409). Kesch et al. (17) believed that $^{18}$F-PSMA-1007 performed slightly better for near-total agreement regarding sensitivity, specificity, PPV, and accuracy but had a worse sensitivity and NPV for total agreement than the multiparameter MRI (mpMRI). This variance can be explained by the higher resolution and anatomic landmark definition derived from mpMRI. Based on the per-lesion analysis, $^{18}$F-PSMA-1007 PET/CT was superior to mpMRI, having both fewer false negatives and fewer false positives (17). Our findings are consistent with those of Kesch et al. (17). Furthermore, the study by Privé et al. (30) of 53 patients with primary prostate cancer found $^{18}$F-PSMA-1007 to accurately stage seminal vesicle invasion (i.e., pT3b) more often than mpMRI (90% vs. 76%), while mpMRI detected extracapsular extension (i.e., pT3a) better than $^{18}$F-PSMA-1007 (90% vs. 57%).

In this study, the pooled DRs of $^{18}$F-PSMA-1007 PET/CT in the BCR of prostate cancer were 82% (95% CI: 74%–88%) (per patient) and 78% (95% CI: 33%–100%) (per lesion), respectively. Although the combined DR of the two was not statistically different, the confidence intervals based on the lesion were large, so the reliability of the combined results might be slightly less. In addition, we performed a subgroup analysis of serum PSA in patient-based studies. However, due to the limited amount of data, only two studies (15, 16) were included in the analysis. The

pooled DRs of PSA levels >2.0, 1.1–2.0, 0.51–1.0, and ≤0.5 ng/ml detected by $^{18}$F-PSMA-1007 PET/CT in the BCR of PCa patients were 97%, 95%, 79%, and 68%, respectively. In the meta-analysis of Treglia et al. (38), the authors found the DR of $^{18}$F-PSMA PET/CT in the BCR of PCa patients with PSA ≥0.5 ng/ml (pooled DR: 86%; 95% CI: 78%–93%) compared to patients with PSA <0.5 ng/ml (pooled DR: 49%; 95% CI: 23%–74%). Therefore, the accurate timing of $^{18}$F-PSMA PET/CT, based on PSA values, substantially affects its diagnostic value in the BCR of PCa patients, and monitoring of PSA values could be useful for accurate timing of $^{18}$F-PSMA PET/CT (38). Eiber et al. (43) demonstrated that, as with other PET tracers, the detection rate of PSMA PET/CT increases with the blood level of PSA, showing a detection rate >95% in patients with PSA ≥2 ng/ml. Although only two studies were included in our analysis, the results obtained also showed that $^{18}$F-PSMA-1007 PET/CT was also better detected in prostate cancer with increased serum PSA levels. This conclusion is consistent with other studies (15, 16, 38, 43).

PSA kinetics has been proposed to supplement other diagnostic modalities in patient selection, especially with low PSA (44). In a 2019 meta-analysis, Pereira Mestre et al. (45) used different PSA doubling times (PSAdt) to assess the DR of PSMA-PET in the biochemical recurrence of prostate cancer. Their results showed that the pooled DR of PSMA-PET in restaging prostate cancer patients was 72%, increasing to 83% when PSAdt was ≤6 months and decreasing to 60% when PSAdt was >6 months. Therefore, they concluded that PSA kinetics, and in particular shorter PSAdt (≤6 months), may be a predictor of PSMA-PET positivity in patients with biochemically recurrent prostate cancer.

There were three studies simultaneously comparing the application of $^{18}$F-PSMA-1007 and $^{68}$Ga-PSMA-11 PET/CT in the primary stage (21) and biochemical recurrence (11, 37) of prostate cancer. However, data from only two studies (11, 37) could be included in the meta-analysis. The pooled DRs of $^{68}$Ga-PSMA-11 PET/CT in PCa were 47% in a patient-based analysis and 89% in a lesion-based analysis, respectively. In a network meta-analysis of the diagnostic performance of radiotracers in recurrent PCa, the results showed a higher DR $^{18}$F-PSMA-1007 than $^{68}$Ga-PSMA and $^{18}$F-DCFPyl with a surface under the cumulative ranking curve of 0.9997 (40). The authors stated their result with caution because only one study (33) was analyzed. Kuten et al. (21) performed a head-to-head comparison of the findings of $^{18}$F-PSMA-1007 PET/CT and $^{68}$Ga-PSMA-11 PET/CT in the same patients presenting with newly diagnosed intermediate- or high-risk PCa using histopathology and immunohistochemical staining as reference standards. They showed that both $^{18}$F-PSMA-1007 and $^{68}$Ga-PSMA-11 identify all dominant prostatic lesions in patients with intermediate- and high-risk PCa at staging. However, $^{18}$F-PSMA-1007 may detect additional low-grade lesions of limited clinical relevance. Morawitz et al. (37) compared the PSMA PET/CT and CT alone for the detection of biochemical recurrence of PCa and their effect on treatment. They found that both $^{68}$Ga- and $^{18}$F-PSMA PET/CT performed significantly better than CT alone, with almost equivalent *P*-values, suggesting that the diagnostic performances of both tracers are similar. Rauscher et al. (11) showed that the sensitivity of $^{18}$F-PSMA-1007 PET/CT was significantly higher than that of $^{68}$Ga-PSMA-11. However, both had the same detection rate for recurrent prostate cancer in patient-based studies. Researchers found that PET/CT with $^{18}$F-PSMA-1007 detected recurrent lesions more accurately closer to the bladder wall. There was a slightly higher DR for $^{18}$F-PSMA-1007 at low PSA levels, possibly due to the different energy distributions of $^{18}$F and $^{68}$Ga positron emitters (16). Theoretically, the resolution of $^{18}$F is higher than that of $^{68}$GA, especially in human PET systems (46). Therefore, it could be hypothesized that $^{18}$F-labeled PSMA ligands might improve the detection sensitivity for very small tumors (16).

Heterogeneity between studies may represent a potential source of bias in meta-analyses (47). Diversity of patient characteristics, differences in methodology, and overall quality of the study may all be sources of heterogeneity. In our meta-analysis, there was a significant difference between studies ($I^2$ > 50%), so the random effects model was used to combine effect sizes. To reduce possible sources of heterogeneity, subgroup analyses were performed according to different serum PSA levels, imaging agents, and imaging devices. Publication bias is a major issue in all meta-analyses, as studies reporting significantly positive results are more likely to be published than studies reporting negative results (48). Indeed, it is not uncommon for small-scale early studies to report a positive relationship that subsequent large studies cannot replicate (47). In our meta-analysis, funnel plot and Egger's test were used to evaluate publication bias. The funnel plot shows the symmetry of the pooled DR, indicating that there was no publication bias based on the patient and lesion analyses, as confirmed by the results of Egger's test. In addition, we used the QUADAS-2 tool to evaluate the included studies and found that most were of medium to high quality.

It is important to note that our study has some limitations. First, the DRs of $^{18}$F-PSMA-1007 and $^{68}$Ga-PSMA-11 in prostate cancer have only been compared and analyzed in two studies simultaneously, so the combined results need to be interpreted cautiously. Second, in the primary prostate stage group, all included studies had serum PSA >2 ng/ml and did not evaluate the use of $^{18}$F-PSMA-1007 PET/CT in PSA ≤2 mg/ml. Third, although some of the positive lesions detected by $^{18}$F-PSMA-1007 PET/CT were considered as biochemical recurrence, those lesions were merely clinically monitored rather than pathologically confirmed. Hence, false positives were not able to be ruled out. Lastly, the study results were heterogeneous. A subgroup analysis was carried out to reduce heterogeneity, but heterogeneity was present across subgroups.

This may be related to differences in the study population, methods, quality, and the general lack of appropriate reference criteria. In the future, these shortcomings need to be addressed through large-scale, high-quality, and better-reported studies.

## 5 Conclusion

This meta-analysis concluded that $^{18}$F-PSMA-1007 PET/CT had a high application value for prostate cancer, including primary tumors and biochemical recurrence. The DR of $^{18}$F-PSMA-1007 PET/CT was slightly higher in primary prostate tumors than in biochemical recurrence. Our study found that the DR of the $^{18}$F-PSMA-1007 PET/CT was also improved with increasing serum PSA levels.

## Data availability statement

The original contributions presented in the study are included in the article/**Supplementary material**. Further inquiries can be directed to the corresponding author.

## Author contributions

XL, WBZ, and TJ contributed to the conception and design of the study. XL and YS organized the database. CLG performed the statistical analysis. XL wrote the first draft of the manuscript. XL, TJ, CLG, and HTL wrote sections of the manuscript. All authors contributed to the manuscript revision and have read and approved the submitted version.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.911146/full#supplementary-material

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REVIEW
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# Neoadjuvant Treatment in Muscle-Invasive Bladder Cancer: From the Beginning to the Latest Developments

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Urothelial carcinoma of the bladder is one of the most prevalent cancers worldwide, diagnosed as muscle invasive in 25% of cases. Although several studies have demonstrated an overall 5% absolute survival benefit at 5 years with cisplatin-based combination neoadjuvant treatment, administration of chemotherapy prior to radical cystectomy (RC) in muscle-invasive bladder cancer (MIBC) patients is still a matter of debate. This may be due to the perceived modest survival benefit, cisplatin-based chemotherapy ineligibility, or fear of delaying potentially curative surgery in non-responders. However, immunotherapy and novel targeted therapies have shown to prolong survival in advanced disease and are under investigation in the neoadjuvant and adjuvant settings to reduce systemic relapse and improve cure rates. Genomic characterization of MIBC could help select the most effective chemotherapeutic regimen for the individual patient. Large cohort studies on neoadjuvant treatments with immune checkpoint inhibitors (ICIs) and molecular therapies, alone or combined with chemotherapy, are ongoing. In this review, we trace the development of neoadjuvant therapy in MIBC and explore recent advances that may soon change clinical practice.

**Keywords: muscle-invasive bladder cancer, neoadjuvant chemotherapy, immunotherapy, combined therapy, biomarkers, molecular subtypes**

## INTRODUCTION

Bladder cancer (BC) accounts for almost 600,000 new cases and over 200,000 deaths worldwide (1). Muscle-invasive bladder cancer (MIBC) constitutes 25% of newly diagnosed BC cases (2), and in approximately 50% of these patients treated with radical cystectomy (RC), the disease recurs within two years (3). To date, cisplatin-based neoadjuvant chemotherapy (NAC) is the standard of care for MIBC and is associated with a 5% absolute survival benefit at 5 years and a 14% relative risk

reduction for death (4). Chemotherapy prior to RC has long been a matter of debate. Although administration of NAC for MIBC has increased over the years, it still does not meet actual needs (5), particularly in cT2 BC for which it is currently recommended in clinical guidelines (6, 7). Multidisciplinary management is of paramount importance in this disease setting. Indeed, with the development of new cytotoxic and targeted therapies, and specifically immune checkpoint inhibitors (ICIs), large ongoing prospective studies have been designed to test their efficacy either alone or in combination in the neoadjuvant setting. Furthermore, identification of biomarkers, such as molecular phenotype and DNA damage repair, appears to predict response to cisplatin-based NAC. In this article, we review data in support of chemotherapy, molecular therapy and immunotherapy in early-stage MIBC, and discuss the impact of molecular biology in clinical practice.

## METHODS

From October 2021 to February 2022, we searched PubMed database for studies containing the keywords "neoadjuvant chemotherapy", "muscle-invasive bladder cancer", "neoadjuvant immunotherapy", "biomarkers of response", and "neoadjuvant combination therapy". Several results were analyzed for review; all studies involved MIBC patients who were candidates for surgery upfront or after neoadjuvant therapy. We also searched the clinical trial.gov database for all phase II/III "active" or "active, not recruiting" studies on neoadjuvant therapy for MIBC.

## NEOADJUVANT CHEMOTHERAPY IN MIBC

Cisplatin-based NAC is the treatment recommended by the National Comprehensive Cancer Network (NCCN) and the European Association of Urology (EAU) for patients with MIBC (cT2-4a or positive lymph nodes, N1) and fit for cisplatin (6, 7). Compared with RC alone, neoadjuvant cisplatin-based combination chemotherapy has improved overall survival (OS) and lowered the risk of recurrence. The clinical benefits of NAC in MIBC have been highlighted by several randomized phase III studies, although the ideal NAC regimen has not yet been established (8–10). Cisplatin-based NAC was first tested in the 1980s as a potential treatment strategy for MIBC. NAC based on methotrexate, vinblastine, doxorubicin and cisplatin (MVAC) was administered to 30 MIBC patients treated with RC, achieving a 33% pathologic complete response (pCR) and 17% disease downstaging to <pT2N0 (11).

A combined analysis of two separate trials with similar patient populations showed an 8% improvement in the 5-year OS rate with NAC (56%) compared with the control group (48%), and a 20% reduction in the relative probability of death (9). Regarding local radical treatment alone versus neoadjuvant cisplatin, methotrexate and vinblastine (CMV), an international multicenter study (BA06 30894 trial) demonstrated on first analysis a non-significant 15% reduction in the risk of death with neoadjuvant CMV (8). Updated results revealed a statistically significant 16% reduction in the risk of death and a 6% increase in the 10-year survival rate with neoadjuvant CMV compared to the control group (12). Further meta-analyses assessing the clinical benefits of NAC confirmed a 5% improvement of OS in MIBC (13–15).

In the SWOG trial, MVAC-based NAC was tested against surgery alone in 317 patients with cT2-4aN0M0 BC. Median OS was 77 months in the NAC group and 46 months in the surgery alone group, while 5-year survival rate was 57% and 43%, respectively (10).

Two small, single-arm phase II trials investigated a modified MVAC regimen consisting of dose dense MVAC (dd-MVAC) with granulocyte colony-stimulating factor (G-CSF) support, evaluating NAC efficacy and safety in cT2-4N0 BC (16, 17). Of 39 patients, 49% achieved pathologic response, defined as downstaging to ≤pT1N0M0, with 10% showing grade 3 or higher treatment-related toxicities (16). Likewise, 38% pCR (pT0) rates and 52% downstaging to non-muscle-invasive disease (NMIBC) were observed by Plimack et al., with the majority of patients (82%) experiencing only grade 1–2 treatment-related toxicities (17).

The combination of gemcitabine and cisplatin (GC) is another regimen utilized in the neoadjuvant setting, showing similar OS, progression-free survival (PFS), and downstaging to pT0/pT1, but lower toxicity when compared to conventional MVAC (18–23). A randomized phase III trial assessed the efficacy of neoadjuvant treatment with GC and dd-MVAC in 537 patients (24). Overall, pCR was seen in 36% and 42% of GC and dd-MVAC patients (p=0.2), while downstaging to organ-confined disease (<ypT3pN0) was achieved in 63% and 77% (p=0.001), respectively. Grade 3 or higher hematologic toxicities were similar, 55% in the GC group and 52% in the dd-MVAC group. Contrariwise, grade 3 or higher gastrointestinal toxicities (p=0.003) and asthenia (p<0.001) were more frequent in the dd-MVAC arm. Results of NAC trials in MIBC are summarized in **Table 1**.

### Cystectomy and Lymphadenectomy in Patients Treated With Neoadjuvant Therapy

Surgery is the standard approach for patients with MIBC or refractory NMIBC. Selection of MIBC patients as candidates for NAC requires careful consideration. It has recently emerged that impaired nutritional status due to neoadjuvant therapy is a key factor. In a study led by Cohen et al., variations in nutritional status were assessed by changes in smooth muscle index (SMI), calculated through cross-sectional imaging of psoas muscle area (25). These authors reported that SMI decline after neoadjuvant therapy was significantly associated with the risk of post-RC complications, including ileus and infections.

With the intent of determining the outcome of patients subjected to RC following NAC, Mir et al. developed and internally validated a nomogram predicting BC-specific mortality (BCSM) in MIBC patients (26). At multivariate analysis, lymph node metastasis (hazard ratio [HR] 1.90, 95% CI: 1.4-2.6), positive surgical margins (HR 2.01, 95% CI: 1.3-2.9)

**TABLE 1** | Main clinical trials of neoadjuvant cisplatin-based chemotherapy for MIBC.

| Trial (ref.) | Phase | N of patients | Regimen | Duration of NAC, weeks | pCR (pT0N0) rates, % | Downstaging (<pT2), % |
|---|---|---|---|---|---|---|
| **SWOG-8710** (10) | III | 317 | MVAC | 14 | 38 | 44 |
| **BA0630894** (8) | III | 976 | CMV | NA | NA | NA |
| **Choueiri et al.** (16) | II | 651 | dd-MVAC | 8 | 26 | 49 |
| **Plimack et al.** (17) | II | 44 | dd-MVAC | 6 | 38 | 53 |
| **Iyer et al.** (18) | Retrospective | 154 | GC | 12 | 21 | 46 |
| **Dash et al.** (20) | Retrospective | 42 | GC | 12 | 26 | 36 |

*Neoadjuvant chemotherapy (NAC), muscle-invasive Bladder cancer (MIBC), gemcitabine-cisplatin (GC), number (N), pathologic complete response (pCR), dose dense methotrexate-vinblastine-doxorubicin-cisplatin (dd-MVAC), cisplatin, methotrexate, and vinblastine (CMV).*

and pathologic stage ypT3-4 (HR 5.9, 95% CI: 3.8-9.3) were correlated with reduced BCSM, thus suggesting the potential use of this nomogram to identify patients eligible for adjuvant approaches or personalized follow-up.

Pre-surgical evaluation through [18F] Fluoro-Deoxy-Glucose Positron Emission Tomography (FDG-PET) is reserved for patients with suspected lymph node involvement at computed tomography (CT) scan. In patients receiving neoadjuvant anti-programmed cell death-1 (PD-1) immunotherapy by pembrolizumab, the sensitivity and specificity of PET/CT to predict lymph node metastasis was investigated before and after treatment (27). In this study, 4 of 7 patients (57%) with baseline FDG-uptake showed pathologic lymph node involvement versus 11 of 101 (11%) with no baseline FDG-uptake. Six of the 7 patients responded to neoadjuvant pembrolizumab, implying the necessity to further investigate and validate the use of PET/CT to determine those MIBC patients who are better candidates for neoadjuvant immunotherapy. Briganti et al. were the first to demonstrate the surgical safety of RC and pelvic lymph node dissection (PLND) in non-metastatic MIBC patients receiving neoadjuvant therapy with checkpoint inhibitors (28). They found that 77% and 34% of patients experienced any-grade and high-grade complications, respectively. The most frequent complications were fever (52%) and ileus (31%), with no perioperative mortality cases observed at 90 days.

According to the EAU guidelines, the high specificity of DWI-MRI seems to accurately predict pCR and allow better patient selection for bladder-sparing protocols (7). Pre-operative MRI in different settings may therefore provide useful information regarding treatment response.

## Predictive Biomarkers of Response in Cisplatin-Based Chemotherapy

Cisplatin-based chemotherapy remains the standard treatment for advanced disease and perioperative (neoadjuvant) treatment of BC (29). Cisplatin crosslinks DNA in different ways, mainly forming adducts that prevent cell replication and induce cell death. DNA damage can manifest as single-strand breaks (SSBs), double-strand breaks (DSBs) or interstrand-crosslinks (30). Cancer cells rely on various mechanisms to repair DNA damage: excision repair, mismatch repair (MMR) or nucleotide excision repair (NER) for SSBs, while non-homologous end joining or homologous recombination (HR) can correct DSBs.

There are several reports on the genes involved in DNA damage repair (DDR) pathways, highlighting their predictive role as biomarkers of response to cisplatin (31) (**Table 2**). A panel of 34 DDR genes was analyzed in a study enrolling 100 advanced BC patients treated with platinum-based chemotherapy. Overall, 47 patients had at least one alteration, and median OS was significantly higher in these patients than in those without (23.7 vs. 13.0 months, p=0.006). A recent phase II trial, investigating a panel of 29 DDR genes in 49 patients administered neoadjuvant dose-dense GC, showed a greater response to chemotherapy, with a positive predictive value of 89% and a 2-year relapse-free survival of 100%, in patients with deleterious mutations (39).

Excision repair 1 and 2 (ERCC1 and ERCC2) proteins, belonging to the NER pathway, have been correlated with cisplatin-based chemotherapy response. High ERCC1 expression has been associated with gain of NER pathway function that leads to increased DNA repair capacity and platinum resistance (40, 41). In preclinical studies, ERCC2 mutations have been linked to loss of NER pathway function that confers sensitivity to cisplatin and carboplatin, but not to doxorubicin and ionizing radiation or poly (ADP-ribose) polymerase (PARP) inhibitors (42). Van Allen et al. detected ERCC2 mutations in 36% of patients who responded effectively to chemotherapy (<ypT1) but not in non-responders (>ypT2) (32). Further studies reported ERCC2 mutations in 38% (17/45) of responders and in only 6% (3/53) of non-responders (30). Recently, ERCC2 mutations were observed more frequently in

**TABLE 2** | Association between biomarkers and response to NAC in MIBC.

| Biomarker | ERCC2 mutation | ERCC2 mutation | ATM/RB1/FANCC mutations | ERBB2 mutations | DDR gene alterations | High expression ERCC1 | BRCA1 mutation |
|---|---|---|---|---|---|---|---|
| **Number of patients** | 50 | 48+54 | 34 | 71 | 34 | 57 | 57 |
| **Response to cisplatin-based NAC** | Increased pathologic response | Improved OS | Improved pT<2 response and OS | Increased pT0 response | Increased pT0/ pTis response | Association with worse prognosis | Negative correlation with pCR and OS |
| **Reference** | (32) | (33, 34) | (33) | (35) | (36) | (37) | (38) |

*Neoadjuvant chemotherapy (NAC), muscle-invasive bladder cancer (MIBC), excision repair (ERCC), breast cancer gene (BRCA), ATM serine/threonine kinase (ATM), RB transcriptional corepressor 1 (RB1), FA complementation group C (FANCC).*

primary than in secondary MIBC (12% vs. 1.2%), and patients with primary MIBC attained higher pathologic response rates following NAC (42).

Efficacy of NAC in MIBC has also been related to mutations in the ATM serine/threonine kinase (ATM), RB transcriptional corepressor 1 (RB1) and FA complementation group C (FANCC) repair genes. Plimack et al. detected genomic alterations in these genes in 13 of 15 cisplatin-responders (87%), while none of the non-responders harbored these mutations (33). A recent update of this study revealed a statistically significant improved 5-year disease-specific survival in carriers of at least one mutation compared to patients with no mutation (90% vs. 49%, p=0.0015) (43). The phase II RETAIN trial is currently evaluating bladder preservation in patients with ATM, RB1, FANCC or ERCC2 mutations who have achieved complete response with NAC (44). The presence of DDR genomic alterations could well identify those patients likely to respond to NAC and benefit from a bladder-sparing approach.

Breast cancer type 1 and 2 (BRCA1 and BRCA2) are among frequently mutated homologous recombination (HR) genes in urothelial carcinoma (45). According to Font et al., increased BRCA1 mRNA expression is negatively associated with pathologic response and survival in MIBC patients receiving NAC (38).

Current evidence indicates that alterations in DNA repair pathways can provide prognostic and predictive information in cisplatin-treated BC patients. Prospective studies including a large number of patients are needed to confirm these findings, which could pave the way for novel treatments, such as PARP inhibitors in HR-deficient cancers (46).

## NEOADJUVANT IMMUNOTHERAPY IN MIBC

Lately, immunotherapy has become an integral part of advanced and metastatic BC treatment (47–56). Between 2016 and 2017, monoclonal antibodies against the negative immunoregulatory human cell surface receptor PD-1 (nivolumab and pembrolizumab), and its ligand programmed death ligan 1 (PD-L1) (atezolizumab, avelumab and durvalumab) have been approved for metastatic urothelial cancer by the United States Food and Drug Administration (FDA). Owing to their clinical benefits in the metastatic setting, several ICIs are being investigated in neoadjuvant (**Table 3**) and adjuvant settings (65).

### ICIs as Single Agents

In two single-arm phase II trials, pembrolizumab and atezolizumab have been tested in the neoadjuvant setting. The PURE-01 trial assessed the activity of pembrolizumab (200 mg every 3 weeks) for three cycles as neoadjuvant treatment before RC in patients with cT2-3bN0 MIBC and predominant urothelial cancer histology (57). Of these patients, 92% were eligible for cisplatin. Neoadjuvant pembrolizumab yielded 42% pCR and 54% downstaging to NMIBC. In addition, pCR was recorded in 54.3% of patients with PD-L1 combined positive score (CPS) ≥10 and in 13.3% of patients with PD-L1 CPS <10. High-grade complications, defined according to the Clavien-Dindo classification, were observed in 34% of patients, with no perioperative mortality at 90 days (7). Pembrolizumab response was maintained after cystectomy in most patients, with 1- and 2-year event-free survival (EFS) rates of 84.5% and 71.7%, respectively (58). A statistically significant longer EFS was found in patients with complete response and high PD-L1 CPS.

The ABACUS trial investigated the efficacy and safety of two cycles of neoadjuvant atezolizumab (1200 mg every 3 weeks) prior to RC for MIBC (59). Contrary to the PURE-01 trial, all patients were ineligible for or refused cisplatin-based NAC. The rates of pCR and downstaging to NMIBC were 31% and 39%, respectively. Treatment-related grade 3-4 toxicities occurred in 12% of patients, and grade 3 or 4 surgical complications in 31% of cases.

### ICIs as Combination Therapy

ICI combination has proved promising in different settings and types of cancer (66). Indeed, combined anti-PD-1 and anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) blockade prompts complementary mechanisms of therapeutic checkpoint inhibition, leading to greater antitumor activity than *via* a single pathway (67–69).

In the NABUCCO study, 24 patients with stage III urothelial cancer were administered 3 mg/kg ipilimumab (day 1), 1 mg/kg nivolumab plus 3 mg/kg ipilimumab (day 22), and 3 mg/kg nivolumab (day 43) in the neoadjuvant setting (60). The primary endpoint was feasibility to resect within 12 weeks from start of treatment. A total of 23 (96%) patients underwent surgery within 12 weeks, and grade 3-4 immune-related adverse events (iAEs) manifested in 55% of cases. Furthermore, 46% of patients showed pCR, and 58% had no remaining invasive disease (pCR or pTisN0/pTaN0).

**TABLE 3** | Main clinical trials of neoadjuvant immunotherapy for MIBC.

| Trial (ref.) | Phase | N of patients | Regimen | Cycles of NAC | pCR (pT0N0) rates, % | Downstaging (<pT2), % |
|---|---|---|---|---|---|---|
| **PURE-01** (57, 58) | II | 114 | Pembrolizumab | 3 | 39 | 56 |
| **ABACUS** (59) | II | 95 | Atezolizumab | 2 | 31 | 39 |
| **NABUCCO** (60) | I | 24 | Ipilimumab/nivolumab | 2 | 46 | 58 |
| **DUTRENEO** (61) | II | 61 | Durvalumab/tremelimumab | 3 | 35 | 57 |
| **BLASST-1** (62) | II | 41 | Nivolumab + GC | 4 | 49 | 66 |
| **HCRN GU14-188** (63, 64) | Ib/II | 12/70 | Pem + GC (cohort 1) | 4 | 44 | 61 |
| | | | Pem + Gem (cohort 2) | | 54 | 52 |

*Neoadjuvant chemotherapy (NAC), number (N), muscle-invasive bladder cancer (MIBC), gemcitabine-cisplatin (GC), pathologic complete response (pCR), pembrolizumab (Pem), gemcitabine (Gem).*

Another randomized phase II trial (DUTRENEO) compared neoadjuvant durvalumab plus tremelimumab versus chemotherapy in cisplatin-eligible patients with cT2-4aN0-1 BC, classified as immunologically "hot" or "cold" according to the tumor immune score devised by NanoString Technologies (61). Patients with "hot" tumors were randomized to three cycles of durvalumab 1500 mg plus tremelimumab 75 mg every 4 weeks or standard cisplatin-based NAC, while patients in the "cold" arm received cisplatin-based NAC. In the "hot" arm, pCR was recorded in 36.4% of patients treated with NAC and in 34.8% of patients receiving durvalumab/tremelimumab. In the "cold" arm, as many as 68.8% of patients achieved pCR. Grade 3-4 toxicities occurred more frequently in the NAC group.

## ICIs and Chemotherapy

Conventional chemotherapy can elicit a tumor-specific immune response by inducing immunogenic cell death of neoplastic cells or engaging immune effector mechanisms (70). The combination of chemotherapy with immunotherapy has been extensively investigated. A phase II, single-arm trial, BLASST-1, examined the efficacy and safety of neoadjuvant nivolumab with GC for MIBC (cT2-T4aN ≤ 1M0) (62). Patients received four cycles of GC with nivolumab every 21 days, followed by RC within 8 weeks. Pathologic response (≤pT1N0) was observed in 65.8% of patients, including those presenting N1 disease. Safety profile was favorable, with 20% of grade 3-4 AEs mainly due to GC.

In patients with operable MIBC (cT2-4aN0-1), the open-label single-arm phase II trial, SAKK 06/17, tested neoadjuvant durvalumab plus GC (4 cycles every 21 days) followed by durvalumab monotherapy (10 cycles every 28 days) after surgery. Pathologic response was observed in 60% of patients, with 18 (34%) achieving pCR. Treatment demonstrated acceptable safety, and data regarding the primary endpoint, i.e. event-free survival (EFS) at 2 years, are awaited (71). Another multicenter, single-arm phase II trial enrolled eligible patients with MIBC (cT2-4aN0M0) to receive a dose of atezolizumab, followed 2 weeks later by GC plus atezolizumab every 21 days for 4 cycles, and after a further 3 weeks by a dose of atezolizumab prior to RC. The primary endpoint, downstaging to < pT2N0, was met in 27 (69%) patients including 16 (41%) pT0N0, all of whom experienced improved relapse-free survival. Grade 3 iAEs occurred in 5 (11%) patients with 2 (5%) requiring systemic steroids (72).

Efficacy and tolerability of neoadjuvant pembrolizumab and GC were assessed in a phase I/II trial, HCRN GU14-188, where patients with MIBC (cT2-4aN0M0) were subdivided into two cohorts: cisplatin-eligible (cohort 1) and cisplatin-ineligible (cohort 2) (63, 64). In cohort 1, pathologic response (≤pT1N0) and pCR were seen in 61.1% and 44.4% of patients, respectively. Median time from last dose to RC was 5.3 weeks; the 36-month relapse-free survival and OS were 63% and 82%, respectively. One death from mesenteric ischemia was recorded.

Phase III trials of neoadjuvant immunotherapy, comprising nivolumab, pembrolizumab and toripalimab, in combination with cisplatin-based chemotherapy are ongoing, and results are eagerly awaited (NCT03732677, NCT03661320, NCT03924856, NCT04861584). Several trials are also evaluating immunotherapy with non-cisplatin-based chemotherapy, including nab-paclitaxel and gemcitabine as neoadjuvant treatment. Among these, tislelizumab (BGB-A317), a humanized monoclonal antibody against PD-1, is being tested with nab-paclitaxel in MIBC (NCT04730219) (**Table 4**).

## ICIs and Antibody-Drug Conjugates

Antibody-drug conjugates (ADCs), i.e. enfortumab vedotin and sacituzumab govitecan, are complex engineered therapeutics consisting of monoclonal antibodies directed toward tumor-associated antigens, to which highly potent cytotoxic agents are attached by chemical linkers (73). Enfortumab vedotin, a fully human monoclonal antibody conjugated to a clinically validated microtubule-disrupting agent, has shown encouraging results. Accordingly, the FDA has granted its accelerated approval in patients with locally advanced or metastatic urothelial carcinoma, formerly treated with PD-1/PD-L1 inhibitors and platinum-containing chemotherapy (74). At the 2022 American Society of Clinical Oncology (ASCO) meeting, Petrylak et al. presented the EV-103 phase Ib/II study evaluating antitumor activity of neoadjuvant treatment with enfortumab vedotin monotherapy in cisplatin-ineligible MIBC patients (75). Two randomized phase III trials are currently comparing perioperative enfortumab vedotin plus pembrolizumab with chemotherapy in cisplatin-eligible patients (NCT04700124) and with cystectomy alone in cisplatin-ineligible patients (NCT03924895). Sacituzumab govitecan is a humanized anti-trophoblast surface antigen 2 (Trop-2) antibody conjugated with SN-38, the active metabolite of irinotecan (76). The FDA has recently approved sacituzumab govitecan for patients with locally advanced or metastatic BC, previously administered platinum-based chemotherapy and PD-1 or PD-L1 inhibitors. At the 2021 GU ASCO Annual Meeting, Necchi et al. presented the design for the SURE trial assessing the efficacy of neoadjuvant sacituzumab govitecan, either as a single-agent (SURE-01) or combined with pembrolizumab (SURE-02), prior to RC in MIBC patients unfit for or refusing cisplatin-based chemotherapy (77).

## ICIs and Emerging Agents

A single-arm, phase II trial (NEODURVARIB) explored the impact of neoadjuvant durvalumab plus olaparib, a poly ADP-ribose polymerase inhibitor, in cT2-4N0 urothelial carcinoma (78). Patients received durvalumab 1500 mg every 4 weeks for a 2-month maximum (up to 2 doses/cycle) plus olaparib 300 mg for up to 56 days (2 cycles of 28 days each cycle). The pCR rate was 44.5% and grade 3-4 AEs occurred in 8.3% of patients, with one death related to postoperative complications. In the ongoing ABATE trial, the efficacy and safety of cabozantinib (a multikinase inhibitor of c-MET, AXL and VEGFR2) plus atezolizumab is being tested as neoadjuvant therapy for cT2-T4N0M0 BC patients who are either ineligible for or decline cisplatin (79).

# PREDICTIVE BIOMARKERS OF IMMUNOTHERAPY RESPONSE

With the emergence of immunotherapy, attempts have been made to identify biomarkers to predict clinical response. To date,

**TABLE 4 |** Recruiting or active, not recruiting phase II and III clinical trials with neoadjuvant therapy for MIBC.

| Trial | Status | Phase | N of Patients | Neoadjuvant Treatment | Primary endpoint |
|---|---|---|---|---|---|
| **NCT04861584** | Recruiting | II/III | 41 | Toripalimab with gemcitabine and cisplatin | Pathologic RR evaluated after 4 cycles |
| **NCT04060459** | Recruiting | II | 50 | Paclitaxel-binding albumin + cisplatin | pCR (<pT0) |
| **NCT03472274** | Recruiting | II | 99 | Durvalumab + tremelimumab | Antitumor activity |
| **NCT03674424** | Recruiting | II/III | 166 | Avelumab± chemotherapy | pCR (ypT0/TisN0) |
| **NCT04700124** | Recruiting | III | 784 | Perioperative enfortumab vedotin plus pembrolizumab vs. NAC | pCR<br>EFS |
| **NCT04730219** | Recruiting | II | 69 | Tislelizumab with nab-paclitaxel | pCR |
| **NCT04543110** | Recruiting | II | 25 | Radiation + durvalumab | pCR |
| **NCT02690558** | Active, not recruiting | II | 39 | Pembrolizumab with gemcitabine and cisplatin | Pathologic downstaging |
| **NCT03732677** | Active, not recruiting | III | 988 | Durvalumab + gemcitabine/cisplatin (neoadjuvant treatment) and durvalumab (adjuvant treatment) | pCR<br>EFS |
| **NCT04209114** | Recruiting | III | 540 | Nivolumab plus bempegaldesleukin vs. nivolumab alone vs. standard of care cisplatin ineligible | pCR<br>EFS |
| **NCT02736266** | Recruiting | II | 90 | Pembrolizumab | pCR |
| **NCT04430036** | Recruiting | II | 42 | AGEN1884 +AGEN2034 with cisplatin-gemcitabine | Pathologic tumor downstaging of >T2 to pT0 |
| **NCT03924856** | Recruiting | III | 870 | Perioperative pembrolizumab + NAC vs. perioperative placebo + NAC | pCR<br>EFS |
| **NCT03294304** | Active, not recruiting | II | 43 | Nivolumab + gemcitabine and cisplatin | PaR |
| **NCT03558087** | Active, not recruiting | II | 76 | Gemcitabine and cisplatin + nivolumab | Clinical CR rate (cT0-Ta) |
| **NCT04289779** | Recruiting | II | 42 | Cabozantinib with atezolizumab | pRR |
| **NCT04047693** | Recruiting | II | 32 | Dose dense MVAC in MIBC and locally advanced urothelial carcinoma | pCR |
| **NCT02365766** | Active, not recruiting | I/II | 83 | Neoadjuvant pembrolizumab with gemcitabine | Rate of pathologic muscle invasive response |
| **NCT04383743** | Recruiting | II | 17 | Pembrolizumab and chemotherapy | pCR |
| **NCT02451423** | Recruiting | II | 42 | Atezolizumab | Change in CD3+ T cell count/μm2 in multi-dose cohorts;<br>pathologic T0 rate in expansion cohorts |
| **NCT03061630** | Recruiting | II | 48 | Chemotherapy with gemcitabine/platinum | PaR |
| **NCT03768570** | Recruiting | II | 238 | Trimodality therapy with/out durvalumab | DFS |
| **NCT00777491** | Active, not recruiting | II | 70 | Chemotherapy and radiation therapy in stage II-III BC | Percentage of patients without distant metastases at 3 years |
| **NCT02845323** | Recruiting | II | 44 | Nivolumab ± urelumab in cisplatin-ineligible or chemotherapy-refusing patients | Immune response measured by tumor infiltrating CD8+ T cell density at cystectomy |

*Muscle invasive bladder cancer (MIBC), number (N), response rate (RR), pathologic complete response (pCR), event-free survival (EFS), pathologic response (PaR), disease-free survival (DFS), methotrexate-vinblastine-doxorubicin-cisplatin (MVAC).*

potential biomarkers such as PD-L1 expression, CD8+ T-cell infiltration, DDR gene alterations, tumor mutational burden (TMB) and immune and stromal gene expression signatures have been correlated with immunotherapy response (80, 81). Nevertheless, none of these markers has shown consistent findings to warrant incorporation into BC routine management.

Controversial results on the role of PD-L1 as a predictive biomarker have been reported (82). PD-L1 positivity, detected in 20-30% of bladder tumors, appears to correlate with more advanced disease and poor prognosis (83). However, PD-L1 expression may depend on biopsy site and previous treatments, but primarily on the assays to test PD-L1 status (i.e. Dako 22C3, Ventana SP142, and Dako 28.8) based on the different ICI used. It should therefore be interpreted in the context of a broader biomarker panel, including neutrophil/lymphocyte ratio, albumin levels, high C-reactive protein and interleukin-6 (IL-6) levels. These can be easily integrated into clinical practice, unlike other more complex biomarkers such as gene expression signatures (84).

Investigation into the DDR pathways could be valuable for establishing the potential utility of immunotherapy. In a study analyzing a panel of 34 DDR genes, patients with advanced BC and deleterious mutations in these genes significantly benefitted from immunotherapy compared to those without DDR alterations (85). Moreover, ICIs have recently been reported to be highly effective in tumors with defects in the MMR/microsatellite instability pathway (86).

In the early phases of BC, the integrity of the immune system seems to induce a greater T-cell expansion than in advanced stages, characterized by increased impairment of T-cell function and cancer-associated inflammation. As a consequence, immunotherapy efficacy with checkpoint inhibitors has been explored in early-stage disease (87).

Recently, Mariathasan et al. demonstrated that inflamed and desert immune phenotypes were associated with response and resistance to atezolizumab, respectively (88). In this study, CD8 levels were higher in responding tumors, while elevated levels of

fibroblast activation protein (FAP) were linked to immunotherapy resistance. In the PURE-01 study, PD-L1 expression, TMB, DDR and RB1 gene alterations were significantly related to pCR (57). Conversely, in the ABACUS trial, pCR correlated with granzyme B (GZMB) expression, a surrogate marker of activated CD8+ T cells (59).

Since several biomarkers, such as TMB and DDR alterations, are associated with the efficacy of both chemotherapy and immunotherapy, it may be difficult to select cisplatin-eligible patients and decide upon integration and sequencing of different therapeutic options in the multimodal management of MIBC (89, 90). Notably, cytotoxicity induced by NAC can elicit an immune effect by activating CD8+ T cells and decreasing Tregs (91). This would impede T-cell response when NAC and immunotherapy are administered concurrently, and partly explain the limited benefit of NAC plus immunotherapy compared with NAC alone in advanced urothelial cancer (92). NAC followed by immunotherapy could therefore be a more effective approach.

## POTENTIAL NEOADJUVANT AGENTS IN MIBC

Emerging neoadjuvant agents are illustrated in **Figure 1**. The genetic alteration of the fibroblast growth factor receptor (FGFR) pathway has been widely investigated in BC with subsequent approval of FGFR inhibitors in advanced and metastatic settings. Infigratinib (FGFR1-3-selective tyrosine kinase inhibitor) monotherapy is currently being tested as neoadjuvant treatment for locally advanced urothelial cancer (NCT0422804).

Bempegaldesleukin (BEMPEG/NKTR-214) is a PEGylated interleukin-2 (IL-2) designed to activate and proliferate CD8+ T cells and natural killer (NK) cells. An in-progress randomized study is comparing BEMPEG plus nivolumab with nivolumab alone for neoadjuvant and adjuvant treatment of cisplatin-ineligible resectable MIBC patients (NCT04209114).

Urelumab is a fully human IgG4 monoclonal antibody that targets the CD137 extracellular domain stimulating cytotoxic T cell responses against tumor cells. A trial assessing the efficacy of nivolumab monotherapy or combined with urelumab in cisplatin-ineligible or chemotherapy-refusing MIBC patients is ongoing (NCT02845323). Further trials are assessing novel agents, namely CD73 inhibitor (NCT03773666), replication-competent oncolytic adenovirus (NCT04610671) and synthetic benzamide-derivative histone deacetylase inhibitor (NCT03978624).

## RADIOTHERAPY IN MIBC

Neoadjuvant radiation should not be used in patients with MIBC prior to RC. Although preoperative radiotherapy, as a single modality, can eradicate disease in a small proportion of patients undergoing cystectomy, it seems to improve local control rather than survival when compared with RC alone (93). However, radiation can synergize with immunotherapy to improve clinical outcomes by causing immunogenic cell death and increasing expression of immune markers (94). Following this hypothesis, several trials, such as RADIANT (durvalumab + radiotherapy) (NCT04543110) and RACE IT (nivolumab + radiotherapy) (NCT03529890) prior to cystectomy in MIBC, are still active. The efficacy of chemotherapy and radiation therapy in stage II and III bladder carcinoma patients is also being tested (NCT00777491).

## BLADDER CANCER MOLECULAR SUBTYPES AND THERAPEUTIC IMPLICATIONS

Potential markers and gene expression models have been correlated to chemotherapy response in BC (32, 95, 96), but none have been approved for clinical practice as yet. However, new insights into BC molecular pathology could lead to a shift

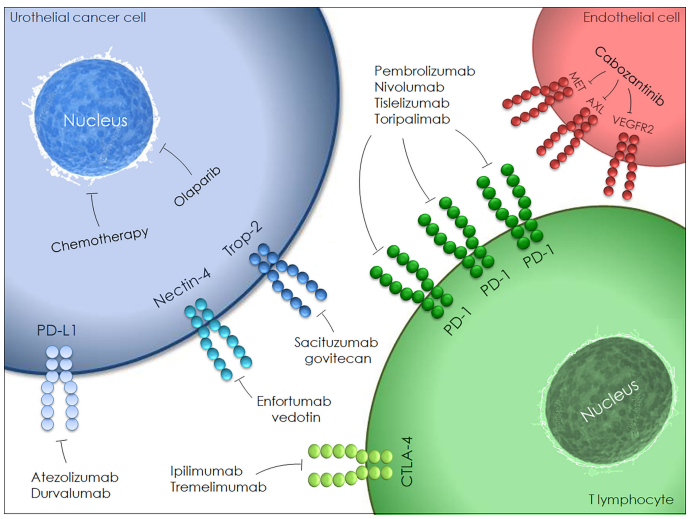


**FIGURE 1** | Emerging agents in the context of neoadjuvant setting for patients with MIBC.

toward individualized treatment and consequently better patient outcomes (**Table 5** and **Figure 2**).

Basal/squamous tumors, defined by the Cancer Genome Atlas (TCGA), University of North Carolina (UNC) and MD Anderson classifications (98, 99, 102), are associated with more advanced stages and worse prognosis, whereas luminal tumors appear to be less aggressive (37, 100). Patients with basal tumors seem to profit more from NAC than those with luminal tumors who derive little or no benefit. Irrespective of treatment strategy, luminal-papillary tumors bear the best prognosis, unlike luminal-infiltrated tumors that have an unfavorable prognosis regardless of NAC (103, 104).

Furthermore, these molecular classifications can balance standard histologic classifications burdened by intra- and intertumoral heterogeneity of primary MIBC, with relevant clinical implications. Compared with transcriptome analysis, immunohistochemistry (IHC) is a simpler and more accessible method to classify urothelial carcinoma into molecular subtypes, comprising basal (KRT5/6, KRT14, and p63) and luminal (GATA3, FOXA1, uroplakins and HER2) phenotypes.

To stratify BC patients, Makboul et al. utilized a simple IHC panel of five biomarkers, i.e. FGFR3, KRT5, cyclin B1, HER2 and p53 (105). The molecular classes identified were correlated with clinico-pathologic variables and patient survival: basal/squamous tumors showed the lowest OS (38.5%), while urobasal A (UroA) tumors, expressing luminal markers, had the best prognosis with OS of 75% and no metastatic events. In addition, Choi et al. found that basal tumors had a significantly higher response rate to cisplatin-based chemotherapy, and all chemoresistant tumors exhibited a p53-like phenotype (99).

A recent study by Font et al. stratified MIBC patients receiving NAC into three subgroups, i.e. basal/squamous (KRT5/6 and KRT14 high; FOXA1 and GATA3 low), luminal (FOXA1 and GATA3 high; KRT5/6 and KRT14 low) and mixed (FOXA1 and GATA3 high; KRT5/6 high and KRT14 low), using

**TABLE 5** | Subtypes of bladder carcinoma according to different molecular classifications.

| Classification | N of patients | Patients | Subtypes | Molecular characteristics |
|---|---|---|---|---|
| **Lund University (2012)** (97) | 308 | BC | Urobasal A<br>Urobasal B | High FGFR3, CCND1 and P63 expression |
| | | | Genomically unstable | TP53 mutations; high CCNE and ERBB2 expression; low cytokeratin expression |
| | | | Squamous cell carcinoma-like | High expression of basal keratins |
| | | | Infiltrated | Stromal and immune cell infiltration |
| **UNC (2014)** (98) | 262 | High grade MIBC | Luminal | Expression of E-cadherin/CDH1 and miR-200; FGFR3 alterations |
| | | | Basal | High EGFR expression |
| **MDA (2014)** (99) | 73 | MIBC | Luminal<br>Basal<br>P53-like | FGFR3 mutations<br>P63 activation<br>P53 signature activation |
| **TCGA (2012)** (97) | 131 | High grade MIBC | Cluster I<br>Cluster II | Luminal phenotype<br>Luminal phenotype with P53-like features |
| | | | Cluster III<br>Cluster IV | Corresponding to basal subtype of UNC and MD Anderson classifications |
| **TCGA (2017)** (100) | 412 | MIBC (T2-4, N0-3, M0-1) | Luminal-papillary | FOXA1, GATA3 and PPARG expression, FGFR3 alterations |
| | | | Luminal-infiltrated | Expression of FOXA1, GATA3, PPARG, EMT and immune markers |
| | | | Luminal | Expression of FOXA1, GATA3, PPARG, KRT20 |
| | | | Basal/squamous | CD44 and KRT5/6 expression; TP53 mutations |
| | | | Neuronal | Neuroendocrine and neuronal marker expression |
| **BCMTG (2020)** (101) | 1750 | MIBC | Luminal-papillary | FGFR3 and PPARG expression; FGFR3, ELF3 and KDM6A mutations |
| | | | Luminal non-specified | PPARG, E2F3 and ERBB2 expression; TP53 and ERCC2 mutations |
| | | | Luminal unstable | EGFR expression; TP53 and RB1 mutations |
| | | | Stroma-rich | Neuroendocrine differentiation; loss or mutations of TP53 and RB1 |

*Muscle invasive bladder cancer (MIBC), bladder cancer (BC), number (N), University of North Carolina (UNC), MD Anderson (MDA), The Cancer Genome Atlas (TCGA) (Bladder Cancer Molecular Taxonomy Group (BCMTG).*

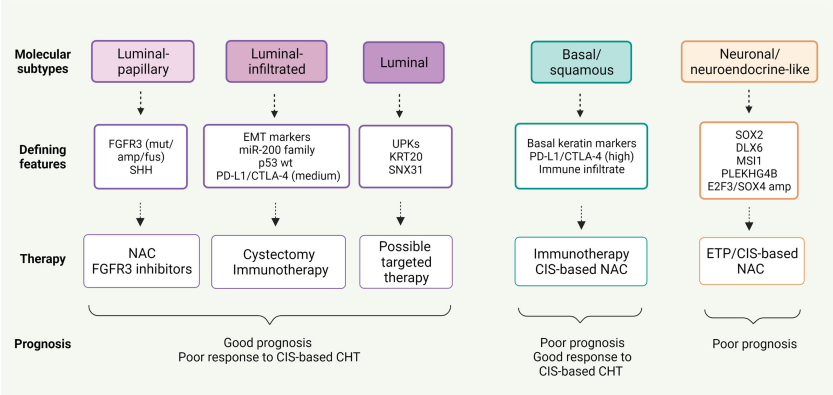


**FIGURE 2** | Correlation between five mRNA-based expression subtypes according to The Cancer Genome Atlas (TCGA) analysis and response to neoadjuvant therapy in MIBC.

IHC combined with hierarchical clustering analysis. Overall, pathologic response to NAC was significantly higher in patients with basal/squamous tumors (p=0.017) (106).

Through the use of transcriptome-wide gene expression and IHC, Seiler et al. categorized the residual tumor at cystectomy after NAC, and outlined the greatest potential benefit from second-line treatments, such as checkpoint inhibition, in tumors with high immune infiltration, elevated expression of immune-associated genes (i.e. CTLA4, MPEG1 and CD27) and low expression of basal or luminal markers (107).

Likewise, correlation between tumor subtypes and efficacy of immunotherapy has recently been explored. The revised TCGA classification suggested that patients with luminal-infiltrated tumors benefit most from immunotherapy (100). In the IMvigor 210 study, treatment with atezolizumab was most beneficial in advanced BC classified as TCGA cluster II (108), whereas basal tumors were more likely to respond to nivolumab in the CheckMate 275 study (49). Despite the high immune infiltration, response to immunotherapy was poor in claudin-low tumors, defined by biologic characteristics of the claudin-low subtype of breast cancer, probably due to more effective T cell suppression in cluster IV than cluster II tumors (109). IMvigor 210 trial also showed that survival advantage of atezolizumab was greater in TCGA neuronal-subtype tumors, without this being related to other predictors of immunotherapy response, such as TMB and tumor neo-antigen load (110).

Molecular classification of BC according to gene expression profiles can play a crucial role in determining the most suitable treatment. Immunotherapy and chemotherapy appear to be advantageous in complementary patient populations. Patients with luminal tumors show better prognosis but poor response to cisplatin-based chemotherapy. Cystectomy is the best option in these patients, however, immunotherapy may be beneficial in luminal-infiltrated tumors. On the contrary, chemotherapy proves to be the treatment of choice in basal tumors.

## CONCLUSIONS

Despite guideline recommendations, NAC prior to cystectomy is still seldom adopted. Newly developed therapies, such as immunotherapy, targeted therapy and combination strategies, are being tested in clinical trials. The use of biomarkers to predict response to cisplatin-based NAC or ICIs is largely investigational, but molecular signatures are showing promise in reshaping selection for tailored treatment and disease monitoring.

## AUTHOR CONTRIBUTIONS

Study concepts: GR, GN. Study design: GR, MC. Data acquisition: RS, MS, IG. Quality control of data and algorithms: UG. Manuscript preparation: MC, GR, GN. Manuscript editing: MS, WP, AA. Manuscript review: UG, AA. All authors contributed to the article and approved the submitted version.

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# Advancements in the treatment of metastatic hormone-sensitive prostate cancer

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In the last decade, there have been substantial improvements in the outcome of the management of metastatic hormone-sensitive prostate cancer (mHSPC) following the development of several novel agents as well as by combining several therapeutic strategies. Although the overall survival (OS) of mHSPC is shown to improve with intense androgen deprivation therapy (ADT), combined with docetaxel, as well as other novel hormonal therapy agents, or alongside local intervention to the primary neoplasm. Notably, luteinizing hormone-releasing hormone (LHRH) antagonists are known to cause fewer cardiovascular side effects compared with LHRH agonists. Thus, in this mini review, we explore the different approaches in the management of mHSPC, with the aim that we may provide useful information for both basic scientists and clinicians when managing relevant clinical situations.

KEYWORDS

**prostate cancer, novel hormone therapy, chemotherapy, ADT, mHSPC**

## Introduction

Prostate cancer (PCa) is one of the most common cancers of the urino-genital system; its associated morbidity has progressively increased in the last decade (1). The morbidity and mortality were approximately 1.4 million and 375,000, respectively, in 2020 (2). The incidence of PCa in China has also significantly increased, accounting for 34.2% of the total PCa in Asia (3, 4). Metastatic hormone-sensitive prostate cancer (mHSPC) is responsive to androgen deprivation therapy (ADT) with overall survival (OS) of 42 months following ADT treatment (5). To improve the OS and quality of life (QoL) of mHSPC patients, many novel approaches to the management of mHSPC have been identified in the last decade. Our review aims to outline the advances in the treatment of mHSPC.

## Androgen deprivation therapy

Recent advances in ADT drug therapy predominantly relate to the manufacturing of luteinizing hormone-releasing hormone (LHRH) antagonists, such as degarelix and relugolix.

Degarelix is a luteinizing hormone-releasing hormone (LHRH) antagonist for castration and testosterone suppression administered *via* a subcutaneous injection. In a randomized, parallel-group, phase III clinical study, Klotz et al. reported that testosterone suppression (≤0.5 ng/ml) was achieved in 97.2%, 98.3%, and 96.4% of the intention-to-treat population in degarelix 240/80 mg, degarelix 240/160 mg, and leuprolide groups, respectively. On Day 3, testosterone suppression was achieved in 96.1% and 95.5% of these patients, with a median testosterone (0.24–0.26) response to the degarelix 240/80 mg and 240/160 mg groups, respectively. Moreover, testosterone suppression was increased by 65% in the leuprolide group (6). These data suggest that degarelix is similar to leuprolide in achieving castration level (6). Compared to the 3-month formulation of goserelin, the 1-month formulation of degarelix has a limited clinical application (7). Another phase III study explored formulations with more convenient clinical applications and reported that the cumulative castration rate was 95.1% in the degarelix group and 100.0% in the goserelin group. This indicated that the 3-month formulation of degarelix was not inferior to goserelin in relation to testosterone suppression; degarelix decreased the testosterone level to a castration level on Day 3, while testosterone surged by 52.74% in the goserelin group and did not reach the castration level until Day 28 (8).

Relugolix is an oral LHRH antagonist. A multinational, randomized, phase III study showed that castration was maintained in 96.7% of the patients in the relugolix group compared with 88.8% in the leuprolide group. This indicated that relugolix was superior to leuprolide in all endpoints (all $p < 0.001$). The major cardiovascular adverse effects were reported by 2.9% of the patients in the relugolix group vs 6.2% of those in the leuprolide group, indicating that relugolix was superior to leuprolide in relation to sustained testosterone suppression with lower cardiovascular adverse effects (7).

### Comparison of luteinizing hormone-releasing hormone antagonists and agonists

A phase II study investigated the impact of LHRH antagonists on cardiovascular disorders (CVDs) and reported that major cardiovascular and cerebrovascular events developed in 20% of patients in the LHRH agonist group vs only 3% of those in the LHRH antagonist group ($p = 0.013$); the absolute risk reduction in cardiovascular-related events in the antagonist group was 18.1% (9). These results suggest that the choice of using an antagonist or agonist may in PCa patients, with preexisting CVD, may differentially affect CVD (9). To provide more evidence, four eligible studies (n = 2,059) were discussed in a recent systematic review and network meta-analysis of antagonists and agonists, which demonstrated that compared to agonists, the relugolix and degarelix antagonists showed no significant difference in relation to the 12-month castration rate and that relugolix was ranked first in maintaining castration, suggesting that the two antagonists have similar efficacies but that the antagonists induced less cardiovascular events than the agonists (10). Although no head-to-head comparative study of the two LHRH antagonists has been conducted, degarelix injection is associated with a higher rate of injection-site reactions (40%) and is more difficult to administer, whereas oral relugolix is convenient for patients (7). Thus, these results suggest that LHRH antagonists may be the most efficacious drugs for ADT in the future and that relugolix is more suitable for purposes of clinical application because of its oral route of administration, daily.

## Novel hormonal therapy drugs

Existing Novel hormonal therapy (NHT) drugs for treating mHSPC include abiraterone, enzalutamide, apalutamide, and darolutamide. ADT, combined with some of the aforementioned NHT drugs, is approved for the treatment of mHSPC as recommended by the National Comprehensive Cancer Network (NCCN), American Urological Association (AUA), and European Association of Urology (EAU) guidelines (11–13).

Abiraterone is an inhibitor of 17 alpha-hydroxylase/C17, 20-lyase (CYP17), which is produced during androgen synthesis. To manage adverse effects related to mineralocorticoid excess, such as hypokalemia, hypertension, and fluid retention, which can occur as a result of CYP17 inhibition, the administration of abiraterone with prednisone or prednisolone at a low dose of 5 mg twice daily is necessary (14). The LATITUDE trial reported that the median OS was significantly prolonged in the abiraterone+ADT group compared with the placebo+ADT group [NA vs 34.7 months, hazard ratio (HR): 0.62, 95% CI 0.51–0.76; $p < 0.001$] and that abiraterone significantly benefitted the median radiographic progression-free survival (rPFS) (33 vs 14.8 months, HR: 0.47, 95% CI 0.39–0.55; $p < 0.001$) in mHSPC; the final follow-up results showed that the median OS was significantly prolonged in the abiraterone group (53.3 vs 36.5 months, HR: 0.66, 95% CI 0.56–0.78; $p < 0.0001$) (15–17), which is consistent with the findings of the STAMPEDE study (18). The LATITUDE study also reported that the median OS of patients with the high-volume disease was 49.7 months in the abiraterone group and 33.3 months in the placebo group (HR: 0.62, 95% CI 0.52–0.74; $p < 0.0001$) but that

the median OS showed no significant difference in the patients with the low-volume disease (16). The *post-hoc* analysis of the LATITUDE trial showed that the median time to prostate-specific antigen (PSA) progression was 33.2 months in the abiraterone group and 7.4 months in the placebo group (HR: 0.3; $p < 0.001$). Moreover, a significantly higher $PSA_{50/90}$ was achieved in the abiraterone group than in the placebo group (RR: 1.36/2.30; $p < 0.001$), and the risk of death was significantly reduced in patients who had a $PSA_{50/90}$ response compared with patients who did not have a PSA response (19). A *post-hoc* exploratory analysis of the LATITUDE trial also suggested that abiraterone treatment improved both rPFS and OS in men with mHSPC and visceral disease, especially those with lung metastases, and that men with liver metastases had a poorer prognosis (20). A *post-hoc* subgroup analysis was performed on the STAMPEDE study, in which the mHSPC patients in the STAMPEDE study underwent stratification using the LATITUDE risk criteria/the CHAARTED volume criteria revealed different outcomes showing that the OS and failure-free survival (FFS) were significantly prolonged by abiraterone compared with the placebo in the low-risk group (HR: 0.66, 95% CI 0.44–0.98, HR: 0.24, 95% CI 0.17–0.33). Also, the same conclusion was drawn in the other subgroups. No significant difference was found in the OS or FFS between the high-risk and low-risk groups, but the number of patients retreated in the low-risk group was fourfold higher than that in the high-risk group (21). The STAMPEDE and LATITUDE studies revealed conflicting conclusions on mHSPC with low-volume disease, which may be associated with the characteristics of the enrolled patients or the number of patients in the low-risk group.

Enzalutamide is a pharmaceutical that blocks an androgen receptor (AR) activity at three levels: 1) AR nuclear translocation, 2) DNA binding, and 3) coactivator recruitment. The ENZAMET study estimated a 3-year OS using the Kaplan–Meier estimator and reported that the 3-year OS was 80% in the enzalutamide+ADT group (based on 94 events) and 72% in the first-generation anti-androgen+ADT group (based on 130 events) in mHSPC patients; enzalutamide also significantly benefitted secondary endpoints, but a high incidence of lassitude, epilepsy, or other adverse effects was observed in the enzalutamide group (22). The ARCHES study also showed that compared with the placebo, enzalutamide significantly reduced the risks of death (HR: 0.39, 95% CI 0.30–0.50; $p < 0.001$), as well as a first symptomatic skeletal event, castration resistance, and pain progression (23). *Post-hoc* analysis of the ARCHES study further clarified that compared to the placebo, enzalutamide reduced the risks of radiographic progression of bone metastases (HR: 0.33, 95% CI 0.22–0.49) and bone metastases with lymphatic metastasis (HR: 0.31, 95% CI 0.21–0.47) but did not significantly reduce the risk of lymph node metastases (24). The analysis of health-related quality of life (HRQoL) showed that enzalutamide maintained a high QoL and a low symptom burden in mHSPC patients (25). In brief, enzalutamide has clinical benefits for all mHSPC patients who have or have not received local or systemic therapy, regardless of disease burden and risk (26).

Apalutamide is an anti-androgen drug similar to enzalutamide, but it has a higher affinity to AR (23). The TITAN study showed that at 24 months, the apalutamide +ADT group, as well as the placebo+ADT group, had an rPFS of 68.2% vs 47.5% (HR: 0.48, 95% CI 0.39–0.60; $p < 0.001$) and an OS of 82.4% vs 73.5%, respectively (HR: 0.67, 95% CI 0.51–0.89; $p = 0.005$), which suggests that rPFS was significantly prolonged in the apalutamide group compared with the placebo group (27). Subgroup analysis showed that the time to pain progression was significantly prolonged in the apalutamide group compared to the placebo group ($p < 0.0146$), but no significant difference was noted regarding the incidence of lassitude between the two groups (28). As the treatment of PCa has racial disparities, the therapeutic results of the East Asian populations in the TITAN study were analyzed, which demonstrated consistent results with participants involved worldwide. The only inconsistency was that the main adverse effect was a rash (29, 30). According to the final OS results in the TITAN study, apalutamide reduced the risk of death by 35% before crossover (HR: 0.65, 95% CI 0.53–0.79; $p < 0.0001$) vs 48% after crossover in 208 patients (HR: 0.52, 95% CI 0.42–0.64; $p < 0.0001$) (31, 32).

Darolutamide is another AR inhibitor. In the latest ARASENS study in mHSPC, the results showed that compared to the placebo+ADT+docetaxel group, the risk of death was significantly reduced by 32.5% in the darolutamide+ADT +docetaxel group (HR: 0.68,95% CI 0.57–0.80; $p < 0.001$) and that darolutamide was beneficial for all secondary endpoints and subgroups (33). The ARANOTE study is a randomized, double-blinded, placebo-controlled clinical study currently in progress, designed to compare the efficacy of darolutamide+ADT vs ADT alone, in mHSPC treatment.

Although combination therapy with any of the above four NHT drugs provides a significant OS benefit compared with ADT alone, the best therapeutic sequences are still unclear. Regarding adverse effects on the central nervous system (CNS), the available evidence suggests that CNS-related adverse effects are less prevalent with darolutamide than with enzalutamide and apalutamide due to the moderate blood–brain barrier penetration of apalutamide and enzalutamide compared with the lower blood–brain barrier penetration of darolutamide and abiraterone (34, 35).

## Chemotherapy

The GETUG-AFU15 study showed no difference in the median OS between docetaxel and ADT alone in mHSPC (36), but the *post-hoc* analysis suggested that no statistically significant OS benefit was achieved in the high-volume disease in the

docetaxel group (37). Subsequently, the CHAARTED study showed that the median OS was prolonged by 13.6 months when using docetaxel compared with using ADT alone (HR: 0.61, 95% CI 0.47–0.80; p < 0.001) in all mHSPC patients. Meanwhile, the median OS was prolonged by 17 months in the subgroup with high-volume disease (HR: 0.60, 95% CI 0.45–0.81; p < 0.001). There was no significant difference in the subgroup with low-volume disease (38). The updated data of the CHAARTED study showed that the median OS was prolonged by 10.4 months in all enrolled patients (HR: 0.72, 95% CI 0.59–0.89; p = 0.0018) and by 16.8 months in patients with high-volume disease in the docetaxel group compared with patients receiving ADT alone (HR: 0.63 95% CI 0.50–0.79; p < 0.001). However, no OS benefit was achieved in the group of patients with a low-volume disease (39). The subgroup analysis of the STAMPEDE study also showed that, compared to the standard of care (SOC) alone, docetaxel+SOC significantly prolonged the median OS in mHSPC patients (81 vs NA HR: 0.78, 95% CI 0.66–0.93; p = 0.006) (40). The long-term follow-up in the STAMPEDE study further indicated that docetaxel was beneficial for the median OS of mHSPC patients with either high or low burden (41). The conclusion of the STAMPEDE study regarding the benefits of docetaxel for patients with low-volume disease contradicts the results of the CHAARTED study, which may be associated with the characteristics of the enrolled patients. The Cochrane review revealed that compared with ADT alone, early docetaxel treatment reduced the risks of death by any cause for mHSPC patients (HR: 0.77, 95% CI 0.68–0.87, $I^2$ = 0%) (42). As treatment with docetaxel is beneficial for patients with mHSPC, according to the STAMPEDE study and the Cochrane review, docetaxel is recommended by the NCCN, AUA, and EUA guidelines (11–13).

## Local intervention of the primary neoplasm

Although systemic therapy is important for mHSPC patients, accumulating evidence suggests that for mHSPC patients with low-volume disease, cytoreductive procedures, combined with systemic therapy, such as radiotherapy (RT) to the primary tumor and cytoreductive prostatectomy, can significantly improve the OS. However, such procedures need to be supported by a large number of randomized controlled trials (43). The HORRAD study identified no significant difference in the OS between the RT+ADT and ADT-alone groups, but the time to PSA progression was 15 months in the RT group vs 12 months in the ADT-alone group (HR: 0.78, 95% CI 0.63–0.97; p = 0.02). Subgroup analysis of the HORRAD study suggested that RT tended to be beneficial for HSPC patients with the low-volume disease compared to those with high-volume disease, but the difference was not statistically significant (44). Furthermore, the STAMPEDE study showed that RT significantly improved the FFS (HR: 0.76, 95% CI 0.68–0.84; p < 0.0001) but not the OS in all mHSPC patients; however, RT significantly improved the OS in the low-volume disease group compared to the high-volume disease group (HR: 0.68, 95% CI 0.52–0.90; p = 0.007) (45). In a retrospective analysis, Morgan et al. found that the RT significantly benefitted the median OS (47.7 vs 26.3 months, HR: 0.69, 95% CI 0.50–0.94; p = 0.02) compared to ADT alone, and such benefit was more remarkable in patients who had survived for at least 1 year (52.2 vs 39.8 months, HR: 0.73, 95% CI 0.54–0.98; p = 0.04) (46). The STOPCAP meta-analysis also demonstrated that a 3-year survival benefit was achieved in 7% of patients with less than five bone metastases (HR: 1.47 95% CI 1.11–1.94; p = 0.007), suggesting that RT for prostate should be considered for mHSPC patients with the low-volume disease (47). Cytoreductive prostatectomy is another method for the local interventions of mHSPC. Heidenreich et al. found that cytoreductive prostatectomy+ADT prolonged the PFS by 12.1 months (p = 0.032), increased the disease-specific survival rate by 11.4%, and increased the OS rate by 12.4%, compared with therapy with ADT alone (48). The TRoMbone clinical trial, which was designed to investigate the efficacy of cytoreductive prostatectomy+ADT vs ADT alone for the treatment of mHSPC patients with low-volume disease, is currently in progress (49). Cytoreductive cryotherapy also shows a survival benefit in mHSPC patients with low-volume disease. Sheng et al. reported that cytoreductive cryosurgery+ADT significantly prolonged the PFS compared to ADT alone in mHSPC patients with the low-volume disease (35 vs 25 months; p = 0.0027) (50). In conclusion, based on the above benefits, local interventions of primary neoplasm are recommended for mHSPC with the low-volume disease according to the AUA guidelines (13).

## Comparison of combination therapy

Although NHT drugs and chemotherapy have significant efficacy in the treatment of mHSPC patients, the optimal drugs for the best therapeutic option should be determined. Sathianathen et al. performed a meta-analysis by focusing on papers published from January 2014 up to June 2019 and reported that the combination of ADT+docetaxel/abiraterone/enzalutamide/apalutamide is superior to ADT alone and that enzalutamide+ADT has the lowest absolute risk among all studied combination therapies (HR: 0.53, 95% CI 0.37–0.75) (51). Wang et al. performed a network meta-analysis and reported that the improvement in OS was achieved with the use of (from largest to smallest improvement) abiraterone, apalutamide, and docetaxel (HR: 0.61, 95% CI 0.54–0.70; HR: 0.67 95% CI 0.51–0.89; HR: 0.79 95% CI 0.71–0.89), whereas the improvement in rPFS was achieved with the use of (from largest

to smallest improvement) enzalutamide, apalutamide, abiraterone, and docetaxel (HR: 0.39, 95% CI 0.30–0.50; HR: 0.48, 95% CI 0.39–0.60; HR: 0.51, 95% CI 0.45–0.58; HR: 0.67, 95% CI 0.60–0.74). Docetaxel had the largest risk of adverse effects, while abiraterone had a slightly increased risk; however, the other drugs had no significantly increased risk (52). Similarly, Ferro et al. performed a network meta-analysis and suggested that compared to chemotherapy, NHT significantly improved the OS of mHSPC patients (HR: 0.78, 95% CI 0.67–0.91) (53). The volume of disease also affects the prognosis of mHSPC. In a meta-analysis conducted by Sathianathen et al., focusing on papers published from January 2014 up to June 2019, the subgroup analysis revealed that each combination therapy significantly improved OS compared with ADT alone in the high-volume disease group; however, no significant difference was observed between the combination therapies. Enzalutamide combination therapy improved the OS to a great extent compared with the other combination therapies in the low-volume disease group (HR: 0.38, 95% CI 0.20–0.68) (51). Wenzel et al. also performed a network meta-analysis of the systemic treatment for mHSPC patients and reported that abiraterone, apalutamide, and docetaxel prolonged the OS (HR: 0.59, 95% CI 0.50–0.69; HR: 0.68, 95% CI 0.50–0.69; HR: 0.73, 95% CI 0.62–0.85) but that enzalutamide did not prolong the OS compared to ADT. Moreover, the ranking analysis showed that the improvement in the OS was achieved with the use of (from largest to smallest improvement) abiraterone, apalutamide, and docetaxel; however, only abiraterone and enzalutamide prolonged the OS in the high-volume disease mHSPC subgroup (HR: 0.61, 95% CI 0.47–0.79; HR: 0.43, 95% CI 0.26–0.72), while apalutamide and docetaxel did not prolong the OS compared to ADT alone. In addition, the ranking analysis showed that the OS benefit was achieved with the use of (from largest to smallest improvement) enzalutamide and abiraterone in the low-volume disease mHSPC subgroup (54). Thus, the best OS was achieved with abiraterone in high-volume disease mHSPC patients and enzalutamide in low-volume disease mHSPC patients (54).

Many comparative studies on abiraterone and chemotherapy have been conducted. Kassem et al. performed a network meta-analysis to compare abiraterone and docetaxel in the treatment of mHSPC, and they suggested that abiraterone therapy had a better PFS and lower drug toxicity than docetaxel but that there was a trend for the abiraterone therapy to benefit OS without statistical significance (55). In a meta-analysis conducted by Wenzel et al., abiraterone treatment of mHSPC patients with high-volume disease resulted in a median OS of 50.1 months, which exceeded that of docetaxel (45.9 months) and ADT alone (34.0 months); no significant difference in the median OS was identified between docetaxel and ADT alone in the low-volume disease group (54). In a retrospective, multicenter study that compared the efficacy and safety of abiraterone and docetaxel in the treatment of mHSPC, the abiraterone+ADT group had a significantly longer PFS1, PFS2 (PFS1/PFS2, time from start of ADT to clinical, biochemical, or radiographic progression during first/second line or death from any cause) and OS as compared with the docetaxel+ADT group (23 vs 13 months; $p < 0.001$; 48 vs 33 months; $p = 0.006$; 80 vs 61 months; $p = 0.040$); according to a multivariate analysis of PFS1 (HR = 0.34, 95% CI 0.183–0.623; $p = 0.001$) and PFS2 (HR = 0.33, 95% CI 0.128–0.827, $p = 0.018$), abiraterone+ADT was significantly better than docetaxel+ADT, but both resulted in similar OS and toxic effects (56). The STAMPEDE clinical study demonstrated that the mean QoL score, over the period of 2 years, was +3.9 points higher in patients treated with abiraterone than in those treated with docetaxel, which fails to meet the predefined criterion for a clinically meaningful difference of >4.0 points; the mean QoL score was +5.7 points higher over 1 year, +7.0 points higher at 12 months, and +8.3 points higher at 24 months, suggesting that the patient-reported QoL was better in patients treated with abiraterone compared with those treated with docetaxel over a 2-year period (57).

## Precision treatment

As gene mutations are significantly associated with the prognosis of metastatic castration-resistant prostate cancer (mCRPC) (58–60), previous studies have also reported the relationship between gene mutation and mHSPC (61–63). Velez et al. retrospectively detected the TP53, PTEN, and RB1 mutations in 97 patients in a single center and identified tumor suppressor gene (TSG) mutations in 48 (49%) patients treated with abiraterone+ADT and in 49 (51%) patients treated with docetaxel+ADT. Velez et al. found that the median PFS was 13.1 months in the TSG-normal group vs 7.8 months in the TSG-altered group ($p = 0.005$); subgroup analysis showed that the median PFS was lower in TSG-altered patients compared to TSG-normal patients in the abiraterone+ADT group (8.0 months, 95% CI 5.8–13.8; 23.2 months, 95% CI 13.1–NA), but no difference was observed between the docetaxel+ADT subgroups. Using multivariable analysis, Velez et al. reported that altered TSG predicted the prognosis of mHSPC in early first-line treatment (HR: 2.37, 95% CI 1.42–3.96; $p < 0.001$) and that detection of the TSG mutation was superior to the clinical criteria (61). However, several gene mutations benefit from hormonal therapy. In a retrospective study, Swami et al. investigated PCa patients who received standard ADT only and who were identified with SPOP gene mutations [n = 121 total patients, 25 patients with mutant SPOP (mtSPOP) and 96 patients with wild-type SPOP (wtSPOP)]; the study reported that standard ADT therapy resulted in a longer median PFS and median OS in patients with mtSPOP compared to patients with wtSPOP (35 vs 13 months, HR: 0.47; $p = 0.016$; 97 vs 69 months, HR: 0.32; $p = 0.027$) (63).

## Summary and prospect

Revolutionary progress has been made in the development of mHSPC treatment options, notably including LHRH antagonists, NHT drugs, chemotherapy, and local intervention of low-volume disease of mHSPC, as well as in certain combined treatments. This has provided major benefits for mHSPC patients over the last decade. Nonetheless, further research is still needed to determine the optimal combinational therapies of these drugs. Therefore, multicenter prospective studies with larger sample sizes will inevitably be conducted in the future.

## Author contributions

XP-L, YL, XR-W participated in drafting the manuscript. MZ was responsible for revising the manuscript. HP-L designed the study and was responsible for revising the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# IL-8 and its role as a potential biomarker of resistance to anti-angiogenic agents and immune checkpoint inhibitors in metastatic renal cell carcinoma

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The therapeutic armamentarium of metastatic Renal Cell Carcinoma (mRCC) has consistently expanded in recent years, with the introduction of VEGF/VEGFR (Vascular Endothelial Growth Factor/Vascular Endothelial Growth Factor Receptor) inhibitors, mTOR (mammalian Target Of Rapamycin) inhibitors and Immune Checkpoint (IC) inhibitors. Currently, for the first-tline treatment of mRCC it is possible to choose between a VEGFR-TKI (VEGFR-Tyrosine Kinase Inhibitor) monotherapy, an ICI-ICI (Immune Checkpoint Inhibitor) combination and an ICI-VEGFRTKI combination. However, a consistent part of patients does not derive benefit from first-line therapy with ICIs; moreover, the use of combination regimens exposes patients to significant toxicities. Therefore, there is a critical need to develop prognostic and predictive biomarkers of response to VEGFR-TKIs and ICIs, and measurement of serum IL-8 is emerging as a potential candidate in this field. Recent retrospective analyses of large phase II and phase III trials found that elevated baseline serum IL-8 correlated with higher levels of tumor and circulating immunosuppressive myeloid cells, decreased T cell activation and poor response to treatment. These findings must be confirmed in prospective clinical trials; however, they provide evidence for a potential use of serum IL-8 as biomarker of resistance to VEGFR-TKIs and ICIs. Considering the amount of new agents and treatment regimens which are transforming the management of metastatic renal cell carcinoma, serum IL-8 could become a precious resource in tailoring the best therapy for each individual patient with the disease.

KEYWORDS

**IL-8, biomarker of resistance, anti-angiogenic agent, immune checkpoint inhibitors, kidney cancer**

## Introduction

Renal cell carcinoma (RCC) accounts for 80% of cases of kidney cancer, and it represents a major cause of morbidity and mortality worldwide (1). Up to 30% of RCC patients present with metastatic disease at diagnosis, and a similar percentage of patients with localized disease successfully removed through surgery will develop subsequent metachronous metastasis (2).

Clear cell renal cell carcinoma (ccRCC) is the most common histologic subtype, making up about 70% of cases of RCC (3), and for such reason, it has been extensively studied from a molecular point of view. The vast majority of cases of ccRCC presents with loss of function of the Von Hippel Lindau (VHL) gene (4), leading to unrestrained Hypoxia-Inducible Factor (HIF)-1α and HIF-2α activity and consequent enhanced cell growth and angiogenesis (5). However, VHL loss alone is not sufficient to induce tumor formation (3, 6). Several genes involved in chromatin remodeling (PBRM1, KDM5C, UTX, JARID1CM. SETD2), as well as genes involved in the PI3K-AKT-mTOR axis were proven to be mutated in RCC (7). Alterations in the PI3K-AKT-mTOR pathway increase tumor cell growth and proliferation as well as induce a metabolic rewiring in cancer cells (8). The high prevalence of these mutations underlies the current view on RCC ontogeny involving inactivation of pVHL as initiating step, followed by additional mutations in the aforementioned genes as subsequent events in tumor formation (4). The elucidation of such biological mechanisms has translated into clinical practice through the introduction of tyrosine kinase inhibitors, directed against VEGFR and similar proteins, and mTOR inhibitors, shutting down the mTOR complex 1 (mTORC1).

Before the introduction of such agents, first-line therapies for RCC relied upon the highly immunogenic nature of the tumor, which could be targeted with the use of high-dose interleukins and interferon (IFN)-alpha (9). This form of treatment generally had really poor response rates and survival benefits, but the presence of a small group of long-term responders underpinned the potentiality of immunotherapy in RCC. As immune checkpoint inhibitors (ICIs) revolutionized the management of several different cancers, their use has become a standard of care in advanced renal cell carcinoma too (1). The double ICI combination ipilimumab plus nivolumab has been approved as first-line treatment in IMDC intermediate and poor-risk patients (1, 10, 11). Several clinical trials tested ICIs in combination with VEGFR-TKIs, and results so far are showing unprecedented response rates and survival benefits across all patients' risk groups (12, 13). As a result, the FDA has approved three combinations of an ICI and a VEGFR-TKI (pembrolizumab and axitinib, pembrolizumab and lenvatinib, nivolumab and cabozantinib) as first-line therapy across all patients' risk groups (14). As the treatments are quickly expanding, physicians are facing new challenges in determining which therapeutic regimens is the most suitable for patients. The two major risk stratification models, the MSKCC and IMDC, are becoming obsolete as the new treatment regimens confer clinical benefits across all risk groups (12, 13). A great amount of work has focused on determining whether tumour and/or tumour-infiltrating immune cell protein expression of programmed cell death ligand 1 (PD-L1) could predict response to ICI therapy (11, 13, 15–17), but the results were controversial (18, 19). Similarly, other deeply-investigated biomarkers, such as CD8+ T cell density (13, 20, 21), tumor mutational burden (TMB) (21–23), PBRM1 mutation (21, 23, 24), have failed to yield uniform predictive results.

Ideally, biomarkers should be assessed in a minimally-invasive manner. In this respect measurement of serum IL-8 might represent a novel prognostic and predictive parameter in immunotherapy. Serum IL-8 has been recently analyzed in several different ICI trials for different cancers, including mRCC, and results suggest that IL-8 might represent a negative prognostic biomarker for solid tumors, but that it might also represent a biomarker of resistance to ICI treatment, hence aiding in predicting response to therapy (25, 26). In this review, we report the physiologic role of IL-8, its involvement in the process of carcinogenesis, its initial assessment as a clinical biomarker in cancer, and how these recent analyses of IL-8 in clinical trials may pave the way for a more thorough investigation of IL-8 as a prognostic and predictive biomarker of response to ICI and/or TKI therapy in mRCC.

## Physiology of IL-8

CXCL8, also known as interleukin (IL)-8, is one of the most extensively studied chemokines. It was first described in the late 1980s, where it was initially called neutrophil activating factor (NAF) due to its role in neutrophil exocytosis and oxidative burst (27, 28). IL-8 is a 6-8 KDa protein secreted by different cell types including blood monocytes, alveolar macrophages, fibroblasts, endothelial cells, and epithelial cells (29, 30). IL-8 expression is induced by various cytokines (IL-1, IL-6, CXCL12, TNF-α), hypoxic states, reactive oxygen species (ROS), bacterial particles, and other environmental stresses (29). Through the binding with its two receptors, CXCR1 and CXCR2, IL-8 exerts its major physiologic functions: promoting a pro-inflammatory state and stimulating angiogenesis. IL-8 is a potent chemoattractant molecule that drives mainly neutrophils but also monocytes to the site of inflammation (31, 32). Moreover, IL-8 favors the resolution of infections by acting mostly on neutrophils and promoting neutrophils-mediated phagocytosis, oxidative bursts, and release of neutrophil extracellular traps (32). In addition to its pro-inflammatory function, IL-8 acts to favor angiogenesis by promoting endothelial cells proliferation, survival, and migration, culminating in the formation of new

blood vessels. This pro-angiogenic property favors the process of tissue healing from the inflammatory state (32).

## IL-8 and its role in carcinogenesis

IL-8 has been extensively explored in cancer research. Tumor cells shape the surrounding microenvironment through the expression and release of cytokines and chemokines. The IL-8/IL-8R axis plays an important role in such context; tumor cell acquisition of CXCR1 and CXCR2 and/or IL-8 is known to be a common event during tumor progression (29, 32), and, similarly, IL-8 and its receptors are widely expressed by a variety of non-malignant cells present in the tumor microenvironment, including tumor-associated macrophages, neutrophils and endothelial cells (33). The pro-tumorigenic effect of IL-8 within the TME is exerted *via* both autocrine and paracrine ways. Autocrine loops form on the surface of tumor cells, which concomitantly produce IL-8 and express its receptors. IL-8 signaling stimulates tumor growth by enhancing tumor cell growth (33, 34). Moreover, IL-8 signaling is emerging as an important factor in tumor cell survival, by promoting the expression of anti-apoptotic genes, particularly in the context of environmental (e.g. hypoxia) or treatment-induced stresses (35, 36). IL-8 has been directly involved in the process of epithelial-mesenchymal transition (EMT), where acquisition of a mesenchymal phenotype enhances tumor cell aggressiveness and invasion capacity, hence favoring metastasis (37).

In addition, by acting in a paracrine manner, IL-8/IL-8R axis has a prominent role in promoting a favorable tumor microenvironment by recruiting immune cells characterized by permissive phenotype for tumor growth, such as N2-neutrophils and myeloid-derived suppressor cells (MDSCs) (32). The presence of such cells in cancer has been associated with a more defective anti-tumor immune response within the TME, particularly by inhibiting T-cells (38, 39). (Figure 1)

Finally, high levels of IL-8 in the TME are potent stimulants for tumor angiogenesis. This is achieved through multiple mechanisms, including directly promoting endothelial cell proliferation and survival, up-regulating VEGF-A and its receptor VEGFR2, and inducing expression of matrix metalloproteases, which are capable of mobilizing sequestered pro-angiogenic factors (40, 41).

Overall, IL-8 pathways play an active part in promoting carcinogenesis, representing a potential therapeutic target in clinical setting (25, 42).

## Analysis of IL-8 as a biomarker in cancer

Measurement of serum IL-8, due to its short half-life, may represent a good candidate to accurately estimate the number of tumor cells producing this chemokine at any given time (43). These values might be exploited both pre-treatment, to estimate tumor burden, and on treatment, where changes in serum concentration could then reflect variations in tumor burden or tumor composition as well.

High IL-8 concentrations have been detected in serum and tissue specimens from patients with different cancers, and have been shown to correlate with a worse clinical stage of tumors (26, 43, 44), a more prominent tumor burden (45), presence of metastasis, and worse overall survival (44, 46). Vlachostergios et al. demonstrated that IL-8 levels, at baseline and after LPS-

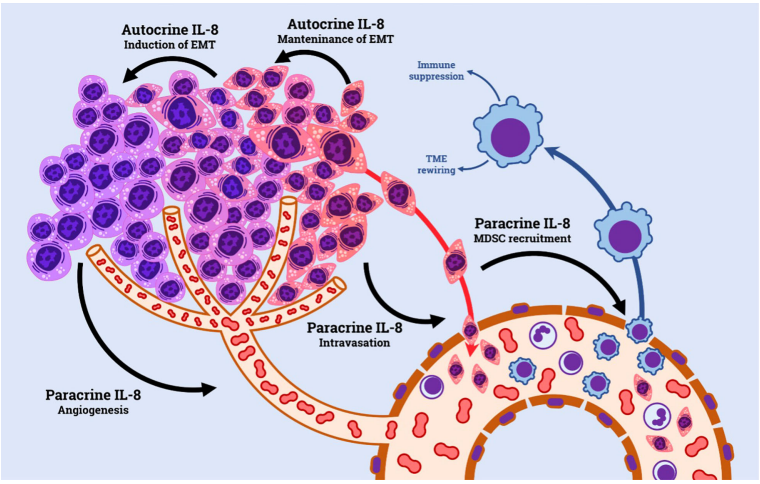


FIGURE 1
Principal mechanisms involved in IL-8-induced resistance to ICI and anti-angiogenic TKI.

stimulation, are independent predictors of both PFS and OS in non-small-cell lung cancer (47).

In renal cell carcinoma, elevated serum IL-8 was associated with higher tumor burden and worse overall survival (43). Several studies also associated IL-8 up-regulation in cancers with resistance to chemotherapy (35, 48, 49) and targeted therapy (50, 51).

## IL-8 and resistance to immune checkpoint inhibitors

Evaluation of IL-8 in the context of immunotherapy has gained interest recently. Analysis of a small sample of patients with advanced melanoma and NSCLC treated with anti-PD1 monoclonal antibodies has shown that changes in serum IL-8 were associated with response to treatment (52). Of note, early changes in serum IL-8 levels, measured only 2-3 weeks after starting therapy, could predict response to treatment and overall survival, with patients witnessing a drop in IL-8 levels having better clinical outcomes compared to patients experiencing rising IL-8 levels (52). In this study, only changes in serum IL-8 were associated with significant clinical response, thereby strengthening the potential role of IL-8 as ICI biomarker (52). (Table 1)

The role of baseline and on-treatment serum IL-8 has been evaluated in two major retrospective analyses of large phase II and phase III trials. The trials spanned several different cancers (53, 57), but a focus on the results related to the RCC trials is presented here. Schaper et al. analyzed the checkmate 025 trial, where patients with advanced renal cell carcinoma were randomized to either nivolumab or everolimus 16; using overall survival to obtain 23 pg/mL as clinically relevant stratification cut-off for serum IL-8 concentration, patients were independently stratified and analyzed in terms of progression-free survival (PFS), overall survival (OS) and objective response rate (ORR) (53). Results showed that in the group of patients treated with the ICI nivolumab, an OS hazard ratio of 2.56 (95% confidence interval 1.07-1.72, P < 0.0001) between patients with high (>23 pg/ml) and low (<23 pg/ml) baseline serum IL-8 was found. Similarly, the PFS (1.36, 95% CI 1.89-3.45, P <0.0001) was worse in patients with higher serum IL-8 levels; moreover, nivolumab-treated patients with serum IL-8 < 23 pg/mL had an ORR of 27.9%, as opposed to ORR of 19.5% in patients with serum IL-8>23 pg/mL. Interestingly, the association between elevated baseline serum IL-8 level and reduced survival was also observed in everolimus arm (HR=2.40, 95% CI 1.78-3.22, P < 0.0001). These results were consistent across treatment and tumor types, supporting the view of IL-8 as a global biomarker of poor prognosis in cancer. In addition, serum IL-8 was positively correlated with tumor IL-8 gene expression. High tumoral IL-8 was associated with tumoral infiltration of specific subsets of inflammatory cells, including neutrophils and monocytes, while and IFN-γ and T-cell transcripts signatures were downregulated. These findings point towards a link between IL-8 and an immunosuppressive tumor microenvironment highly infiltrated by myeloid cells with decreased antitumoral adaptive T-cell response.

The analysis performed by Yuen et al. focused on the possible correlation between plasma, peripheral blood mononuclear cell (PBMCs), and intratumoral IL-8 and clinical outcomes in the phase II IMmotion 150 trial, where patients with treatment-naïve mRCC were randomized to atezolizumab monotherapy or atezolizumab plus bevacizumab versus sunitinib (57). Elevated plasma IL-8 was associated with worse OS in the atezolizumab monotherapy arm (HR, 2.55, 95% CI

TABLE 1 Principal retrospective data in literature exploiting the role of IL-8 as a predictor of resistance to ICI and anti-angiogenic TKI.

| Reference | Study population | Study design | Outcome |
|---|---|---|---|
| Schalper et al. (52) | Retrospective analysis of 392 mRCC patients of the Checkmate-025 trial | Nivolumab vs Everolimus in mRCC patients progressed beyond ≥1 anti-angiogenic therapy | Longer OS (HR 2.56) and PFS (HR 1.36) in patients with low (<23 pg/ml) baseline serum IL-8 (p < 0.0001) in the Nivolumab arm.<br>Longer OS (HR 2.40) in patients with low IL-8 (p< 0.0001) in the Everolimus arm. |
| Yuen et al. (53) | Retrospective analysis of 915 mRCC patients of the IMmotion 150 trial | Atezolizumab vs Atezolizumab + Bevacizumab vs Sunitinib in mRCC treatment-naïve patients | Longer OS (HR 2.55) in patients with low baseline serum IL-8 (p =0.017) in the Atezolizumab arm.<br>Trend toward longer OS in patients with low baseline serum IL-8 the Atezolizumab à Bevacizumab and Sunitinib arm. |
| Tran et al. (54) | Retrospective analysis of 344 mRCC patients in the Pazopanib phase III trial | Pazopanib vs placebo in mRCC treatment-naïve and cytokine-pretreated patients | Longer PFS in patients with high concentrations (relative to median) of interleukin 8 (p=0·006) in the Pazopanib arm. |
| Iacovelli et al. (55) | Retrospective analysis of 36 patients with mRCC treated with targeted agents | mRCC patients treated with either Sunitinib, Pazopanib, Sorafenib, Bevacizumab, Temsirolimus | Higher 12 months-PFS in patients without immunohistochemical espression of interleukin 8 (p=0.009) |
| Sepe et al. (56) | Prospective analysis of 25 patients treated with Pazopanib | mRCC patients treated with Pazopanib as a first-line therapy | Low levels of baseline interleukin 8 associated with higher OR rate (p=0.047) and longer OS (p=0.04). |

1.18-2.55, P = 0.017), while a trend towards worse OS in the atezolizumab + bevacizumab (HR, 1.25, 95% CI 0.61-2.60, P = 0.535) and sunitinib (HR, 1.48; 95% CI 0.69-3.20, P = 0.314) was observed (57). Using single-cell RNA sequencing, IL-8 expression was shown to be more prominent in the peripheral mononuclear myeloid cluster compared to the mononuclear lymphoid cluster and, concomitantly, within individual myeloid subsets, including monocyte, dendritic cells, and DC-like clusters, increased IL-8 expression was associated with both enrichment of myeloid inflammatory genes and downregulation of genes associated with the antigen-presentation machinery, such as HLA genes and interferon-γ-induced genes. A similar gene signature was seen in myeloid cells infiltrating the tumor. This fact may underlie a defective anti-tumoral antigen presentation machinery in the presence of overexpressed IL-8. Elevated IL-8 gene expression in the tumor correlated with higher neutrophils within the tumor (53, 57),. Additionally, high tumor IL-8 gene expression was associated with worse OS in mRCC treated with atezolizumab monotherapy; importantly, high tumor IL-8 expression remained associated with worse OS even in T cell-infiltrated tumors in mRCC patients treated with atezolizumab (HR, 15.6; 95% CI, 3.15, 77.6; P = 0.0004), but not in the atezolizumab + bevacizumab group (HR, 0.96; 95% CI, 0.29, 3.2; P = 0.945) and sunitinib group (HR, 1.94; 95% CI, 0.67, 5.6; P = 0.225).

## IL-8 and resistance to anti-angiogenic TKI

The idea that the changes in tumor microenvironment induced by TKI could improve the efficacy of ICI has been suggested by many preclinical data (58). The results from recent clinical data seem to confirm this hypothesis (12, 59). Hence the tendency toward ICI plus TKI combinations. (Table 1)

IL-8 could be useful in this setting, since it could predict resistance to TKI (60). In 2010, Huang et al. observed that tumors developing alternative angiogenic pathways are often those with increased expression of tumor-derived IL-8. Up-regulation of IL-8 may thus activate proangiogenic pathways that may functionally compensate for the inhibition of VEGF-VEGFR-dependent angiogenesis (61). It has been documented that the hyper-expression of IL-8 leads to VEGF mRNA transcription and autocrine VEGFR-2 activation (62). Moreover, this cytokine can induce the epithelial-to-mesenchimal transition *via* AKT activation in RCC cells, thus rendering them more resistant to VEGFR inhibition (54).

Exploration of plasma IL-8 as a potential prognostic biomarker in patients treated with the anti-angiogenic agent pazopanib has been performed both retrospectively (55, 56) and prospectively in a small cohort of patients with mRCC (63). Similarly, a prospective study analyzed baseline serum IL-8 and clinical outcomes in patients with mRCC receiving sunitinib (64). Results obtained suggest a potential negative prognostic value for plasma IL-8, with elevated plasma concentration associated with worse clinical outcomes upon treatment with anti-angiogenic TKIs as compared to lower plasma concentration (55, 63)

## Discussion

Overall, this review highlights the potential role of IL-8 as a driver of resistance to immune checkpoint inhibitors. While the findings reported in the Checkmate-025 trial point towards a generalized role of IL-8 as a negative prognostic biomarker, both in ICIs and TKI regimens, Yuen et al. found that the effect of plasma IL-8 on clinical outcomes appeared to be more pronounced in single-agent ICI. These findings suggest that higher baseline IL-8 may be selectively predictive of which patients are less likely to benefit from ICI monotherapy. This point can be particularly relevant in the management of metastatic renal cell carcinoma, where continuously expanding therapeutic options calls for the rapid development of new biomarkers that could allow selection of the proper treatment regimen for each individual patient, thereby maximizing survival and concomitantly limiting toxic adverse effects. Besides its direct stimulation of cancer cell proliferation and promotion of angiogenesis, high tumoral IL-8 levels reflect a unique, unfavorable tumor microenvironment characterized by prominent myeloid-cell infiltration and suppression of adaptive T-cell anti-tumor response (53). High-tumoral IL-8 expression is associated with recruitment of several myeloid cells lines, including MDSCs, CD15+ monocytes, and neutrophils, which have all been demonstrated to impair adaptive T cell antitumor immunity by several different mechanisms (39, 65, 66). Transcriptomic characterization of circulating and tumor-infiltrating IL-8-producing MDSCs demonstrated an increased expression of myeloid pro-inflammatory genes and downregulation of antigen-presentation and interferon-inducible genes, underlying impairment of adaptive immunity (57).

This deleterious effect of MDSCs on anti-tumor adaptive immunity might directly affect resistance to immunotherapies in cancer (67). In the phase II IMmotion 150 trial, the authors conducted exploratory analyses of molecular biomarkers relevant to the disease and tumor immune biology in mRCC, and their potential association with clinical outcomes within each treatment group and across treatment groups (68). Distinct biological subgroups were obtained, based on the relative expression of angiogenesis, lymphocitic, and myeloid inflammation-associated genes. Atezolizumab monotherapy was effective on tumor with pre-existing immunity and a relatively lower expression of myeloid inflammation-associated genes (Teff-high/Myeloid-low), but less so in immunogenic tumors with concomitantly high myeloid inflammation (Teff-

high/Myeloid-high) (21). These findings underscore the relevance of a myeloid inflammatory milieu in determining resistance to ICI, even in the presence of a strong T cell inflammatory response, which is normally associated with better outcomes with ICIs therapy (69).

Conversely, the combination atezolizumab plus bevacizumab showed improved PFS compared to atezolizumab monotherapy in the Teff-high/Myeloid-high biological subgroups (HR 0.25; 95% CI, 0.10-0.60). This is in line with previous findings delineating an immunosuppressive role for VEGF, on top of its pro-angiogenic function, by impairing dendritic cell maturation, T-cell function, and promoting the proliferation of MDSCs (70). Consequently, VEGF/VEGFR blockade is thought to exert an anti-tumor immunomodulatory effect and has been shown to reduce MDSCs in tumors and blood in both preclinical tumor models and human cancers (71). In the context of a highly inflamed TME with infiltration of both T cell and MDSCs (Teff-high/Myeloid-high), the addition of an anti-VEGF/VEGFR agent (bevacizumab) to an immune checkpoint inhibitor (atezolizumab) may overcome innate inflammation-mediated resistance in these tumors, and synergistically enhance the reinvigorating effects of ICI on adaptive antitumor immunity. As increased serum IL-8 levels correlate with bulk tumor IL-8 gene expression and with tumor and circulating MDSCs (66), measurement of its serum concentration might be indicative of the myeloid inflammatory state of tumors, and therefore whether ICI monotherapy could be effective or the addition of a VEGF/VEGFR inhibitor should be considered. In this regard, IL-8 could be used as a predictive marker of response to immunotherapy and might be part of a comprehensive biomarker signature, that could contribute to personalized therapy in patients with mRCC. The current findings provide a rationale for the use of IL-8 as a potential prognostic and predictive biomarker in the use of ICIs and TKI in mRCC, but a limitation of this conclusion relies on the retrospective nature of the results reported. To ensure that the relevance of such data translates soon into clinical practice, IL-8 must be evaluated in prospective biomarker clinical trials.

## Author contributions

MR, LV, and CP wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Bibliometric analysis of the global research development of bone metastases in prostate cancer: A 22-year study

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**Background:** Prostate cancer (PCa) is the second most diagnosed cancer in men. Most PCa-related deaths result from metastatic disease. Metastases occur most often in the bones (90%). However, the current treatments for bone metastases in PCa are not very effective. Here we present an overview of the current research situation of bone metastases in PCa, focusing on hotspots and trends.

**Methods:** We searched the Web of Science Core Collection database for publications related to bone metastases in PCa published between 1999 and 2021. We used VOSviewer, CiteSpace, and a bibliometric online platform to perform a bibliometric analysis of countries, institutions, authors, journals, references, and keywords.

**Results:** A total of 4,832 related articles were included in the present study. The USA published the most articles in the field, followed by China and England. The University of Texas MD Anderson Cancer Center is the leading institution in the research field of bone metastases in PCa. Saad F, from Canada, has made great achievements in this area by publishing 91 related articles. *Prostate* is the journal which published most related articles, and Mundy GR, 2002, *Nat Rev Cancer*, is the most cited article in this field. Furthermore, the analysis of author keywords can be divided into five clusters: (1) diagnosis of PCa, (2) mechanism of bone metastasis, (3) drug treatments of bone metastases, (4) radiotherapy of bone metastases, and (5) treatments and prognosis of PCa.

**Conclusions:** mCRPC has been the hottest topic in PCa in recent years. CT is the most common diagnostic method for bone metastases. Enzalutamide and radium-223, as important treatments for bone metastases in PCa, bring about widespread attention. Furthermore, the researchers focus on the tumor

microenvironment and biomarkers to explore the mechanism and the therapeutic targets of bone metastases in PCa.

KEYWORDS

prostate cancer, bone metastases, bibliometric, VOSviewer, Citespace

# Introduction

Prostate cancer (PCa) is the second most diagnosed cancer type in men (1). The survival rate of patients with localized or regional PCa is relatively good, with a 5-year survival rate of 100%. However, once metastases develop, prognosis is poor, and the 5-year survival rate declines to 29.8% (2). Among the metastatic sites of PCa, bone, especially the axial skeleton, is the most frequently occurring metastatic site, accounting for 90% (3, 4). Bone metastases (BM) can be characterized morphologically as osteolytic, osteoblastic, or mixed. BM in PCa are predominantly osteoblastic (5). Skeletal-related events (SREs) are the major complications of BM and can affect the prognosis of BM patients. SREs include pathological fractures, spinal cord compression, hypercalcemia, the need for radiotherapy to relieve bone pain or reduce structural bone damage, and surgery to bone to prevent or repair a fracture (6).

As yet, the exact mechanisms causing BM remain unknown. It is known that the bone marrow has a high blood flow, and PCa cells release adhesive molecules that bind them to marrow stromal cells and bone matrix (7, 8). Additionally, bone stores a lot of growth factors, including fibroblast growth factors, insulin-like growth factors I and II, transforming growth factor β, platelet-derived growth factors, and bone morphogenetic proteins, and calcium (9). Bone will release these growth factors to provide a fertile ground for the growth of tumor cells during bone resorption (10). This "seed-and-soil hypothesis", proposed by Stephan Paget in 1889 (11), can roughly explain the mechanism of the process of BM, but more specific research is required.

The current treatments of BM are mainly aimed at preventing disease progression and symptom palliation but not cure—for example, bone-targeted agents, such as bisphosphonates and denosumab, have been proved to improve bone structure and quality to minimize the risk of skeletal morbidity (12–14). The clinical burden on patients and healthcare systems is very large due to the high incidence of BM. According to the World Health Organization (WHO), there were 1.3 million PCa patients, and 359,000 of them died of cancer in 2018 (1). Therefore, it is critical to develop strategies to prevent and treat BM and improve the quality of life and survival of BM patients.

Bibliometric analysis is an information visualization method to identify and summarize the frontiers or hot spots in a certain area. The quantification of literature in this field is based on mathematical and statistical methods through the analysis of literature and metrological characteristics. Moreover, we can compare the research status among various institutions, authors, or journals, evaluate the latest cutting-edge research, understand the scientific articles, and visualize their trends through this method.

We aimed to show the developments and trends in the research field of BM in PCa in the past 22 years by bibliometric analysis. Moreover, our study provides an overview of the research of BM in PCa that can serve as a reference for researchers.

# Methods

## Database

The data source of our research is the Science Citation Index Expanded (SCI-Expanded) of Clarivate Analytics' Web of Science Core Collection (WoSCC). The WoSCC is a widely used database for bibliometric studies which contains publications from nearly 9,000 high-impact journals.

## Search strategy

A search for publications related to PCa and BM was performed on April 25, 2022. The search query was formulated as follows: topic = "(prostate OR prostatic) NEAR/1 (cancer OR tumor OR tumor OR oncology OR neoplasm OR carcinoma)" AND "bone metastasis OR bone metastases" AND "publication date = (January 1, 1999–December 31, 2021)". The search results were downloaded in plain text format and in the tab separator format of "Full Records and cited References". Furthermore, the publication types were limited to original articles and reviews, and the language was limited to English.

## Data analysis and visualization

To ensure the accuracy of data and the reliability of the research, two researchers independently downloaded and

analyzed the data. Co-authorship, co-occurrence, and co-citation analysis are the most significant indicators in bibliometric analysis. Co-authorship analysis is conducted to analyze the relationships among authors, countries, or institutions. Co-occurrence analysis is a quantitative method to analyze the most frequently appearing items in articles. A co-citation analysis was conducted by comparing the ranked results with the co-citation score.

Microsoft Excel 2019 was used to analyze the annual publications and generate the line graph and to produce the tables of top-cited or productive countries/regions, publications, journals, and authors.

VOSviewer (version 1.6.18), CiteSpace (version 5.8.R3), and a widely used online platform for bibliometric analysis were used to visualize the data. With VOSviewer, one can map network data based on computer programs. It can be used to create a co-authorship, keyword co-occurrence, citation, bibliographic coupling, or co-citation map based on bibliographic data. Furthermore, The VOSviewer shows not only how the subjects are related to each other but also how far away in time they are in different colors. Therefore, we can use this visualization to predict future research hotspots. CiteSpace is mainly used to analyze the potential knowledge contained in the scientific literature and for data visualization. We used CiteSpace to analyze the co-authorship of institution and author, the co-authorship of reference and journal, and the co-citation of authors. We can learn about current and even future research hotspots from the reference's citation bursts. In addition, we generated a dual map overlay of journals by CiteSpace. The online platform for bibliometric analysis was applied to analyze the country/region's publications and co-authorship.

## Research ethics

All data were obtained from the public database. As a result, there was no need to seek ethics approval.

# Results

## Data collection and the trend of publication outputs

We obtained 6,162 related publications from the WoSCC database (Figure 1). However, 1,149 papers of them, including 752 meeting abstracts, 153 editorial materials, 151 conferences, 40 letters, 19 corrections, 17 book sections, and 17 other types of publications, were excluded. Moreover, 181 articles were also excluded because they were not written in English. Finally, a total of 4,832 articles, including 3,827 original articles and 1, 005 reviews, were included.

The quantitative analysis of published articles about BM in PCa is shown in Figure 2. We can see that the number of published articles increased from 1999 to 2016, and the increase was fastest from 2004 to 2014. However, the number of published articles increased less rapidly from 2017 to 2021, when it remained stable at a level of 300 to 400 articles per year.

## Analysis of published papers per country/region

In total, 4,832 articles were published by 4,872 organizations in 92 countries (Figure 3A). The USA published the most articles in this period (1,890, 39.1%) (Figure 3B; Table 1). China, England, Germany, Japan, and Italy also published more than 400 articles each. In addition, USA's total number of citations (98,773) and H-index (142) both ranked first. The country collaboration network is shown in Figure 3C. Figure 3C, which was generated by VOSviewer. In total, 37 countries that published more than 15 articles were included. The thickness of the line demonstrates the strength of cooperation among countries (named as total link strength, TLS). The top five TLSs were the USA, England, Germany, Canada, and Italy.

## Top productive institutions

In total, 4,872 institutions produced related articles. The most productive institutions were from the USA. As shown in Table 2, among the top 10 productive institutions, eight come from the USA and two are from the UK. The University of Texas MD Anderson Cancer Center (145) was the most productive institution, followed by the University of Michigan (139), the University of Washington (120), the Memorial Sloan-Kettering Cancer Center (97), and Amgen Inc. (89). We used VOSviewer to analyze the cooperation among institutions (Figure 4). With a threshold of ≥20 articles published, 110 institutions were included, and the top five TLSs were Amgen Inc., Massachusetts General Hospital, the University of Michigan, Inst Cancer Research, and the University of Washington.

## Analysis of authors and co-cited authors

In total, 23,495 authors and 64,811 co-cited authors were identified. The top 10 productive authors and the top 10 co-cited authors are shown in Table 3. Saad F (91), Logothetis CJ (50), Chung LWK (49), Fizazi K (47), and Keller ET (47) were the top five productive authors in the past 22 years. Saad F was the most cited author with a total number of 7,703 citations, and his H-index was the highest among the authors. However, Smith MR's average article citations (AAC) ranked first (138.74). Moreover, the top five co-cited authors were Coleman RE (1,813), Saad F

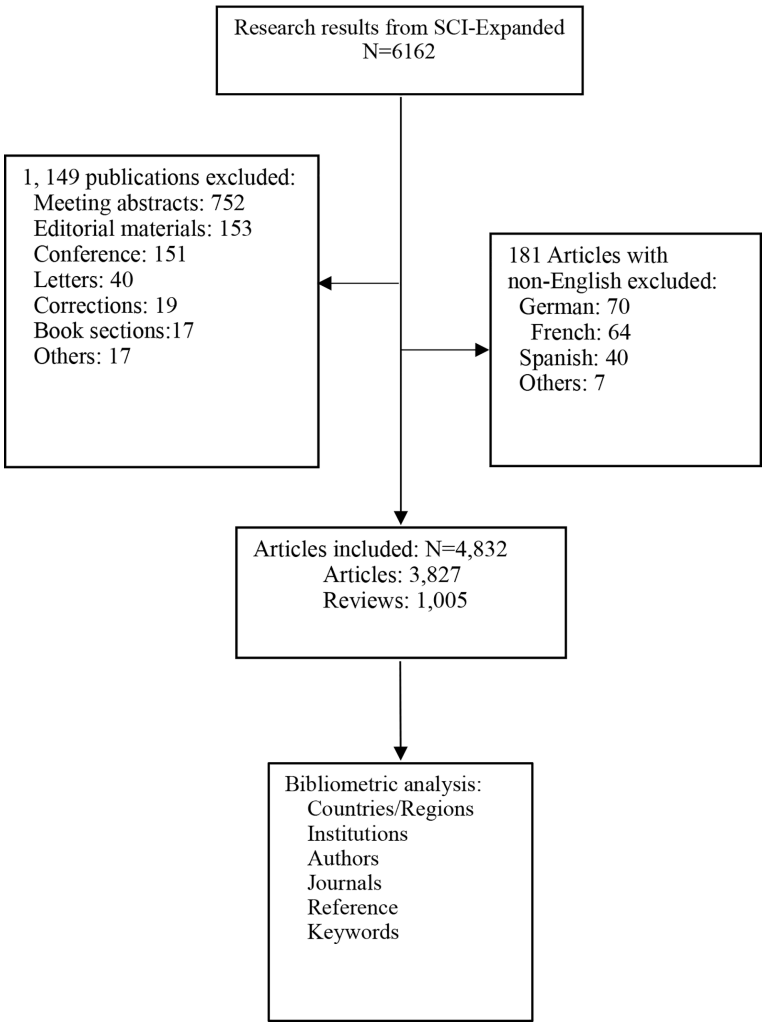


FIGURE 1
Flow chart of articles selection.

(1,598), Fizazi K (1,148), Smith MR (1,131), and Scher HI (988). The cooperation among co-cited authors who were cited more than 150 times is shown in Figure 5.

## Analysis of the top publishing journals

A total of 873 journals have published related articles. The top 10 publishing journals are shown in Table 4. *Prostate* (149), *European Journal of Nuclear Medicine and Molecular Imaging* (92), *Cancer Research* (89), *Clinical Cancer Research* (80), and *Anticancer Research* (74) were the most contributing journals. Among the top 10 journals, 60% were in JCR Q1, and their impact factors were relatively high, with 70% being higher than 5.0. Besides this, *Cancer Research* had both the highest number of citations and the highest H-index. A dual map reflecting the relationship between citing and cited journals is shown in Figure 6. There are four citation pathways in the dual map. The citing papers were classified into two research areas: (1) medicine, medical, and clinical and (2) molecular biology and immunology. Furthermore, the cited papers were also classified into two fields: (1) health, nursing, and medicine and (2) molecular biology and genetics.

## Analysis of the top co-cited references and the references with the strongest citation bursts

We used VOSviewer to analyze the co-cited references. The threshold was set as 100 cited times, and 74 references were included. The co-cited collaboration network is shown in

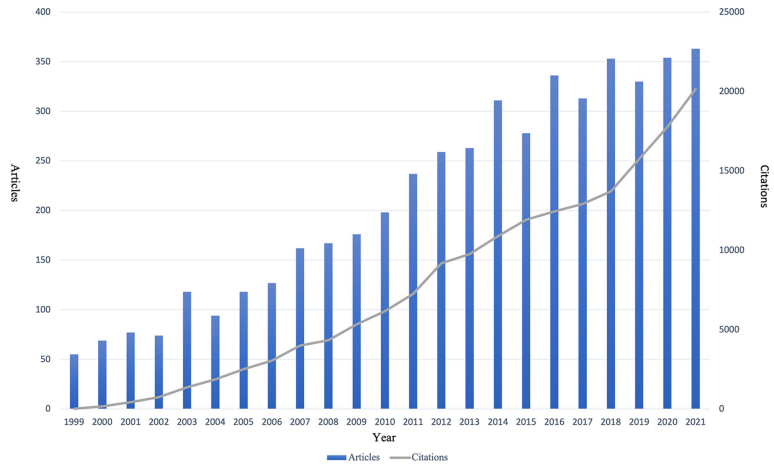


FIGURE 2
Quantitative analysis of the published articles per year.

Figure 7. The top 10 co-cited references are shown in Table 5. The top five co-cited references were Mundy GR, 2002, *Nat Rev Cancer* (464), Parker C, 2013, *New Engl J Med* (454), Saad F, 2002, *Jnci-J Natl Cancer I* (430), Roodman GD, 2004, *New Engl J Med* (406), and Fizazi K, 2011, *Lancet* (405). Interestingly, these top co-cited references were published relatively long ago, and most of them were published in journals with a high impact factor, which indicates that they are authoritative articles in the research field of BM in PCa. The top 25 references with the strongest citation bursts are shown in Figure 8. The first citation

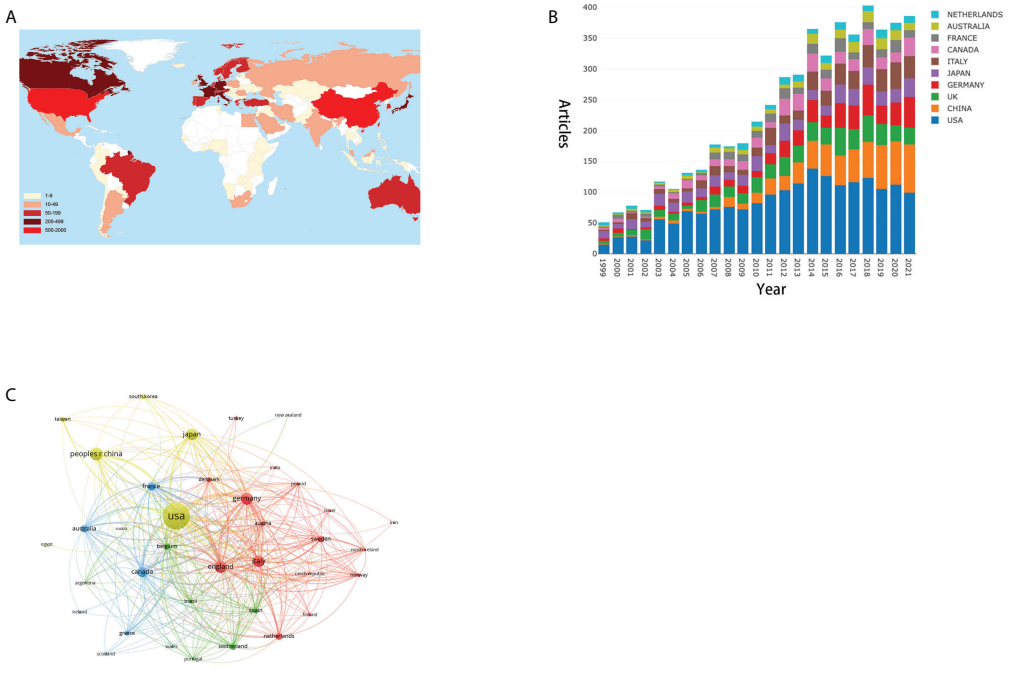


FIGURE 3
**(A)** Total articles per country/region. A different color indicates a different output. **(B)** Bar graph of the top 10 productive countries/regions. **(C)** Correlations among the countries/regions with more than 15 articles. The graph was generated by VOSviewer. Line thickness indicates the citation strength.

TABLE 1 Top 10 productive countries/regions associated with the bone metastases in prostate cancer.

| Rank | Country | Documents | Percentage | TC | AAC | H-index |
|---|---|---|---|---|---|---|
| 1 | USA | 1,890 | 39.11% | 98,773 | 52.26 | 142 |
| 2 | China | 535 | 11.07% | 10,733 | 20.06 | 51 |
| 3 | England | 473 | 9.79% | 28,719 | 60.72 | 82 |
| 4 | Germany | 453 | 9.38% | 20,014 | 44.18 | 76 |
| 5 | Japan | 441 | 9.13% | 14,353 | 32.55 | 55 |
| 6 | Italy | 428 | 8.86% | 16,017 | 37.42 | 66 |
| 7 | Canada | 343 | 7.10% | 19,385 | 56.52 | 70 |
| 8 | France | 234 | 4.84% | 16,162 | 69.07 | 69 |
| 9 | Australia | 197 | 4.08% | 14,220 | 72.18 | 57 |
| 10 | Netherlands | 180 | 3.73% | 8,793 | 48.85 | 47 |

BM, bone metastases; PCa, prostate cancer; TC, total citations; AAC, average article citations.

burst started in 2002, and there have been a lot of explosions since 2011. Notably, many citation bursts are still ongoing, which means that the field of BM in PCa is still a research hotspot.

## Analysis of the top co-occurrences of keywords

The analysis of the co-occurrences of keywords was performed by VOSviewer. A total of 6,150 author keywords were identified, and 72 keyword co-occurrences were found more than 20 times; their relationship network is shown in Figure 9. The network displays five colors, representing five clusters, and the thickness of lines reflects the relationship between keywords. The keyword "prostate cancer", with a TLS of 1,867, is located in the central position of the red cluster, which concentrates on the diagnosis of PCa with the keywords "diagnosis", "immunohistochemistry", "staging", and so on. The blue cluster, with the central keyword "bone metastasis", is focused on the mechanism of BM with the keywords "tumor microenvironment", "angiogenesis", "EMT", "osteoblast", and so on. The yellow cluster concentrates on the drug treatment of BM, the purple cluster reflects the radiotherapy of BM, and the green cluster shows the treatments and the prognosis of PCa. Furthermore, the overlay visualization of keywords, which shows the relationship of keywords and time, is shown in Figure 9B. The top 20 occurrences of keywords are shown in Table 6. Interestingly, "breast cancer" ranks fifth with 253 occurrences.

# Discussion

To our knowledge, this is the first bibliometric analysis about BM in PCa. We analyzed the research situation and provided a reference to researchers in the field. According to data from the WoSCC database on April 25, 2022, a total of 4,832 articles associated with BM in PCa have been published between 1999 and 2021. The number of publications is an essential indicator of the trends in the research area (15). The number of publications per year gradually increased from 55 articles in 1999 to 363 articles in 2021. The fastest increase was observed in the period of 2004 to 2014. Saad F, 2004, *J Natl Cancer I* (16) and Roodman

TABLE 2 Top 10 productive institutions in publications related to the research on bone metastases in prostate cancer.

| Rank | Institution | Countries/regions | Articles | Citations | TLS |
|---|---|---|---|---|---|
| 1 | University of Texas MD Anderson Cancer Center | USA | 145 | 6,477 | 2,827 |
| 2 | University of Michigan | USA | 139 | 8,902 | 3,194 |
| 3 | University of Washington | USA | 120 | 6,640 | 2,891 |
| 4 | Memorial Sloan-Kettering Cancer Center | USA | 97 | 7,583 | 2,655 |
| 5 | Amgen Inc | USA | 89 | 7,510 | 3,813 |
| 6 | University of Sheffield | UK | 81 | 4,509 | 2,544 |
| 7 | Inst Cancer Research | UK | 79 | 5,278 | 3,119 |
| 8 | California State University, Los Angeles | USA | 69 | 3,486 | 974 |
| 9 | Massachusetts General Hospital | USA | 68 | 8,262 | 3,297 |
| 10 | Wayne State University | USA | 68 | 3,823 | 965 |

TLS, total link strength.

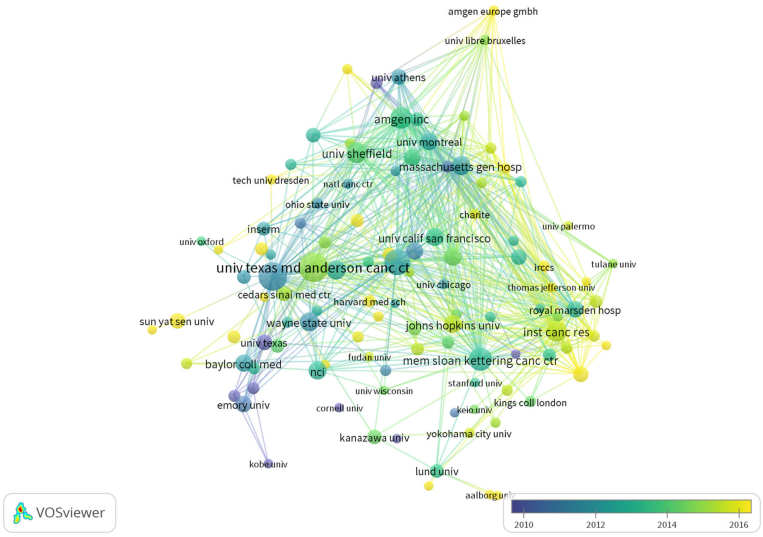


FIGURE 4
Overlay visualization map of the institutions with more than 20 articles.

G, 2004, *New Engl J Med* (17) played critical roles in the research of BM in PCa (Figure 8).

With respect to countries, the USA leads the way in research in this field. The USA has published 1,890 related articles in the past 22 years, while the second place is occupied by China with only 535 articles. Furthermore, USA has an H-index of 142 and a total number of 98,773 citations. China, ranking second in the output, has made great achievements in the field in the past 10 years (Figure 3B). However, the H-index (51) and AAC (20.06 times) of China fall behind those of the USA and European countries, which demonstrates that the quality of the research needs to be continually improved. Moreover, 80% of the top 10 productive institutions come from the USA, and all top five productive institutions are American. Although the University of Texas MD Anderson Cancer Center published most articles, the University of Michigan, ranking second in the production, has the most citations (8,902). As regards the quality of articles, Massachusetts General Hospital had the highest AAC with 68 articles and a total of 8,262 citations.

Furthermore, nearly all authors with authority in the field come from the USA and the UK (Table 3). Of the top 10 productive authors, 70% are American and 30% come from the UK. Saad F, coming from the USA, has published the most articles (almost twice as many as the second most productive author). However, Fizazi K and Smith MR have much higher AAC values than other authors.

*Prostate*, as a specialty journal, has published the most related articles in the past 22 years. *European Urology* (EU), from the Netherlands, is the most influential journal in the field of urology. EU has published 66 related articles in this period,

TABLE 3 The 10 most productive authors and the top 10 co-cited authors with the highest number of citations.

| Rank | Author | Country | Documents | Citation | Average article citations | H-Index | Co-cited author | Country | Total citations |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Saad, F | Canada | 91 | 7,703 | 84.65 | 39 | Coleman, RE | UK | 1,813 |
| 2 | Logothetis, CJ | USA | 50 | 3,132 | 62.64 | 29 | Saad, F | USA | 1,598 |
| 3 | Chung, LWK | USA | 49 | 3,049 | 62.22 | 33 | Fizazi, K | UK | 1,148 |
| 4 | Fizazi, K | UK | 47 | 6,327 | 134.62 | 32 | Smith, MR | USA | 1,131 |
| 5 | Keller, ET | USA | 47 | 2,728 | 58.04 | 30 | Scher, HI | USA | 988 |
| 6 | Pienta, KJ | USA | 47 | 2,890 | 61.49 | 28 | Lipton, A | USA | 947 |
| 7 | Smith, MR | USA | 46 | 6,382 | 138.74 | 34 | Mundy, GR | USA | 689 |
| 8 | Coleman, RE | UK | 45 | 3,478 | 77.28 | 33 | Parker, C | UK | 684 |
| 9 | Lin, SH | USA | 43 | 1,439 | 33.47 | 23 | Body, JJ | Belgium | 625 |
| 10 | Sartor, O | UK | 43 | 3,703 | 114 | 25 | Nilsson, S | Sweden | 625 |

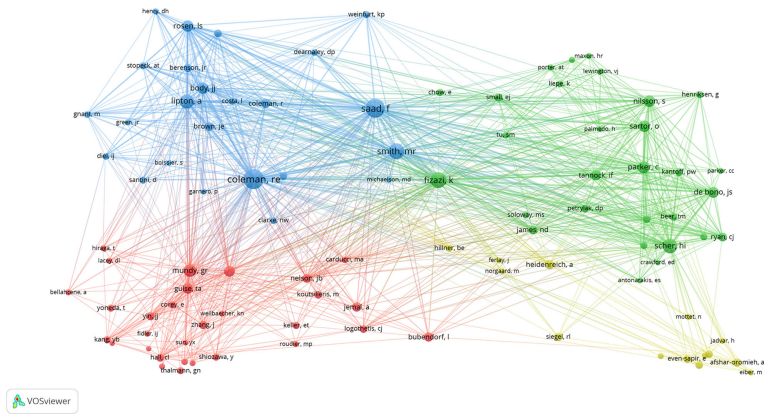


FIGURE 5
Visualization map of co-cited authors generated by VOSviewer.

which also indicates that BM in PCa is a hotspot in urology. Besides this, there are also some journals with low impact factors, such as *Plos One*, having published lots of related articles.

The concept of document co-citation in co-citation analysis was first proposed by Small and Marsakova. Document co-citation means that, if two documents A and B are jointly cited by a later published document C, then A has a co-citation relationship between B, which implies that they have a topic similarity relationship. As shown in Table 5, most of the top co-cited references were published during the first decade of the 21st century. According to Figure 8, Saad F, 2002, *Jnci-J Natl Cancer I* (18) initiated the first strong citation burst in 2002. Interestingly, there was no newly published reference causing a strong citation burst from 2004 to 2009. With the publication of Fizazi K, 2009, *Journal of Clinical Oncology*, the second wave of citation burst began, and it persists to the present.

In recent years, the most frequently used author keywords were "CT", "tumor microenvironment", "biomarkers", "mCRPC", "enzalutamide", "radium-223", and "bone-targeted agents" (Figure 9B). This demonstrates that CT, which is relatively precise and cheap, is the most frequently used diagnosis modality in BM. Furthermore, "tumor microenvironment" and "biomarkers" are the two hotspots in the research of the mechanism of BM in PCa. The immune microenvironment of primary PCa is considered "cold" while BM is relatively "hot" (19). According to Galon J's study (20), the immune microenvironment of BM is not inherently "hot", but it can be targeted by inhibiting the CCL20–CCR6 axis, which takes part in immune suppression and a lot of inflammatory and immune-activated states, including autoimmune disease (21). Consequently, through the research of "tumor microenvironment" and "biomarkers", finding suitable and effective therapeutic targets has a general prospect in the treatment of BM. "mCRPC" is a difficult and long-standing problem in PCa. BM is found in 90% of mCRPC patients, which can be explained by the tropism of PCa to bone (22). The first-line treatments of mCRPC include the androgen synthesis inhibitor abiraterone, the androgen receptor (AR) inhibitor

TABLE 4 The top 10 journals with the most articles associated with bone metastases in prostate cancer.

| Rank | Journal | Country | IF (2021) | JCR (2021) | Articles | Citations | ACC | H-Index |
|---|---|---|---|---|---|---|---|---|
| 1 | *Prostate* | USA | 4.104 | Q3 | 149 | 5,927 | 39.78 | 43 |
| 2 | *European Journal of Nuclear Medicine and Molecular Imaging* | Germany | 9.236 | Q1 | 92 | 4,578 | 49.76 | 39 |
| 3 | *Cancer Research* | USA | 12.701 | Q1 | 89 | 8,455 | 95.00 | 53 |
| 4 | *Clinical Cancer Research* | USA | 12.531 | Q1 | 80 | 6,001 | 75.01 | 46 |
| 5 | *Anticancer Research* | Greece | 2.480 | Q4 | 74 | 1,080 | 14.59 | 18 |
| 6 | *Journal Of Nuclear Medicine* | USA | 10.057 | Q1 | 73 | 4,252 | 58.23 | 35 |
| 7 | *Plos One* | USA | 3.240 | Q2 | 72 | 2,178 | 30.25 | 24 |
| 8 | *European Urology* | Netherlands | 20.096 | Q1 | 66 | 4,367 | 66.17 | 41 |
| 9 | *Bju International* | UK | 5.588 | Q1 | 62 | 1,800 | 29.03 | 27 |
| 10 | *Clinical & Experimental Metastasis* | Netherlands | 5.150 | Q3 | 61 | 2,236 | 36.66 | 26 |

IF, impact factor.

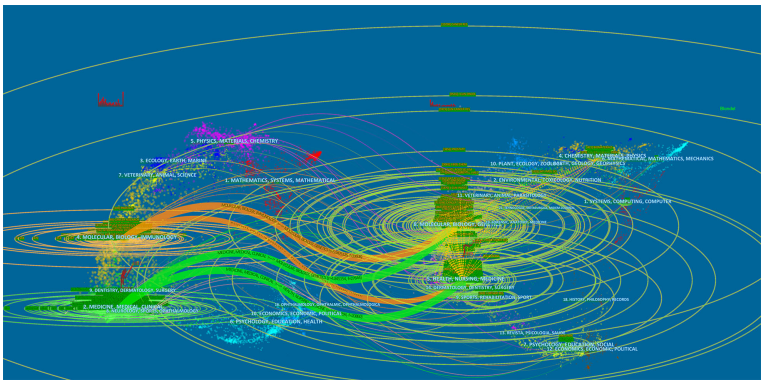

FIGURE 6
A dual map overlap of journals publishing articles on bone metastases in prostate cancer generated by CiteSpace.

"enzalutamide", the chemotherapeutic docetaxel, and the immunotherapeutic sipuleucel-T (23). It was reported that abiraterone can improve the overall survival by 4.4 months compared with the controlled group (34.7 *vs.* 30.3 months, $p$ = 0.0033) (24). Enzalutamide, a second-generation nonsteroidal AR inhibitor, affects the AR pathway in three ways: it binds to the AR with greater relative affinity than bicalutamide, it reduces the efficiency of AR nuclear translocation, and it impairs both DNA binding to androgen response elements and the recruitment of co-activators (25). According to Fizazi K and Scher HI's research (26, 27), enzalutamide can improve the median overall survival by 4.8 months. Docetaxel is another important treatment option for mCRPC, with 2–2.9 months of improvement in median survival compared with mitoxantrone plus prednisone therapy (28, 29). A phase III trial of sipuleucel-T showed 4.1 months of improvement of survival in 512 asymptomatic or minimally symptomatic mCRPC patients compared with the placebo group (30). Bone-targeted agents, including bisphosphonates, denosumab, and radium-223, are important for BM patients because they can prevent SREs to improve the quality of life and survival. However, despite progress in the development of new drugs, mCRPC is still incurable, and more targeted research is needed. The noncurative therapy for BM just aims to prolong survival, palliate symptoms, improve and maintain the quality of life, and prevent complications. In addition, the psychological impact of PCa treatments on patients is also a matter of concern. It has been reported that psychological disease, depression, and anxiety have incidence rates of 20%–28%, 4%–19%, and 7%–32%, respectively, in patients receiving radical prostatectomy. Interestingly, the psychological impact on patients under active surveillance is much more serious than

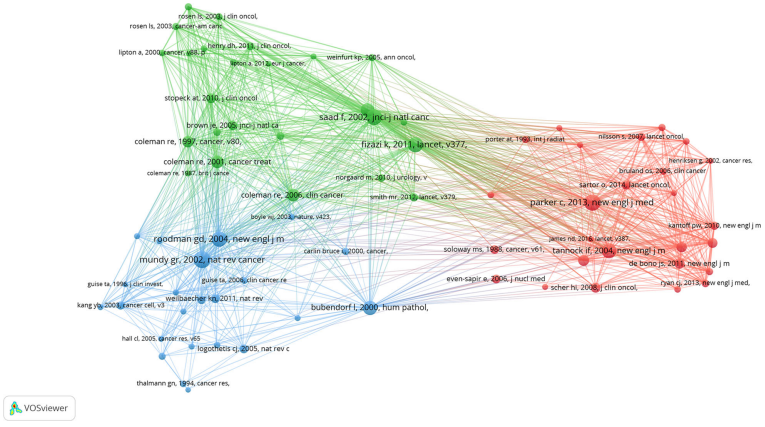


FIGURE 7
Co-cited reference collaboration network visualized by VOSviewer.

TABLE 5 Top 10 co-cited references concerning the research of bone metastases in prostate cancer.

| Rank | Title | Journals | Authors | Year | Citations |
|---|---|---|---|---|---|
| 1 | Metastasis to bone: causes, consequences and therapeutic opportunities | *Nat Rev Cancer* | Mundy GR, et al. | 2002 | 464 |
| 2 | Alpha emitter radium-223 and survival in metastatic prostate cancer | *New Engl J Med* | Parker C, et al. | 2013 | 454 |
| 3 | A randomized, placebo-controlled trial of zoledronic acid in patients with hormone-refractory metastatic prostate carcinoma | *Journal Of the National Cancer Institute* | Saad F, et al. | 2002 | 430 |
| 4 | Mechanisms of bone metastasis | *New Engl J Med* | Roodman GD, et al. | 2004 | 406 |
| 5 | Denosumab *versus* zoledronic acid for treatment of bone metastases in men with castration-resistant prostate cancer: a randomised, double-blind study | *Lancet* | Fizazi K, et al. | 2011 | 405 |
| 6 | Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer | *New Engl J Med* | Tannock IF, et al. | 2004 | 394 |
| 7 | Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients | *Hum Pathol* | Bubendorf L, et al. | 2000 | 368 |
| 8 | Long-term efficacy of zoledronic acid for the prevention of skeletal complications in patients with metastatic hormone-refractory prostate cancer | *J Natl Cancer Inst* | Saad F, et al. | 2004 | 339 |
| 9 | Metastatic bone disease: clinical features, pathophysiology and treatment strategies | *Cancer Treat Rev* | Coleman RE, et al. | 2001 | 316 |
| 10 | Clinical features of metastatic bone disease and risk of skeletal morbidity | *Clin Cancer Res* | Coleman RE, et al. | 2006 | 316 |

the impact on patients receiving radiotherapy (31). Actually, artificial intelligence, an emerging research field in PCa, may be able to better predict the side effects of different treatments in the future (32).

Bibliometric analysis, because of its function of summarizing research hotspots in a certain research field, can provide directions for future research in this field. Through a bibliometric analysis of prostate cancer bone metastases, we were able to summarize the research in this field over the past few decades. More importantly, we can learn about the research hotspots in this field in recent years, and researchers will benefit from it. Scientific research not only needs to keep moving forward but also needs to summarize the past and gain experience and direction from past research. Therefore, the field of prostate cancer bone metastases research would benefit from the bibliometric analysis of clinical and biomedical

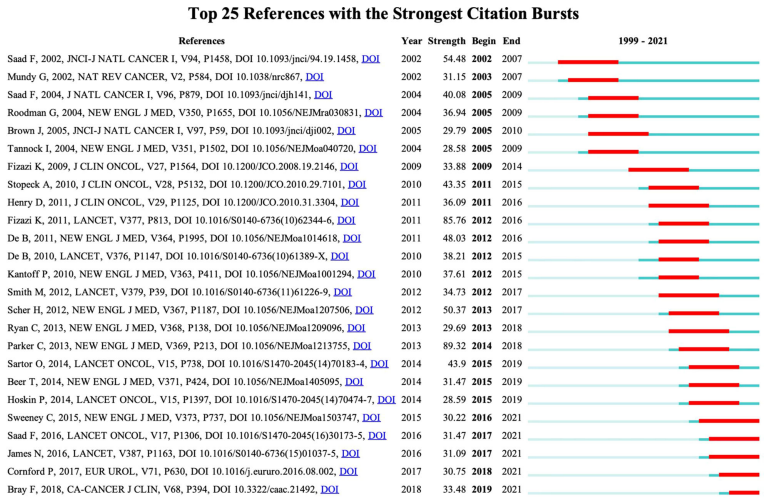


FIGURE 8
Visualization map of top 25 references with the strongest citation bursts from 1999 to 2021 generated by CiteSpace.

TABLE 6 Top 20 author keywords with the most occurrences in the included articles.

| Keyword | Occurrences | Total link strength (TLS) | Keyword | Occurrences | TLS |
|---|---|---|---|---|---|
| Prostate cancer | 1,703 | 1,867 | Bone | 165 | 348 |
| Bone metastasis | 1,535 | 1,890 | Denosumab | 164 | 380 |
| Metastasis | 327 | 513 | Osteoclast | 140 | 280 |
| Bisphosphonates | 279 | 533 | Radium-223 | 137 | 197 |
| Breast cancer | 253 | 470 | SRE | 135 | 299 |
| Zoledronic acid | 248 | 507 | CRPC | 134 | 154 |
| PSA | 206 | 309 | Radiotherapy | 126 | 172 |
| Cancer | 194 | 301 | Prostate | 111 | 176 |
| Prognosis | 182 | 274 | Osteoblast | 98 | 205 |
| PET | 174 | 277 | MRI | 83 | 136 |

PSA, prostate-specific antigen; PET, positron emission tomography; SRE, skeletal-related events; CRPC, castration-resistant prostate cancer; MRI, magnetic resonance imaging.

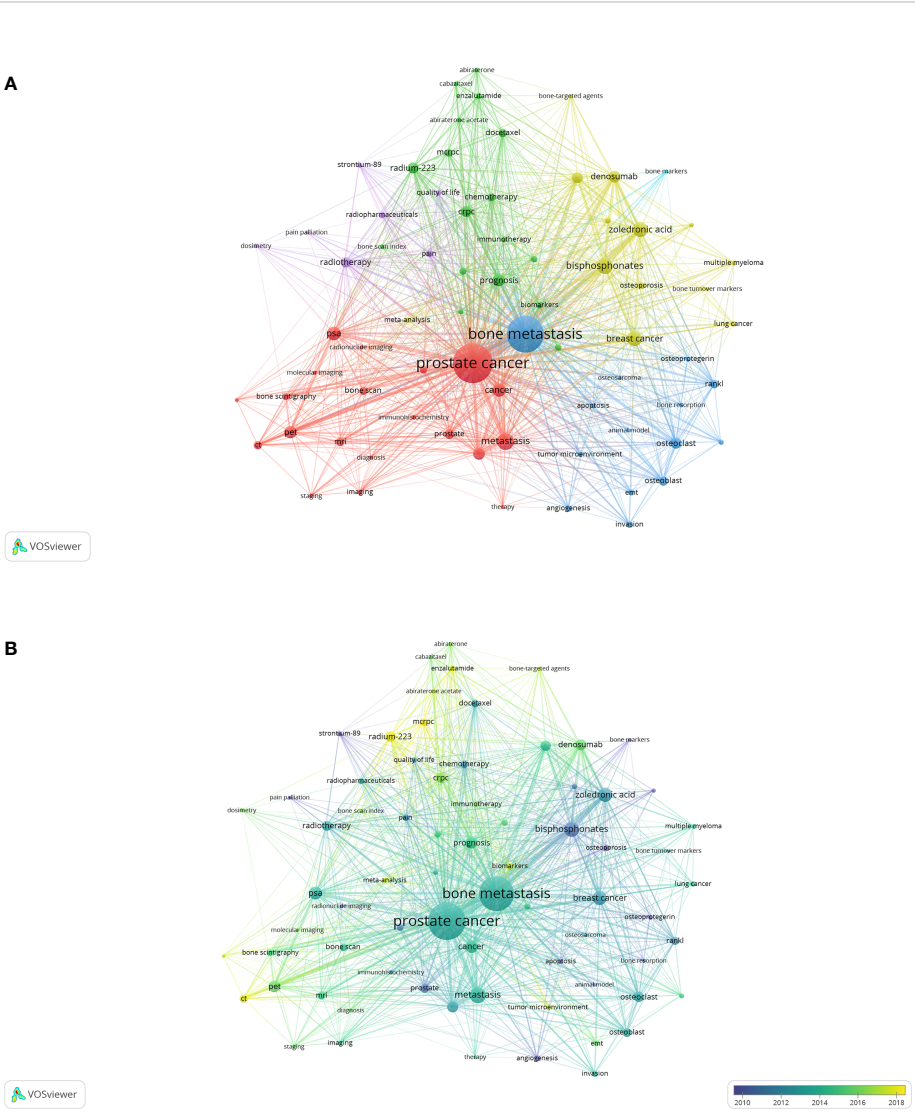


FIGURE 9
Networks generated by VOSviewer. **(A)** Visualization map of the author keywords with more than 20 occurrences. **(B)** Overlay visualization map of the author keywords with more than 20 occurrences.

exploration—for example, clinical bibliometric analysis can tell us the research hotspots for clinical diagnosis or treatments of prostate cancer bone metastases. In addition, from the bibliometric analysis of biomedicine, we can learn the hotspots in the research on the mechanism of bone metastasis in prostate cancer, and researchers will be guided to pay attention to this aspect. In sum, these could advance the field of prostate cancer bone metastases research.

### Limitations

There are still some limitations in our study. First, we cannot ensure that all related articles were included with our search strategy, which may cause a bias of the results. Second, recently published articles have not had enough time to be cited.

## Conclusion

Based on the present bibliometric analysis of BM in PCa, we conclude that the research field of BM in PCa has been getting hot since 1999. USA leads the way in nearly all aspects of the research field, such as the most productive institutions and authors. Notably, mCRPC has been the hottest topic in PCa research in recent years. CT is the most common diagnostic method for BM. Enzalutamide and radium-223, as important treatment modalities for BM in PCa, have attracted widespread attention. Furthermore, studies focus on the tumor microenvironment and biomarkers to explore the mechanism and the therapeutic targets of BM in PCa. In the future, perhaps mCRPC therapies will be the most critical research field of BM in PCa.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Author contributions

ZG, HH and YL conceived and designed the study. YC and CT analyzed the data by using Excel and wrote the manuscript. ZS and JZ visualized the data by using VOSviewer and Citespace. ZL and LL searched and downloaded the data from WoSCC. SP and WW revised and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

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# Radioresistance in rhabdomyosarcomas: Much more than a question of dose

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Management of rhabdomyosarcoma (RMS), the most common soft tissue sarcoma in children, frequently accounting the genitourinary tract is complex and requires a multimodal therapy. In particular, as a consequence of the advancement in dose conformity technology, radiation therapy (RT) has now become the standard therapeutic option for patients with RMS. In the clinical practice, dose and timing of RT are adjusted on the basis of patients' risk stratification to reduce late toxicity and side effects on normal tissues. However, despite the substantial improvement in cure rates, local failure and recurrence frequently occur. In this review, we summarize the general principles of the treatment of RMS, focusing on RT, and the main molecular pathways and specific proteins involved into radioresistance in RMS tumors. Specifically, we focused on DNA damage/repair, reactive oxygen species, cancer stem cells, and epigenetic modifications that have been reported in the context of RMS neoplasia in both *in vitro* and *in vivo* studies. The precise elucidation of the radioresistance-related molecular mechanisms is of pivotal importance to set up new more effective and tolerable combined therapeutic approaches that can radiosensitize cancer cells to finally ameliorate the overall survival of patients with RMS, especially for the most aggressive subtypes.

KEYWORDS

**rhabdomyosarcoma, radiotherapy, radiation therapy, radioresistance, radiosensitizers**

# Introduction

## Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is a highly aggressive soft tissue sarcoma (STS) that primarily affects pediatric patients, accounting for 5% of all childhood cancers and representing 3% of STS in adult, for whom it has a worse prognosis. As shown in Figure 1, the most common RMS location is in the head and neck region (35%–40%), genitourinary tract (bladder/prostate, 11%), genitourinary tract non-bladder/prostate (male, 12%; female, 5%), and limbs (16%). Signs and symptoms at presentation will depend on the site of the primary tumor, whether there is extension into contiguous organs, and, in some cases, the presence of metastatic disease (1). Originally, two major subtypes of RMS were recognized: embryonal RMS (ERMS), preferring male children, and alveolar RMS (ARMS), which remains constant throughout childhood and adolescence, showing the worse prognosis. Others two rarer RMS subtypes are the pleomorphic RMS and the spindle cell/sclerosing RMS, which typically occur in adults and children, respectively (2, 3). ARMSs more frequently carry t (2;13)(q35;q14) or t(1;13)(p36; q14) chromosomal translocations that, juxtaposing Paired box gene 3 (PAX3) on chromosome 2 or PAX7 on chromosome 1 with Forkhead box protein O1 (FOXO1) on chromosome 13, generate PAX3–FOXO1 and PAX7–FOXO1 fusion genes, respectively, and finally transcribe/translate into pro-oncogenic fusion proteins with an aberrantly enhanced transcriptional activity (4, 5). Because fusion protein presence correlates with a poorer prognosis, nowadays, the preferred RMS classification is expressing, i.e., "fusion positive" (FP-RMS), or not expressing fusion protein, i.e., "fusion negative" (FN-RMS) (6). ERMSs (FN-RMS tumors), more frequently present various mutations largely converging on a limited number of pathways, also perturbed in FP-RMSs, indicating some commonality in the molecular driving forces in RMS (2). FN-RMSs often harbor a mutation affecting mitogen-activated protein kinases and/or PI3K–AKT–mTOR pathways (7, 8), aberrantly activated also in FP-RMS, to the ability of fusion proteins to activate several cell surface receptor tyrosine kinases upstream of these pathways (5). Notably, patients with FN-ARMS are clinically and molecularly indistinguishable from ERMS (9).

This review presents a brief overview of the guidelines for the diagnosis and treatment of RMS, with particular emphasis on the role of radiotherapy (RT) and on the molecular mechanisms mainly responsible for radioresistance, focusing on possible candidate radiosensitizing strategies in RMS. In particular, after summarizing the key principles of the management of the patient with RMS, from diagnosis to treatment, focusing on the role of RT and on the novelties in terms of indications, therapy schemes, and treatment techniques, we will analyze the principles of radiobiology and of the RMS and, therefore, the molecular mechanisms of radioresistance.

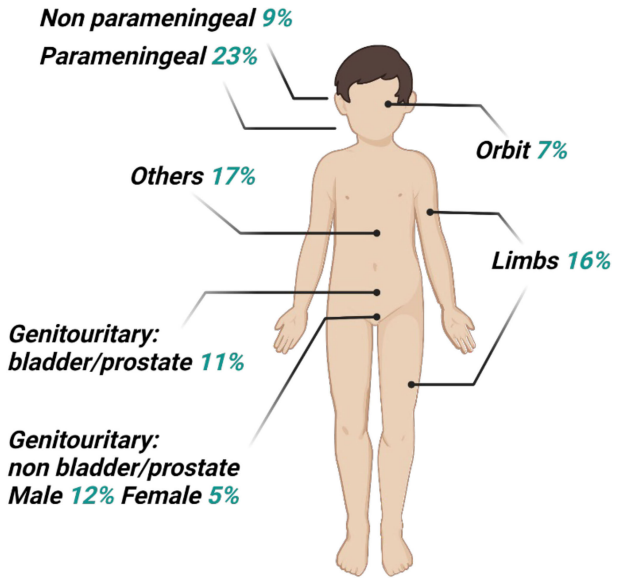


FIGURE 1
Distribution of primary sites for rhabdomyosarcoma. The head and neck site may be subdivided as 7% orbit, 8% other head, 23% parameningeal, and 9% non-parameningeal. The pelvic sites may be subdivided as 11% bladder and prostate, and 5% female genital or 12% male non-bladder/prostate.

## RMS diagnosis and staging

After the head and neck site (35%–50%), the genitourinary tract represents the primary site for around 20%–25% of RMS pediatric patients, resulting as exceedingly rare in adults (10, 11). Vagina or uterus, favorable sites, are more frequently involved, followed by the kidney, bladder, or prostate, considered as unfavorable sites (12, 13). At the onset, hematuria and urinary obstruction represent the signs and symptoms more frequent, whereas 10%–20% of pediatric and 40% of adult patients present distant metastases. RMS diagnosis requires, in addition to standard laboratory (complete blood counts, electrolytes, renal function tests, liver function tests, and urinalysis), the direct evaluation of tumor tissue derived from either an incisional/excisional biopsy or a core needle biopsy (1) magnetic resonance imaging, for local staging, and computed tomography (CT) and [F-18]2-fluoro-2-deoxyglucose positron emission tomography (18F-FDG PET)/CT), for systemic staging and the risk stratification (14, 15). TNM staging is based on the anatomic location and invasiveness of the primary tumor, tumor size, nodal status, and extent of metastasis (Tables 1, 2). Intergroup Rhabdomyosarcoma Study Group (IRSG) establishes risk group stratification, identifying low-, standard-, high, or very high–risk patients. The clinical subgroup is primarily determined by IRSG group, lymph node involvement, fusion protein expression, site, and age (16, 17). Risk stratification is summarized in Table 3. The bioptic samples must be subjected to a series of histology and molecular pathology studies aimed at measuring myogenic markers like desmin, skeletal alpha-actin, myosin, and myoglobin and early myogenesis transcription factors like MyoD and myogenin (18). The analysis of these markers is achieved with immunohistochemical assays, and it is combined with cell morphological assessment in light microscopy, used to distinguish RMS from other childhood neoplasms also expressing myogenic proteins (18). More recently, molecular analysis has become an essential tool for differential diagnosis and classification of RMS (Figure 2). Specifically, Real-Time PCR (RT-PCR) and Fluorescence in situ hybridization (FISH) assays designed to measure the expression of fusion gene PAX3–FOXO1 or PAX7–FOXO1 are very useful to identify subsets of ARMS, and microarray genome-wide RNA expression techniques have been shown to generate, through various statistical algorithms, "diagnostic signatures" of the FP-RMS and FN-RMS categories (2).

## RMS treatments

Treatment of locally and locally advanced RMS is mainly based on surgery (14, 15), although, aggressive surgery, often necessary to achieve tumor debulking and negative microscopic margins, is no longer recommended (19). This is particularly true for genitourinary RMS, to avoid significant long-term morbidities such as urinary diversion, infertility, and sexual dysfunction particularly. Therefore, except for paratesticular tumors (20), the standard care of RMS, genitourinary and non-genitourinary, usually provides neoadjuvant chemotherapy (CHT) followed by RT or concomitant CHT/RT followed or not by excision (14, 15). In 20% of patients with genitourinary RMS, a close follow-up with imaging is a reasonable alternative to aggressive surgery (14, 15).

TABLE 1 TNM classification for rhabdomyosarcoma.

| T: Tumor Stage | | |
|---|---|---|
| $T_1$: Confined to anatomic site of origin | $T_{1a}$: ≤5 cm | $T_{1b}$: >5 cm |
| $T_2$: Extension and/fixative to surrounding tissue | $T_{1a}$: ≤5 cm | $T_{1b}$: >5 cm |
| **N: Regional Nodes** | | |
| $N_0$: Not clinically involved | $N_1$: Clinically involved | $N_X$: Clinical status unknown |
| **M: Metastases** | | |
| $M_0$: No distant metastases | $M_1$: Distant metastases present | |

TABLE 2 TNM stage for rhabdomyosarcoma.

| stage | Primary site | TNM stage | Tumor size | Regional nodes | Distant metastasis |
|---|---|---|---|---|---|
| 1 | Favorable* | $T_1$ or $T_2$ | Any size | $N_0$ - $N_1$ - $N_x$ | $M_0$ |
| 2 | Unfavorable | $T_1$ or $T_2$ | ≤5 cm | $N_0$ - $N_x$ | $M_0$ |
| 3 | Unfavorable | $T_1$ or $T_2$ | ≤5 cm | $N_1$ | $M_0$ |
| | | | >5 cm | $N_0$ - $N_1$ - $N_x$ | |
| 4 | Any | $T_1$ or $T_2$ | Any size | $N_0$ - $N_1$ - $N_x$ | $M_1$ |

*Favorable sites: orbit; non-parameningeal head and neck; genitourinary tract other than kidney, bladder, and prostate; and biliary tract.

TABLE 3 European Paediatric Soft Tissue Sarcoma Study Group staging of rhabdomyosarcoma.

| Risk Group | Subgroups | FP (+) FN (-) | IRS Group | | Site | Node Stage | Age / Size |
|---|---|---|---|---|---|---|---|
| | | | I = R0 or Complete | | Favorable | N0 | Favorable <10y / <5cm |
| | | | II = R1 or Microscopic disease or primary complete resection but N1 | | | | |
| | | | III = R2 or Macroscopic Disease | | Unfavorable | N1 | Unfavorable >10y / >5cm |
| | | | IV = Distant Metastases | | | | |
| Low | A | – | I | R0 | Any | N0 | A(F)+S(F) |
| Standard | B | – | I | R0 | Any | N0 | A(F) or S(F) |
| Standard | C | – | II | R1 | Favorable | N0 | Any |
| | | | III | R2 | | | |
| High | D | – | II | R1 | Unfavorable | N0 | Any |
| | | | III | R2 | | | |
| High | E | – | II | R1 | Any | N1 | Any |
| | | | III | R2 | | | |
| High | F | + | I | R0 | Any | N0 | Any |
| | | | II | R1 | | | |
| | | | III | R2 | | | |
| Very High | G | + | II | R1 | Any | N1 | Any |
| | | | III | R2 | | | |
| Very High | H | Any | IV | Metastases | Any | Any | Any |

In support of a delayed surgery, it has been shown that, despite RMS can persist after a neoadjuvant approach (21, 22), the rhabdomyoblasts found in subsequent biopsies progressively decrease and their presence do not necessarily predict local recurrence (23, 24). Thus, aggressive surgical resection, followed or not by RT, is usually performed for recurrent and metastatic RMS (25, 26). CHT is based on IVA (ifosfamide, vincristine, and actinomycin D) or VAC (vincristine, actinomycin D, and cyclophosphamide), respectively used, with no difference, in Europe or in North America (27, 28). Combining doxorubicin improves IVA, inducing several treatment-related adverse events (29). Low-dose maintenance CHT has been shown to improve outcome (30). Trabectedin is commonly used as a second line (31), whereas several clinical trials conducted to test the effects of several molecular targeted drugs combined or not with other targeted therapies or CHT had not shown significant clinical improvement (32–40). However, because of CHT-induced toxicities, pharmacological treatments are often interrupted (41).

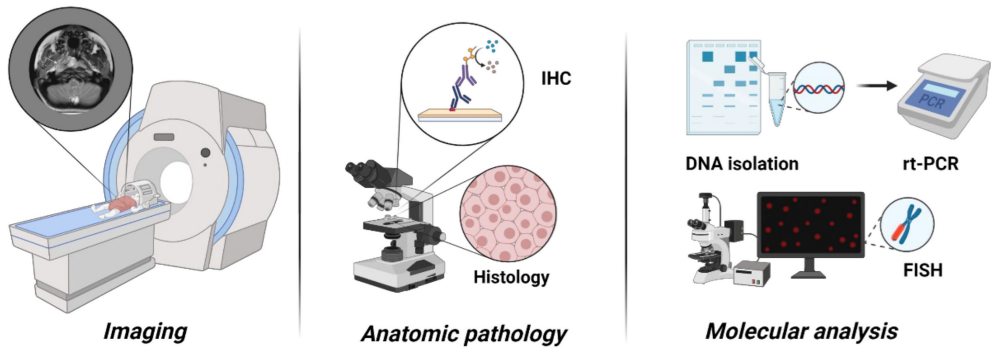


FIGURE 2
The role of molecular analysis for differential diagnosis and classification of RMS. Molecular analysis helps to identify the subtypes and classify RMS.

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TABLE 4 Doses and fractions of radiotherapy for patients over 3 years of age.

| Conventional Radiotherapy (Age > 3 years) | | |
|---|---|---|
| **IRS Group** | **ERMS** | **ARMS** |
| **I = R0 or Complete** | | |
| **II = R1 or Microscopic Disease** | | |
| **III = R2 or Macroscopic Disease** | | |
| **I** | No radiotherapy | 41.4 Gy in 23 fractions |
| **II** | 41.4 Gy in 23 fractions | 41.4 Gy in 23 fractions |
| **III** | 50.4 Gy in 28 fractions | 50.4 Gy in 28 fractions |
| **III → R0 after reoperation** | 36 Gy in 20 fractions | 41.4 Gy in 23 fractions |
| | 41.4 Gy in 23 fractions | |
| **Complete clinical response to Chemotherapy and no surgery** | 41.4 Gy in 23 fractions | 50.4 Gy in 28 fractions |
| **Partial clinical response to Chemotherapy and no surgery** | 45 Gy in 25 fractions | 50.4 Gy in 28 fractions + boost 5.4 Gy in 3 fractions |
| **Stable clinical response to Chemotherapy and no surgery** | 50.4 Gy in 28 fractions + boost 5.4 Gy in 3 fractions | 50.4 Gy in 28 fractions + boost 5.4 Gy in 3 fractions |
| **Orbital** | 45 Gy in 25 fractions | |

## Current status of radiation treatment in RMS

Achieving local tumor control with the first-line treatment is crucial for patients with RMS (42). RT plays an increasingly critical role in the management of RMS for local control both at primary and metastatic sites and continues to be a major treatment modality for genitourinary RMS (43–49). Because routine use has been encouraged by the EpSSG RMS 2005 study (44), RT has been shown to improve the event-free survival rate of patients, whereas local failures have been shown to be more frequent when the irradiation is omitted (50). The dose, duration, and timing of external beam radiation therapy, in which x-rays can penetrate deeper in the tissues while minimizing skin irradiation and side effects (51), depend on the patient's age, RMS type and histology, the site of origin of the tumor, how much tumor remained after surgery, and the local lymph nodes involvement. Table 4 resumes treatment schedules (15, 52, 53). In case of adult patients with RMS, RT can provide a total dose from 50 up to 70 Gy, conventionally delivered (54–56). The use of hyperfractionated (hFRT) regimen, smaller doses per single fraction, performed by the large, randomized IRS-IV study, failed (57). This failure, however, has permitted to revise the radiobiology of RMS, as later discussed. On the other hand, the use of hyperfractionated (HFRT) regimen, larger doses per single fraction, delivered in combination or not with CHT, did not give significant advantages (58–70). Thus, the RMS response to RT appears to go far beyond the simple dose problem because, as for other highly radioresistant tumor types, RMS appears capable of activating a complex biological response supporting radioresistance.

## The radiobiology of RMS: The linear quadratic model and the question of dose

Radiobiology has been classically focused on achieving the greatest possible difference between a high probability of local tumor control [tumor control probability (TCP)] and a low risk of normal tissue complications [normal tissue complication probability (NTCP)], namely, therapeutic window. In fact, whether increasing the dose improves TCP, because of the lack of technology able to spare normal tissues, it also increases NTCP. Thus, RT has been long delivered by using daily fractions of 1.8–2.2 Gy, the conventional fractionation, which is still largely used today. The reason why conventional fractionation guarantees the best therapeutic window depends on the concept that normal cells repair sublethal damages more efficiently than cancerous cells, as shown from the linear quadratic model (LQ) (71) and from of "4Rs" of radiobiology (72). Briefly, LQ is a mathematical model describing the relationship between cell survival and delivered dose, and it is represented by the equation $S = e^{-}$ (71). The probability to survive ($S$) of a cell/tissue type to a single dose of radiation depends on the ratio between two factors: i) the number of cells directly killed by double-strand breaks (DSBs), namely, a; and ii) the number of cells that, having saturated the repair mechanisms, die for the accumulation of sublethal unrepaired single-strand breaks (SSBs), namely, b. The a/b ratio indicates the fraction size sensitivity of a tissue, with b indicating the ability of cell to repair SSBs. Hence, cells with a low a/b ratio efficiently repair SSBs, contrary to cells with a high a/b ratio (71). Notably, doses of RT close to 1.8–2.2 Gy induce thousands of repairable SSBs and few DSBs (73–75), thus

indicating that the proportion of cells surviving to conventional fractionation strictly depends on the ability to repair SSBs. Thus, because cancer cells less efficiently "R"epair SSBs than normal cells, cancer cells slower "R"edistribute cell cycle from RT-induced $G_2$/M arrest, less efficiently "R"epopulate killed cancer cells, and result more affected by the "R"eoxygenation of the central portions of the tumor induced by the progressive reduction of the peripheral regions. Those are the "4Rs" of radiobiology, historically supporting the efficiency of the conventional fractionation (72). However, after a long time, it was shown that not all cells within cancer population and not all patients with the same tumor have the same sensitivity to RT, introducing the fifth "R", the "R"adiosensitivity (76). Thus, considering the technological evolution of RT that nowadays permits the safety delivery of larger fractions (77), the use of HFRT or Stereotactic Body Radiation Therapy (SBRT), ablative dose of radiation, has been proposed as strategy to overcome the intrinsic radioresistance of cancer. Furthermore, this choice is also supported by the fact that increasing evidence shows the ability of HFRT and SBRT to "R"eactivate the anti-tumor immune response, the sixth "R" of radiobiology (78). As previously discussed, the use of higher dose per fraction has been also proposed for RMS as a consequence of the low a/b ratio (2.8 Gy) shown for this cancer type (79). However, the use of HFRT for the treatment of RMS did not lead to any improvement in efficacy (58–70), as already described for other cancer types (80–84). Despite being a milestone in the multimodal treatment of pediatric RMS, RT is still significantly associated with local failure in most cases of tumor relapse, and the RMS response to radiation appears to go far beyond the simple dose problem. Indeed, together with the development of more sophisticated and effective technologies, overcoming radioresistance seems to be not just a question of dose but rather of understanding the cellular mechanisms that support radioresistance to identify future radiosensitizing strategies.

## Mechanisms of radioresistance in RMS

As other highly radioresistant tumor types, RMS appears capable of activating a complex biological response that makes them capable of resisting even high radiation doses. Therefore, it is necessary to deeply elucidate the precise mechanisms that are responsible for the radioresistance in RMS to identify new radiosensitizing therapeutic strategies. Over the last years, different studies have identified several key cellular and molecular factors, including DNA damage and repair, oxidative stress, tumor microenvironment, cancer stem cells (CSCs), and tumor heterogeneity (Figure 3), which are implied in RMS radioresistance and are discussed in detail in the following subsections.

### RT and DNA breaks/damage response-related pathways

Ionizing radiations used in RT are electrically charged particles, which deposit energy in the tissues that they pass through, killing cancer cells or causing genetic changes that lead to cancer cell death (85). On a cellular level, the biological target of radiation is DNA, by inducing several types of DNA damages involving one strand (SSBs) or both strands of DNA (DSBs) (86, 87). Although radiations damage both cancer and normal cells,

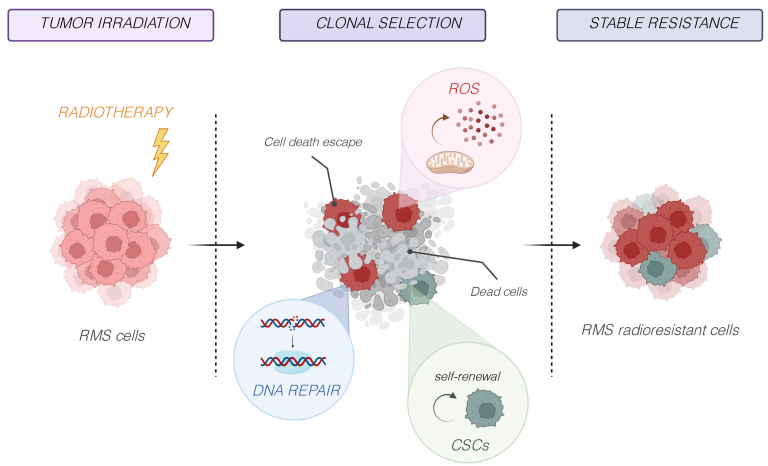


FIGURE 3
Molecular mechanisms responsible of radioresistance. Several key cellular and molecular factors, including DNA damage and repair, oxidative stress, tumor microenvironment, cancer stem cells (CSCs), and tumor heterogeneity, are implied in RMS radioresistance.

cancer cells are generally less efficient in repairing damages caused by RT, resulting in differential killing outcomes (88). DSBs, the most lethal form of DNA damage and a primary cause of cell death induced by RT, can be divided in simple and complex types. Simple DSBs are two-ended breaks of DNA, usually directly consequent to the action of radiation, whereas complex DSBs are clusters of different DNA damages including single-base mutations, insertions, and deletions and/or SSBs around DSBs, generally indirectly induced by radiation through the production of reactive oxygen species (ROS) (89–93). Thus, contrary to SSBs and simplex DSBs, complex DSBs are usually inefficiently repaired, determining genomic instability and cell death (94–96) although cancer cells can activate specific DNA damage repair mechanisms, thus surviving the following irradiation (92). The homologous recombination (HR) and the non-homologous end joining (NHEJ) mechanisms represent the most prominent pathways, orchestrating the DNA damage response (DDR) in eukaryotic cells. HR uses homologous sequences of undamaged sister chromatid as a template to repair DSBs, thus resulting in an error-free DDR mechanism (97). HR is mainly regulated by the MRN complex (Mre11–Rad50–Nbs1), which recognizes DSBs recruiting activated ataxia-telangiectasia mutated (ATM) that, in turn, orchestrates the activity of Breast Cancer gene 1 (BRCA1), Breast Cancer gene 2 (BRCA2), Checkpoint kinase 2 (CHK2), RAD51, and tumor protein 53 (p53)—key factors involved in HR. Parallelly, ATR (ataxia-telangiectasia mutated and Rad3-related) kinase attenuates DSB-induced ATM activation by switching DSB ends from an ATM-activating mode to an ATR-activating model (98) and activates CHK1 that slows or arrests cell cycle progression, thus allowing more time for DNA repair (99). Mutation inactivating BRCA1 and/or BRCA2 and/or other(s) gene(s) of the HR pathway, namely, the "BRCAness" status, permits to stratify HR-deficient (HRD) from HR-proficient (HRP) cancers (100) and to identify HRD as more sensitive to "synthetic lethality" mediated by PARP inhibitor (PARPi) (101, 102). The "BRCAness" phenotype has been shown in several types of sarcomas (103–105), including RMS (106, 107), although the ability of RMS to activate HR-mediated DDR is not excluded (108, 109). On the benchside, RMS biopsies overexpressing PARP1, PARP2, and PARP3 mRNAs compared with normal skeletal muscle and PARPi have been demonstrated to affect growth, survival, and radiation susceptibility of human ARMS and ERMS cell lines (110, 111). However, on the bedside, clinical trials testing PARPi on sarcomas, not including RMS, failed (112, 113), whereas a recent phase I trial (NCT02787642) combining the PARPi with RT in locally advanced/unresectable STS, including RMS, is going to give encouraging downstaging and survival rates (114). Therefore, using PARPi could radiosensitize RMS independently of HRD or HRP phenotype because conventional RT, causing thousands of SSBs, would saturate the HR mechanisms inducing, in the presence of PARPi, RMS death, as already shown for other cancer types (115).

Another potential target to affect ERMS radiosensitivity is c-Myc, whose downregulation through the inhibition of the MEK/ERK pathway has been demonstrated to *in vitro* and *in vivo* cause cell death by promoting the radiation-induced DNA DSB damage and impairing the DNA DSB repair machinery (116). NHEJ is the major DDR pathway activated by RT (117). Unlike HR, NHEJ re-ligates two broken DNA strands mainly through DNA-dependent Protein Kinase catalitic subunit (DNA-PKcs) that, complexing with Ku70/Ku80 heterodimer and DNA polymerase μ (Pol μ) and Pol λ, and in collaboration with XRCC4, XLF, LIG4, and PAXX, orchestrates this prone-error repair process. Moreover, DNA-PKcs has been shown to interplay with HR pathway, suggesting its pleiotropic role in regulating DDR (118). Targeting DNA-PKcs has been supposed to be a critical radiosensitizing strategy (89, 119), and, nowadays, several inhibitors, with a high selectivity and a valid pharmacokinetics, are available (118), including peposertib that is investigated by several clinical trials, in combination with RT or CHT plus RT, across a variety of cancer types (NCT02516813, NCT02316197, NCT03770689, NCT04555577, NCT04533750, and NCT03907969). Preclinical evidence shows that inhibiting DNA-PKcs sensitizes sarcoma to RT (120, 121), although no RMS cells have been investigated. However, several studies suggest a role for DNA-PKcs in RMS radioresistance. Specifically, DNA-PKcs has been shown to promote sarcomagenesis (122) and to sustain the activity of c-Myc (123) and AKTs (124), which are known to foster radioresistance in ERMS (116, 125–127) and ARMS tumors (128). Thus, it seems unlikely that DNA-PKcs targeting will not lead to an RMS radiosensitization. Notably, several molecules have been identified as upstream regulators of DDR in RMS including ERKs (126, 129), DNA methyltransferases 3A (DNMT3A) and DNMT3B (130), BET proteins (131), ephrin-A2 (132), caveolin-1 (CAV-1) (128), nuclear factor erythroid 2–related factor 2 (NRF-2) (133), c-Myc (116), SNAI2 (134), FAK (135), androgen receptor (136), and HDAC (137–139). Thus, another strategy to target DDR could be inhibiting these upstream molecules.

## RT and antioxidant response

RT mainly kills cancer cells by inducing the generation of ROS, which, in turn, represents the main induction mechanism of DSBs (140). Furthermore, the production of ROS can persist for several months after RT, thus enhancing the curative effects of treatment (141). However, cancer cells can activate an antioxidant stress response able to protect cells against ROS injury during RT exposure (142, 143). Kelch-like ECH-associated protein 1 and NRF2, respectively, inhibits and promotes the antioxidant response by upregulating the expression of downstream genes, such as peroxiredoxins (PRDXs), superoxide dismutases (SODs), catalase (CAT), and

glutathione peroxidases 4 (GPx4) (144). The radiosensitizing effects of targeting antioxidant response in cancer cells shown on the benchside (145) have been recently confirmed on the bedside (146–148). ROS levels are critical for RMS homeostasis (149), and their modulation results are critical for the response to therapies (150). Irradiated RMS upregulates NRF2, SODs, CAT, and GPx4 expression, whereas NRF2 silencing counteracts RMS radioresistance by increasing DSBs and impairing DDR (133). Furthermore, we have recently shown that CAV-1, a tumor promoter sustaining rhabdomyosarcomagenesis (151–153), promotes radioresistance in RMS through increased oxidative stress protection (128) and that RMS surviving to RT more efficiently detoxifies from ROS (109). Increasing oxidative stress has been shown to efficiently kill RMS (154). RMS antioxidant response is finely regulated by molecular epigenetic mechanisms (137–139), known to be critical regulator of adaptive responses to stress (155, 156), including RT (157). Interestingly, the ability of RMS to detoxify from ROS increases in parallel with the acquisition of a more radioresistant phenotype (109), suggesting that, for these cells, ROS detoxification is critical to survive to radiation. However, clinical trials working by inducing ROS levels (158–160) have not included RMS. No preclinical and/or clinical data related to the use of directly targeting redox proteins drugs have been collected on RMS. However, several pieces of evidence suggest the use of drugs able to increase ROS beyond targeting redox proteins (137–139). On this regard, ROS generation has been identified as a mediator of histone deacetylase (HDAC) inhibitor (HDACi)–induced cell death (161), and the combination of HDACi with RT brings to RMS radiosensitization through increased ROS accumulation (137–139).

## RT and cell death, autophagy, and senescence

Radiobiology defines cell death as the loss of replicative capacity determined by clonogenic assays, thus including apoptosis, necrosis, mitotic catastrophe, and mitotic death, autophagy, and tumor dormancy (162, 163), although increasing evidence indicates that RT-induced tumor dormancy may not be reversible (164, 165). Apoptosis caused by RT can be mediated by the following: i) intrinsic apoptotic pathway, through the activation of the cytochrome c-caspase 9/8/3 cascade (166); ii) extrinsic apoptotic pathway, through TNF-α/TNF-R1– (167) or TRAIL/Apo2L/TRAIL–receptor–caspase 8/3 cascade (168); and iii) ceramide accumulation that, acting as second messenger, initiates a complex apoptotic program (169). Mitotic catastrophe and mitotic death, defined as the failure to undergo complete mitosis after DNA damage, coupled to defective checkpoints, are usually mediated by intrinsic apoptosis (170). In addition, cell death can be induced by inducing necroptosis, pyroptosis, and ferroptosis (162, 163). Necroptosis, mediated by TNF-α/TNF-R1/RIP1/RIP3/MLKL cascade, in the absence of caspase 8 activation (171) and pyroptosis, triggered by cytoplasmic damaged-associated molecular patterns (DAMPs) and mediated by NLRP1/NLRP3/NLRC4/caspase 1/gasdermin cascade, leads to pore formation at the cytoplasmatic membrane. Ferroptosis, induced by excessive lipid peroxidation that leads to $Fe^{3+}$ accumulation-induced oxidative stress, is mediated by SLC11A2 and negatively regulated by GSH/GPx4 cascade (172). RMS is resistant to apoptosis (173) and necrosis (174), including from RT, as we have shown in preclinical *in vitro* and *in vivo* models (130, 137–139, 175). The tumor suppressor p53, a master promoter of apoptosis (176) and programmed necrosis (177), is frequently mutated in ERMS (178) and downregulated in ARMS (179). Recently, p53 mutations and/or pathway alterations have been associated with the increase of RMS radioresistance (180). Furthermore, RMS expresses high levels of anti-apoptotic Bcl-2 family members (181) and inactivation of caspase 8 expression by hypermethylation (182–184). On the other hand, RMS has been shown to differently modulate the expression of several factors, restraining the activation of programmed necrosis (185–188), whereas a programmed necrosis–related gene signature has been recently identified as novel prognostic biomarker for sarcoma (189). Thus, altogether, these alterations could explain the RMS resistance to RT-induced apoptosis and necrosis. Targeting cell death pathways regulating molecules has been supposed to be an opportunity for the development of innovative treatment strategies also in RMS (190). Targeting TRAIL (184, 191) and Bcl-2 (192) and reactivating caspase 8 expression (183) have been shown to promote apoptosis in RMS, alone or in combination with cytotoxic agents. The depletion of endogenous GSH by sorafenib has shown encouraging result *in vitro* and *in vivo* (193) but failed on the bedside (37), whereas others GSH inhibitors have shown anti-RMS therapeutic potential (194, 195). No data have been collected on combining pro-apoptotic or pro-necrotic agents with RT in treating RMS; however, our group has recently showed that pre-treatment with the BET inhibitor (BETi) OTX015 radiosensitizes RMS cells by inhibiting DDR and concomitantly inducing cell death as demonstrated by the strong activation of the apoptotic marker cleaved PARP (131). Death is not the only response from irradiated cells. Autophagy is a catabolic pathway for lysosomal-mediated cellular components degradation, basally inhibited by the mammalian target of rapamycin (TOR) complex 1 (mTORC1) pathway and tightly regulated by autophagy-related proteins (196). Physiologically considered as a cell survival mechanism that can also promote cell death (197), autophagy plays dual roles in cancer (198), including in RMS (199–204). Similarly, RT-induced autophagy has been shown to be cytoprotective or not (205, 206). However, increasing evidence suggests that autophagy cannot be restricted to a single cytoprotective or cytotoxic function, although it is more correct to speak about

"autophagic switch". Thus, autophagy can switch its function even within the context of a specific cancer type and/or with the respect to external stress type (207). Notably, RMS aberrantly expresses guanine nucleotide exchange factor T (208) recently shown to protects cells by inhibiting autophagy and apoptosis (204). Thus, the reduction in autophagy, which we recently shown on irradiated RMS (175), could be a mechanism of radioresistance. There is a lack of evidence on the effects of combining autophagy and RT promoters or inhibitors in the treatment of RMS. Senescence is classically defined as an irreversible form of growth arrest, mainly induced by p53, $p21^{WAF1}$, $p27^{KIP1}$, and $p16^{INK4A}$ and the inhibition of cyclin-dependent kinases and RB (209). The induction of senescence represents a therapeutic advantage, thus preventing further proliferation. However, senescent cells can escape from the irreversible growth arrest status and re-enter the cell cycle, boosting tumor growth (210). In particular, these "post-senescent" cells retain stem cell–related features, also known as senescence-associated stemness, suggesting a more aggressive behavior and favoring tumor relapse (210). Senescence represents the most common cellular response after RT (211–213). However, it has been recently shown that RT-induced accumulation of senescent cells can interfere with the therapy and encourage tumor regrowth (211). Thus, the use of senolytics, small molecules that can selectively induce apoptosis of senescent cells, has been supposed to be a valid radiosensitizing strategy (214). The role of RT-induced senescence in RMS is under investigation. Our group has recently shown that DNMT3A promotes radioresistance of RMS by restraining RT-induced senescence (130), probably for the ability of DNMTs to promote DNA repair activity (215), as summarized in Figure 4. Furthermore, we have also found that the expression of CAV-1 protects against RT-induced cell senescence (128), thus indicating the modulation of specific regulators of cellular senescence as a promising tool to set up new and effective therapeutic intervention against RMS, mainly for overcoming tumor radioresistance-related mechanisms.

## RT and immune response

Immunotherapies, largely used in several cancer types, resulting effective in a significant fraction of standard therapy refractory patients (216), have not shown objective response in patients with RMS (217, 218). Sarcoma and particularly RMS are considered "cold" to underline the immunologically inert nature of these tumors. Thus, identifying new strategies to turn "cold" in "hot" tumors could be the way to induce immunogenic cell death (ICD) and promote the use of immunotherapies for treating sarcoma (219). RT has been shown to convert malignant cells into endogenous anticancer vaccines, thus resulting in the main strategy able to trigger ICD and boost immunotherapies. RT promotes the release of DAMPs, triggering the chemotaxis of antigen-presenting cells (APCs), dendritic cells, macrophages, and B cells, finally determining cross priming of CD8+ effector T cells. Parallelly, RT-induced cell death modifies the tumor microenvironment through the cytokine release and the expression on endothelium of cell adhesion molecules. In response, cancer cells can rapidly trigger anti-immune response by different signals including PD1/PD-L1 and CTLA4/B7-1 or B7-2 (220, 221), resulting in an opportunity for combining immunotherapies (222). However, cancer cells surviving to RT express mutated genes, increasing the presentation to APCs of neoantigens potentially able to refresh the immune response (223). This process could lead to an "antigen recycling" potentially able to re-boost the ICD-induced anticancer immune response. In this context, repeated exposure to tumor antigens released by "pulsed-RT" has been recently shown to amplify the adaptive immune response by expanding the tumor-specific T-cell receptor repertoire, the production of high-affinity tumor antibodies, and the generation of memory lymphocytes and thereby improve immune control of systemic disease (224). Therefore, the dose and fractionation seem to be the variables mainly affecting pro-immunogenic effects of RT (225, 226). Actually, HFRT and SBRT seem to improve the ability of RT to promote immune responses to tumors (225, 226). A very small percentage of RMS or RMS-infiltrating immune cells express PD-L1, more frequently in low-stage RMS and related to an increased 5-year overall survival rate (227–229). The use of immunotherapies for the treatment of pediatric advanced solid tumors has been limited (227–229), and no patients with RMS have been included. We have recently shown that Transforming growth factor beta (TGF-β), Macrophage migration inhibitory factor (MIF), C-C Motif Chemokine Ligand 2 (CCL2), C-X-C motif chemokine 5 (CXCL5), CXCL8, and CXCL12, are key players of both intrinsic and acquired radioresistance in RMS (109). Thus, targeting these cytokines, known to be mediators of radioresistance (230), could be another strategy to radiosensitize RMS tumors.

## RT and cancer stem cells

An important evidence of cancer management is the impact of RT-mediated strategies on CSCs, which are characterized by a slowly dividing subpopulation of tumoral cells capable of self-renewal features that have a critical role in tumor maintenance and metastasis as well as in resistance phenomena to conventional treatments in many cancer types (231). Recent evidence suggests that CSCs of several malignancies, also comprehending RMS, can resist ionizing radiation because of their peculiar metabolic status, associated with high expression of genes and pathways related to stem-like features, activated DNA repair mechanisms (232), and altered levels of free radical scavenger levels (233). Specifically, several studies have demonstrated the specific molecular pathways contributing to

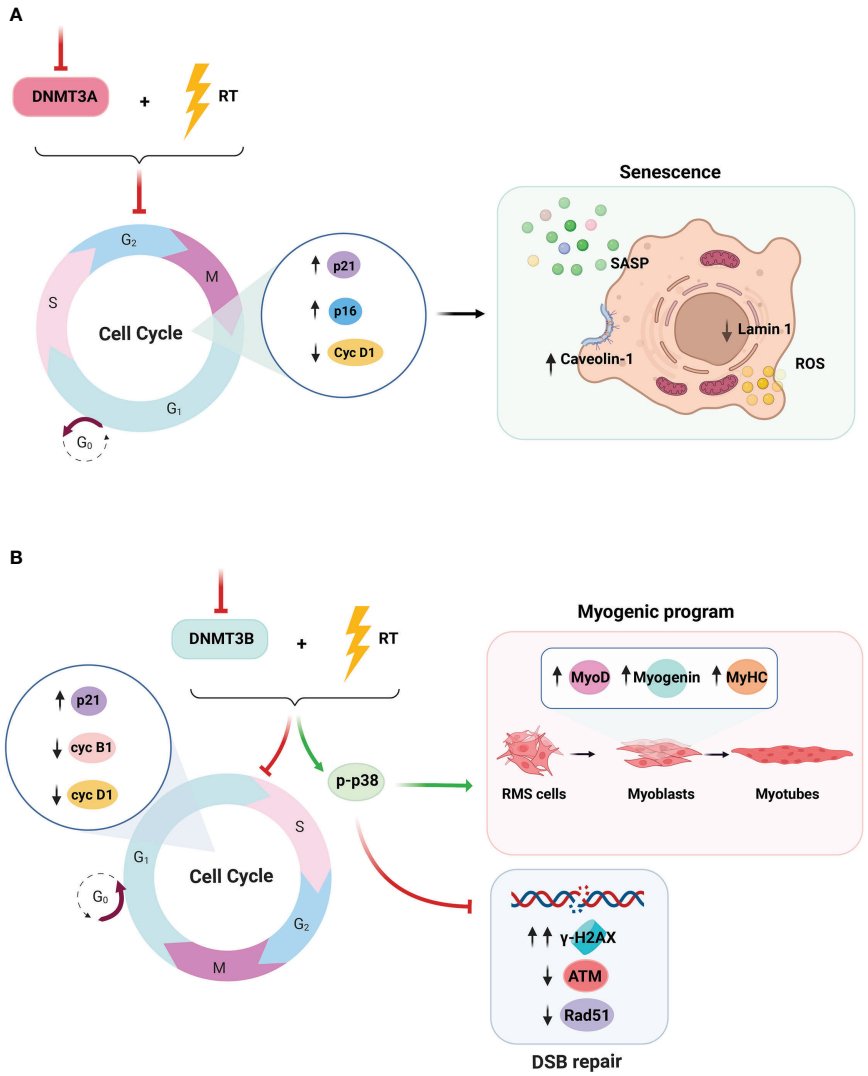


FIGURE 4
Effects of the combined treatment with DNMT3A/3B silencing and radiotherapy. Visual representation of the different radiosensitizing mechanisms observed upon **(A)** DNMT3A and **(B)** DNMT3B knocking down and RT co-treatment.

the CSC intrinsic radioresistance, such as PI3K/Akt/mTOR and NOTCH ones (234–237), which upregulates ROS scavenging enzymes (238). Thus, inhibiting NOTCH could be the efficient strategy to radiosensitize CSCs, bypassing their ability to detoxify from ROS. To date, clinical trials testing NOTCH inhibitors have not included RMS tumors as well as the combination with RT, but their use as radiosensitizers has been recently encouraged (239). More recently, boron neutron capture therapy and carbon-ion particle therapy have been proposed in combination with PARPi as effective strategies for the treatment of radioresistant clear cell sarcoma and osteosarcoma, opening up the possibility of successfully treating patients with RMS by combined treatment with RT and PARPi.

## RT and epigenetic remodeling.

Epigenetic alterations, mainly DNA methylation and histone modifications, characterize various cancers (240), including RMS (8, 241, 242). This evidence raises the question about whether the regulation of DNA methylation activity might represent a useful target for radiation sensitization. Targeting epigenetic molecules may therefore be significant in development of novel therapies, including the development of radiosensitizers (243, 244). Notably, deregulated epigenetic mechanisms have been shown to sustain different mechanisms of radioresistance including DNA repair (245, 246), antioxidant response (247, 248), cancer cell life and death decisions (249), as well as anti-cancer immune response (250). Therefore, identifying the molecules and epigenetic reprogramming pathways

used by cancer cells could lead to the development of promising targeted therapies able of weakening the different mechanisms of radioresistance, also in the context of RMS. Indeed, targeting specific DNMTs or HDACs has been demonstrated to reverse RMS phenotype, counteracting stemness and inducing radiosensitization (137–139). Specifically, our group has recently demonstrated the overexpression of DNMT3A and DNMT3B in ERMS primary tumor biopsies (251) and highlighted the synergic impact of DNMT3A or DNMT3B silencing and irradiation on viability and aggressiveness of RMS cells, suggesting that DNMT inhibitors could have a clinical application in combination with standard RT in the clinical management of patients with RMS. Other strategies to reduce radioresistance-mediated mechanisms have been recently shown in ARMS tumor, the most aggressive type of RMS (137, 252). The BETi OTX015, an orally drug able to bind and block histones' acetylated lysines, can downregulate GNL3 gene, encoding for the G nucleolar 3 protein, which is overexpressed in different malignancies, and it has been associated with uncontrolled proliferation, inhibition of programmed cell death, and resistance to therapies. Interestingly, our preclinical data also indicate that OTX015 exposure can enhance the radiosensitivity of ARMS cells by inducing a drastic G2 cell cycle arrest, which was correlated to a permanent DNA damage (upregulation of γ-H2AX) and to the inability of tumoral cells to repair it (alteration of RAD51, ATM, and DNA-PK protein expression). Moreover, OTX015 and irradiation (IR) synergistically downregulated the expression of GNL3 gene, thus suggesting a potential role of BETi in reducing cell cycle progression and maintenance of cell stemness with the potential to counteract the radioresistance phenomena. Similar remarkable radiosensitizing effects were exerted on FP-RMS cells by targeting class I and IV HDACs through MS-275 *in vitro* and *in vivo* treatment (137), confirming the crucial role of epigenetic deregulation in RMS onset and progression. Interestingly, the immunological effects of epigenetic modifiers could be used for stimulating therapeutically relevant anticancer immunity when used as stand-alone treatments or in combination with established immunotherapies, favoring the RT-induced presentation of new antigens. Thus, it is possible to assume that the antigenic recycling induced by pulsed radiotherapy, for example, could be further enhanced in presence of an epigenetic remodulation and that, therefore, the tumor can be, in this way, more easily "heated". To this date, growing evidence suggests that radiation exposure is also related to substantial epigenetics changes of cancer cells (253). Different studies demonstrated that RT could affect DNA methylation patterns and promote a decrease in the expression level of DNMTs (254), with that genomic hypomethylation resulting in enhanced radiation sensitivity in colon carcinoma (255).

## Discussion

Years of oncology research have demonstrated the great ability of cancer cells to adapt to various therapies, including RT. Technological advances in delivering radiation have improved tumor targeting, limiting radiation exposure of healthy tissues. Thus, nowadays, the largest part of cancer patients receives RT, which results to be curative in the most cases. RT is integrated into the primary treatment of most patients with RMS. However, despite more than 90% of children with non-metastatic RMS achieves complete remission, up to one-third of them experiences a recurrence, whereas the outcome of adult patients treated with RT has not been improved. Thus, RMS remains a very deadly cancer. The use of HFRT, based on single larger dose fractions, has not led to the desired results, suggesting that the improvement of the therapeutic potential of RT goes far beyond the question of the dose, requiring knowledge and counteracting of the molecular mechanisms responsible for radioresistance. Thus, radiosensitizers remain a viable option for improving the outcome of therapy in RMS. More research is necessary to fully understand the mechanisms of RMS radioresistance and improve the outcomes of patients with this deadly disease.

## Author contributions

SCa, MC, and SP: study design, data analysis, data collection, and writing; LM, FV, AP, GB, CM, SCo, and MT: data analysis and data collection; RR and AF: review and editing; FMa and FMe: review and editing, and supervision. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

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# A review of the biology and therapeutic implications of cancer-associated fibroblasts (CAFs) in muscle-invasive bladder cancer

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Cancer-associated fibroblasts (CAFs) play a fundamental role in the development of cancers and their response to therapy. In recent years, CAFs have returned to the spotlight as researchers work to unpick the mechanisms by which they impact tumour evolution and therapy responses. However, study of CAFs has largely been restricted to a select number of common cancers, whereas research into CAF biology in bladder cancer has been relatively neglected. In this review, we explore the basics of CAF biology including the numerous potential cellular origins of CAFs, alongside mechanisms of CAF activation and their diverse functionality. We find CAFs play an important role in the progression of bladder cancer with significant implications on tumour cell signaling, epithelial to mesenchymal transition and the capacity to modify components of the immune system. In addition, we highlight some of the landmark papers describing CAF heterogeneity and find trends in the literature to suggest that the iCAF and myCAF subtypes defined in bladder cancer share common characteristics with CAF subtypes described in other settings such as breast and pancreatic cancer. Moreover, based on findings in other common cancers we identify key therapeutic challenges associated with CAFs, such as the lack of specific CAF markers, the paucity of research into bladder-specific CAFs and their relationship with therapies such as radiotherapy. Of relevance, we describe a variety of strategies used to target CAFs in several common cancers, paying particular attention to TGFβ signaling as a prominent regulator of CAF activation. In doing so, we find parallels with bladder cancer that suggest CAF targeting may advance therapeutic options in this setting and improve the current poor survival outcomes in bladder cancer which sadly remain largely unchanged over recent decades.

KEYWORDS

**cancer-associated fibroblast, muscle-invasive bladder cancer, bladder cancer, CAF, immunotherapy, radiotherapy**

# 1 Introduction

## 1.1 Brief overview of bladder cancer

Approximately 20,000 people are diagnosed with bladder cancer each year in the UK (1). Typically, patients are aged 75 years or greater at diagnosis and are predominantly male. However, bladder cancer affects all genders and also occurs in younger patients, and the most clearly established risk factor for bladder cancer is smoking (2). In the past 30 to 40 years, there have been minimal advances in the survival outcomes of patients diagnosed with bladder cancer. Indeed, the 5-year survival following radical cystectomy or chemo-radiation remains at approximately 50% (3). Therefore, there is an urgent need to better understand bladder cancer biology to help find more effective curative treatments.

The most common form of bladder cancer arises from cells in the bladder lining, or urothelium, and is consequently known as urothelial bladder carcinoma. The anatomy of the bladder and stages of bladder cancer, defined according to how far the cancer cells have invaded into the bladder wall, are shown in Figure 1. Early stage, non-muscle-invasive bladder cancers (NMIBC) reside within the bladder lining and are risk-stratified depending on the tumour grade, a measure of tumour cell growth rate. In contrast, muscle-invasive bladder cancer (MIBC) describes tumours that have grown into the muscle wall of the bladder, referred to as the muscularis propria.

Comprehensive analysis of gene expression data resulted in the molecular classification of MIBC and the identification of molecular subtypes by several research groups (4–9). In a recent consensus classification, six subtypes have been defined: luminal papillary, luminal non-specified, luminal unstable, stroma-rich,

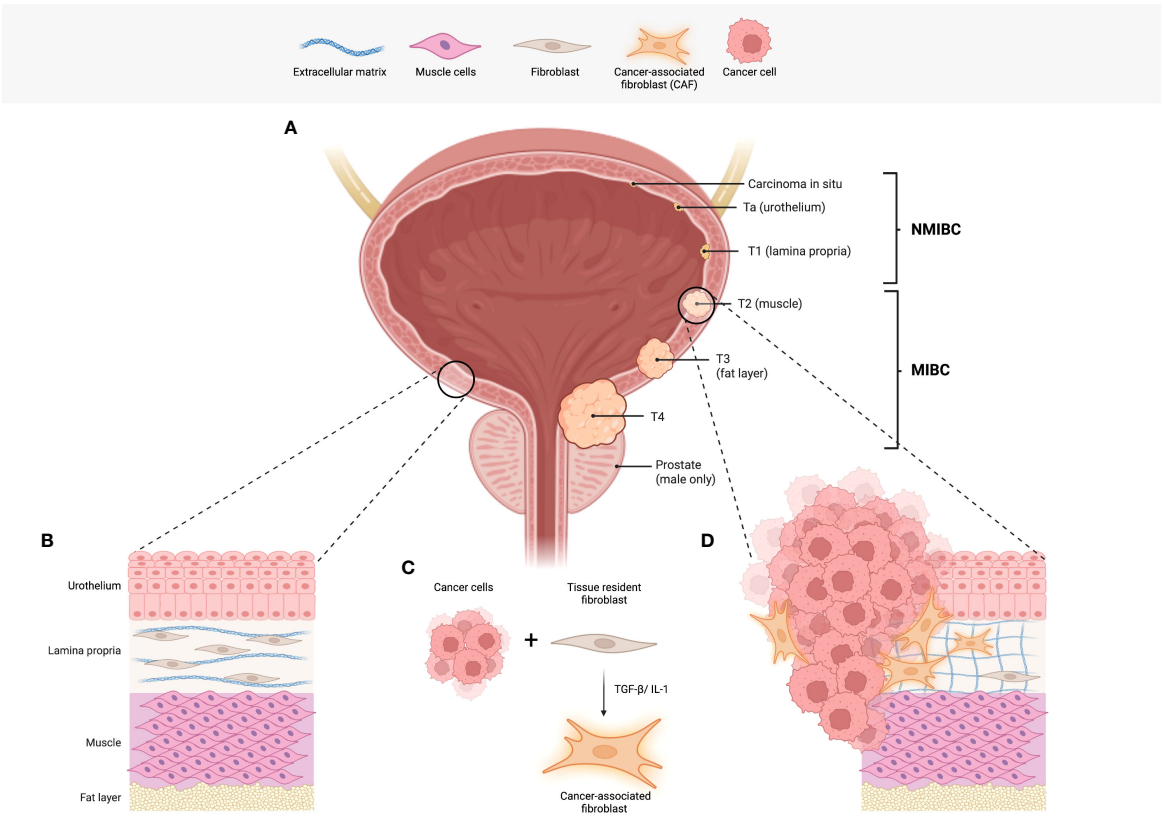


FIGURE 1
Bladder cancer staging and the role of fibroblasts. **(A)** An illustration of the human bladder, including the tumour (T stages) of bladder cancer. Commencing in the urothelium, NMIBCs describe carcinoma *in situ* (in the inner most layer of the urothelium) Ta (urothelium only) and T1 tumours. T1 tumours have infiltrated the first sub-urothelial layer known as the lamina propria. To progress to stages T2-4, invasion into the muscle layer is required and is the determining factor in the diagnosis of MIBC. **(B)** A representation of the physiological histology observed in the human bladder. Of note, fibroblasts found in the lamina propria function to produce and maintain collagen and other fibres that make up the loose connective tissue. **(C)** Interactions between tumour cells and tissue resident fibroblasts are one of the likely origins of cancer-associated fibroblasts (CAFs). **(D)** The illustration depicts the histological changes observed in bladder cancer including the presence of CAFs. In addition, there is an increase in extracellular matrix deposition and remodelling. Abbreviations: non-muscle invasive bladder cancer (NMIBC), muscle invasive bladder cancer (MIBC), cancer-associated fibroblasts (CAFs). Figure created with Biorender.com.

basal/squamous, and neuroendocrine-like (10). The molecular classification of MIBC has helped to increase knowledge of bladder cancer and find potential associations between subtypes, clinical characteristics, response to therapies, and survival. While the neuroendocrine-like subtype is associated with the worst prognosis, it is interesting to note that the stroma-rich subtype appears to have a particularly poor response to neo-adjuvant chemotherapy (10). Moreover, the stroma-rich subtype has an over-expression of gene signatures associated with smooth muscle, endothelial cells, fibroblasts and myofibroblasts (10).

## 1.2 Treatment of NMIBC vs MIBC

Currently, all patients with bladder cancer undergo a biopsy-like procedure known as a transurethral resection of bladder tumour (TURBT) which acts as both a diagnostic tool to clarify the presence or absence of tumour cell invasion into muscle and a debulking procedure to remove as much of the visible tumour mass as possible. In patients with high grade NMIBC, immunotherapeutic agents such as Bacillus Calmette–Guérin (BCG) are commonly used after TURBT; BCG works to stimulate an anti-tumour immune response to tackle residual cancer cells. Intriguingly, BCG remains one of the longest-established immunotherapies ever used in cancer treatment.

If muscle invasion is identified during the pathological assessment of the TURBT tissue, MIBC patients are treated surgically with a cystectomy or bladder-sparing treatments (BST) such as radiotherapy. Following the improved locoregional control seen with the addition of concomitant 5-fluorouracil and mitomycin C to radiotherapy in the phase III UK BC2001 trial, led by James et al., provision of BST typically includes radiotherapy in combination with chemotherapy (11). This combinatorial approach can offer quality of life benefits in selected patients and can also be a useful option for patients who are unable to undergo cystectomy (11, 12). Of relevance, following BST, patients will require lifelong cystoscopic surveillance due to the risk of recurrence (13), alongside the cross-sectional imaging and clinical surveillance carried out following treatment in all patients.

## 1.3 Research challenges and scope of this review

At present, we lack predictive biomarkers to identify which patients may be more likely to relapse following BST and could therefore be better surgical candidates or require treatment intensification. A strong predictive biomarker is therefore a key unmet clinical need to aid radical treatment decisions.

Recently, stromal cell populations in the tumour microenvironment of solid tumours, known as cancer-associated fibroblasts (CAFs), have gained prominence as an important cell type influencing bladder cancer survival outcomes.

In metastatic bladder cancer, poor responses to immune checkpoint inhibitors have been associated with TGFβ signaling in fibroblasts (14) and high EMT/stromal related gene expression (15). Moreover, in cases with poor responses, CD8 T-cells are frequently found trapped in the peri-tumoural regions amongst fibroblasts and collagen-rich matrix (14). High EMT/stromal gene expression and low tumour-infiltrating T cell abundance have both also been associated with inferior outcomes following cystectomy (15). Unfortunately, research into the effect of CAFs on radiotherapy responses, and, similarly, the impact of radiotherapy on CAF biology, is in its infancy and warrants further investigation in the setting of bladder cancer.

In this review we will outline the latest consensus views on CAF biology with a specific focus on bladder cancer. We will introduce some of the current controversies regarding CAF heterogeneity and disease-specific CAF subtypes. This review will discuss the gaps in our current understanding of the biological relevance of CAFs in bladder cancer – here, we will draw from studies of CAFs in other cancers to explore longitudinal changes in CAF biology during therapy. Finally, we will discuss whether the addition of therapeutic approaches to specifically target CAFs may improve survival in bladder cancer.

# 2 The tumour microenvironment and the role of CAFs

The tumour microenvironment (TME) describes the ecosystem formed by many different cell populations coexisting within the tumour. Within the TME, CAFs help to create the structural framework, including the extracellular matrix (ECM), within which all other cells in the TME reside. Such cells include, but are not limited to, cancer cells, cytotoxic and regulatory immune cells, antigen-presenting cells and cells of the vasculature including endothelial cells.

## 2.1 Origins and activation of CAFs

### 2.1.1 Origins

CAFs are often classified as non-neoplastic, not epithelial, endothelial, or immune cells. As opposed to normal fibroblasts that can be temporarily activated, CAFs are persistently activated, usually *via* epigenetic reprogramming (16).

CAFs are derived from several potential sources: the most commonly cited and likely origin is from normal fibroblasts resident in the tissue of tumour origin that have undergone activation *via* processes outlined below (17) (see Figure 1); secondly, CAFs may be mesenchymal stem cells derived from

the bone marrow (18). Some anecdotal studies suggest that CAFs can arise from cells of the vasculature such as endothelial cells, as well as pericytes and adipocytes (19). Furthermore, the specific cell of origin may be indicative of the CAFs eventual function and role in the TME (20). A fascinating recent study utilised single cell RNA sequencing (scRNAseq) data atlases for healthy and disease state tissues of human and mouse derivation to trace the lineage of fibroblasts (21). In doing so, they suggest that all fibroblasts, whether healthy, tissue-specific, or activated in disease, may all share a common ancestor. From such an ancestor, tissue-specific functions are subsequently acquired *in situ*, typically *via* changes to their epigenetic and transcriptional profile (21).

### 2.1.2 Activation

In addition to their cellular origin, the mechanism by which CAFs are activated seemingly imparts an additional layer of information that can provide functional and spatial stratification of CAFs. Activation of CAFs by cancer cells has been explored in several studies. For example, using *in vitro* and *in vivo* experiments, Strell et al. showed direct cell to cell contact between ductal breast carcinoma *in situ* (DCIS) cancer cells and fibroblasts induces changes in the expression profile of fibroblasts, eventually resulting in a platelet-derived growth factor (PDGF) receptor (PDGFR)α low, PDGFRβ high subset of CAFs (22). Interestingly, assessment of PDGFRα and PDGFRβ expression in tissue specimens from 458 primary DCIS patients showed an increased proportion of the PDGFRα low PDGFRβ high CAFs was associated with a higher risk of recurrence (22).

In addition to the direct cell contact driving activation of CAFs described above, paracrine crosstalk between cancer cells and fibroblasts can also lead to acquisition of the CAF phenotype. *In vitro* studies of bladder cancer cell lines revealed a high content of interleukin (IL)-1α in tumour conditioned media (CM) (23). When fibroblasts were cultured in IL-1α rich CM, this led to the release of cytokines and pro-tumour factors such as IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1) and hepatocyte growth factor (HGF), i.e. acquisition of an "activated CAF phenotype" which subsequently promoted cancer cell migration in Boyden chamber assays (23).

In a further study, Öhlund et al. demonstrated that both cell to cell contact and paracrine crosstalk between cancer cells and CAFs occur in distinct regions of pancreatic tumours with differing results (24). Contact between fibroblast activation protein (FAP)+ pancreatic stellate cells (PSC) and pancreatic cancer cells resulted in activation and conversion of PSC into CAFs, represented by an increase in expression of alpha smooth muscle actin (αSMA). These CAFs were subsequently classified as myofibroblastic CAFs (myCAFs) with the ability to deposit components of the ECM. In contrast, PSCs with less proximity to cancer cells were activated in a paracrine manner resulting in a reduced expression of αSMA and an increase in the secretion of IL-6; these CAFs were designated as inflammatory CAFs (iCAFs). Under differing conditions, CAFs appear to have the capacity to be directed into either myCAFs or iCAFs by modifying their expression profiles, suggesting that different, spatially distinct CAF subtypes exist, with functional characteristics dependent on activation (24). Of further interest, using scRNAseq, the same group identified an additional CD74+ MHC class II+ CAF subtype, denoted as antigen-presenting CAFs (apCAF) (17).

Studying single cell sequencing outputs of fibroblasts extracted from normal and pancreatic ductal adenocarcinoma (PDAC) mouse models, Dominguez et al. developed an evolutionary model to characterise CAF heterogeneity (25). Using dimensionality reduction and pseudotime analysis, they demonstrated that the previously described iCAF and myCAF populations were derived from a common early CAF subtype (25). This supported earlier work that showed mechanistically how signalling *via* IL-1 and transforming growth factor beta (TGFβ) drives the differentiation of iCAFs and myCAFs, respectively, from a common "early CAF" ancestor (26). The diversity of CAF subtypes is of considerable biological importance, and we will return to this concept in greater detail below.

## 2.2 Functions of CAFs

Pathologically, CAFs exhibit a wide range of functions that can modify the TME *via* diverse non-immune and immune mechanisms. This section will explore the functions of CAFs with reference to both bladder cancer and wider tumour biology. To help understand pathological CAF function, we will first describe the role of physiologically normal fibroblasts in the bladder: Such fibroblasts are situated in the sub-urothelial lamina propria layer of the bladder where their function includes the production of extracellular matrix components such as collagen and fibronectin (27). Uniquely, in the bladder, myofibroblasts help to transmit signals from the urothelium in response to bladder filling or pathological stressors (28). As discussed earlier, these normal fibroblasts are likely to be an important source of persistently activated CAFs with functions as outlined below:

### 2.2.1 Production and remodeling of the ECM

One of the key functions of CAFs is to deposit and modify the filamentous ECM which facilitates tumour cell invasion (29). This has several biomechanical consequences including the generation of greater contractile strength and tension in the tissue and increased tumour stiffness. Such increased stiffness can result in further activation of CAFs, thus creating a self-

perpetuating positive feedback loop. This loop is thought to arise following the activation of transcription factor Yes-associated protein (YAP) which subsequently regulates the downstream expression of genes associated with cytoskeletal and matrix remodeling (29). The increased stiffness can also impact oncogenic signalling within the tumour cell, including *via* the mitogen-activated protein kinase (MAPK) pathway (30).

### 2.2.2 Impact on tumour cell signalling

CAFs have been shown to directly impact the proliferation and invasiveness of bladder cancer cells *via* several mechanisms including the exosomal transfer of non-coding RNA fragments such as LINC00355 (31). Components of the CAF secretome, such as microfibrillar-associated protein 5 (MFAP5), have a similar pro-tumour effect (32). Additionally, conducting co-culture studies of CAFs with bladder cancer cell lines showed that cancer cell proliferation and invasion was enhanced in conditions where CAFs were induced to undergo autophagy (33).

Further *in vitro* studies support the proposed pro-tumour impact of CAFs on bladder cancer cells. One such study showed that CAFs, but not normal fibroblasts, secrete pro-tumour cytokines such as IL-1β which activates the Wnt signalling pathway in bladder cancer cell lines. Indeed, inhibition of Wnt signalling abolished this pro-tumour effect (34). Similar effects were seen following the exosomal transfer of miR-148b-3p which induced increased invasive behaviour of bladder cancer cells *via* Wnt signalling, an effect which could be attenuated *via* the upregulation of phosphatase and tensin homolog (PTEN) (35). Overall, this suggests CAFs may impart a pro-tumour effect in bladder cancer *via* Wnt signalling pathway activation.

In addition to the pro-tumour signaling described above, CAFs have also been shown to contribute to metabolic reprogramming of tumours (36). As with the Warburg effect witnessed in cancer cells, CAFs are capable of upregulating components of the glycolytic pathway leading to an upregulation in lactate production (37). Moreover, exposure to CAF conditioned media has been shown to enhance the glycolytic activity of pancreatic cancer cells (38) helping to support the metabolic needs of surrounding cancer cells.

### 2.2.3 Induction of epithelial to mesenchymal transition

CAFs can also have a pro-tumour effect *via* the induction of epithelial to mesenchymal transition (EMT). Indeed, secretion of IL-6 by tumour-activated CAFs was shown to induce EMT in bladder cancer cell lines (39). Here, observations included the upregulation of proteins associated with EMT such as N-cadherin and vimentin and an increase in EMT-inducing transcription factors SNAIL, TWIST and ZEB1 in bladder cancer cell lines (39). These findings are further supported by immunohistochemistry analysis of urinary bladder carcinoma which revealed the upregulation of several CAF markers was associated with an increase in markers of EMT on multiple cell types within the TME (40).

### 2.2.4 Immunomodulatory functions of CAFs

CAFs have multiple immunomodulatory functions, which are generally, but not exclusively, considered to be immunosuppressive in nature. Such functions can be driven by the *physical* properties of CAFs for example the sequestration or trapping of immune cells in the matrix or by *secretion* of immunomodulatory chemokines for example TGFβ (41, 42). Obviously, both physical and secretory immunomodulatory functions may co-exist within a single tumour.

In a landmark paper published in 2018, Mariathasan et al. were one of the first authors to describe the role of CAFs in the creation of an immune excluded phenotype, and the associated lack of response to anti-programmed death-ligand 1 (PD-L1) checkpoint inhibitor, atezolizumab (14). Of note, this finding was demonstrated in the setting of metastatic urothelial bladder carcinoma and therefore highlights the fundamental role CAFs play in this disease (14). We will explore additional findings from this paper in subsequent sections. Below we have detailed additional studies which have explored the interplay between CAFs and cells of the immune system.

In bladder cancer, FAP+ CAFs have been associated with immune cold TMEs with poor infiltration of CD8+ T-cells and with considerable loss of human leukocyte antigen (HLA-I) expression on tumour cells (43). Poor CD8+ T cell infiltration is also likely to be due to the deposition of a heavily crosslinked ECM which acts as a physical barrier to exclude lymphocytes in peritumour regions, preventing them from reaching the tumours cells and unleashing their full cytotoxic potential (44).

To note, CAFs impact many immune populations other than lymphocytes. For example, in lung squamous cell carcinoma, CAFs have been associated with a higher infiltrate of immunosuppressive cells such as tumour-associated macrophages (45).

Although the immunomodulatory effects of CAFs are predominantly immunosuppressive and tumour-promoting, in melanoma a podoplanin (PDPN)+, FAP- CAF subtype has been shown to act in an immunostimulatory manner *via* development of tumour-associated tertiary lymphoid structures (TA-TLS). In this setting, an increase of TA-TLS was positively correlated with improved patient survival and response to immune checkpoint therapies (46). Tertiary lymphoid structures (TLS) are equally relevant in bladder cancer. In a study of immune checkpoint inhibitors targeting PD-L1 and CTLA-4, Gao et al. report the enhanced treatment response associated with a high density of TLS in the pre-treatment biopsies of patients with high grade urothelial carcinoma (47). It would be very interesting to determine the phenotype of CAFs in these patients, including the CAFs specifically present in the TLS.

Considering the diversity in the functions of CAFs described above, it is of great importance that we correctly characterise CAF subtypes and consider any implications that may follow depletion of some or all CAF populations.

## 2.3 CAF heterogeneity

Some markers such as FAP and αSMA are used frequently in CAF research and are often considered to be "canonical". Using individual canonical CAF markers, many studies have attempted to identify the total CAF population leading to conflicting results - this is likely to be due to the heterogeneous nature of CAFs. Indeed, at present there is no known single marker that is capable of identifying all CAF populations or of segregating them from other stromal populations.

To explore CAF heterogeneity and characterise distinct CAF subtypes a variety of methods have been used across multiple cancer types, including scRNASeq, spatial transcriptomics, flow cytometry and immunohistochemistry. Each method offers a unique opportunity to study the underlying biology but can come with disadvantages. Briefly, bulk RNA sequencing facilitates a broad overview of gene signatures and signalling pathways that may be up or down regulated in the transcriptome but lacks the single cell resolution that is provided by scRNAseq. Protein expression can be studied at single cell resolution using flow cytometry, however spatial information is lost in all three of these methods. Techniques such as multiplex immunofluorescence and imaging mass cytometry help to overcome these challenges and provide insights into the interactions between different cell types. However, these techniques come with time and cost limitations that can restrict high throughput analysis.

Across many studies, the concept of CAF heterogeneity, i.e. different CAF subtypes in a single tumour, generally holds true and typically shows the distinction between iCAFs and myCAFs described above. However, beyond this, subtype classification, descriptions of functionality and biomarker selection are inconsistent and vary greatly depending on the method of CAF profiling, tumour sample quality and disease type. The type of biopsy used to collect tissue and thus stratify CAFs can also have implications on the interpretations of results. In bladder cancer, a TURBT may only provide access to superficial regions of tumour and thus not reveal the full extent of the CAF infiltrated tumour layers, resulting in a loss of heterogeneity. In contrast, a full cystectomy will typically provide access to the whole tumour and surrounding healthy tissue. These limitations surrounding inter and intra tumoural heterogeneity are not restricted to bladder cancer; the renal and lung TRACERx studies (48–50) have demonstrated the importance of evaluation of biopsies from multiple different sites within a tumour to comprehensively profile cancer biology, rather than a single snapshot.

To study inter-tissue CAF heterogeneity, Galbo et al. utilised scRNAseq data from melanoma, head and neck, and lung cancer to create a series of gene signatures describing pan-cancer CAF subtypes (pan-CAF) (51). In total they described 5 pan-CAFs, each with distinct gene expression patterns; myofibroblast (myCAF), desmoplastic (dCAF), inflammatory like (iCAF and iCAF-2) and proliferating (pCAF) - each named to represent their predicted function. Applying the newly defined pan-CAF gene sets to bulk RNA sequencing data across a variety of cancer types not only confirmed the presence of multiple CAF subtypes across different tumours, but survival analysis also revealed that in different settings, different pan-CAFs subtypes were predictive of poor outcomes. In bladder cancer, a high infiltrate of the myCAF signature correlated with poor prognosis in The Cancer Genome Atlas (TCGA) dataset, which consists primarily of cystectomy specimens (51).

Studying CAF heterogeneity in breast cancer, Cremasco et al. identified two distinct populations in mouse and human tumours; FAP+ PDPN+ CAFs and FAP+ PDPN- cancer-associated pericytes (CAPs) (52). They showed that FAP+ PDPN+ CAFs, but not CAPs, were capable of supressing T cell proliferation and, *via* deposition of ECM, trapping infiltrating immune cells in the peritumoral regions - a process which appears to be driven by TGFβ (52).

A further key study exploring CAFs subtypes in breast cancer by Costa et al. used flow cytometry with several common CAF markers to reveal the presence of four distinct CAFs (53). These subtypes were differentially expressed in luminal A, triple negative (TNBC) and human epidermal growth factor receptor 2 (HER2)+ breast cancer and showed distinct protein expression signatures and spatial patterns. Two of the identified CAFs were αSMA+ and were classified as CAF-S1 and CAF-S4; each had distinct transcriptomic profiles and pro-tumour functions. Of note, the CAF-S1 ($CD29^{Med}$, $FAP^{Hi}$, fibroblast-specific protein 1 $(FSP1)^{Low-Hi}$, $\alpha SMA^{Hi}$, $PDGFR\beta^{Med-Hi}$, caveolin 1 $(CAV1)^{Low}$) subset was associated with an immunosuppressive phenotype *via* the recruitment of Foxp3+ CD4+ CD25+ regulatory T-cells (Tregs) and inhibition of cytotoxic CD8+ T-cells. In contrast CAF-S4 did not play a role in the immune response and instead was found to be associated with muscle contraction, regulation of the actin cytoskeleton and oxidative metabolism (53).

CAF-S1 and CAF-S4 were also identified in high grade serous ovarian cancer where the immunosuppressive function of CAF-S1 was ascribed to the expression of CXCL12β which recruits Tregs *via* CXCR4, this cell-cell contact subsequently enhances survival and differentiation of Tregs (54). CAF-S4 cells on the other hand, did not express CXCL12β, likely due to silencing by micro RNAs miR-141 and miR-200a (54), reinforcing the functional difference between CAF-S1 and CAF-S4.

Obradovic et al. recapitulated Costa's findings in head and neck squamous cell carcinoma identifying all CAF-S1 to

CAF-S4 subsets (55). In addition, they showed that clustering CAFs by flow cytometry lacks resolution and instead inferred protein activity from gene expression data and the enrichment of transcriptional targets using the Virtual Inference of Protein-activity by Enriched Regulon (VIPER) algorithm. In doing so, this added depth to CAF subtype classification and showed that the CAF-S1 subtypes could be further divided into 3 groups with differing effects on response to programmed cell death protein 1 (PD-1) checkpoint inhibition (55). This work highlights potential tissue-specific differences in CAF biology, but, crucially, helps to establish consistency in the results produced by independent groups in different tissue types (Figure 2).

In addition to diversity driven by the cell of origin, plasticity in CAF profiles has been shown to change temporally throughout tumour development (57). Not only does the amount of stroma increase with stage, but Elwakeel et al. showed a dramatic increase in the proportion of regulator of G protein signalling 5 (RGS5)+ CAFs, denoted as vasculature CAFs (vCAFs), in the TME of late-stage mouse mammary tumours (57). In contrast, matrix CAFs (mCAFs) were more prevalent in untransformed mammary glands and indeed declined with tumorigenesis. The contribution of a third subset of CAFs was unchanged (57). A temporal shift in CAF biology may be indicative of environmental cues that change within the TME as the tumour progresses. It is therefore of great interest to explore how different therapeutic options can alter signals in the TME and potentially exploit CAF plasticity.

# 3 CAFs in bladder cancer

## 3.1 Bladder cancer-specific CAF heterogeneity – current knowledge

In recent years, with the aid of single cell sequencing technologies, our understanding of the cell populations in the TME of bladder cancer and their respective contributions to disease progression has substantially developed. The application of this technology in bladder cancer is limited to a handful of studies with conservative patient numbers, but, interestingly, findings to date suggest a prominent role for CAFs in bladder cancer biology.

Chen et al. performed scRNAseq on 8 bladder cancer patients and found that the fibroblast population could be

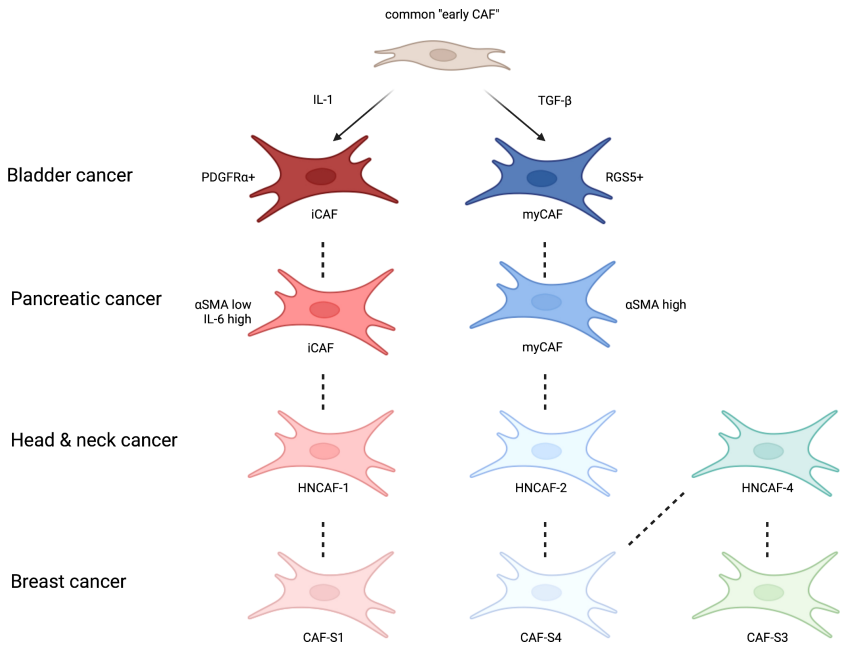


FIGURE 2
A simplified schematic of CAF subtypes centred around bladder cancer and their similarity with CAFs reported in other common cancers. Derived from a common CAF progenitor (25) and activated by IL-1 and TGFβ (26), in bladder cancer inflammatory CAFs (iCAFs) and myofibroblast CAFs (myCAFs) are delineated by PDGFRα and RGS5 (56). These CAF subtypes and others have been described in other common cancers, such as pancreatic (24), head and neck (55) and breast (53) with their own respective protein markers or transcriptional profiles. Figure adapted from Obradovic et al., 2022. Figure created with Biorender.com.

stratified into iCAFs or myCAFs by the expression of PDGFRα and RGS5 respectively (56). Comparing differentially expressed genes revealed distinct functions of the two CAF subgroups and suggested that iCAFs are likely to be more pro-tumorigenic *via* functions associated with migration, proliferation, and angiogenesis. Moreover, when evaluated in the TCGA bladder dataset, abundance of iCAFs was associated with poor survival outcomes (56). Wang et al. were also able to segregate fibroblasts in this manner and found RGS5+ myCAFs and PDGFRα+ iCAFs in tissue from healthy, NMIBC, MIBC and metastatic bladder cancer tissue from 13 patients (58) thus corroborating the work by Chen et al.

## 3.2 A potential prognostic/predictive biomarker?

Acknowledging the existence of distinct CAF subtypes, Mesheyeuski et al. studied the expression of several canonical CAF markers (FAP, αSMA, CD90, PDGFRα or PDGFRβ) alone, and in combination, using immunohistochemistry, in a mixed cohort of NMIBC and MIBC, to explore associations with survival (59). Unlike other groups (Table 1) that have studied survival in the context of a single marker, none of the CAF markers studied by Mezheyeuski et al. were significantly associated with survival when studied alone. Instead, by combining the CAF markers, they showed statistically significant associations with survival and specifically demonstrated that patients with a FAP dominant stromal score had the shortest 5-year survival. Further emphasising the need to explore multiple components of the TME in combination, Mezheyeuski et al. also found that patients with a CD90 dominant stroma and a high CD8+ T-cell infiltrate had a longer 5-year survival rate (59).

## 3.3 The impact of CAFs on response to treatment

### 3.3.1 CAFs and the treatment of bladder cancer

Disease progression following bladder-sparing treatments is a real barrier to success in the treatment of bladder cancer. As outlined in the introduction, the survival rates for bladder cancer have remained unchanged for the last five decades. In contrast other common cancers such as prostate and breast cancer have seen large improvements in survival (65).

In metastatic bladder cancer, anti-PD-1/PD-L1 immune checkpoint inhibitors such as nivolumab, pembrolizumab and atezolizumab are routinely used following key phase II and III trials that showed improved overall survival with these agents (66, 67). One of such studies was the CheckMate 275 phase II trial of the anti-PD-1 antibody nivolumab in which biological profiling of tumours was carried out by Wang et al. (15). By exploring stromal parameters in combination with CD8+ T cell infiltration, the study showed that tumours with a high CD8 infiltrate but low stromal scores had the best response to nivolumab. In contrast, a combination of high CD8 infiltrate and high stromal score was detrimental to outcome, and was attributed to an immune excluded phenotype generated by the CAFs and ECM, as discussed above (15).

Having demonstrated the benefits of checkpoint inhibition in the metastatic setting, a separate phase III trial, IMvigor010, considered the use of the anti-PD-L1 monoclonal antibody, atezolizumab, as an adjuvant therapy for patients with high-risk muscle-invasive urothelial carcinoma (68). Despite being generally well tolerated, the trial didn't reach the intended efficacy endpoints and did not support the use of atezolizumab in this setting (68). However, further investigation of samples produced in the IMvigor010 trial showed that patients with blood samples that were positive for circulating tumour DNA

TABLE 1 An overview of research studies that have evaluated individual markers of CAFs in bladder cancer.

| Marker | Notes | Method | Tissue type | Ref |
|---|---|---|---|---|
| PDPN | Patients with over 1% PDPN+ stromal cells had significantly worse RFS following cystectomy. PDPN didn't predict response to chemotherapy. | IHC | Cystectomy | Okajima et al., 2020 (60) |
| PDPN | PDPN+ cells found to be infiltrating tumour regions were associated with worse survival. Tumours with less PDPN+ CAFs were likely to have a better response to chemotherapy. | IHC | Cystectomy | Zhou et al., 2020 (61) |
| CALD1 | High CALD1 expression was associated with shorter overall survival in bladder cancer. | TCGA gene expression | Cystectomy | Du et al., 2021 (62) |
| FAP | When co-expressed with basal markers CK5/6 and CD44, FAP was a strong prognosticator of disease-specific survival in bladder cancer. | IHC | Cystectomy | Calvete et al., 2019 (63) |
| LRRC15 | LRRC15+ myCAFs were associated with poor response to anti-PD-L1 treatment in bladder cancer trial of atezolizumab. | RNAseq | Not stated | Dominguez et al., 2021 (25) Mariathasan et al., 2018 (14) |
| Kindlin-2 | In a study of 203 bladder cancer patients, high Kindlin-2 expression was associated with shorter patient survival. | IHC | Cystectomy and TURBT | Wu et al., 2017 (64) |

Podoplanin (PDPN), relapse-free survival (RFS), immunohistochemistry (IHC), Caldesmon 1 (CALD1), Cytokeratin 5/6 (CK5/6), Fibroblast activation protein (FAP), leucine rich repeat containing 15 (LRRC15), single cell RNA sequencing (scRNAseq).

(ctDNA), were better responders to atezolizumab (69). With further validation, ctDNA could help to predict which candidates are likely to respond to adjuvant immune checkpoint inhibition (69). As researchers continue to explore the ways in which immune checkpoint inhibition may improve treatment options for patients with MIBC, perhaps more attention to dual targeting of both CAFs and immune cells would be beneficial.

One of the very few studies to specifically investigate the impact of CAFs on the treatment of bladder cancer found that, of the 28 MIBC patients treated with neo-adjuvant chemotherapy, those with less CAFs in their pre-treatment biopsy were more likely to have a pathological complete response (70). In addition, the tumour cell expression of oestrogen receptor β (ERβ) was lower in tumours with a complete response compared to those with a partial or non-response. Furthermore, the patients with both a higher infiltrate of CAFs and increased expression of ERβ in their post-treatment tumours had a particularly poor response. The same group demonstrated a chemoprotective effect and enhanced proliferation in bladder cancer cell lines when co-cultured with CAFs, versus monoculture, and attributed this effect to the secretion of insulin-like growth factor (IGF) 1 (70).

### 3.3.2 CAFs in the treatment of other cancers

From the few studies in bladder cancer, it appears that CAFs play a broad role in reducing the efficacy of cancer therapies. However, there is a lack of bladder cancer-specific data on the impact of CAFs on some important therapies including radiotherapy. To expand our understanding of CAFs and their impact on treatments such as radiotherapy, we must look to studies in other cancer settings.

A protective effect of CAFs has been identified in response to radiotherapy for pancreatic cancer. *In vitro* and *in vivo* studies of pancreatic cancer cell lines showed that in monoculture, the growth rate of various pancreatic cancer cells markedly slows following treatment with single dose or fractionated radiotherapy. However, when co-cultured with PSC, the growth rate of pancreatic cancer cells is less affected by irradiation (71). Moreover, co-culturing pancreatic cancer cells with PSC resulted in an increase in the expression of markers associated with EMT and cancer cell stemness induced by TGFβ (72). In a separate *in vivo* study of radiotherapy in PDAC, treatment with radiotherapy resulted in elevated levels of inducible nitric oxide synthase (iNOS) and nitric oxide secreted by CAFs. A combination of radiotherapy with iNOS inhibition delayed PDAC tumour growth in PDAC mouse models (73), which is thought to have occurred because iNOS inhibition reduced CAF-induced pro-tumour effects.

In colorectal cancer, radiotherapy has also been shown to induce the secretion of paracrine factors from CAFs which subsequently enhanced metabolic changes and activation of IGF receptor (IGFR) in neighbouring tumour cells (74). Signalling *via* the IGF1R–Akt–mTOR pathway provides a survival advantage to colorectal tumour cells targeted with radiotherapy. Neutralising IGFR is therefore a potential therapeutic strategy to enhance sensitivity to radiotherapy in patients with CAF-enriched tumours (74).

In rectal cancer, patients with inferior responses to chemoradiotherapy had higher numbers of CAFs in their tumours, and low levels of IL-1 receptor α (IL-1RA) in their serum (75). In searching for the mechanistic basis of these clinical observations, Nicolas et al. showed that reciprocal signalling between CAFs and tumour cells enhanced radiotherapy resistance (76). In an array of studies, they show that IL-1α present in the *ex vivo* culture media of murine tumour cells was responsible for the pre-conditioning of CAFs, resulting in polarisation to an inflammatory iCAF phenotype. Accordingly, they found that increasing levels of nitrite production and signs of oxidative DNA damage made the iCAFs more vulnerable to conversion into a senescent state when treated with irradiation. In this state, iCAFs maintained their capacity to deposit ECM and so continued to create an immune cold and hostile TME that is markedly resistant to therapy. Importantly, reversal of iCAF polarisation *via* IL-1α inhibition seemingly sensitised tumours to radiotherapy and therefore may be a useful combination therapy (76).

Lastly, exploring differences in responder and non-responder breast cancer patients treated with neo-adjuvant chemotherapy, Su et al. found that responders had less CAFs (77). In contrast, the non-responders had a markedly higher CAF population that could be uniquely identified by the surface expression of CD10 and G protein-coupled receptor 77(GPR77). Not only did they find this CAF subtype to be chemoresistant in itself, but the subtype also reduced tumour cell sensitivity to chemotherapy. Furthermore, targeting this CAF subtype with antibodies against GPR77 reversed the chemoresistant effects (77).

Collectively, the evidence reported here suggests that CAFs play a role in the resistance to radiotherapy, chemotherapy, and combinatorial approaches. Likewise, it is apparent that in some cases radiotherapy may exacerbate the pro-tumour CAF phenotype.

## 4 Targeting CAFs – the future of bladder cancer treatment?

The findings discussed earlier in this review indicate that a number of factors are associated with the activation and functionality of distinct CAFs in the TME. This pleiotropic biology, and the lack of a single unifying CAF marker, poses a

challenge for therapeutic targeting. However, recent reports have focused on IL-1 and TGFβ.

In this section we will explore the role of TGFβ, a pathway of particular relevance in bladder cancer. We have also indicated a number of studies that have attempted to target CAFs using several strategies in a broad spectrum of different cancers. Although we acknowledge the existence of tumour site specific biology that may affect CAF functionality and expression of phenotypic markers, we also recognise consistent trends throughout the literature that suggest that targeting CAFs in bladder cancer may result in improvements to outcomes, particularly when we consider the impact of CAFs on therapy responses.

## 4.1 The role of TGFβ in bladder cancer

TGFβ has a complex role in the progression of cancer which has been eloquently summarised by Barcellos-Hoff (78). TGFβ has a number of extracellular and intracellular sources, including the ECM which harbours large deposits of latent TGFβ in the form of latency-associated peptide. Under a variety of stimuli, including irradiation of tissue (79), TGFβ is released from the latency complex, resulting in activation of downstream signalling (80). Other TGFβ sources include cancer cells exosomes which can be an important mechanism to activate CAFs (81).

### 4.1.1 Epithelial mesenchymal transition

In bladder cancer, TGFβ signalling has been shown to be an important stimulus for the induction of EMT (82). Several studies have attempted to define precisely how TGFβ contributes to this outcome and point towards a two-part mechanism whereby TGFβ acts both up and downstream of CAFs.

Comparing the effect of multiple growth factors; TGFβ, acidic fibroblast growth factor (aFGF) and PDGF, Schulte et al. showed that only TGFβ was responsible for the upregulation of markers associated with stromal activation such as αSMA, FSP1 and FAP (40). Furthermore, fibroblasts activated by TGFβ alone, or TGFβ in combination with aFGF, were the most effective at inducing the invasion of RT112 bladder cancer cell lines. Likewise, the combination of TGFβ and aFGF led to a marked increase in the expression of markers associated with EMT (40). To uncover the mechanisms by which CAFs induce EMT, Zhuang et al. showed TGFβ was a vital component of the CAF conditioned media responsible for the induction of EMT in bladder cancer cells (82). They also identified that expression of a long non-coding RNA, ZEB2NAT, in bladder cancer cells, was essential for TGFβ-induced EMT to occur (82).

### 4.1.2 Immune exclusion

As discussed earlier, TGFβ is a key factor in the stromal exclusion of infiltrating immune cells. Intriguingly, in metastatic bladder cancer, TGFβ signalling status can be used in combination with PD-L1 expression and a score of tumour mutational burden to predict patient responses to the anti-PD-L1 antibody atezolizumab, illustrating how important TGFβ biology is in determining the response to immunotherapy (14). Furthermore, preliminary mouse models, representative of the immune excluded TMEs typically found in metastatic bladder cancer, have been used to demonstrate the effectiveness of TGFβ inhibition in combination with anti-PD-L1. With this combination therapy, a reduction in tumour burden was accompanied by an increase in the number of tumour-infiltrating CD8+ T lymphocytes and crucially such lymphocytes were released from stromal traps to unleash anti-tumour immunity in the appropriate tumour regions (14).

### 4.1.3 Reprogramming of CAFs

As described earlier, RGS5 has recently emerged as an important marker of the myCAF population in bladder cancer (56). Interestingly, in the pancreas, a high expression of RGS5 on pericytes is typically associated with apoptosis of these cells (83). In contrast, Dasgupta et al. found RGS5+ CAFs in the TME of pancreatic cancers do not undergo apoptosis as expected and instead survive and expand (83). In further exploring this observation, they found TGFβ plays a pivotal role in the reprogramming of CAFs *via* conversion of signalling pathways to allow RGS5 to interact with pSmad2/3 - this resulted in the transcription of genes associated with pro-tumour and proliferative functions (83). If TGFβ is capable of inducing profound reprogramming of RGS5+ CAFs towards a pro-tumour phenotype, it would be interesting to explore how manipulation of TGFβ signalling may affect the RGS5+ myCAF population identified in bladder cancer. We will continue to explore this concept below.

## 4.2 Improvements to therapy responses *via* targeting of CAFs

### 4.2.1 Manipulation of TGFβ signalling

As we have indicated throughout this review, TGFβ has a fundamental role in the activation and differentiation of CAFs where it typically exerts profound pro-tumour effects. In some cancers, there is evidence that "natural" TGFβ downregulation corresponds with favourable outcomes. This was evident in patients with HPV+ Head and neck squamous cell carcinoma who had less CAFs in their TME and an improved prognosis (84). Wang et al. suggest that miRNA exosomes derived from the tumour cells of these patients were capable of infiltrating CAFs and altering active signalling pathways by reducing the

expression of NADPH oxidase (NOX)4 and the presence of reactive oxygen species (ROS). As a result, TGFβ signalling is inhibited (84).

Although the pleiotropic effects of TGFβ make it a challenging target, the above observations encourage further research into novel strategies for manipulation of TGFβ signalling to reduce the pro-tumour impact of CAFs (Table 2). It should be noted that TGFβ signaling is important for the regulation of many non-stromal cell types, indeed TGFβ can contribute to both tumour suppression and promotion (91). Therefore targeting TGFβ may result in undesirable consequences in some situations and should be approached with caution. As more of the specific biology associated with TGFβ signalling in CAFs is uncovered, there is hope that a more precise therapeutic approach may become available (25).

One novel method to manipulate TGFβ uses a dual TGFβ and PD-L1 targeting approach with a novel fusion protein called bintrafusp alpha. In combination with radiotherapy, bintrafusp alpha proved effective at reducing the fibrotic networks induced by TGFβ-activated CAFs, and increased the infiltration of cytotoxic CD8+ T-cells in multiple immunocompetent mouse modes of PDAC, glioblastoma, neuroendocrine colonic carcinoma and mouse mammary carcinoma (85).

Similar findings have been observed in the phase Ib/IIa MP-VAC-204 trial which involved a combination of the TGFβ receptor type 1 inhibitor Vactosertib with the PD-1 inhibitor pembrolizumab to treat patients with microsatellite stable metastatic colorectal carcinoma (mCRC) (86). The initial results of MP-VAC-204 are promising and show a decrease in biomarkers of TGFβ signalling and crucially an increase in CD8 + T-cell infiltration. Where previously pembrolizumab was effective for a small percentage of patients with microsatellite instability mCRC, the addition of TGFβ inhibition appears to increase the number of patients who may benefit from checkpoint inhibition (86).

Taking a different approach, Yegodayev et al. found a stromal increase in TGFβ signalling to be associated with a poor response to the epidermal growth factor receptor (EGFR) antibody cetuximab in patient-derived xenograft models of head and neck cancers (87). Moreover, cetuximab efficacy could be greatly improved with the combined use of a SMAD3 inhibitor to block the downstream effects of TGFβ (87).

Promising strategies to target the NOX4/TGFβ axis have also been explored. They include the NOX1/NOX4 inhibitor GKT137831, which reduced the production of ROS in TGFβ-activated CAFs in prostate cancer and further dissipated the pro-tumour functions of CAFs (92). In a number of mouse models, GKT137831 "normalised" CAFs and, as a result, stranded CD8+ T-cells were able to infiltrate tumours and were more responsive to immunotherapeutic agents such as anti-PD-1 (88). Similarly, *in vitro* studies of bladder cancer have demonstrated the efficacy of the NOX4 inhibitor diphenylene iodonium, which slowed cancer cell growth *via* a reduction in the expression of ROS (89). Lastly, magnesium isoglycyrrhizinate has also been tested *in vitro* and *in vivo* in the bladder cancer setting and showed promise as a potential NOX4 inhibitor that prevents tumour growth (90).

### 4.2.2 Alternative strategies to target CAFs

Strategies to combine CAF targeting agents other than TGFβ inhibitors with immune checkpoint blockade have also shown pre-clinical promise. Nintedanib is a tyrosine kinase inhibitor which has anti-fibrotic effects in the TME and contributes to an anti-tumour response *via* the release of previously excluded immune cells (93). The addition of anti-PD-1 treatment enhanced responses to treatment with nintedanib and slowed the growth of B16-F10 melanoma tumours *in vivo* (93). Intriguingly, nintedanib has recently shown promise when combined with neo-adjuvant chemotherapy in localised MIBC. In the NEOBLADE trial, addition of nintedanib to standard radical treatment significantly improved overall survival, suggesting this drug warrants further consideration in bladder cancer (94).

Alternative attempts to target CAFs, and therefore slow the progression of cancer, have included the use of FAP-specific chimeric antigen receptor (CAR) T-cells. *In vitro* and *in vivo*

TABLE 2 A summary of research studies that have attempted to manipulate TGFβ signalling.

| Target | Summary | Stage of development | Ref |
|---|---|---|---|
| TGFβ and PD-L1 | Bintrafusp alpha in combination with radiotherapy. | Pre-clinical | Lan et al., 2021 (85) |
| TGFβR1 and PD-1 | MP-VAC-204 trial of Vactosertib with pembrolizumab in metastatic colorectal carcinoma. | Phase Ib/IIa | Kim et al., 2021 (86) |
| EGFR and SMAD3 | Cetuximab in combination with SMAD3 inhibition to downregulate TGFβ in head and neck cancer. | Pre-clinical | Yegodayev et al., 2020 (87) |
| NOX4/TGFβ axis | GKT137831 reduced ROS in TGFβ-activated CAFs in prostate cancer. | Pre-clinical | Ford et al., 2020 (88) |
| NOX4 | Diphenylene iodonium reduced ROS and slowed bladder cancer cell growth. | Pre-clinical | Shimada et al., 2011 (89) |
| NOX4 | Magnesium isoglycyrrhizinate prevents tumour cell growth in bladder cancer. | Pre-clinical | Yuan at al. 2022 (90) |

transforming growth factor β (TGFβ), TGFβ receptor 1 (TGFβR1), programmed death-ligand 1 (PD-L1), epidermal growth factor receptor (EGFR), NADPH oxidase 4 (NOX4), reactive oxygen species (ROS).

studies have demonstrated the feasibility of targeting CAFs using this approach, and show early signs of promise that FAP-specific CAR T-cells can reduce tumour burden (95, 96). However, by targeting CAFs we have continued to learn about crucial elements of their biology. Several attempts to deplete the fibroblast component of the TME and the associated ECM have unfortunately resulted in enhanced tumour progression. This finding not only supports the notion that CAFs are a heterogenous population, but that CAFs can also function in a manner that restrains tumour growth in specific contexts. Although we do not wish to over-emphasize this point, it is important we include this for completion.

One such example was demonstrated in a genetically modified mouse model of PDAC in which αSMA+ myofibroblasts could be depleted at a given time point in a drug dependent manner. Depletion at both early and late stages of PDAC development resulted in reorganisation of the associated ECM, a reduction in infiltrating effector T-cells, and an increase in regulatory T-cells, all of which combined to create significantly worse survival outcomes compared to control mice (97).

Similarly, progression of PDAC tumours was observed when the stromal driving factor sonic hedgehog (Shh) was deleted from tumour cells. Under these conditions, the PDAC TME contained less stromal cells, but was more vascular and proliferative (98). Deletion of stromal components such as type one collagen, a crucial part of the ECM, had a similar effect (99).

Although there have been very few studies targeting CAFs in bladder cancer, we can apply our knowledge of bladder cancer biology to infer similarities with tumour types such as PDAC (97). For example, Shin et al. showed that in mouse and human tumours loss of Shh was associated with progression from carcinoma *in situ* to MIBC (100). As such tumour cells were no longer able to induce stromal cell differentiation *via* Shh, this resulted in a proliferative tumour lacking restraint from neighbouring stromal cells (100).

Rather than deplete specific CAF populations, a new trial reported by Mizutani et al. considered the plasticity of CAFs and proposed a new treatment regimen for patients with advanced pancreatic cancer that could convert tumour-promoting CAFs (pCAFs) into tumour-restraining CAFs (rCAFs) *via* the addition of AM80 (101). In combination with chemotherapy, AM80, a synthetic retinoid, converted pCAFs into rCAFs marked by the upregulation of Meflin and downregulation of the canonical CAF marker αSMA. Although this phase I trial is in the initial stages, it offers an insight into the potential direction of research into CAFs and the future strategies that may be undertaken to target them (101).

In a similar manner, manipulation and reversal of CAF activation has been attempted using Vitamin D (102, 103). Indeed, in colorectal carcinoma (CRC) an increase in the expression of Vitamin D receptors (VDR) on fibroblasts in the TME was associated with improved survival outcomes (102). Ferrer-Mayorga et al. subsequently found that an increase in the active form of Vitamin D ($1,25(OH)_2D_3$) prevented activation of normal fibroblasts into CAFs and induced a gene signature that was associated with improved survival outcomes in CRC patients (102). Experiments using the VDR ligand calcipotriol had a similar effect in PDAC (103).

Understanding CAF biology not only helps to develop complementary treatment options, but it can also help to optimise current ones. One CAF targeting strategy takes an alternative approach and uses a 68Ga-radiolabeled inhibitor of FAP (FAPI) to visualise CAFs on PET/CT scans for patients with head and neck squamous cell carcinoma (104). In this novel study, visualisation of FAP+ CAFs acts as both a diagnostic tool and a therapeutic guide helping to risk stratify patients and improve the delivery of radiotherapy (104).

## 5 Conclusion

This review has discussed how further research into the role of CAFs in bladder cancer is necessary to fill important gaps in our knowledge of this disease and expand therapeutic options for patients. Additionally, it is particularly important to understand how CAFs impact the response to therapies currently available to patients. Based on the evidence presented in this review, we hypothesise that CAF enrichment of bladder tumours is associated with inferior responses to radical radiotherapy for MIBC. Poor responses to radiotherapy are seen in other tumour types with a high stromal score and an increased number of CAFs, such as rectal cancer (75). If, as we hypothesize, MIBC enriched for CAFs show similar behavior, this highlights a cohort of patients that we propose would benefit from a combinatorial approach that includes targeting of CAFs. In addition, CAF targeting approaches may have a role in metastatic disease. In the United Kingdom, the RE-ARM trial is exploring the combination of immune checkpoint blockade with "experimental" palliative radiotherapy in patients with metastatic urothelial cancer (105). The clinical outcomes of this trial, including the comprehensive translational profiling that is embedded in RE-ARM, may help to identify groups of patients that could benefit from CAF targeting approaches. In doing so, this could expand the number of patients that ultimately benefit from immune checkpoint inhibition.

In conclusion, we propose that the various strategies to target CAFs discussed in this review have considerable therapeutic potential, both as single agents, and in combination with existing therapies, to improve survival outcomes for patients with bladder cancer. In order to inform treatment advances, we consider that it is a research priority to better understand bladder cancer-specific CAF subtypes and their spatial relationship with other cells of the TME at baseline, as well as longitudinal changes in CAF biology following treatments such as radiotherapy.

## Author contributions

AB wrote and edited the manuscript and created the figures. AW proposed the conception, edited and reviewed the manuscript. AR edited and reviewed the manuscript. All authors listed in the paper have made a substantial, direct, and intellectual contribution to the work and approved it for publication. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Cellular milieu in clear cell renal cell carcinoma

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Clear cell renal cell carcinoma (ccRCC) is globally the most prevalent renal cancer. The cells of origin in ccRCC have been identified as proximal tubular epithelial cells (PTEC); however, the transcriptomic pathways resulting in the transition from normal to malignant PTEC state have remained unclear. Immunotherapy targeting checkpoints have revolutionized the management of ccRCC, but a sustained clinical response is achieved in only a minority of ccRCC patients. This indicates that our understanding of the mechanisms involved in the malignant transition and resistance to immune checkpoint therapy in ccRCC is unclear. This review examines recent single-cell transcriptomics studies of ccRCC to clarify the transition of PTEC in ccRCC development, and the immune cell types, states, and interactions that may limit the response to targeted immune therapy, and finally suggests stromal cells as key drivers in recurrent and locally invasive ccRCC. These and future single-cell transcriptomics studies will continue to clarify the cellular milieu in the ccRCC microenvironment, thus defining actional clinical, therapeutic, and prognostic characteristics of ccRCC.

KEYWORDS

**clear cell renal cell carcinoma, proximal tubular epithelial cells, tumor associate macrophages, CD8+ T cells, cancer associated fibroblasts**

## Introduction

Kidney cancer is the seventh most common adult-onset cancer in Australia (1). At diagnosis, 75% of these kidney cancers will be subtyped as clear cell renal cell carcinoma (ccRCC) with a 5-year survival rate of 50%–69% (2–4). However, if at diagnosis the ccRCC tumor measures greater than 7 cm or has metastasized, then 5-year survival decreases to 10% (3, 5). Clinical outcomes of ccRCC are variable and prediction of survival based on available clinical parameters has been attempted (6). Variability within similar clinical categories occurs, likely due to a combination of limited biomarkers and tumor heterogeneity which hampers more precise prognostication (7). The challenges of poor survival and clinical variation have resulted in numerous detailed cellular profiling studies for ccRCC, providing mechanistic insight for targeted therapeutics. However, gaps persist in our understanding of the complex and variable cell types and states in ccRCC.

Understanding the complex cellular milieu in ccRCC requires knowledge of both individual and integrated cell types and their states. The key cell types in ccRCC are tubular epithelial, immune, and stromal cells that can each attain variable cell states. Individually, these cellular phenotypes have been profiled within ccRCC by various analytical methods. In this review, we summarize the reported individual cellular phenotypes from single-cell transcriptomics studies of ccRCC, to provide an integrated view of key cell types and states that reside in the ccRCC microenvironment.

## Cellular origin of ccRCC—all paths lead to ccRCC

The development of ccRCC is initiated at the gene level. Multiple genomic studies in human ccRCC have revealed a complete or partial biallelic loss in chromosome 3p encoding *VHL* (von Hippel–Lindau tumor suppressor gene) (8). The loss in chromosome 3p has been attributed to faulty chromothripsis, forming micronuclei during mitosis in normal proximal tubular epithelial cells (PTEC) (9, 10). The trigger for the micronuclei formation in normal PTEC has been attributed to their susceptibility to hypoxic microenvironments, a hallmark in ccRCC progression (11, 12). Alteration in *VHL* expression, present in 80%–93% of primary ccRCC cases, forms a self-perpetuating hypoxic PTEC microenvironment (13–17).

### ccRCC development in mouse models

However, singularly this altered *VHL* expression lacks the capacity to induce ccRCC development in mouse models (12, 17–19). Verification of additional genetic alterations in ccRCC was demonstrated in a mouse model study that combined deletion of *Vhl*, transformation-related protein 53 (*Trp53*), and retinoblastoma (*Rb1*) genes to induce ccRCC development (18). In this study, two key aspects of ccRCC were demonstrated. First, the positive staining of malignant cells by proximal tubule protein markers (CD10, AQP1, or NAP12A) confirmed PTEC as the cellular origin of ccRCC (18). Second, the multiple genetic deletions in this mouse model demonstrated a combined genetic variability underlying the development of ccRCC. Similarly, in humans, the development of ccRCC has been reported in PTEC with altered *VHL*, following additional inactivation of polybromo 1 (*PBRM1*), BRCA-associated protein 1 (*BAP1*), and/or SET domain containing 2 (*SETD2*) genes (12, 17, 19).

### ccRCC cellular origin in human studies

Further support for PTEC as the cellular origin of ccRCC has been provided by two human single-cell transcriptomics studies, matching the captured ccRCC PTEC transcriptome to single and/or bulk normal and ccRCC transcriptomes (20, 21). Collectively, these two transcriptomics studies identified the expression of carbonic anhydrase 9 (*CA9*), vascular cell adhesion molecule-1 (*VCAM1*), solute carrier family 17 member 3 (*SLC17A3*), intercellular adhesion molecule 1 (*ICAM1*), integrin subunit beta 8 (*ITGB8*), alpha kinase 2 (*ALPK2*), and vimentin (*VIM*) in ccRCC PTEC (20, 21). Surprisingly, in ccRCC patients the adjacent morphologically normal kidney tissue demonstrated protein marker staining for VCAM1 within CA9-positive PTEC. The VCAM1 and CA9-positive PTEC were termed precursor PTEC and defined as morphologically normal PTEC with $VHL^{+/-}$ mutation (20). This identification of precursor PTEC in morphologically normal kidney implicates identifiable transcriptomic alteration following genomic alteration. Additionally, this precedes morphological change in ccRCC development and supports a proposed transition from normal to precursor and finally malignant PTEC states.

### An inflamed PTEC state

Intriguingly, the precursor PTEC expressing VCAM1 and CA9 appear transcriptomically similar to inflamed PTEC with *VCAM1*, but without *CA9* expression. The transcriptomic profile of inflamed PTEC was identified by a multi-omics study performed on normal human kidney tissues (22). These inflamed PTEC are defined with *VCAM1, ICAM1, CD24, CD133*, and *HAVCR1* expression resulting in response to acute and/or chronic tubular injury. Again, this tubular injury is perpetuated by the susceptibility of tubules to hypoxic

conditions. Indeed, trajectory inference modeling of the captured PTEC transcriptome revealed a continuum from normal to inflamed PTEC that expanded in tubular injury-related inflammation (21). Since the transcriptomic profile of inflamed PTEC provides the strongest similarity to malignant PTEC, an alternative PTEC transition from normal to inflamed to precursor and finally malignant PTEC state can be proposed in ccRCC development (Figure 1).

## Alternative transcriptomics pathway in ccRCC development

The above-mentioned transcriptomic studies validated earlier mouse models by confirming PTEC as the cellular origin of ccRCC in humans (20–22). However, they proposed an alternative inflamed PTEC transcriptomic state under hypoxic injury that perpetuates normal to malignant transition of PTEC

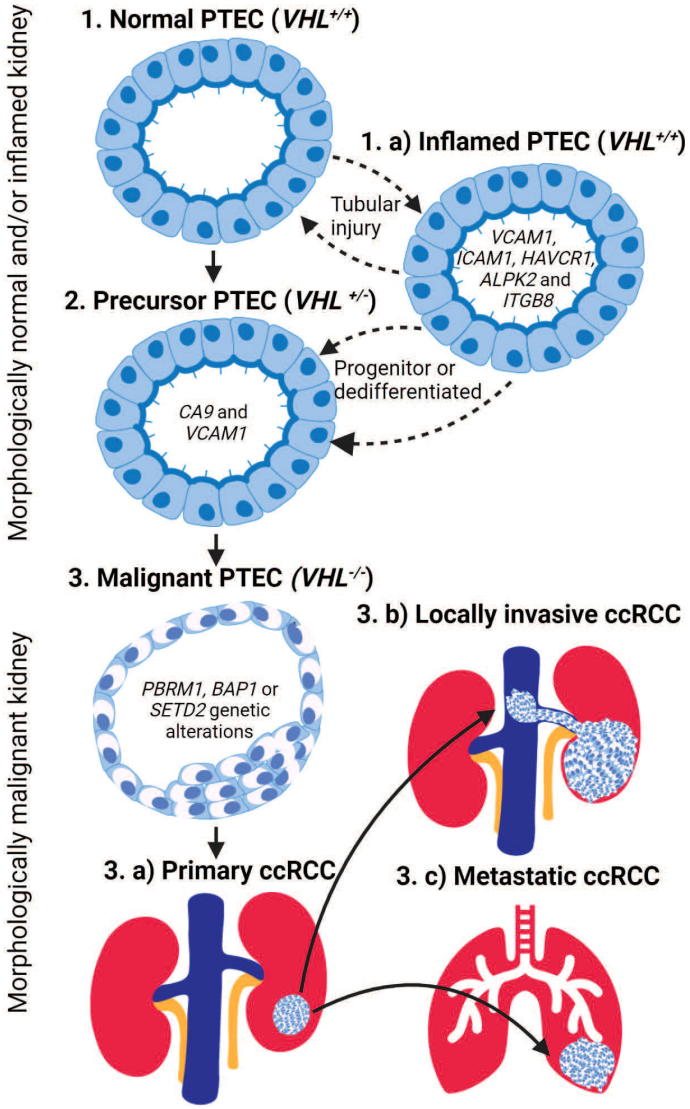


FIGURE 1
The transition of proximal tubular epithelial cells (PTEC) in the development of ccRCC. PTEC are the cell of origin in ccRCC that under hypoxic conditions transition from normal to malignant state. PTEC transition from normal (Step 1) to precursor $VHL^{+/-}$ (Step 2) and finally malignant $VHL^{-/-}$ (Step 3) after additional genetic alterations are acquired. Alternatively, under hypoxic conditions, PTEC may transition to an inflamed PTEC *VCAM* (Step 1.a) state due to tubular injury. This inflamed PTEC state is bidirectional until a loss of $VHL^{+/-}$ within the inflamed PTEC is acquired, resulting in the non-reversible transitions to the precursor PTEC (Step 2) state and finally the malignant (Step 3) state after the loss of both $VHL^{-/-}$ and additional genetic alterations are acquired. Once PTEC transition to the malignant state, they can develop primary ccRCC (Step 3.a) lesions within the kidney or locally invasive ccRCC (Step 3.b) lesions that extend into adjacent large vessels and/or metastatic ccRCC (Step 3.c) lesions that spread to distant organs.

in ccRCC development (Figure 1). Furthermore, transcriptomic profiling in adjacent normal kidney demonstrates that normal to precursor PTEC transition initiates within morphologically normal kidney. Here, the normal ($VHL^{+/+}$) and precursor ($VHL^{+/-}$) PTEC transition bidirectionally between these two states. The precursor ($VHL^{+/-}$) PTEC transition to irreversible malignant PTEC, if further necessary genetic alterations like $VHL^{-/-}$ with *PBRM1, BAP1*, and/or *SETD2* mutations are somatically acquired. The proposed alternative transcriptome pathway suggesting normal to inflamed to precursor and finally malignant PTEC transition also initiates within morphologically normal kidney. Here, the normal and inflamed ($VHL^{+/+}$) PTEC transition between these two states, as progenitor stem-like PTEC and/or dedifferentiated mature PTEC, to repair the injury that has resulted from transient hypoxic conditions (23, 24). However, during this repair process, mitotic activity increases in the inflamed PTEC, making somatic loss in $VHL^{+/-}$ plausible and thus allowing irreversible transition to the precursor PTEC state. The final transition to malignant PTEC still requires further necessary ccRCC associated genetic alterations and mutations. While uncertainty remains on whether inflamed PTEC transitioning to the precursor state are progenitor stem-like or dedifferentiated mature (*CD133* and *CD24*) PTEC (22, 24–27), it does raise the possibility that within a subset of ccRCC, the origin may be progenitor stem-like PTEC rather than dedifferentiated mature PTEC (28).

## Locally invasive and metastatic ccRCC

Once PTEC transition to a malignant ccRCC state, they can form (1) a tumor lesion limited to the kidney (2); a locally invasive lesion extending into adjacent medium and/or large vessels; or (3) a metastatic lesion spreading to distant organs. The above-mentioned single-cell transcriptomics profiles are from primary ccRCC lesions. However, a recent single-cell transcriptomics study of ccRCC primary, locally invasive, and adjacent normal tissue identified enhanced extracellular matrix (ECM) remodeling by malignant PTEC in locally invasive lesions (29). This indicates that while locally invasive ccRCC lesions may result from opportunistic extension into vasculature due to proximity, the extending malignant PTEC also require supporting ECM (29, 30). This ECM remodeling can be profiled by the collagen gene markers *COL20A1, COL28A1*, *TGFB1*, *COL6A2, COL1A2*, and *COL4A2.* Similarly, metastatic ccRCC progression has been profiled by 17 metastasis-associated gene (MAG) markers identified in a single-cell transcriptomics study conducted on 121 single cells (31, 32). These single cells were captured from parental metastatic and patient-derived xenografted primary and metastatic ccRCC samples (31, 32). These MAGs include chemokines (*CCL20* and *CXCL1*), and mitochondrial (*MT-ND3, MT-ND4*, and *MT-RNR2*) and cancer (*NDUFA5, NNMT*, *BHLHE41, ALDH1A1*, and *BNIP3*) markers. Expression of these MAG markers is correlated with a higher likelihood of ccRCC recurrence.

In summary, single-cell transcriptomics studies confirm PTEC as the cellular origin of ccRCC. However, the transition from normal to malignant PTEC states may occur *via* several transcriptomics pathways, which are important to define for potential clinical, therapeutic, and prognostic reasons.

# Immune cells in ccRCC—exhausted when things get bad

ccRCCs are defined as immunogenic cancers, which has further been reconfirmed transcriptomically. An immunogenic transcriptome profile initiates with upregulation of gene sets associated with inflammatory cytokines, interferon gamma, and antigen processing on major histocompatibility complex (MHC) by inflamed and malignant PTEC, recruiting immune cells to the ccRCC microenvironment (21, 22, 33). Recruited monocytes enter the kidney tissue and differentiate to macrophages, activating the innate immune response through phagocytosis, exogenous antigen presentation, and immunomodulation (34). The activated innate immune response further recruits T cells, activating the adaptive immune response. In this manner, abundant myeloid and lymphoid cell types and states are recruited to the ccRCC microenvironment, characterizing ccRCC as immunogenic (35–39).

## A dysfunctional immune response in ccRCC

Multiple studies in human ccRCC however, reveal an inverse correlation between abundant immune infiltrate in ccRCC and patient survival, suggesting a dysfunctional immune response (33, 39–43). Understanding this dysfunctional immune response requires an understanding of the infiltrating immune cell types, states, and interactions in the ccRCC microenvironment. Several single-cell transcriptomics and clonal studies have been performed in human ccRCC (21, 33, 42–46), which profile the captured immune cell populations from ccRCC tumor (primary, metastatic, treated, and non-treated), adjacent normal kidney, and/or peripheral blood samples. These provide insight into the transcriptomic profiles of myeloid and lymphoid cell types, states, and their interactions in ccRCC (47–50), with particular emphasis on tumor-associated macrophages (TAMs) and $CD8^+$ T cells in the ccRCC microenvironment as both drive the tumor progression and evasion.

## TAM in ccRCC

Single-cell transcriptomics studies with ccRCC samples have identified synchronous pro-inflammatory M1-like TAMs and

anti-inflammatory M2-like TAMs (Figure 2). The former are defined by high expression levels of MHC class II molecules and cytokines *IL1B, IL6, IL8*, and *TNF*, while the latter are defined by high expression levels of MHC class II molecules and *CD163, FOLR2, MS4A4A, SEPP1*, and *MSR1* (21, 43). Furthermore, identification of TAM within metastatic ccRCC is defined by high expression of both HLA class I and II genes in conjunction with *IFI27, CTSL, CTSS, C1QA, C1QB, SERPING1, APOE*, and *PLTP* (43, 44). These TAM populations within ccRCC demonstrate high plasticity covering a continuum from M1-like to M2-like states; thus, intermediate TAM subpopulations are defined based on HLA-DR or interferon signaling gene expression levels (21, 33, 43, 53, 54). This continuum of TAM states across different stages of ccRCC has been inferred by trajectory analysis to commence from classic/non-classic monocyte to M1-like to M2-like and finally metastatic TAMs across normal, early, locally advanced, and metastatic ccRCC tissue (43). Indeed, a general shift in the TAM states with ccRCC progression is typified by an increase in dysfunctional M2-like TAMs with a simultaneous decrease in M1-like TAMs (43).

## CD8$^+$ T cells in ccRCC

Similarly, the transcriptome expression of CD8$^+$ T cells in ccRCC samples demonstrates a heterogeneous population and a continuum progressing to terminally exhausted clonotypes (Figure 2) (38, 42). Transcriptome expression and/or inferred cell activity has identified naïve, cytotoxic, exhausted, progenitor, and terminally exhausted CD8$^+$ T cells (33, 42–45). Transcriptomics and clonotyping profiles in conjunction with inferred pseudotime trajectory analysis of CD8$^+$ T cells in ccRCC suggest higher exhausted CD8$^+$ T cells with low TCR diversity in advanced and metastatic ccRCC microenvironments compared to those of normal kidney tissues and peripheral blood (39, 42–44). The identification of immune inhibitory

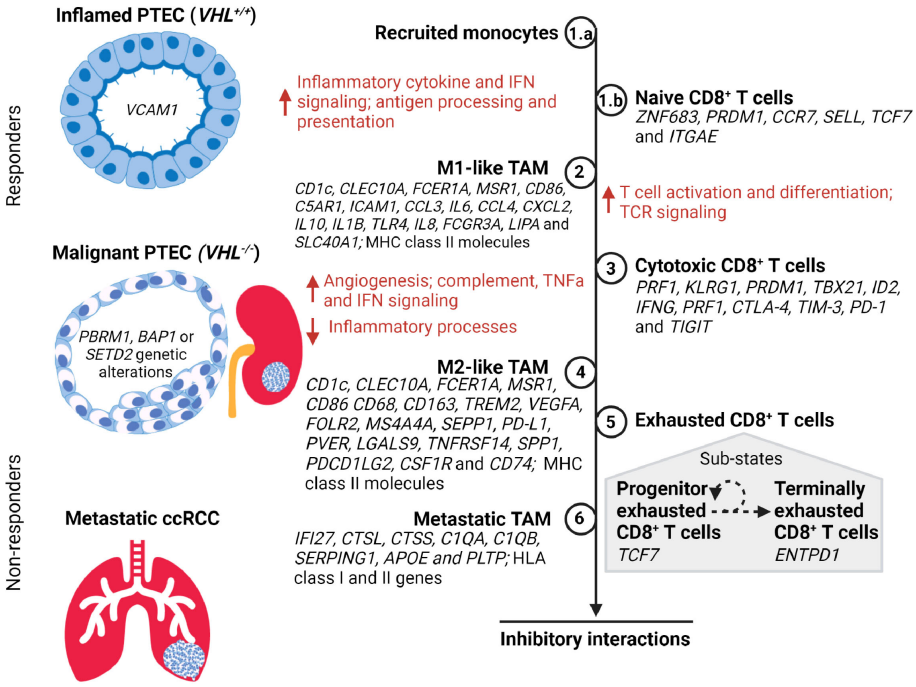


FIGURE 2
A timeline of tumor-associated macrophage (TAM) and CD8$^+$ T cell states during the transition of PTEC in the development of ccRCC. During hypoxic injury, the inflamed PTEC increase the expression of inflammatory cytokine and interferon gamma (IFN) signaling to recruit monocytes (Step 1.a) from the peripheral circulation. These recruited monocytes transition to M1-like TAM states (Step 2) and commence antigen processing and presentation, thus activating the naïve CD8$^+$ T cells (Step 1.b) within the kidney to transition to the cytotoxic CD8$^+$ T cell state (Step 3). During the malignant PTEC state, the M1-like TAMs increase angiogenesis, complement, tumor necrosis factor alpha (TNFα), and IFN signaling. However, as the ccRCC lesion progresses, the M1-like TAMs transition to the M2-like TAM state (Step 4). The cytotoxic CD8$^+$ T cells transition to an exhausted CD8$^+$ T cell state (Step 5) composed of a heterogeneous mix of progenitor and terminally exhausted states (51, 52). The subpopulation of exhausted CD8$^+$ T cells that exhibit progenitor transcriptome may respond to immune checkpoint therapy until they attain a terminally exhausted CD8$^+$ T cell state. In metastatic ccRCC, the M2-like TAM (Step 6) attain metastatic TAM transcriptome profile expressing both the HLA class I and II genes. However, in advancing ccRCC lesions, the M2-like TAMs and exhausted CD8$^+$ T cells co-occur and form inhibitory interactions that limit response to immune checkpoint therapy.

markers on $CD8^+$ T cells has been concordant with bulk RNA-seq studies demonstrating potential epigenetic reprogramming resulting in exhaustive states *via* TOX2 (39, 55–57). Within the exhausted $CD8^+$ T cell population, the identification of progenitor and terminally exhausted subpopulations suggests a spectrum of exhausted states that may transition from progenitor (*TCF7*) to terminally exhausted (*ENTPD1*) state (33, 43, 51, 52).

## Inhibitory interaction between M2-like TAMs and exhausted $CD8^+$ T cells

Unlike most $CD8^+$ T cells, exhausted $CD8^+$ T cells appear to develop many inhibitory interactions with M2-like TAMs, suggesting that ccRCC progression might result from their co-occurrence in advancing ccRCC (43). Due to the loss of spatial information with single-cell transcriptomics, protein marker staining with CD163 (for M2-like TAMs), PD-1, and TIM-3 (for exhausted $CD8^+$) has been used to confirm virtual co-localization within the ccRCC microenvironment (43). Further ligand–receptor gene inferencing revealed an increase in immune checkpoint interactions such as *PD-L1-PD-1*, *CD80/CD86-CTLA4*, *NECTIN2/PVR-TIGIT*, *LGALS9-TIM-3*, *TNFRSF14-BTLA*, and *SPP1-CD44* between M2-like TAMs and exhausted $CD8^+$ T cells, in advanced ccRCC (43). Inversely, the identification of *CSF1* and *MIF* ligands on exhausted $CD8^+$ T cells suggests M2-like polarization *via* interactions with *CSF1R* and *CD74* receptors on TAMs. These inferred ligand–receptor interactions between M2-like TAMs and exhausted $CD8^+$ T cells suggest that their inhibitory interaction increases as ccRCC progresses.

## M2-CD8 exhaustion gene signature correlates with worse survival in ccRCC

Therefore, both TAMs and $CD8^+$ T cells transition to an anti-inflammatory and exhaustive state within the tumor microenvironment as ccRCC progresses (Figure 2). These dysfunctional M2-like TAMs and exhausted $CD8^+$ T cells additionally form inhibitory interactions that further perpetuate a dysfunctional immune response. To confirm immune dysfunction resulting from the inferred inhibitory interactions, there has been further investigation of the generated M2-CD8 exhaustion gene signature. First, the expression of this M2-CD8 exhaustion signature was confirmed by mass cytometry (54) and the Cancer Genome Atlas (TCGA) (58) ccRCC datasets to be present in advanced ccRCC. Next, the effect that M2-CD8 exhaustion has on treatment outcomes was investigated in advanced and/or metastatic ccRCC patients treated with either PD-1 blockade or mTOR inhibition (43, 59). This showed no association between response or progression-free survival with the expression of M2-CD8 exhaustion signature with either treatment. In fact, increased M2-CD8 exhaustion signature in TCGA and treatment datasets correlated with worse overall survival in ccRCC patients (43). This suggests that M2-like TAMs and exhausted $CD8^+$ T cells may not respond to PD-1 blockade in ccRCC as is clinically expected.

## A subset of progenitor exhausted $CD8^+$ T cells in ccRCC

Clinical trial data suggest that ccRCC does otherwise respond to immune checkpoint blockade (ICB), like PD-1 inhibitor (60). The function of ICB is to block inhibitory signals that limit immune cell activation, thus allowing tumor reactive immune cells to overcome this pro-tumor regulatory mechanism and initiate an effective anti-tumor immune response (61). Indeed, other studies demonstrate that exhausted $CD8^+$ T cells include a subset of progenitor exhausted $CD8^+$ T cells (*TCF7*) within the tumor microenvironment that respond to PD-1 blockade and then transition to a terminal exhausted (*ENTPD1*) state (Figure 2) (43, 51, 52, 62–66). Further investigation of ccRCC transcriptomics data has identified this terminal exhausted subset within the progenitor exhausted $CD8^+$ T cell population with *TNFRSF9* (or *4-1BB*$^{Low}$) and upregulated *GZMA* and *FASLG*, confirming the presence of progenitor exhausted $CD8^+$ T cells (33). Based on this, it can be concluded that effective response to immune checkpoint therapy in advanced and metastatic ccRCC requires an absolute or relative absence of dysfunctional M2-like TAMs and exhausted $CD8^+$ T cells, or the presence of progenitor exhausted $CD8^+$ T cells. Resistance to immune checkpoint therapy in ccRCC can additionally be attributed to failed reversal or reinvigoration of dysfunctional M2-like TAMs and exhausted $CD8^+$ T cells, as has been suggested in other cancers (42–45, 56, 57, 67–71). Therefore, it is controversial whether additional checkpoint therapies, targeting additional immune checkpoints, will confer clinical benefit to ccRCC patients with an immune profile composed of M2-like TAMs and terminally exhausted $CD8^+$ T cell states. Unlike other solid malignancies, tumor mutation burden and PD-L1 status in ccRCC are not predictive indicators of immune checkpoint therapy outcome (46), suggesting that dysfunctional immune responses associated with infiltrating immune cell types and states may be better predictors of clinical response and therapeutic resistance to immune checkpoint therapy in ccRCC.

# Stromal cells in the ccRCC microenvironment—an elusive tumor driver

In ccRCC, the biallelic loss of *VHL* alleles in malignant PTEC activates and stabilizes the hypoxia-inducible factors

(HIFs), further supporting the transcription and secretion of HIF target genes, like vascular endothelial growth factor (*VEGF*) (21). This *VEGF* upregulation in the ccRCC microenvironment supports proangiogenic and immunosuppressive processes. The above-mentioned single-cell transcriptomics studies have inferred angiogenic activity with the secretion of *VEGFA* ligand by malignant PTEC and macrophages, which interact with the VEGF-signaling receptors on endothelial cells (*KDR, FLT1*, *NRP2, NRP1*, and *ACKR1*), macrophages (*NRP2* and *NRP1*), and fibroblasts (*NRP1*) (20, 21, 39, 45). Furthermore, angiogenic and proliferative activity in ccRCC has been inferred *via PGF* and *EFNA1* ligands secreted by malignant PTEC, which interact with the receptors on endothelial cells, TAMs, and fibroblasts (39). These transcriptome profiles support the well-known proangiogenic activities within the ccRCC microenvironment, morphologically characterized as a highly vascular tumor with favorable response to antiangiogenic treatments (72).

## Cancer-associated fibroblasts in recurrent and locally invasive ccRCC

However, like immune checkpoint therapies, antiangiogenic treatments fail to maintain a sustained clinical response in ccRCC patients. Thus, some focus and attention has turned to non-malignant and non-immune stromal cells. These include cancer-associated fibroblasts (CAF) within the ccRCC microenvironment due to their possible immunosuppressive functions. The recruitment of CAF within the ccRCC microenvironment is proposed to occur *via* interactions with malignant PTEC that upregulate *COL20A1, COL28A1*, and *TGFB1* (29). These infiltrating CAF are able to reduce $CD8^+$ T cell infiltration within ccRCC microenvironments, particularly within recurrent ccRCC as identified by a recent single-cell transcriptomics study (Figure 3) (73). In this study, the immunosuppressive behavior mediated by CAF was attributed to the secretion of Galectin-1 (Gal1), which was noted within the captured transcriptome by the substantial expression of *LGALS1*. Gal1, a well-known immunosuppressor within various tumor microenvironments, mediates apoptosis of cytotoxic $CD8^+$ T cells. This apoptotic activity of CAF was demonstrated within both *in vitro* and *in vivo* Gal1 knockdown models. Furthermore, the immunosuppressive nature of CAF was confirmed by reduced progression-free survival in ccRCC patients whose malignancies were observed to have high CAF infiltration and who had received immune checkpoint therapy (73). In addition to these immunosuppressive properties within the tumor microenvironments, the secreted Gal1 by CAF has been reported to promote epithelial–mesenchymal transition (EMT) in gastric cancer. Interestingly, CAF mediated EMT has been proposed within locally invasive ccRCC that rapidly extends into surrounding large vessels (Figure 3). Here, a single-cell transcriptomics study in locally invasive ccRCC identified CAF-mediated extracellular matrix remodeling by the increased gene signature for the EMT pathway (29). Therefore, in both recurrent and locally invasive ccRCC, CAF infiltrate should be considered as an additional key cell type driving tumor progression and immunosuppression (29, 73).

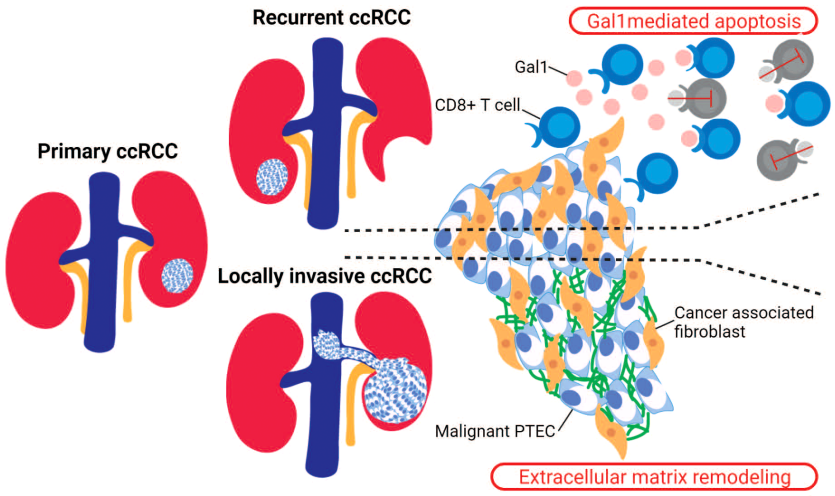


FIGURE 3
Stromal cells in recurrent and locally invasive ccRCC. Cancer-associated fibroblasts (CAF) are stromal cells that have been identified as drivers in the recurrent and locally invasive ccRCC microenvironment. In recurrent ccRCC, the infiltrating CAF secrete Gal1 that binds to the activated $CD8^+$ T cells and thus mediating T cell apoptosis. In locally invasive ccRCC, the infiltrating CAF increase the expression of genes within the epithelial–mesenchymal transition (EMT) pathway, leading to an increased extracellular matrix remodeling within the invasive ccRCC lesion.

## Conclusion

ccRCCs are characterized as hypoxic, immunogenic, and angiogenic tumors. An understanding of ccRCC requires investigation of all these characteristics not only within tumor cells but also in immune and stromal cells that infiltrate the ccRCC microenvironment. Recent application of single-cell transcriptomics within the ccRCC tumor (primary, metastatic, treated, and non-treated), adjacent normal kidney, and/or peripheral blood samples has expanded our understanding of the divergent cell types and states of ccRCC. The identification of inflamed PTEC poses the possibility of an alternative transcriptomic pathway in the development of ccRCC. There is growing evidence suggesting a dysfunctional interaction between M2-like TAMs and exhausted $CD8^+$ T cells and/or the lack of progenitor exhausted $CD8^+$ T cells in advanced and metastatic ccRCC might play a major role in resistance to available immune checkpoint therapies. Recent identification of immunosuppressive and extracellular matrix remodeling activities by CAF suggests stromal cells as additional elusive drivers in recurrent and locally invasive ccRCC. Therefore, a complete account of PTEC, immune and stromal cell types and states within the ccRCC microenvironment is shedding light on tumor progression and evasion in early, local, and metastatic ccRCC and informing future clinical management, therapeutics, and prognostics.

## Author contributions

AR, MR, SW, and AM conceived the review. AR, MR, and AK undertook the literature research and write-up. MR, SW, HH, AK, and AM reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Systematic review and meta-analysis of multiparametric MRI clear cell likelihood scores for classification of small renal masses

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**Purpose:** To systematically assess the multiparametric MRI clear cell likelihood score (ccLS) algorithm for the classification of small renal masses (SRM).

**Methods:** We conducted an electronic literature search on Web of Science, MEDLINE (Ovid and PubMed), Cochrane Library, EMBASE, and Google Scholar to identify relevant articles from 2017 up to June 30, 2022. We included studies reporting the diagnostic performance of the ccLS for characterization of solid SRM. The bivariate model and hierarchical summary receiver operating characteristic (HSROC) model were used to pool sensitivity, specificity, positive likelihood ratio (LR+), negative likelihood ratio (LR–), and diagnostic odds ratio (DOR). The quality evaluation was performed with the Quality Assessment of Diagnostic Accuracy Studies-2 tool.

**Results:** A total of 6 studies with 825 renal masses (785 patients) were included in the current meta-analysis. The pooled sensitivity and specificity for cT1a renal masses were 0.80 (95% CI 0.75–0.85) and 0.74 (95% CI 0.65–0.81) at the threshold of ccLS ≥4, the pooled LR+, LR–, and DOR were 3.04 (95% CI 2.34-3.95), 0.27 (95% CI 0.22–0.33), and 11.4 (95% CI 8.2-15.9), respectively. The area under the HSROC curve was 0.84 (95% CI 0.81–0.87). For all cT1 renal masses, the pooled sensitivity and specificity were 0.80 (95% CI 0.74–0.85) and 0.76 (95% CI 0.67–0.83).

**Conclusions:** The ccLS had moderate to high accuracy for identifying ccRCC from other RCC subtypes and with a moderate inter-reader agreement. However, its diagnostic performance remain needs multi-center, large cohort studies to validate in the future.

KEYWORDS

**clear cell likelihood score, MRI, classification, meta-analysis, small renal mass**

# Introduction

Over the past couple of decades, the incidence of renal cell carcinoma (RCC) has steadily increased in the United States and worldwide, in which cross-sectional imaging is play an important role (1–4). Indeed, as many as 70% of RCCs are detected incidentally for unrelated medical conditions (5). Higher detection of small renal lesions results in at least 80% increase in the number of surgical resections but does not bring considerable benefit to cancer-specific mortality at the population level (6, 7). Additionally, many renal masses exhibit an indolent behavior or grow very slowly and need no intervention (8, 9). Renal mass biopsies are recommended by several groups to facilitate personalized management; however, its nondiagnostic is up to 20% and not feasible in all patients (10). Thus, using non-invasive imaging examinations such as MRI and CT represents an alternative to biopsy to assist location, staging, and management of renal masses (1, 11, 12).

For cystic renal masses, the Bosniak classification provides standardized risk stratification and has been widely utilized in clinical practice for decades (13, 14). With respect to small solid renal masses, however, there is no widely accepted standardized risk stratification up to date, even though many studies demonstrated that imaging techniques such as US, CT, and MRI may play an important role in prediction of tumor histologic findings (15). Clear cell RCC (ccRCC) is the most common subtype of various RCC, accounting for more than half of cases and associated with worse outcome as compared to papillary and chromophobe tumors (16, 17). In addition, ccRCC is the most common cause of disease progression and metastasis in patients under active surveillance based on the combination of characteristics (18). In 2017, Canvasser et al. proposed the five-category Likert scale named ccLS to evaluate whether an SRM being a ccRCC (from 1 point=very unlikely to 5 points=very likely) (19). To date, several published studies have reported that this scoring system performed well in clinical practice; however, this algorithm has not been systematically assessed. Therefore, the purpose of this study was to evaluate the overall performance of the ccLS algorithm for the classification of ccRCC.

# Methods

This meta-analysis and systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement, with a predefined review and data extraction protocol (20). The primary outcome of our study was the diagnostic accuracy of the ccLS for identifying the cT1a (≤4 cm) solid renal masses. Additionally, considering that some studies applied the ccLS to cT1b (>4 cm and ≤7 cm) masses, we would assess the diagnostic performance of this algorithm for all cT1 (≤7 cm) renal masses.

## Search strategy and selection criteria

We conducted a systematic search of PubMed, EMBASE, Cochrane Library, Web of Science, and Google Scholar online scientific publication databases to identify articles published between January 2017 and June 2022, by using Medical Subject Headings (MeSH) and restricted language to English. The following terms and synonyms was used for literature searching: ([kidney] OR (renal) OR (nephron)] AND [(cancer) OR (mass*) OR (lesion)] AND ([ccLS] OR [clear cell likelihood score]). We supplemented our searches by manually screening the bibliographies of reviews and eligible articles. Two reviewers (*T.J.* and *T.F.X.*) evaluated the results of the literature search independently, and discrepancies were resolved by discussion with a third reviewer (*Z.H.*).

## Inclusion and exclusion criteria

We included studies that satisfied all of the following criteria: 1) using the ccLS for characterization of ccRCC; 2) providing sufficient details for reconstruction of 2×2 contingency tables for determination of the diagnostic accuracy; and 3) with biopsy or surgical pathological results as the reference standard. We excluded studies that met any of the following criteria: 1) not using the ccLS but other scoring systems or subjective assessment; 2) case reports or case series involving less than 20 participants; 3) with insufficient data to assess the diagnostic performance; 4) meta-analyses, guidelines, editorials, reviews, and letters; and 5) with partially overlapping patient populations.

## Data extraction and quality assessment

We extracted the following information from included studies with a standardized form: 1) demographic and clinical characteristics such as sample size of patients and masses, patient age, male-to-female ratio, and tumor size; 2) study characteristics such as authors, study design, year of publication, country and period of the study conducted, number of readers and their experience, inter-reader agreement, blinding to final results, and reference standard; and 3) technical characteristics such as MRI sequences and magnetic field strength. We employed the Quality Assessment of Diagnostic Accuracy Studies–2 to evaluate the study quality (21), in which the risk of bias for each study was assessed according to four domains: patient selection, method of the index test, reference standard, and flow and timing. Data extraction and quality assessment was carried out by two reviewers (*T.J.* and *T.F.X.*) independently.

## Data synthesis and statistical analysis

The bivariate model and HSROC model were used to pool the summary estimate of sensitivity, specificity, LR+, LR−, DOR, and their 95% confidence intervals (CIs) (22, 23). In addition, we constructed the forest plots and HSROC curve to graphically present the results. The Deeks' funnel plot was used to evaluate the publication bias, and the Deeks' asymmetry test was used to decide statistical significance (24). The degree of heterogeneity between studies was measured with Cochran *Q* statistics and Higgins $I^2$: for value of 0%-40%, not important; for value of 30%-60%, moderate; for value of 50%-90%, substantial; for value of 75%-100%, considerable (25). The "metandi" and "midas" modules in STATA 16.0 (StataCorp, Texas, USA) were used for all analyses, with a $P$ <0.05 indicating statistically significant.

# Results

## Literature search and data extraction

The flow chart of the literature selection process is presented in Figure 1. Our search strategy yielded 137 results initially, of which 39 were removed due to duplicates. After screening the titles and abstracts, a total of 65 results were excluded. Full-text reviewing was performed among the remaining 33 potential results and 27 were excluded for insufficient data ($n$=5), not in the field of interest ($n$=22). Ultimately, a total of 6 studies involving 785 patients were included in this meta-analysis (19, 26–30).

## Characteristics of the included studies

The detailed demographic and study characteristics are summarized in Tables 1 and 2. Regarding study design, nearly all studies were retrospective, and the sample size of the study population ranged from 57 to 241 patients. The mean age for patients ranged from 57 to 67 years, with an average tumor size of 24-38 mm. The proportion of ccRCC among studies was 41.7%-65.7%. Regarding the number of radiologists, 1 study reported that images were interpreted by only one reader (28), whereas in the remaining 5 studies images were interpreted by at least 3 readers. The reported radiologists' experience ranged from 1 to 30 years, with inter-reader agreement measured with kappa value of 0.53-0.65. Regarding cutoff values, 3 studies reported that the results were derived from the threshold of ccLS ≥4 (26, 28, 30), whereas the remaining 3 studies reported results from both ccLS ≥3 and ccLS≥4 (19, 27, 29). Concerning technique characteristics, nearly all studies reported that the images acquired from 1.5T or 3.0 T MRI; however, in one study

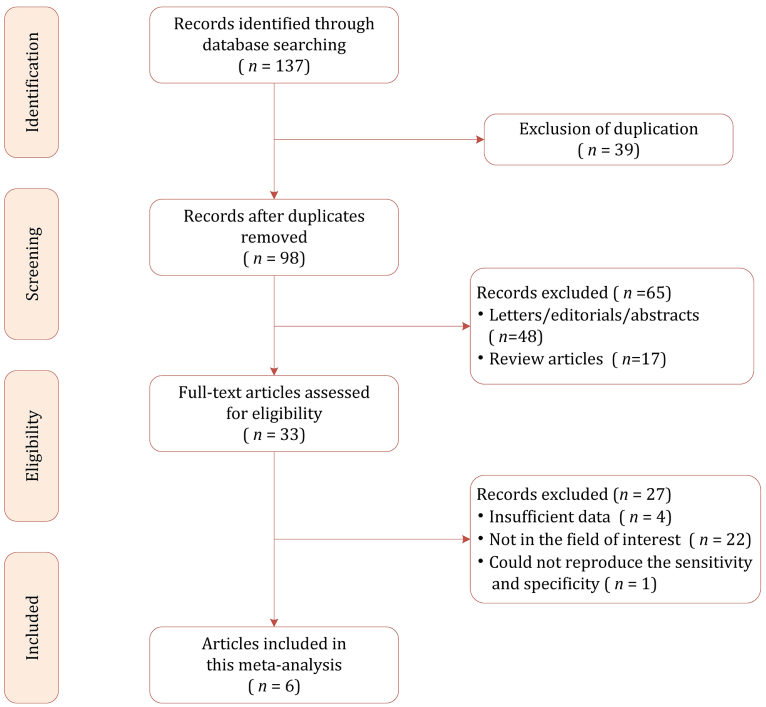


FIGURE 1
Study selection process for this systematic review and meta-analysis.

TABLE 1 Demographic Characteristics of Included Studies.

| Study | Country | Year | No. of patients | No. of lesions | Type of masses | No. ofccRCC | Gender (M/F) | Age(year, mean ±SD/median) | Tumor Size(cm, mean ±SD/median) |
|---|---|---|---|---|---|---|---|---|---|
| **Canvasser et al.** | USA | 2017 | 110 | 121 | cT1a | 61 | 61/39 | 57±14 | 2.4±0.8 |
| **Dunn et al.** | Canada | 2022 | 102 | 108 | cT1a/cT1b | 45 | 67/53 | 56.9±12.8 | 3.0±1.3 |
| **Johnson et al.** | USA | 2019 | 57 | 63 | cT1a | 35 | 38/19 | 61.7±14.9 | 2.7±0.7 |
| **Morgan et al.** | USA | 2021 | 70 | 70 | cT1 | 66 | 45/25 | 67/61-72 | 3.8/2.8-4.8 |
| **Schieda et al.** | USA/ Canada | 2022 | 241 | 250 | cT1a | 119 | 174/76 | 60±13 | 2.5±0.8 |
| **Steinberg et al.** | USA | 2020 | 204 | 213 | cT1a/cT1b | 183 | 110/94 | 59±13 | 2.7±0.8 |

ccRCC, clear cell renal cell carcinoma; SD, standard deviation.

the field strength was not reported (28). As for MRI protocol, only 3 studies used all sequences of T1, T2, dynamic contrast-enhanced (DCE), and diffusion-weighted imaging (DWI) (27, 29, 30). Concerning the reference standard, surgical resection pathological results were used in 4 studies, in the remaining 2 studies the biopsy results also were used in case of pathological results were not available (26, 27).

## Quality assessment

The overall quality assessment of the included studies was high. With respect to the type of renal masses, 1 study included all of the cT1 renal masses, thus was assigned as high risk of bias. In more than half studies, the analysis was restricted to masses with confirmed pathological results, which may lead to selection and verification biases as those masses under surveillance and did not undergo histological confirmation were not included. For the reference standard domain, in 2 studies the biopsy results were also used as the reference standard. Concerning the flow and timing domain, all included studies were assigned low risk of bias, detailed quality assessment is presented in Figure 2.

## Diagnostic performance of the ccLS for renal masses

For 5 studies using the ccLS for risk stratification of cT1a ccRCC, the sensitivity and specificity were 0.75-0.89 and 0.58-0.82 for individual studies. The summary estimates of sensitivity and specificity for cT1a renal masses were 0.80 (95% CI 0.75-0.85) and 0.74 (95% CI 0.65-0.81), respectively, with the area under HSROC of 0.84 (95% CI 0.81-0.87). Coupled forest plots

TABLE 2 Study Characteristics of Included Studies.

| FirstAuthor | StudyDesign | Study Period | No. ofReaders | Experience (Years) | Magnet Field Strength | Blinded | MRISequence | CutoffValue | $\kappa$ Value | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| **Canvasser et al.** | Retrospective | 2011.12-2015.07 | 7 | 1-15 | 1.5 T/3.0 T | Yes | T1/T2/DCE | ≥3/≥4 | 0.53/0.38-0.64 | Histological |
| **Dunn et al.** | Retrospective | 2013.01-2018.02 | 3 | 7-12 | 1.5 T | Yes | T1/T2/DCE | ≥4 | 0.65 | Biopsy/ Histological |
| **Johnson et al.** | Prospective | 2016.06-2018.04 | 14 | NA | 1.5 T/3.0 T | Yes | T1/T2/DCE/DWI | ≥3/≥4 | NA | Biopsy/ Histological |
| **Morgan et al.** | Retrospective | 2016.04-2020.02 | 1 | NA | NA | Yes | NA | ≥4 | NA | Histological |
| **Schieda et al.** | Retrospective | 2012.12-2019.12 | 10 | 5-30 | 1.5 T/3.0 T | Yes | T1/T2/DCE/DWI | ≥3/≥4 | 0.58/0.42-0.75 | Histological |
| **Steinberg et al.** | Retrospective | 2016.06-2019.11 | 16 | NA | 1.5 T/3.0 T | Yes | T1/T2/DCE/DWI | ≥4 | NA | Histological |

DCE, dynamic contrast enhanced; DWI, diffusion weighted imaging; NA, not available; T1, T1 weighted imaging; T2, T2 weighted imaging.

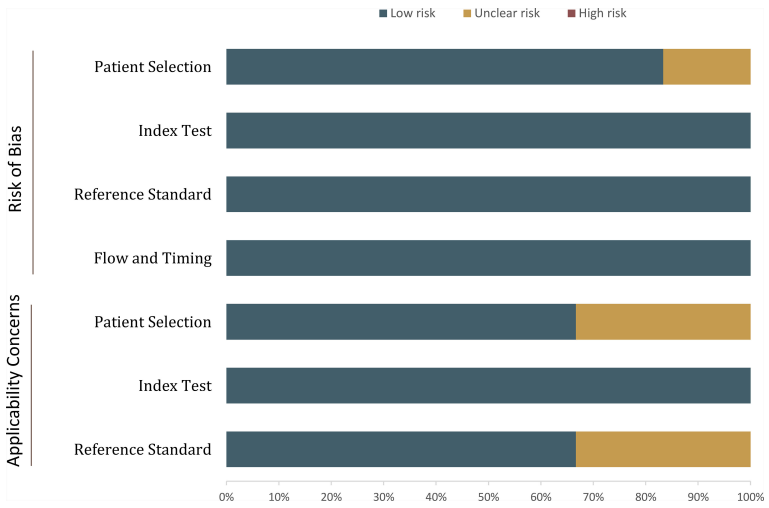


FIGURE 2
Grouped bar charts show the risk of bias and concerns for applicability of included studies.

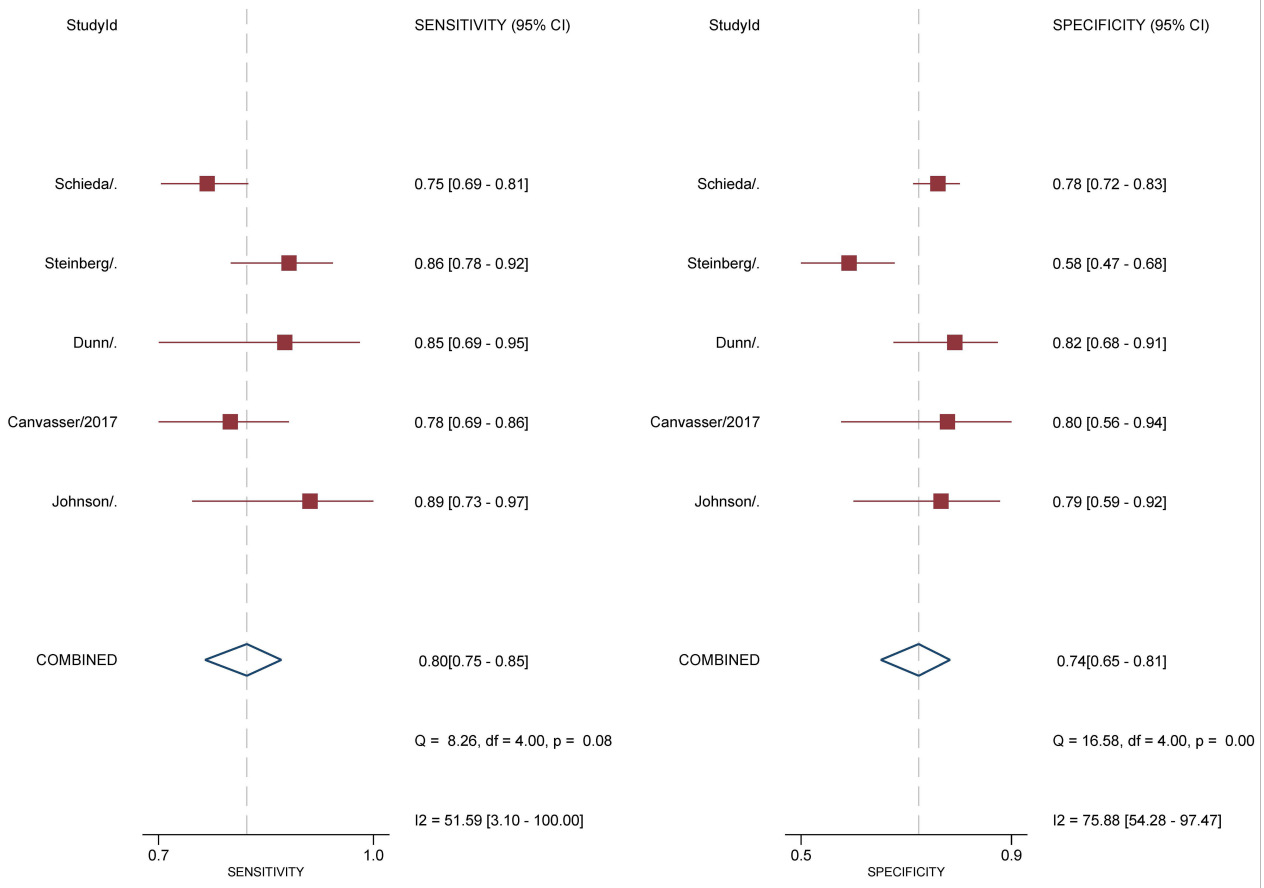


FIGURE 3
Coupled forest plot of pooled sensitivity and specificity. Numbers are pooled estimates with 95% CI in parentheses. Corresponding heterogeneity statistics are provided at bottom right corners. Horizontal lines indicate 95% confidence intervals.

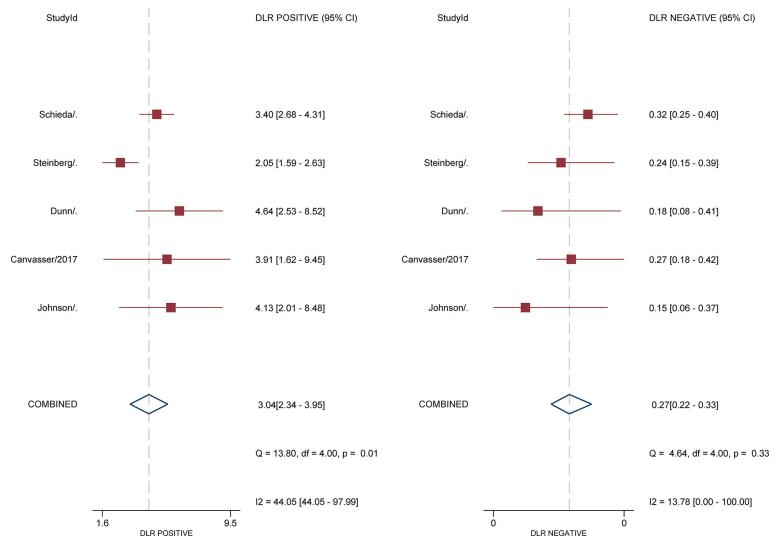


FIGURE 4
Coupled forest plot of pooled negative and positive likelihood ratios.

are presented in Figure 3. The pooled LR+, LR−, and DOR were 3.04 (95% CI 2.34-3.95), 0.27 (95% CI 0.22–0.33), and 11.4 (95% CI 8.2-15.9), respectively (Figure 4). The *Q* test revealed substantial heterogeneity presented throughout studies (P<0.05), and Higgins $I^2$ statistics also indicated substantial heterogeneity in terms of both sensitivity ($I^2$ =51.6%) and specificity ($I^2$ = 75.9%). In the HSROC curve, a large difference between the 95% confidence region and the 95% prediction region suggested substantial heterogeneity among studies (Figure 5). The Deeks funnel plot is presented in Figure 6, the P value of 0.15 for the slope coefficient indicated that the likelihood of publication bias was not statistically significant.

In the light of 3 studies providing the results of using the ccLS for stratification of cT1b renal masses, we then pooled the sensitivity and specificity of diagnostic accuracy for these lesions. The calculated summary estimates were comparable with cT1a masses, with pooled sensitivity and specificity of 0.83 (95% CI 0.71-0.89) and 0.78 (95% CI 0.58-0.91), respectively. For all cT1 renal masses (cT1a and cT1b), the pooled sensitivity and specificity were 0.80 (95% CI 0.74–0.85) and 0.76 (95% CI 0.67–0.83), with the calculated area under HSROC of 0.85 (0.82-0.88).

## Discussion

In this study, we systematically assessed the diagnostic performance of the ccLS for the classification of solid SRMs. Based on 6 studies, the pooled sensitivity and specificity at the threshold of ccLS ≥4 were 0.80 and 0.74, demonstrating moderate accuracy for cT1a renal masses. Despite the primary goal of ccLS is for classification of cT1a masses, some studies have applied it to cT1b masses. Our study suggested that the ccLS could also work well for all cT1 masses, with sensitivity and specificity of 0.80 and 0.76, respectively. Nevertheless, due to the small sample the diagnostic performance of the ccLS for all cT1 still needs large prospective multi-center studies to validate in the future. Reproducibility is critical for the standardized scoring system, as it relates to reducing the variability of interpretation between readers and improving the classification of solid SRMs. In the current meta-analysis, the included studies reported moderate inter-reader agreement between radiologists, with kappa value of 0.53-0.65.

At present, both the American Urologic Association and the American Society of Clinical Oncology recommend active surveillance as an initial management option for small renal masses, which is based on the fact that although approximately 80%-85% of small renal masses are malignant, only a minority showed the aggressive histologic features associated with disease progression and metastasis (31). Moreover, considering the patient morbidity and healthcare costs, active surveillance has been regarded as a viable management option for incidental small renal masses (18). Nevertheless, for ccRCC, the most common cause of disease progression and metastasis, active surveillance may occasionally yield unfavorable outcomes (16). Therefore, the need for better risk stratification strategies for indeterminate small solid renal masses is the main barrier to the wide acceptance of active surveillance in clinical practice (11). The emergence of the ccLS algorithm provides an encouraging start of standardization for solid renal mass, which represents the routine viewing approach from radiologists with less experience. According to the ccLS, assessment of the renal

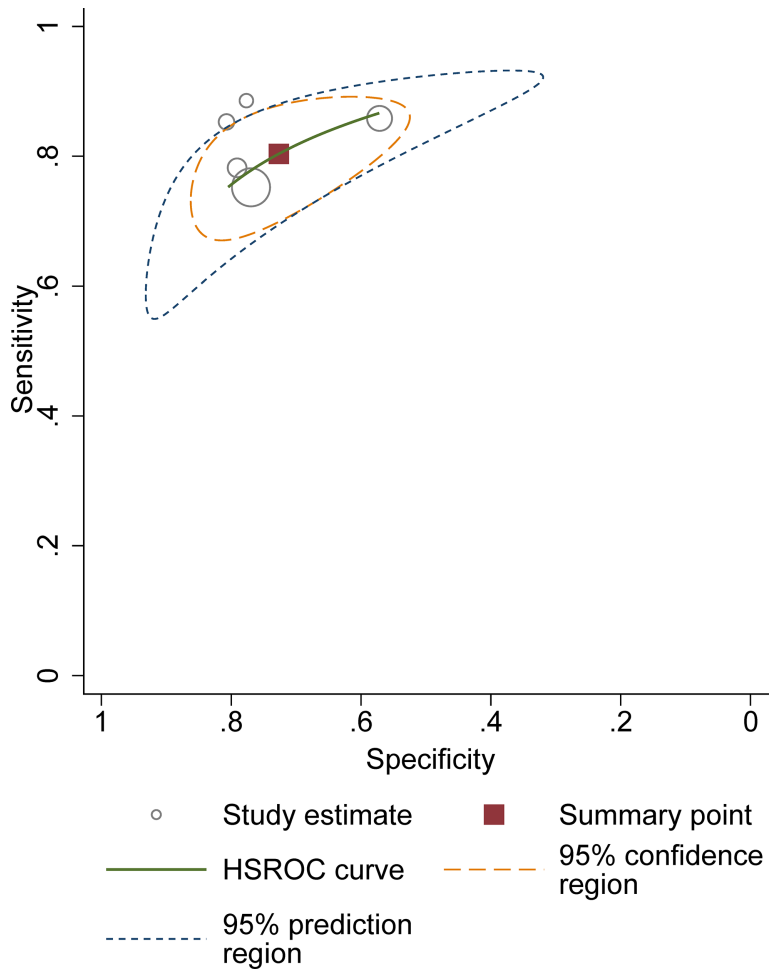


FIGURE 5
Hierarchic summary receiver operating characteristic plots with summary point and 95% confidence area for the overall.

masses includes two primary steps: eligibility criteria, ensuring the absence of macroscopic fat and at least mild (defined as 25%) contrast enhancement; and major criteria, assessing signal on T2-weighted MRI scans, corticomedullary contrast-enhancement degree, and presence of intra-lesion microscopic fat (32). In addition to offer an algorithm for assessing the likelihood of renal masses being ccRCC, Rasmussen et al. found that SRMs assigned ccLS category 4–5 grew at a faster rate than those assigned ccLS category 1–2 or ccLS category 3, which could help avoid pathologic confirmation through biopsy in many patients before recommending active surveillance or other intervention (33).

As compared with CT, MRI provides excellent soft-tissue contrast to differentiate those solid from cystic masses when enhancement is questionable on CT, especially for lesions between 10 and 20 HU (34). Furthermore, with DCE and functional information such as DWI, MRI can provide specific information regarding tumor histology, to acquire multiple postcontrast phases routinely without ironizing radiation (35). Although using the ccLS algorithm yielded similar diagnostic accuracy to radiologists' personal experience, this standardized workflow can assist radiologists with less experience to assess small SRMs with MRI (36). Moreover, the reported inter-reader agreement for this classification seemed moderate and comparable with other existing standardized scoring systems such as PI-RADS and TI-RADS (37, 38). Despite the ccLS has been assessed in several institutions, some improvements should be taken into account in the future version, e.g., the ccLS does not consider the other 2 RCC subtypes of papillary and chromophobe (32).

Our study has limitations that deserve mention. First, regarding study design, nearly all studies included were

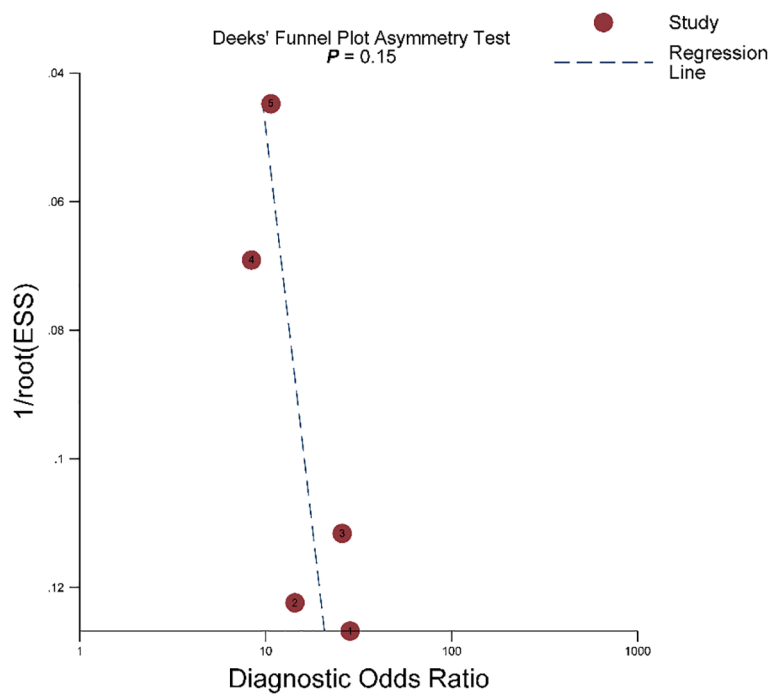


FIGURE 6
The Deeks' funnel plot.

retrospective, which led to high risk of bias in terms of the patient selection domain. Nonetheless, considering that there was only one study of prospective, it was unfeasible to pool data for a single study. Second, considerable heterogeneity was observed between studies, which may lower the applicability of our study. Nevertheless, it is unfeasible to conduct meta-regression to explore the source of heterogeneity because merely 6 studies were included. However, the methodology for this meta-analysis was conducted strictly according to the Cochrane Collaboration guideline. Third, all studies did not report the results of experienced and inexperienced readers separately, therefore whether ccLS could work well among radiologists with less unknown is still unknown.

## Conclusion

Use of the ccLS algorithm could yield moderate sensitivity and specificity for evaluation of ccRCC, with a moderate inter-reader agreement. Considering the complex subtype of RCC, the ccLS offers an encouraging start of standardization for the assessment of ccRCC. However, its diagnostic performance needs multi-center large cohort studies to validate in the future.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Cancer-testis antigen lactate dehydrogenase C4 as a novel biomarker of male infertility and cancer

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A unique lactate dehydrogenase (LDH) isoenzyme designated as lactate dehydrogenase C4 (LDH-C4) is found in mammalian mature testis and spermatozoa. Thus far, LDH-C4 has been well studied with regard to its gene and amino acid sequences, structure, biological properties, and peptide synthesis. Accumulating evidence has shown that LDH-C4 is closely related to spermatic energy metabolism and plays a critical role in sperm motility, capacitation, and fertilization. Defects in the catalytic activity of LDH-C4 are key to pathophysiological abnormalities underlying infertility. LDH-C4 was originally thought to be present only in mature testis and spermatozoa; however, recent studies have implicated LDH-C4 as a cancer-testis antigen (CTA), owing to its aberrant transcription in a broad spectrum of human neoplasms. This review highlights the recent findings on LDH-C4 with particular emphasis on its role in male infertility and tumors.

KEYWORDS

**cancer, infertility, cancer-testis antigen, lactate dehydrogenase C4, biomarker**

## Introduction

Lactate dehydrogenase (LDH) isozymes are widely distributed throughout mammalian tissues and catalyze the interconversion of pyruvate and lactate, representing the last step of anaerobic glycolysis (1, 2). In humans, two subunits of LDH were initially identified and designated as A and B, respectively. Functionally, the A- and B-subunits can assemble into five forms of tetrameric isoenzymes (LDH-1 to LDH-5). In the 1960s, the sixth LDH isoenzyme was identified from the human mature testis and originally named LDH-X (3, 4). Gradual research confirmed LDH-X as a tetrameric protein comprising four identical C-subunits. Thus, it was endowed with another name—LDH-C4 or LDHC (5).

LDH-C4 has been well studied in mammals, particularly in humans and murine models. Interestingly, the catalytic properties of the LDH isoenzymes are reflected through their varying compositions and expression patterns across organs and tissues. For instance, LDHA is abundant in anaerobic tissues including the skeletal muscle, wherein oxygen deficiency during exercise necessitates glycolysis to support the metabolic needs (6). LDHB is predominately found in the brain and heart and plays a critical role in maintaining aerobic metabolism by converting lactate from anaerobic glycolysis (7). In particular, LDH-C4 is mainly present in spermatids and spermatozoa within the mature testis (3–5). LDH-C4 is associated with glucose and plays an essential role in maintaining ATP production in the spermatozoa (8, 9).

Cancer-testis antigen (CTA) belongs to a group of cancer-associated antigens with normal expression in the adult testis but aberrant levels in several types of cancers, particularly in advanced stages exhibiting stem cell–like characteristics (10). Growing evidence suggests that LDH-C4 is a key enzyme for sperm function, and its abnormal activity or function contributes to male infertility (8, 9, 11–14). Moreover, the LDH-C4 isoenzyme is a molecular CTA with aberrant expression in several human cancers (15, 16). In this review, we have focused on gene regulation, tissue distribution, and molecular characteristics of LDH-C4 with particular emphasis on the roles of LDH-C4 in male infertility and tumors.

## *LDHC* gene expression and regulation

### *LDHC* gene and its regulation

In humans, expression of the C-subunit is under the control of the *LDHC* gene located at 11p15.3-15.5. Human *LDHC* yields two transcripts with a full-length mRNA of 2,089 bp for transcript variant 1 (NM_002301.5) and 2,035 bp for transcript variant 2 (NM_017448.5). Both variants encode a protein of 332 amino acids. The coding region of *LDHC* comprises seven exons (exons 2~8), whereby exon 1 plays no functional role in coding polypeptides but functions as a transcriptional regulator. The promoter sequences of human *LDHC* have been identified. The 5′-untranslated region (UTR) possesses several ubiquitous cis-acting elements, including one palindrome (PAL), one GC-box, one TATA-box, and two putative CCAAT elements (17). Specifically, the NF-I (nuclear factor I) protein binding site in PAL is adjacent to the TATA box, whereas three DNase I hypersensitive sites are located upstream of the CCAAT elements (17, 18). The NF-I proteins play a functional regulatory role in *LDHC* expression by binding to NF-I–specific sequences of PAL (18). A 110-bp core promoter region comprising a conserved GC-box and two cAMP-responsive element (CRE) binding sites has been identified (19). The GC-box and CRE sites adjoin each other and are all located upstream of the TATA-box (19). Both GC-box and CRE sites are essential for basal *LDHC* transcription (19). Mutations in GC-box or CRE sites reduce 73% and 74% of the total promoter activity, respectively (17). Further studies have demonstrated that a 60-bp sequence in the core promoter region is sufficient to drive robust transcription of *LDHC* in testis (20, 21). PAL may not be essential for *LDHC* transcription (17), but a mutation in the 31-bp PAL sequence in the core promoter region can abolish *LDHC* transcription in mice (22). Other transcription factors, including MYBL1, also play a critical role in regulating *LDHC* expression. A study highlighted that *LDHC* transcription was lost in 21-day-old testes of MYBL1 mutant mice (23). In fact, MYBL1 activates the transcription of *LDHC* in mice by interacting with the proteins that bind to CRE cis-element in the promoter region (23). The gene structure of *LDHC* includes its cis-regulatory elements and known trans-acting transcriptional regulators as shown in Figures 1A, B.

## Molecular characteristics of LDH-C4 and its expressions in somatic tissues/cells

LDH-C4 reveals distinct enzymatic, physicochemical, and immunological properties that differ significantly from other LDH isoenzymes (5, 24–27). Intriguingly, although human and mouse LDH-C4 share high–amino acid sequence similarity, some biochemical properties are substantially different. As exemplified by a previous study, mouse LDH-C4 is more thermostable than other LDH isozymes, which retain most of the biological activity after incubation at 65°C for 30 min (25); in comparison, the thermostability of human LDH-C4 is inferior. In addition, the protein distribution of LDH-C4 differs in mature sperms of humans versus mice: LDH-C4 mainly concentrates in the neck segment of the human mature sperms, whereas it is most abundant in the sperm principal piece of mice (5, 28).

Originally, the expression of LDH-C4 was thought to be highly tissue-specific and restricted to mature testis and germ

**Abbreviations:** aa, amino acids; ATP, adenosine triphosphate; AR, acrosome reaction; BC, breast cancer; cAMP, cyclic adenosine monophosphate; CTA, cancer-testis antigen; CTC, circulating tumor cell; CRE, cAMP-responsive element; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial–mesenchymal transition; HCC, hepatocellular carcinoma; KO, knockout; HNSCC, head and neck squamous cell carcinoma; LDH, lactate dehydrogenase; LDHC, lactate dehydrogenase C; LDH-C4, lactate dehydrogenase C4; LUAD, lung adenocarcinoma; LUCA, lung cancer; MYBL1, myoblastosis protein L1; NF-I, nuclear factor I; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide hydrogen; NSCLC, non–small cell lung cancer; OXPHOS, oxidative phosphorylation; PAGE, polyacrylamide gel electrophoresis; PAL, palindrome; PTP, protein tyrosine phosphorylation; PKA, protein kinase A; qRT-PCR, quantitative real-time polymerase chain reaction; RCC, renal cell carcinoma; ROS, reactive oxygen species; Sp1, specificity protein; TBT, tributyltin; UTR, untranslated region.

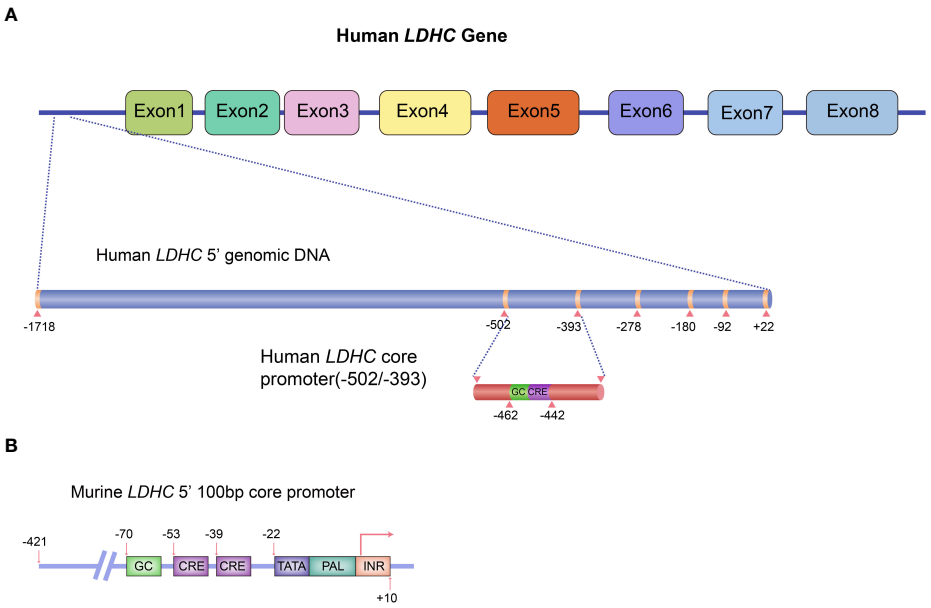


FIGURE 1
Schematic diagram of *LDHC* gene structure and the regulatory elements for gene expression. **(A)** Human *LDHC* gene structure and its cis-regulatory elements and known trans-acting transcriptional regulators. **(B)** The 100-bp core promoter murine LDHC and its elements. The schematic diagram was plotted in line with the published studies (17, 19).

cells, including the spermatocytes, spermatids, and spermatozoa (3, 4, 29). Goldberg et al. confirm that the *LDHC* gene is expressed first in leptotene-zygotene spermatocytes, with the highest mRNA expression in spermatids (5). LDH-C4 is responsible for more than 80% of the total LDH activity in mouse spermatozoa. It is absent in pre-pubertal testes and, thus, can be used as a prospective biomarker for germinal epithelium activity (12, 14, 30). However, the subcellular distribution of LDH-C4 in germ cells especially in spermatozoa is complex. Immunohistochemical studies have demonstrated mouse LDH-C4 in the cytoplasm of spermatocytes or spermatids as well as in the principal and middle pieces of the sperm tail (5, 29). In addition, LDH-C4 is present on the surface of human and murine spermatozoa and is closely related to its immune antiserum binding function (29, 31). In particular, LDH-C4 is present in the matrix of sperm-type mitochondria and forms the mitochondrial sheath in spermatocytes, spermatids, and spermatozoa (29, 32, 33); however, cross-contamination during indirect detection cannot be ruled out, and the role of LDH-C4 in the formation of the mitochondrial sheath in male germ cells remains controversial. Moreover, experiments using the specific antibodies also positioned human LDH-C4 in the post-acrosome area, the neck, and the mid-piece (34). Utilizing the histochemical staining and immunofluorescence techniques, LDH-C4's localization in human spermatozoa shows strong signals in the neck region (with a high concentration of mitochondria), but these are weak in the middle piece (28).

Thus, LDH-C4 is not strictly testis-specific, as evidenced by its presence in other non-testicular tissues or cells. Coonrod et al. have ascertained the transcription of *LDHC* in oocytes and early embryos; LDH-C4 protein is found in the cortex of oocytes, ova, zygotes, and embryonic blastomeres (27). Although no LDH-C4 activity has been detected in oocytes and the *LDHC*-null female mouse is fertile (12, 27), it does not rule out the fact that LDH-C4 may play a role in oogenesis or ovum function. Recent studies suggest that *LDHC* is transcribed in the somatic cells of the plateau pikas, including the liver, heart, lung, kidney, brain, skeletal muscle, and testis (35). The subcellular distribution of LDH-C4 only appears in the cytosol of somatic cells, whereas it is present in both the cytoplasm and mitochondria of the germ cells in the testis (35).

According to the definition of CTA and the specificity of *LDHC*/LDH-C4 expression in somatic cells, its expression in the peripheral blood of normal subjects is not expected. Interestingly, *LDHC* expression can be detected in the peripheral blood of some healthy person who currently have no apparent clinical abnormalities (35–38). We hypothesize that these healthy individuals with positive serum *LDHC* expression are at high risk of developing tumors or are at early stages of *in situ* tumor or carcinoma development that has not been clinically diagnosed. The above findings shed new light on the functional role of *LDHC*/LDH-C4 in mammals and prompt a host of interesting questions that need further investigations.

# *LDHC*/LDH-C4 and male infertility

## *LDHC*/LDH-C4 are involved in the metabolic pathways in spermatozoa

Gene expression of *LDHA*, *LDHB*, and *LDHC* occurs during the differentiation of germ cells during spermatogenesis (39). *LDHA* is present in pachytene spermatocytes, whereas *LDHB* is typically expressed in Sertoli and spermatogonial cells (39). The synthesis of the *LDHC* gene in testis takes place exclusively during meiosis and spermiogenesis, beginning in preleptotene or leptotene spermatocytes, and reaching its peak in pachytene spermatocytes, spermatids, and spermatozoa (3–5, 28, 29, 33); most LDH activity in male germ cells is attributed to that of LDH-C4 (5). Several investigations have documented the critical role of LDH-C4 in sperm motility, adenosine triphosphate (ATP) production, capacitation, and fertilization (5, 8, 9, 12, 13).

Sperm motility requires high levels of ATP with 70% being utilized for the movement of the flagella alone (40, 41). Two metabolic pathways for energy production operate in the mammalian spermatozoa: oxidative phosphorylation (OXPHOS) and glycolysis (42). OXPHOS takes place mainly in the mitochondria of spermatozoa, whereas glycolysis occurs largely in the head and principal piece (42). Because LDH-C4 is involved in both metabolic pathways and accounts for at least 80% of the total LDH activity in spermatozoa (5, 8, 9, 12), abnormal LDH-C4 function may exert a substantial impact on ATP production, which further weakens sperm function, ultimately leading to sterility. A study revealed that spermatic *LDHC* levels in normal donors are significantly higher relative to those in infertile patients with impaired sperm motility through qRT-PCR analyses, thereby suggesting *LDHC*'s involvement in the motility of spermatozoa (43). The *LDHC* knockout and functional studies based on both mating and preliminary sperm function experiments have ascertained the role of LDH-C4 in the maintenance of male fertility. Although the spermatic morphology or density appears normal, *LDHC* (−/−) male mice are sub-fertile, and the fertility is severely compromised due to a rapid reduction in the level of ATP and motility relative to the wild-type mice. *LDHC* (−/−) sperm does not acquire hyperactivated motility and cannot penetrate the zona pellucida *in vitro*, thus failing to undergo the phosphorylation event characteristic of capacitation (12). Interestingly, the dependence on LDH-C4 for fertilization differs markedly among mice of different genetic backgrounds. As exemplified by the study, *LDHC*-null C57BL/6 (B6) male mice are sub-fertile and the ATP content is moderately attenuated, whereas 129S6 male mice are infertile with drastically reduced ATP levels in spermatozoa (9). Another study further interpreted this interesting phenomenon and found that exogenous LDHA rescued sperm function in *LDHC*-deficient B6 mice (44). These results strongly suggest that *LDHA* is responsible for some or most of the LDH activity in *LDHC*-null sperm. However, on the basis of the current evidence, sperm contains substantially more LDH-C4 than what is required to maintain normal fertility (5). Thus, the function of LDHC if LDHA alone can provide the terminal reaction of glycolysis remains elusive. Whether any functional redundancy among LDH isozymes exists in sperm needs to be investigated in the future.

LDH-C4 regulates multiple signaling pathways in response to sperm capacitation and acrosome reaction (AR). However, the detailed mechanism remains poorly understood. It is suggested that the cAMP/protein kinase A (PKA) pathway depends on the ATP produced from glycolysis and further activates the signaling cascade for protein tyrosine phosphorylation (PTP) during capacitation (45). Upon regulation of glycolysis for ATP production, LDH-C4 impacts sperm capacitation through the cAMP/PKA pathway, further resulting in impaired sperm function. Accumulating evidence suggests that *LDHC*-null sperm does not undergo the phosphorylation event characteristic of capacitation, owing to reduced ATP levels in spermatozoa (12). Moreover, when LDH-C4 activity is selectively blocked using its inhibitor, PTP levels during capacitation are also attenuated (45). AR is a well-controlled exocytosis process that requires the participation of redox activity and several protein kinases. LDH-C4 can maintain the redox status in capacitated spermatozoa and participate in the cAMP/PKA pathway necessary for AR (46). LDH-C4 concentration is correlated with the acrosomal contents, suggesting its close association with infertility caused due to abnormal acrosomes (47).

## LDH-C4–specific antibodies and immune-infertility

LDH-C4 is a potent anti-fertility target for developing vaccines for immunocontraception. Notwithstanding, evidence for the notion that LDH-C4–specific antibodies induced in the female reproductive tract are targeted toward spermatozoa to block fertilization is present (24). The anti-sperm LDH-C4 (IgG) levels in 177 infertile patients were tested by ELISA, and 54 subjects were found to be anti–LDH-C4 IgG positive; within the infertile group, the positivity rates among male and female patients were 31.46% and 29.55%, respectively, implying that the presence of anti–LDH-C4 IgG likely indicates immuno-infertility (48). Contraceptive "DNA vaccines" for LDH-C4 have been synthesized, and its immuno-contraceptive effects on mice have been evaluated (49). As expected, the antibody titers were measurable in the serum of vaccinated male mice and the reproductive tract secretions of vaccinated female mice. The DNA vaccines manifest strong suppression of fertilization in both treated male and female mice (49). Goldberg et al. have also presented similar results, whereby a declining birth rate was observed in LDH-C4–immunized male baboons (24). Although the immunized animals could recover their fertility potential

after a period and showed no autoimmune diseases, the induction with high levels of anti–LDH-C4 serum potentially causes male infertility (49).

## LDH-C4 and environment-related male sterility

In particular, some studies have also raised concerns about the adverse effects of environmental factors on LDH-C4 function. Continuous lighting reduces total LDH and the LDH-C4 activity in rat sperms (50). Similarly, radiation impairs the functional capacity of LDH-C4 by unfolding or dissociating the tetramers and further destroying the secondary and tertiary structures of its C-subunit (51). Constant exposure to chromium (VI) results in a visible disruption of germ cell arrangement near the walls of the seminiferous tubules in chromium (VI)–exposed rats. A constant decrease in the concentration of LDH-C4 in seminal plasma further contributes to diminished reproductive function or infertility (52). Some chemicals, including dibromoacetonitrile (DBAN), tributyltin (TBT), and gossypol, also exert substantial impacts on LDH-C4 activity and other spermatic parameters (16, 53–55). DBAN can induce oxidative stress in the testis and decrease nearly 46% of the testicular activity of LDH-C4 in male mice (53). TBT exerts spermatotoxic effects, resulting in increased sperm abnormalities and a decline in sperm count and quality (54). Some studies, however, also propose opposite views. These suggest that LDH-C4 can be utilized as a target of gossypol for developing contraceptive drugs, as long-term treatment with gossypol or its analogs would result in complete atrophy of the seminiferous epithelium and weaken the fertility potential (55). *LDHC* is upregulated in cases of metastatic lung adenocarcinoma (LUAD). The average transcript level of *LDHC* is higher among male patients or smokers as compared with female patients or non-smokers, respectively. *LDHC* is a putative oncogene responsible for smoking-related lung adenocarcinoma, particularly in male patients with pleural effusions, indicating that smoking to some extent contributes to altered LDH-C4 levels in male patients (56).

# *LDHC*/LDH-C4 and tumors

## Pan-cancer expression of LDH-C4

*LDHC* has been detected in various tumor types with varying levels. For instance, the *LDHC* positivity rate reaches 100% (18/18) in lung adenocarcinoma (LUAD), 76% in high-grade serous ovarian carcinomas (HGSC), and 44% in melanoma (56, 57). Koslowski et al. (15) confirm the expression of *LDHC* mRNA in all tested tumor types; the corresponding reported frequencies are as follows: melanoma (7/16), breast (7/20), colon (3/20), prostate (3/8), lung (8/17), renal (4/7), ovarian (3/7), thyroid (1/4), and cervical cancers (5/6); melanoma (5/8) and lung cancer cell lines (2/6). Another study confirms the positivity rate of *LDHC* at 25% in non–small cell lung cancer (NSCLC) samples (including 102 tumor specimens and seven cell lines) (58). Immunohistochemistry (IHC) of LUAD tissues demonstrates the presence of LDH-C4 in 81.8% of samples (72/88), whereas it was absent in the adjacent normal tissues (59). However, Atanackovic et al. have detected the expression of CTAs in 51 cases of head and neck squamous cell carcinoma (HNSCC) and report no positive expression of *LDHC* mRNA (60). Intriguingly, Chen et al. suggest that *LDHC* mRNA levels are significantly downregulated in osteosarcoma samples (38).

The expression of *LDHC*/LDH-C4 is also altered in genitourinary cancers. Hua et al. have evaluated *LDHC* expression levels in 18 pairs of frozen samples from renal cell carcinoma (RCC) patients (18 cancers and 18 corresponding adjacent tissues) by RT-qPCR. Aberrant *LDHC* expression was observed in 22.2% (4/18) RCC tissues, whereas it was absent in all adjacent tissue specimens. LDH-C4 protein is expressed in RCC tissues (27/133), evidenced by IHC analysis (59). Moreover, *LDHC* mRNA and protein levels were higher in CAKI-2 cells relative to CAKI-1 RCC cells; the HK-2 renal tubular epithelial cells show a low level of *LDHC* expression (59). *LDHC* is also specifically expressed in spermatocytic tumors during the prophase of meiosis (61) as well as in type 2 testicular germ cell tumors (TGCTs) (62).

The pan-cancer expression of *LDHC*/LDH-C4 has been assessed, and the results of IHC based on high-throughput tissue microarray analyses suggest the highest positivity rate of LDH-C4 in LUAD (96.7%) (63), followed by breast cancer (BC) (91.55%) (36), and hepatocellular carcinoma (HCC) (55.84%) (37). *LDHC* is expressed in serum and serum exosomes of tumor patients, with serum *LDHC* positivity rates of 91.66%, 68%, and 65% in BC (36), HCC (37), and LUAD (63), respectively. Serum-sourced exosomal *LDHC* in BC and HCC also yield comparable positivity rates as those in the serum (36, 37). The pan-cancer expression of *LDHC*/LDH-C4 based on TIMER (64) and UALCAN (65) suggests high levels in cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), ovarian serous cystadenocarcinoma (OV), uveal melanoma (UVM), TGCTs, skin cutaneous melanoma (SKCM), HNSCC, and BC (Figures 2A, B). At the protein level, LDH-C4 expression is high in lung cancer (LUCA), RCC, and HNSCC relative to paired normal controls (Figure 2C). LDH-C4 has been detected by IHC in CRC, BC, prostate cancer (PC), LUCA, and RCC with corresponding positivity rates of 50.00%, 22.22%, 25.00%, 25.00%, and 90.91% (Figure 2D), respectively, in the Human Protein Atlas (HPA) database (https://www.proteinatlas.org/about/licence). Table 1 summarizes *LDHC*/LDH-C4 expression and functions in human cancer types based on published studies.

## *LDHC*/LDH-C4 in cancer: Biological functions and regulatory mechanisms

LDH-C4 plays an oncogenic role in the majority of malignant tumors. However, reports on the detailed mechanism underlying *LDHC*'s involvement in tumor development are scarce. Much of the research has mainly focused on the correlations of LDH-C4 with epithelial–mesenchymal transition (EMT), MMP-2/MMP-9, and PI3K/AKT/GSK-3β signaling pathways. For instance, suppression of endogenous LDH-C4 significantly inhibits the growth and invasion of CAKI-2 cells, whereas overexpression of *LDHC* enhances the migration and invasion of CAKI-2 cells, in addition to inducing EMT in RCC (59). Wang et al. suggest that *LDHC* correlates substantially with EMT and elevates the expression of MMP-9, thus boosting the migratory ability of RCC cells (59). Chen et al. show that lentivirus-mediated *LDHC* overexpression upregulates MMP-2 and MMP-9 levels in A549 and H1299 LUAD cells (66). Exogenous LDH-C4 upregulation increases the expressions of PI3K and the phosphorylation of Akt (Ser473) and GSK-3β (Ser9) in A549 and H1299 cells, whereas the expressions of Akt and GSK-3β remain unchanged. Conversely, the PI3K inhibitor, LY294002, effectively diminishes the levels of phosphorylated Akt/GSK-3β induced by LDH-C4 overexpression in the two cell lines (66). Consequently, upon treatment with LY294002, the migratory and invasive abilities of LDH-C4–overexpressing A549 cells, EMT, and expression of MMP-2 and MMP-9 are attenuated. Correspondingly, LDH-C4 can be induced by EMT and the expression of MMP-2 and MMP-9 to achieve xenograft tumor growth and metastatic potential by the activation of the PI3K/AKT pathway *in vivo* (66). Collectively, these results demonstrate that LDH-C4 promotes malignant biological activities in LUAD cells by triggering the PI3K/AKT/GSK-3β signaling pathway and promoting EMT (66).

## LDH-C4 and tumor energy metabolism

Cancer cells preferentially convert glucose to lactate and obtain energy by aerobic glycolysis, a phenomenon known as the "Warburg effect" (67). LDH is a key metabolic enzyme for aerobic glycolysis that plays a crucial role in the conversion of pyruvate to lactate (68). *LDHC*/LDH-C4 affects the progression of tumor development mainly by influencing glycolysis. Chen et al. (38) confirm the high abundance of biomarkers in the glycolytic pathway in osteosarcoma, including *LDHC* genes and two metabolites (lactate and pyruvate). Lactate concentration reduces significantly following the blocking of endogenous *LDHC* in CAKI-2 RCC cells (59). *LDHC* overexpression results in increased lactate concentration in CAKI-1 RCC cells (59). In addition to its involvement in metabolism in LUAD cells, *LDHC* overexpression in A549 and H1299 cells results in significantly higher lactate concentrations (66). This results in a consistently high ATP concentration in LUAD cells, suggesting LDH-C4's importance for lactate metabolism in LUAD cells (66).

## LDH-C4—a biomarker for cancer prognosis, diagnosis, and immunotherapy

LDH-C4 is not cancer-type specific, and most studies suggest that *LDHC*/LDH-C4 can be used as a prognostic indicator of tumors. By analyzing LOGpc (https://bioinfo.henu.edu.cn/DatabaseList.jsp) and Kaplan–Meier plotter (https://kmplot.com/analysis/) databases, *LDHC* was found to be oncogenic in BC, RCC, LUCA, uterine corpus endometrial carcinoma (UCEC), thymoma (THYM), and pheochromocytoma and paraganglioma (PCPG), with high *LDHC* expression being associated with poor prognosis. However, *LDHC* was a protective factor for prognosis in HNSCC and CESC (Figures 3A, B). In LUAD (63), LDH-C4 protein was significantly associated with TNM tumor status, and patients with high LDH-C4 expression had a worse prognosis than those with low/negative expression. Similar results were observed in HCC (37) and BC (36). In genitourinary cancer, Hua et al. (59) report that LDH-C4–positive status is significantly associated with advanced tumor stage in RCC ($P$ = 0.042). Moreover, positive LDH-C4 expression is significantly associated with an increased risk for poor clinical prognoses in patients with RCC (log-rank $P$ = 0.0043) and short OS (59). Why might LDHC be a risk factor for some tumor types but a protective factor for prognoses of other tumor types (HNSCC and CESC) remains unclear? The plausible reasons could be as follows: First, the survival analyses of HNSCC and CESC in Kaplan–Meier plotter were based on limited sample size. Several other cancer types were also included, and the overall combined prognostic effects were more likely to be canceled. Second, because of post-translational modifications of proteins, *LDHC* mRNA levels in mRNA datasets might be inconsistent with the corresponding protein levels, resulting in inconsistent prognostic results in databases.

*LDHC* mRNA has been detected in serum and serum-derived exosomes of patients with BC (36), HCC (37), and LUAD (63), and the source may be related to vesicle encapsulation of tumor cells and release of necrotic tumor cells into the peripheral blood. The AUCs of serum *LDHC* for BC (36), HCC (37), and LUAD (63) reached 0.9587, 0.8382, and 0.8121, respectively, whereas those of serum-sourced *LDHC* for BC, HCC, and LUAD reached 0.9464, 0.9451, and 0.8925, respectively, higher than the corresponding values for serum *LDHC*. These data indicate that *LDHC* is a promising novel non-invasive marker for tumor diagnosis.

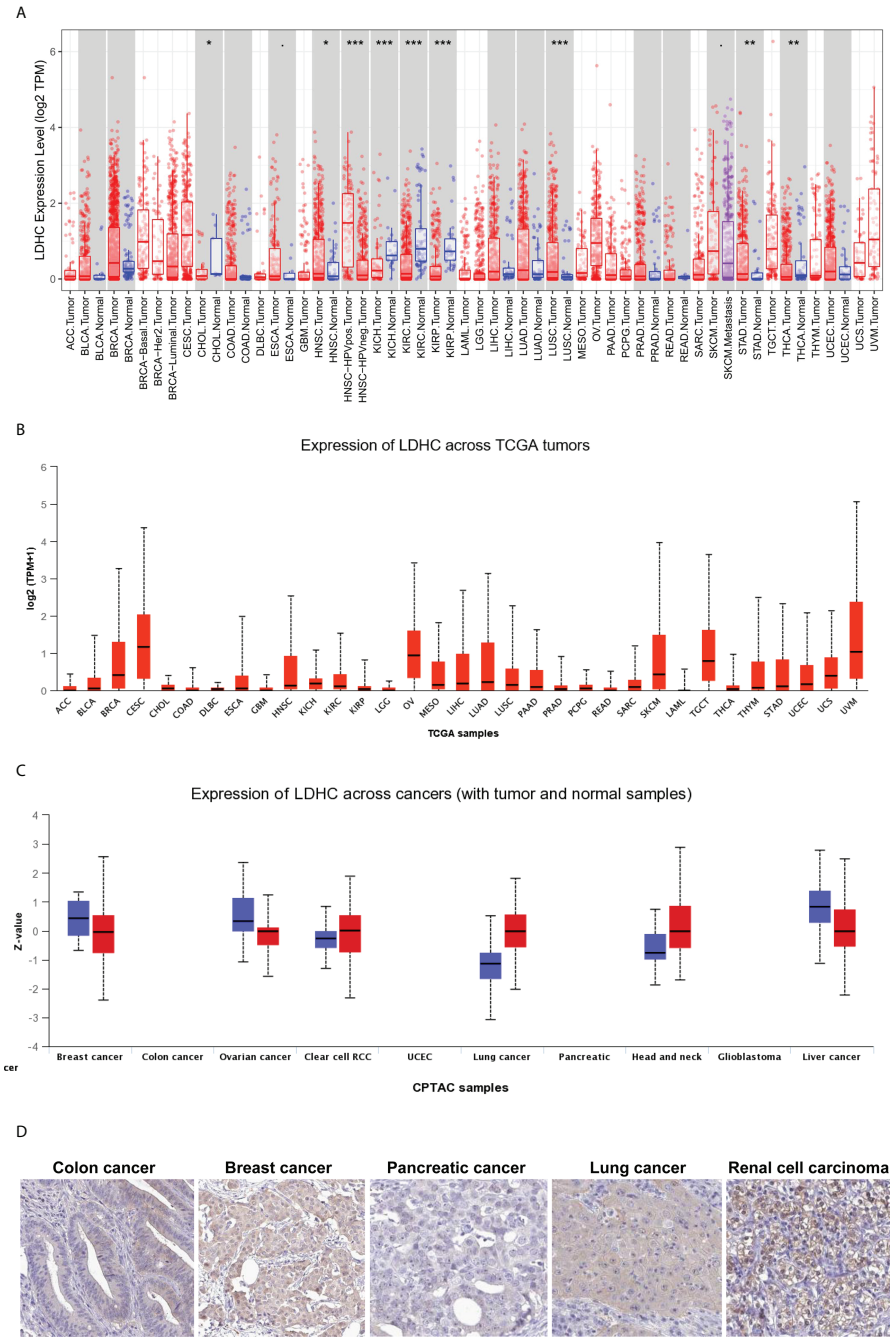


FIGURE 2
Pan-cancer expression of LDHC/LDH-C4 in public databases. Pan-cancer *LDHC* expression in **(A)** TIMER and **(B)** UALCAN databases. **(C)** Pan-cancer expression analysis of LDH-C4 in the UALCAN database. **(D)** IHC analysis of pan-cancer LDH-C4 using the HPA database. ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangio carcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSCC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower-grade glioma; LIHC, liver hepatocellular carcinoma; LUCA, lung cancer; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; RCC, renal cell carcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

TABLE 1 Expression status of *LDHC*/LDH-C4 in human tumors.

| Tumors | Expressions/Clinical Utility/Biological Functions | Biomarker for Disease | Reference |
|---|---|---|---|
| Pan-cancer, melanoma, and lung cancer cell lines | Frequencies of *LDHC* in pan-cancer: melanoma (7/16), breast (7/20), colon (3/20), prostate (3/8), lung (8/17), kidney (4/7), ovary (3/7), thyroid (1/4), and cervix (5/6); melanoma cell lines (5/8) and lung cancer cell lines (2/6); *LDHC* activation was neither mediated by gene promotor demethylation nor induced by hypoxia. | Biomarker | (15) |
| Melanoma cell lines (A375M and C81-61) | It was found that CpG island hypomethylation as well as transcription factors Sp1 and CREB played a major role in *LDHC* transcription. | / | (19) |
| Breast cancer (BC) | It showed 91.66% and 87.50% of serum and exosomal *LDHC*-positive cases, respectively, and 91.55% of LDH-C4–positive patients. *LDHC*/LDH-C4 exhibited high discrimination power in diagnosis, efficacy evaluation, relapse monitoring, and prognosis prediction for BC. | Prognostic and diagnostic biomarker | (36) |
| Hepatocellular carcinoma (HCC) | It showed that 68% and 60% of serum and exosomal *LDHC*-positive cases, respectively, were reported in HCC; patients with high LDH-C4 expression in HCC tissues accounted for 55.84%. *LDHC*/LDH-C4 could be used as a useful biomarker for early diagnosis, efficacy evaluation, relapse monitoring, and prognosis prediction for HCC. | Prognostic and diagnostic biomarker | (37) |
| Lung adenocarcinoma (LUAD) | *LDHC* is a candidate oncogene in the carcinogenesis of smoking-related LUAD.<br>LDH-C4 was positive in 81.8% of LUAD samples (72/88), whereas no LDH-C4 was found in any adjacent tissues; LDHC induced the EMT and the expression of MMP-2 and MMP-9, so as to achieve xenograft tumor growth and metastatic potential by the activation of the PI3K/Akt pathway both *in vitro* and *in vivo*. LDHC is a prognostic biomarker in LUAD | Biomarker<br>Prognostic biomarker | (56)<br>(63, 66) |
| Non–small cell lung cancer (NSCLC) | *LDHC* yielded a frequency of 25% in NSCLC; *LDHC* activation was independent of genomic hypomethylation. | / | (58) |
| Head and neck squamous cell carcinoma (HNSCC) | *LDHC* was negative in 51 cases with HNSCC. | / | (60) |
| Osteosarcoma | *LDHC* mRNA level was significantly downregulated in osteosarcoma samples | Biomarker | (38) |
| Renal cell carcinoma (RCC) | *LDHC* was abnormally expressed in 22.2% (4/18) RCC tissues, whereas no *LDHC* expression was found in adjacent tissues; a high percentage of RCC tissues (27/133) contained LDH-C4 protein; *LDHC* had a significant correlation with EMT, which could elevate the expression level of MMP-9 and enhance the migratory ability of RCC cells. | Prognostic biomarker | (59) |
| Spermatocytic tumor | *LDHC* is specifically expressed in spermatocytic seminomas during the prophase of meiosis. | / | (61) |
| Testicular germ cell tumors | Some cases of testicular germ cell tumors had a relatively high expression of *LDHC*, which may be due to undiagnosed normal testis elements in the tumors. | / | (62) |

Immunotherapy is the fourth most successful treatment strategy for tumors after surgery, chemotherapy, and radiotherapy. Numerous preclinical and clinical investigations focusing on NY-ESO-1 (69), MAGE-A3 (70), and PRAME (71) have shown that CTAs are promising candidates for adoptive T-cell treatment. According to recent research, LDH-C4 may be used as a CTA for targeted immunotherapy (57). The peptide pools (PP1–PP8) comprising 10–11 individual peptides each have been investigated for the immunogenicity of LDH-C4. These are indeed immunogenic; individual peptides within PP2 and PP8 can induce LDH-C4–specific T cell responses in HLA-A*0201 donors. Notably, PP2- and PP8-specific T cells exhibit cytotoxic activity toward BC cells expressing endogenous LDH-C4 within an HLA-A*0201 model. Peptides LDH-C4 amino acid (aa) 41–55 and aa288–303 from PP2 and PP8 elicit a functional cellular immune response. An increase in IFN-γ secretion by $CD8^+$ T cells and cancer cell killing of HLA-A*0201/LDH-C4–positive breast cancer cells by LDH-C4 aa41–55– and aa288–303–induced T cells have been documented (57). This study supports the rationale to assess LDH-C4; in particular, the HLA-A*0201 restricted LDH-C4 (aa41–55) and LDH-C4 (aa288–303) peptides (57), as a CTA for targeted immunotherapy. Another study highlights that *LDHC* expression is upregulated in the responder population among patients with melanoma undergoing treatment with anti–PD-1 therapy, suggesting that LDHC is a potential predictive biomarker of response to immune checkpoint inhibitor therapy (72).

## Conclusions and perspectives

LDH-C4 is a critical isoenzyme required for sperm motility, capacitation, and fertilization. As such, it can be employed as an important parameter in evaluating semen quality and male reproductive function. Owing to its status as a CTA gene, *LDHC* has therapeutic potential for immunotherapy against tumors. *LDHC* as a target CTA for immunotherapy has been exploited in BC; consequently, it is necessary to conduct in-

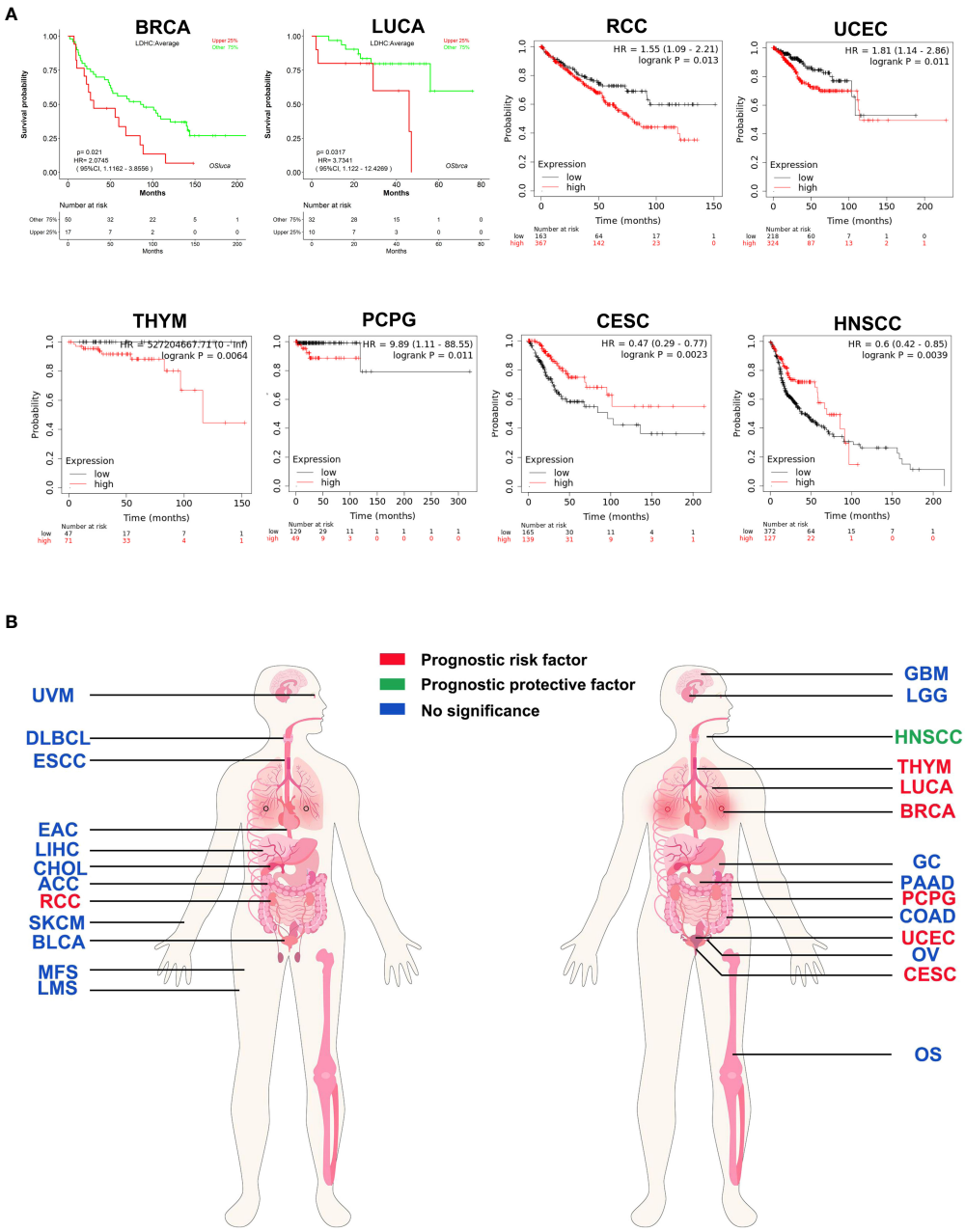


FIGURE 3
Prognostic pan-cancer analysis of *LDHC* using public databases. **(A)** Survival curves plotted using LOGpc and Kaplan–Meier plotter. **(B)** Pan-cancer view of the prognostic feature of *LDHC* based on the data from LOGpc and Kaplan–Meier plotter. The images of panel **(B)** are from the LOGpc database (https://bioinfo.henu.edu.cn/DatabaseList.jsp). ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangio carcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; GC, gastric cancer; HNSCC, head and neck squamous cell carcinoma; RCC, renal cell carcinoma; LGG, brain lower-grade glioma; LIHC, liver hepatocellular carcinoma; LUCA, lung cancer; OS, osteosarcoma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; SKCM, skin cutaneous melanoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UVM, uveal melanoma.

depth research for malignant tumors including BC. Furthermore, a better knowledge of *LDHC*'s aberrant transcription profile in cancer cells will shed light on its role in tumor progression. Further investigations are needed to validate the functional involvement of LDH-C4 in the treatment of carcinomas. The expression of *LDHC*/LDH-C4 in somatic cells and peripheral blood of some healthy people other than in tumors and testis necessitates further evaluation. Circulating *LDHC* has the potential to provide new clues for the early diagnosis of tumors, but these data need to be confirmed further.

## Author contributions

ZC, FL, and YL conceived and designed the study. JW and YC wrote the manuscript, and ZC and YL proofread the manuscript. All authors approved the final version of the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Prognostic and diagnostic value of circRNA expression in prostate cancer: A systematic review and meta-analysis

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**Background:** Circular RNAs (circRNAs) are receiving increasing attention as novel biomarkers. Our goal was to investigate the diagnostic, clinicopathological, and prognostic utility of circRNAs in prostate cancer (PCa).

**Methods:** Relevant literature was searched in PubMed, Web of Science, and EMBASE. Sensitivity, specificity, diagnostic odds ratio (DOR), negative likelihood ratio (NLR), positive likelihood ratio (PLR), and the area under the curve (AUC) were calculated to evaluate the diagnostic accuracy of circRNA expression. circRNAs' clinical, pathological, and prognostic value was examined using pooled odds ratios (ORs) and hazard ratios (HRs).

**Results:** This meta-analysis included 23 studies, with 5 for diagnosis, 16 for clinicopathological parameters, and 10 for prognosis. For diagnostic value, the pooled sensitivity, pooled specificity, PLR, NLR, DOR, and AUC were 0.82, 0.62, 2.17, 0.29, 7.37, and 0.81, respectively. Upregulation of carcinogenic circRNAs was associated with poor clinical parameters (Gleason score: OR = 0.222, 95% CI: 0.145–0.340; T classification: OR = 0.274, 95% CI: 0.175–0.430; lymph node metastasis: OR = 0.353, 95% CI: 0.175–0.716; tumor size: OR = 0.226, 95% CI: 0.099–0.518) and could predict poor survival outcomes (HR = 2.408, 95% CI: 1.559–3.720, $p < 0.001$). Conversely, downregulation of tumor-suppressor circRNAs was also associated with poor clinical parameters (Gleason score: OR = 1.689, 95% CI: 1.144–2.493; T classification: OR = 2.586, 95% CI: 1.779–3.762) and worse prognosis (HR = 1.739, 95% CI: 1.147–2.576, $p = 0.006$).

**Conclusion:** Our results showed that circRNAs might be useful biomarkers for the diagnosis and prognosis of PCa.

**Systematic review registration:** https://www.crd.york.ac.uk/prospero/, identifier CRD42021284785.

KEYWORDS

**circular RNAs, prostate cancer, diagnosis, prognosis, clinicopathological features**

# Introduction

Prostate cancer (PCa) is one of the most common malignancies in men worldwide, accounting for approximately 27% of all cancer cases with a high mortality rate (1). According to the global cancer statistics, in 2018, there were about 1,276,106 new cases and 358,989 death cases every year (2). Although the morbidity of PCa is lower in China than in other countries, the annual incidence has been on the rise (3). Early-stage PCa can be cured by surgery and chemotherapy, with 5-year survival rates exceeding 90%. The inhibition of gonadal testosterone production with androgen deprivation therapy has been the cornerstone of PCa treatment (4). In addition, multiple drugs, including abiraterone, enzalutamide, apalutamide, darolutamide, and docetaxel, have been approved for advanced PCa (4, 5). However, most advanced PCa can turn into castration-resistant PCa after long-term castration treatments, with 5-year survival rates, were below 30% (5, 6). Therefore, biomarkers are needed urgently for early diagnosis and for assessing the prognostic status of patients with PCa.

In recent years, prostate-specific antigen (PSA) has been used for the early determination and staging of PCa. However, its accuracy in predicting prognosis and biochemical recurrence is still questionable; thus, PSA is not recommended by experts (7, 8). Therefore, there is an urgent need to explore new biomarkers with high sensitivity, specificity, and reproducibility in the diagnosis, prognosis, and treatment of PCa.

Circular RNAs (circRNAs) are endogenous non-coding RNAs that range from a few hundred to thousands of nucleotides (9). circRNAs were once thought to be splicing faults; however, their structure was discovered because of the rapid growth of whole-genome sequencing (10). The circRNAs' 3′ and 5′ ends covalently form a loop, without the 5′ cap and 3′ poly(A) tails, making them structurally conserved and stable (11, 12). circRNAs are implicated in a variety of biological activities (13), including microRNA or protein sequestration, transcription regulation, splicing interference, and polypeptide translation (14). In addition, they play a role in physiological illnesses such as cancer cell proliferation, differentiation, apoptosis, and metastasis (15). Meanwhile, circRNAs are increasingly linked to the incidence and progression of PCa. Wang et al. (16) discovered that high levels of hsa_circ 0088233 increased the malignant phenotypes of PCa by sequestering miR-515-5p and inducing FKBP1A expression. Xia et al. explored the diagnostic value of circ_0057558 and circ_0062019 in PCa (17). Moreover, the prognostic role of circ_PSMC3 in PCa has also been explored in other studies (18). However, detailed discussions on the role of circRNAs in diagnostic and prognostic value are still lacking. In the present meta-analysis, we included papers on the involvement of circRNAs in PCa and investigated their potential diagnostic, clinicopathological, and prognostic relevance.

# Materials and methods

## Registration

The protocol was registered on the Prospective Register of Systematic Reviews (PROSPERO) with registration number CRD42021284785.

## Data search strategy

Our literature search was guided by the recently published PRISMA statement (19, 20).

We searched all relevant articles through PubMed, Embase, and Web of Science online databases that were published before 8 October 2021.

To avoid omission, two independent researchers completed the retrieval process by combing Medical Subject Heading (MeSH) terms and text words. For literature retrieval, the following MeSH terms and text words were used: (1) "Prostatic Neoplasms", "Prostate Cancer", or "PCa"; (2) "RNA", "Circular", or "circRNAs". The detailed search strategy is shown in Supplementary Material. The language was limited to English. In addition, the references of the identified studies were also searched for relevant documents. Other details are provided in the Supplementary Material. The authors of the included articles were contacted when deemed necessary.

## Inclusion and exclusion criteria

Two independent investigators assessed the appropriate studies and extracted the imperative data, and disagreements were resolved by discussing with the third researcher. Studies were included if they assessed the accuracy of circRNA for differentiation between PCa and non-PCa patients. To be eligible, studies needed a clearly defined standard of reference. We defined PCa according to the guidelines of the European Association of Urology, the International Society of Geriatric Oncology (21), and the National Comprehensive Cancer Network (22).

The inclusion criteria were as follows: (1) patients with a pathological diagnosis of PCa; (2) expression level of circRNAs could be divided into high and low expression; (3) studies that included data to estimate the diagnostic, prognostic, and clinicopathologic features; and (4) cohort or case-control research.

The exclusion criteria are as follows: (1) duplicate studies; (2) reviews, meta-analyses, letters, conference abstracts, or case reports; (3) articles without complete information; (4) for diagnostic meta, those without clear tests and control group size, and those without true positive (TP), true negative (TN),

false positive (FP), and false negative (FN) or sensitivity (SEN), specificity (SPC), and receiver operating characteristics (ROC); and (5) for the prognostic meta-analysis, those without clear information on the number of trial and control groups, survival information, and Kaplan-Meier (KM) plots.

## Data extraction

Two researchers extracted the data independently. The extracted information was as follows: (1) first author, publication year, type of cancer, circRNAs, numbers of patients, detection methods, and outcomes; (2) follow-up time and outcomes; (3) sensitivity, specificity, and the areas under the curve (AUCs) of circRNAs for diagnosis; and (4) clinicopathological features including age, smoking, drinking, TNM stage, T classification, lymph node metastasis, distant metastasis, and Gleason score. If the HRs with 95% CIs for outcomes were not shown directly in the article, then the survival data were extracted from KM plots by Engauge Digitizer 4.1 software. The HRs and 95% CIs were calculated using the Excel program file provided by Tierney et al. (23). If the parameters of TP, TN, FP, and FN were not offered, then we assessed them according to sample size, SPC, SEN, and AUC.

## Quality assessment

Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) and Newcastle Ottawa Scale (NOS) were used by two independent investigators to assess the quality of the studies for diagnosis and prognosis, respectively. The four domains of QUADAS-2 were as follows: patient selection, index test, reference standard, and flow and timing. Bias risk was graded as high (H), low (L), or unclear (U). The total scores of NOS ranged from 0 to 8, and the studies that were higher than 6 in NOS were considered high-quality studies.

## Statistical analysis

Review Manager 5.3 and Stata 15.0 were used for statistical analysis. $I^2$ statistics were used to perform the heterogeneity test. We determined that there was considerable heterogeneity among the included studies if the $I^2$ value > 50% and the p-value < 0.05. Random-effects model was used to examine the pooled results. If there was no significant heterogeneity in the included studies, then a fixed-effects model was used. A p-value < 0.05 was used to determine statistical significance.

In the diagnostic meta-analysis, the number of TP, FP, FN, and TN was combined to calculate pooled results of sensitivity, specificity, diagnostic odds ratio (DOR), negative likelihood ratio (NLR), and positive likelihood ratio (PLR). The area under the summary ROC (SROC) was calculated to determine the diagnostic accuracy of circRNA expression. Deeks' funnel plot asymmetry test was used to investigate potential publishing bias. If the p-value > 0.1, then we considered that there was no publishing bias. Pooled ORs and 95% CIs were used to explore the association between circRNA expression and clinicopathological features. For the prognostic meta-analysis, HRs and 95% CIs were used to assess the prognostic value of circRNAs. Egger's tests were used to determine the possibility of publication bias. To determine the stability of the pooled HR, a sensitivity analysis was done. If the p-value > 0.1 for Egger's tests, then we considered that there was no publication bias.

# Results

## Search results

The flow diagram for study selection is given in Figure 1. A total of 550 relevant studies were found from PubMed (129 records), Embase (170 records), and Web of Science (251 records) from initial screening. After eliminating duplicate items, 310 articles were obtained. Furthermore, 249 articles were filtered out for inappropriate types (175 irrelevant articles and 74 reviews, letters, or meta-analyses). After the review of full-text articles, 37 articles were excluded for the following reasons: 17 did not include relevant outcomes and 20 did not report complete data. Finally, 23 studies ranging from 2018 to 2021 were screened for meta-analysis, including 5 for diagnosis, 16 for clinicopathological features, and 10 for prognosis (1, 14, 16–18, 24–40).

## Study characteristics and quality assessment

The essential characteristics of the included studies are shown in Tables 1–4. A total of 23 circRNAs were included and published between 2018 and 2021. Quantitative real-time reverse transcription PCR (qRT-PCR) was used to calibrate the expression of circRNAs. The majority of the included studies were from China. Among them, some elements employed in the diagnostic analysis, such as sensitivity, specificity, and AUC, are recorded in Table 1. The range of sample size was between 112 and 324. The quality of the contained literature was evaluated in terms of bias risk and applicability concerns. The results indicated that the quality of our included research was good (Figure 2).

The relationship between clinicopathological characteristics and circRNAs is given in Table 2. As indicated in Table 3, a total of 10 circRNAs were used in 10 investigations, providing some basic information about the prognostic analysis. The patients' follow-up time ranged from 48 to 105 months, and the number of samples collected ranged from 42 to 596. The NOS scores

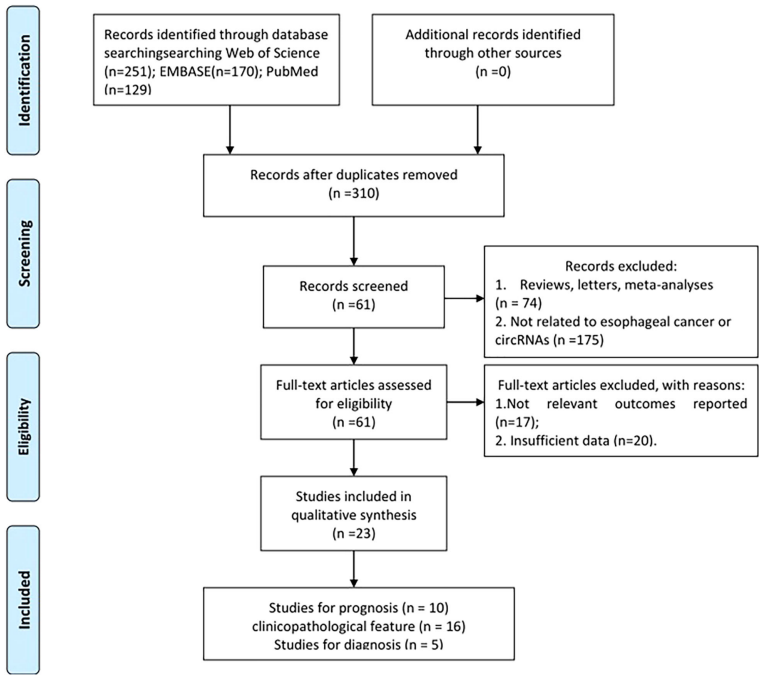


FIGURE 1
Flowchart of trial selection.

TABLE 1 Basic features of studies for diagnosis analysis.

| | | | | | Sample size | | | | Diagnosis power | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Study | Year | Country | circRNA | Cancer type | Case | Control | Sample type | Methods | Sen | Spe | AUC |
| Xia1 et al. | 2018 | China | circ_0057558 | PCa | 95 | 78 | Tissue | qRT-PCR | 74.10 | 63.40 | 0.729 |
| Xia2 et al. | 2018 | China | circ_0062019 | PCa | 95 | 78 | Tissue | qRT-PCR | 80.00 | 74.60 | 0828 |
| Huang et al. | 2019 | China | circ-ITCH | PC | 162 | 162 | Tissue | qRT-PCR | 88.30 | 61.70 | 0.8112 |
| Fan et al. | 2021 | China | circSOBP | PCa | 56 | 56 | Tissue | qRT-PCR | 83.90 | 40.00 | 0.763 |
| Mao et al. | 2020 | China | hsa_circ_0003768 (circPDHX) | PCa | 75 | 75 | Tissue | qRT-PCR | 80.00 | 58.70 | 0.64 |
| Rochow et al. | 2020 | Germany | circATXN10 | PCa | 115 | 79 | Tissue | qRT-PCR | 77.00 | 72.00 | 0.801 |

AUC, area under the ROC curve; qRT-PCR, quantitative real-time polymerase chain reaction; Sen, sensitivity; Spe, specificity; PCa, prostate cancer; circRNAs, circular RNAs.

indicated the excellent quality of studies that were included for clinical parameter analysis and prognostic analysis on each of the eight dimensions (Table 4).

## Diagnosis analysis

The diagnostic meta-analysis included 1,024 patients from five studies that were all qualified. The pooled SEN and SPC were calculated to examine the diagnostic utility of circRNAs, and the findings are shown in Figure 3. A random-effects model was used because of the observable heterogeneity ($I^2$ = 67.42% and $I^2$ = 77.73%). As the results showed, the pooled SPC was 0.82 (95% CI: 0.76–0.86), whereas the pooled SEN was 0.62 (95% CI: 0.53–0.71). Moreover, the AUC was 0.81 (95% CI: 0.77–0.84) according to the SROC curve analysis (Figure 4). Thus, it could be seen that these circRNAs had excellent diagnostic efficacy in distinguishing patients with PCa from the healthy population. As shown in the bivariate boxplot and Galbraith plot (Figures 5A, B), one study fell outside the game and Galbraith diagrams, respectively, which suggested that there was heterogeneity in the analysis. Because of the limited inclusion of articles, a subgroup analysis could not be performed to find the sources of heterogeneity. In addition, the DOR was 7.37 (95% CI: 4.87–11.14). The pooled PLR and NLR were 2.17 (95% CI: 1.73–2.71) and 0.29 (95% CI: 0.23–0.38), respectively

TABLE 2 Clinical parameters of circRNAs in PCa.

| | Tumor promoter | | | Tumor Suppressor | | |
|---|---|---|---|---|---|---|
| | OR | 95% CI | P | OR | 95% CI | P |
| Age (older/younger) | 0.883 | 0.629–1.238 | 0.470 | 1.256 | 0.882–1.788 | 0.206 |
| Gleason score | 0.222 | 0.145–0.340 | 0.000 | 1.689 | 1.144–2.493 | 0.008 |
| TNM stage (III + IV/I + II) | 1.228 | 0.637–2.365 | 0.540 | 1.088 | 0.243–4.861 | 0.912 |
| T classification (T3 + T4/T1 + T2) | 0.274 | 0.175–0.430 | 0.000 | 2.586 | 1.779–3.762 | 0.000 |
| Lymph node metastasis (Y/N) | 0.353 | 0.175–0.716 | 0.004 | 1.502 | 0.988–2.284 | 0.057 |
| Distant metastasis (Y/N) | 0.823 | 0.181–3.741 | 0.801 | 1.300 | 0.330–5.115 | 0.707 |
| PSA | 0.632 | 0.213–1.881 | 0.410 | 1.054 | 0.729–1.524 | 0.778 |
| Smoking | 1.364 | 0.457–4.071 | 0.578 | 2.250 | 0.724–6.989 | 0.161 |
| Tumor size | 0.226 | 0.099–0518 | 0.000 | – | – | – |

CI, confidence interval; N, no; Y, yes; circRNAs, circular RNAs; OR, odds ratio. The results are in bold if $p < 0.05$.

(Figures 6A–C). These results also demonstrated the excellent diagnostic ability of circRNAs in patients with PCa. These findings suggested that circRNAs have good diagnostic accuracy for PCa when taken combined.

## Clinicopathological parameters

The association between circRNAs and clinicopathological features is shown in Table 3. For clinicopathological features, 16 studies were enrolled in our meta-analysis with a total of 1,153 patients. Upregulation of carcinogenic circRNAs was linked to adverse clinical characteristics (Gleason score: OR = 0.222, 95% CI: 0.145–0.340; T classification: OR = 0.274, 95% CI: 0.175–0.430; lymph node metastasis: OR = 0.353, 95% CI: 0.175–0.716; tumor size: OR = 0.226, 95% CI: 0.099–0518). Furthermore, decreased expression of tumor-suppressor circRNAs was also linked to worse clinical outcomes (Gleason score: OR = 1.689, 95% CI: 1.144–2.493; T classification: OR = 2.586, 95% CI: 1.779–3.762);. Remarkably, there was no correlation between circRNA expression and other clinicopathologic factors such as age, TNM stage, distant metastasis, expression of PSA, and smoking.

## Prognosis analysis

In our work, the prognosis meta-analysis included 1,164 participants from 10 investigations, all of which were eligible. The role of upregulated circRNAs in PCa prognosis was estimated using fixed-effect models ($I^2 = 0.0\%$, $p = 0.782$), and the results demonstrated that upregulation of carcinogenic circRNAs was linked to a poor prognosis (HR = 2.408, 95% CI: 1.559–3.720, $p < 0.001$) (Figure 7A). At the same time, downregulation of tumor-suppressor circRNAs was significantly associated with worse PCa prognosis (HR = 1.739, 95% CI: 1.147–2.576, $p = 0.006$). Because there was no heterogeneity between studies ($I^2 = 0\%$, $p = 0.624$), a fixed-effects model was used (Figure 7B).

## Publication bias and sensitivity analysis

We used the Deeks' funnel plot asymmetry test to analyze the potential publication bias of diagnostic meta-analysis and found that there was no clear publication bias ($p = 0.07$) (Figure 8A). Furthermore, the potential publication bias of prognostic meta-analysis was investigated, which showed no publication bias in the included studies (Figure 8B). In addition, the Egger's test also supported the conclusion that there was no publication bias (p = 0:85, Figure 8C). At the same time, in our research, sensitivity analysis revealed that the pooled results in the prognostic meta-analysis were stable (Figure 8D).

# Discussion

In recent years, the high incidence and mortality of PCa have caused worldwide concern (41, 42). The diagnosis and prognosis of PCa are currently determined by tissue biopsy and PSA levels (42). However, tissue biopsy is invasive, and the predicted accuracy of PSA should be furthermore improved. Therefore, PSA has been steadily discouraged from being advised in recent years (43, 44).

Many researchers are looking for new biomarkers to determine the diagnosis and prognosis of patients with PCa to enhance their survival (45). circRNAs are non-coding RNAs playing an important role in the development and progression of cancer (46). At the same time, circRNAs were thought to have promising prospects and advantages as perfect biomarkers for human cancer diagnosis and prognosis (47).

Therefore, as a novel biomarker, circRNAs have several advantages for clinical applications. First, traditional puncture

TABLE 3 Basic features of studies for prognosis analysis.

| Author | Year | Country | circRNA | Cancer type | circRNA expression | | Methods | Sample type | Regulation | Follow-up (month) | HR | CI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | High | Low | | | | | | |
| Dong et al. | 2020 | China | circPSMC3 | PCa | 55 | 55 | qRT-PCR | Tissue | Downregulated | 62 | 2.48 | 1.10–5.63 |
| Liu et al. | 2021 | China | CircFOXM1 | PCa | 26 | 26 | qRT-PCR | Tissue | Upregulated | 62 | 3.01 | 1.36–6.66 |
| Hu et al. | 2020 | China | Circ-MTO1 | PCa | 298 | 298 | qRT-PCR | Tissue | Downregulated | 48 | 1.56 | 0.96–2.53 |
| Liu et al. | 2020 | China | circHIPK3 | PCa | 28 | 28 | qRT-PCR | Tissue | Upregulated | 62 | 1.52 | 0.54–4.30 |
| Huang et al. | 2019 | China | circABCC4 | PCa | 24 | 23 | qRT-PCR | Tissue | Upregulated | 62 | 3.34 | 0.94–12.57 |
| Tao et al. | 2019 | China | circABCC4 | PCa | 21 | 21 | qRT-PCR | Tissue | Upregulated | 100 | 4.94 | 0.84–29.12 |
| Wang et al. | 2019 | China | CircITCH | PCa | 26 | 26 | qRT-PCR | Tissue | Downregulated | 80 | 1.58 | 0.49–5.15 |
| Gao et al. | 2020 | China | hsa_circ_0000735 | PCa | 25 | 25 | qRT-PCR | Tissue | Upregulated | 62 | 3.03 | 1.11–8.22 |
| Mao et al. | 2020 | China | hsa_circ_0003768 (circPDHX) | PCa | 54 | 21 | qRT-PCR | Tissue | Upregulated | 105 | 1.27 | 0.19–8.34 |
| Li et al. | 2020 | China | circ-0016068 | PCa | 42 | 42 | qRT-PCR | Tissue | Upregulated | 62 | 1.22 | 0.33–4.49 |

PCa, prostate cancer; qRT-PCR, quantitative real-time polymerase chain reaction; CI, confidence interval; HR, pooled hazard ratio.

biopsy entails a risk of injuries and is more difficult for the operator to perform. In contrast, circRNAs in plasma are more readily available and harmless. Second, circRNAs are resistant to denaturation because of their stable structures and conservative sequences. Third, circRNAs outperform standard markers in terms of diagnostic and prognostic value, as well as accuracy.

The aberrant expression of circRNAs has been shown in numerous investigations in patients with PCa; with a few studies, few meta-analyses have been published on the role of circRNAs in PCa diagnosis or prognosis. Our study is the first to discuss the link between circRNA expression and diagnostic performance, prognostic value, and clinical characteristics in PCa.

In our meta-analysis, the aggregated data revealed an AUC of 0.81, with a sensitivity of 0.82 and a specificity of 0.62 for diagnostic value, indicating that circRNAs could be employed as diagnostic biomarkers for PCa. In terms of clinical and prognostic importance, abnormal expression of circRNAs was connected to clinical parameters and prognosis.

The sensitivity and specificity results also demonstrated that circRNAs could discriminate between healthy people and patients, but the specificity needs to be enhanced. However, the bivariate boxplot and Galbraith plot results indicated that the studies included in the diagnostic analysis were heterogeneous. Because the number of included papers was insufficient to allow additional subgroup analysis to identify sources of heterogeneity, further research is warranted.

In clinical practice, the PLR and NLR signify diagnostic ability. The PLR > 10 and NLR < 0.1 are considered to indicate good diagnostic ability (47). However, in our study, PLR was 2.17 and NLR was 0.29, which meant that circRNAs' diagnostic accuracy is currently limited. In other words, circRNAs would give a higher rate of FN and FP in clinical applications.

DOR is a measurement index for diagnostic performance that incorporates the advantages of sensitivity and specificity. The higher the value of DOR, the better it can identify test performance (48). In our study, the pooled DOR was 7.37, which supported the use of circRNAs as a viable diagnostic tool for PCa.

As for clinicopathological parameters, T classification and Gleason score were found to be linked with both upregulated and downregulated circRNAs. The aberrant expression of circRNAs in colorectal carcinoma and esophageal cancer was also linked to T classification in the studies by Yuan et al. (49) and Lin et al. (50). Together, this could imply that circRNAs are vital for tumor staging.

The Gleason score is a widely used method for histological grading of PCa and a useful tool for making plans for PCa treatment (51). Our findings revealed that circRNAs were highly linked to a low Gleason score, indirectly reflecting the status of PCa.

In terms of prognostic values, our recent meta-analysis found that aberrant circRNA expression was strongly linked to poor overall survival. As a result, prompt monitoring of circRNA

TABLE 4 Quality assessment of eligible studies for clinical parameter analysis and prognosis analysis according to the Newcastle-Ottawa Scale.

| Study | Selection | | | Comparability | | | Outcome | | Total |
|---|---|---|---|---|---|---|---|---|---|
| | Adequacy of case definition | Number of case | Representativeness of the cases | Ascertainment of relevant cancers | Ascertainment of detection method | circRNA expression | Assessment of outcome | Adequate follow-up | |
| Wang et al. | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 6 |
| Liu et al. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 8 |
| Huang et al. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 7 |
| Tao et al. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 7 |
| Wang et al. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 8 |
| Li et al. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 8 |
| Shi et al. | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 7 |
| Huang et al. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 8 |
| Chao et al. | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 6 |
| Song et al. | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 6 |
| Li et al. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 7 |
| Liu et al. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 7 |
| Cai et al. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 7 |
| Wang et al. | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 6 |
| Chen et al. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 8 |
| Huang et al. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 7 |
| Dong et al. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 8 |
| Hu et al. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 8 |
| Liu et al. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 7 |
| Gao et al. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 8 |



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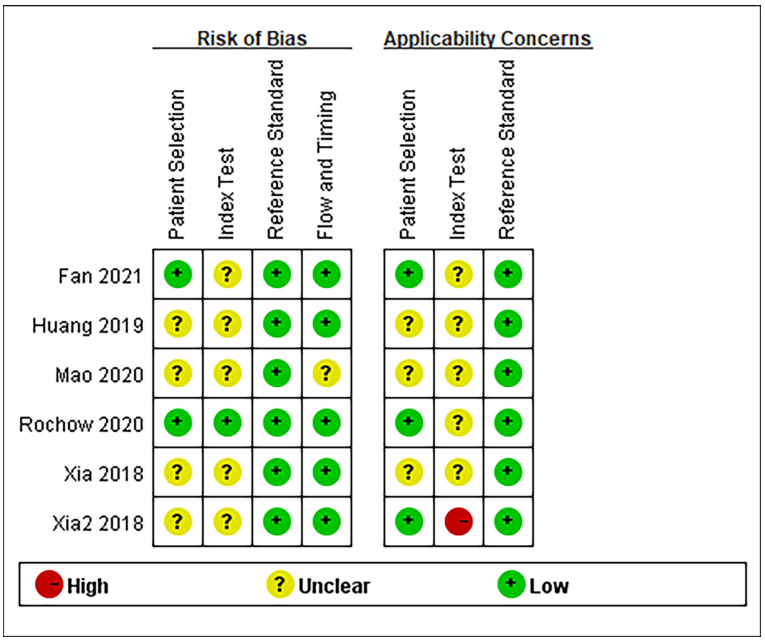


FIGURE 2
Quality assessment of eligible studies for diagnostic meta-analysis.

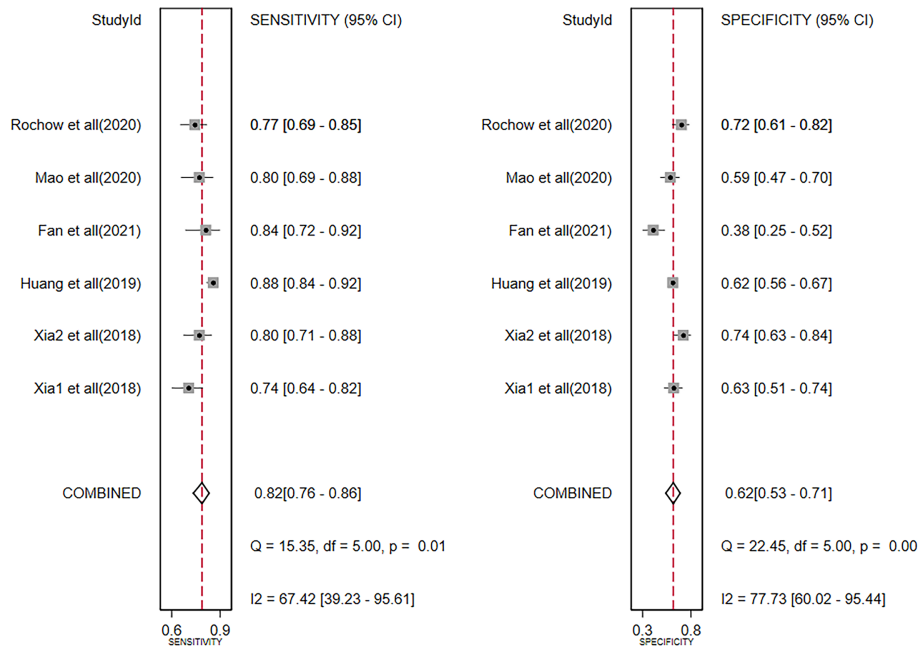


FIGURE 3
Forest plots of summary sensitivity and specificity to illustrate the diagnostic value of circRNAs for PCa. circRNAs, circular RNAs; PCa, prostate cancer.

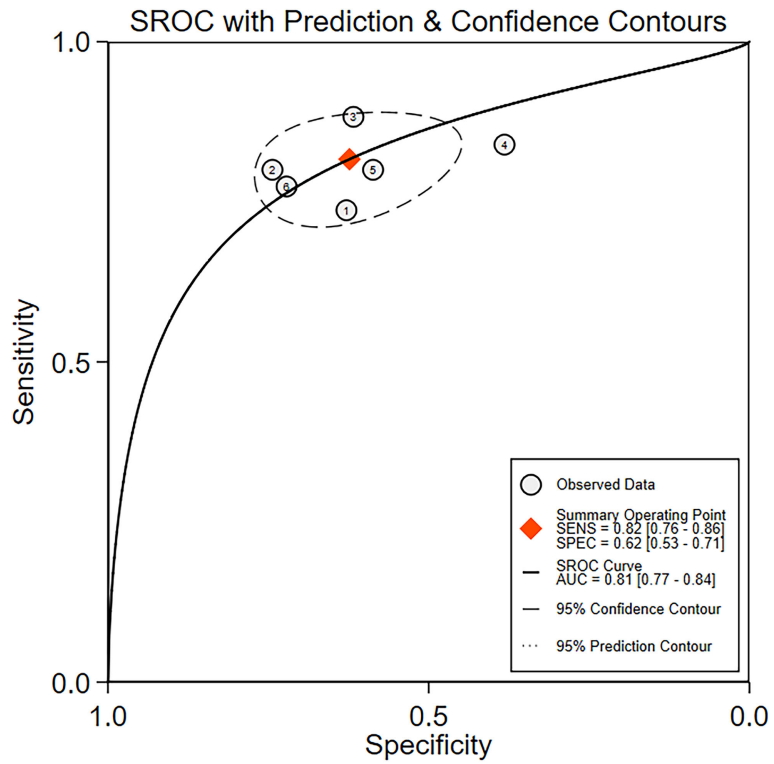


FIGURE 4
The summary receiver operating characteristic (SROC) curve based on circRNAs for diagnosis analysis. ROC, receiver operator characteristic.

alterations in patients with PCa can aid clinical decision-making and help patients live longer.

Some studies focusing on experimental research rather than cohort or case-control research also demonstrated that circRNA does play a role in the diagnosis and prognosis of PCa. As described by Vo et al., multiple upregulated or downregulated circRNAs were shown to be associated with PCa progression (52). Their results are consistent with previous studies showing that circRNAs generally do not directly contribute to the growth of cancer cells but instead do so indirectly through controlling mRNAs (53–55). These studies support the value of circRNA in PCa; however, we did not include them in our meta-analysis

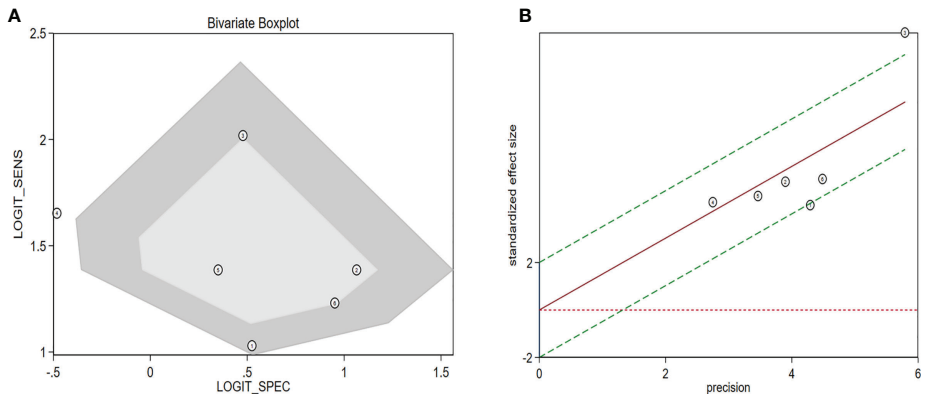


FIGURE 5
Assessment of the diagnostic accuracy of circRNAs in PCa. **(A)** Bivariate boxplot. **(B)** Galbraith plot. circRNAs, circular RNAs; PCa, prostate cancer.

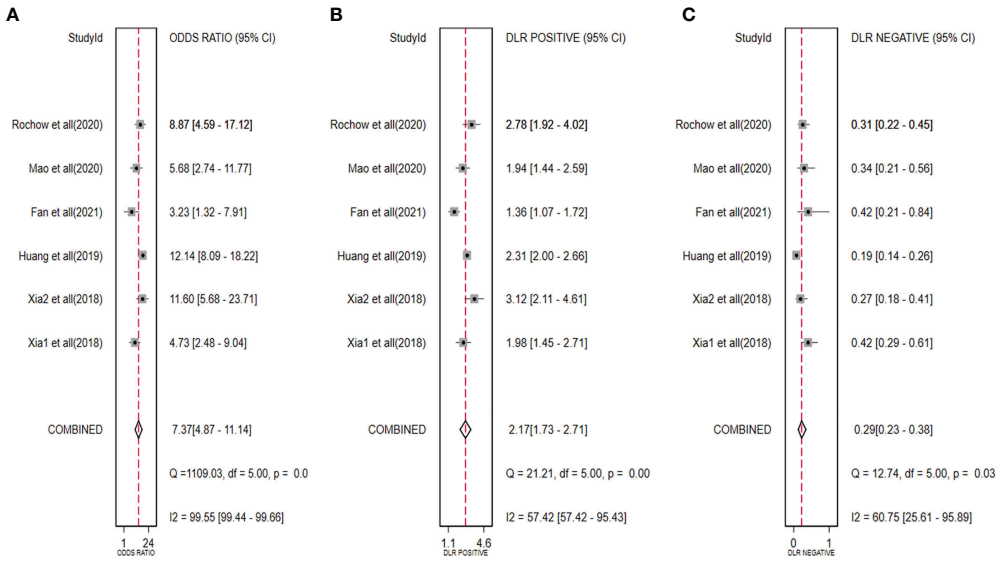


FIGURE 6
Forest plots for pooled DOR **(A)**, PLR **(B)**, and NLR **(C)** of circRNAs for PCa. DOR, diagnostic odds radio; PLR, positive likelihood ratio; NLR, negative likelihood ratio; circRNAs, circular RNAs; PCa, prostate cancer.

because most of them are clustered in experiments that lack data from entire cohort studies.

Numerous studies about the effect of circRNAs on various cancers have been conducted. Some meta-analyses had demonstrated that circRNA had good diagnostic and prognostic value for patients with colorectal carcinoma (49), solid cancers (56), and osteosarcoma (57). However, only several meta-analyses exploring the diagnostic and prognostic value of circRNA in PCa have been conducted to date. Yuan et al. performed a meta-analysis that included only articles from 2015 to 2019 (49). However, in the last 3 years, more studies have focused on the differences in circRNA expression between patients with cancer patients and healthy subjects. Our study fills this gap and covers a wider range of data. In addition, Li et al. included only five studies in their prognosis meta-analysis (56). This meta-analysis was based on a small number of studies, which may have introduced some bias. The present study has been quantitatively supplemented to make the results of the meta-analysis more convincing. In addition, the source of specimens was indicated in our meta-analysis. Compared with

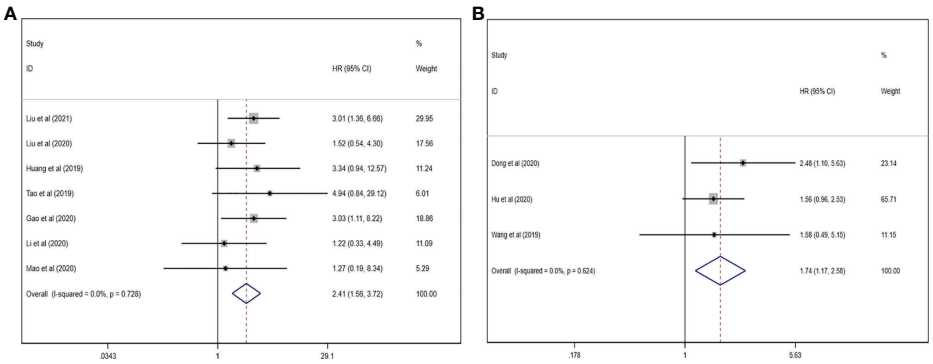


FIGURE 7
Forest plots for the association between circRNAs and estimating HR in PCa. **(A)** Upregulated circRNAs. **(B)** Downregulated circRNAs. HR, hazard ratios; PCa, prostate cancer; circRNAs, circular RNAs.

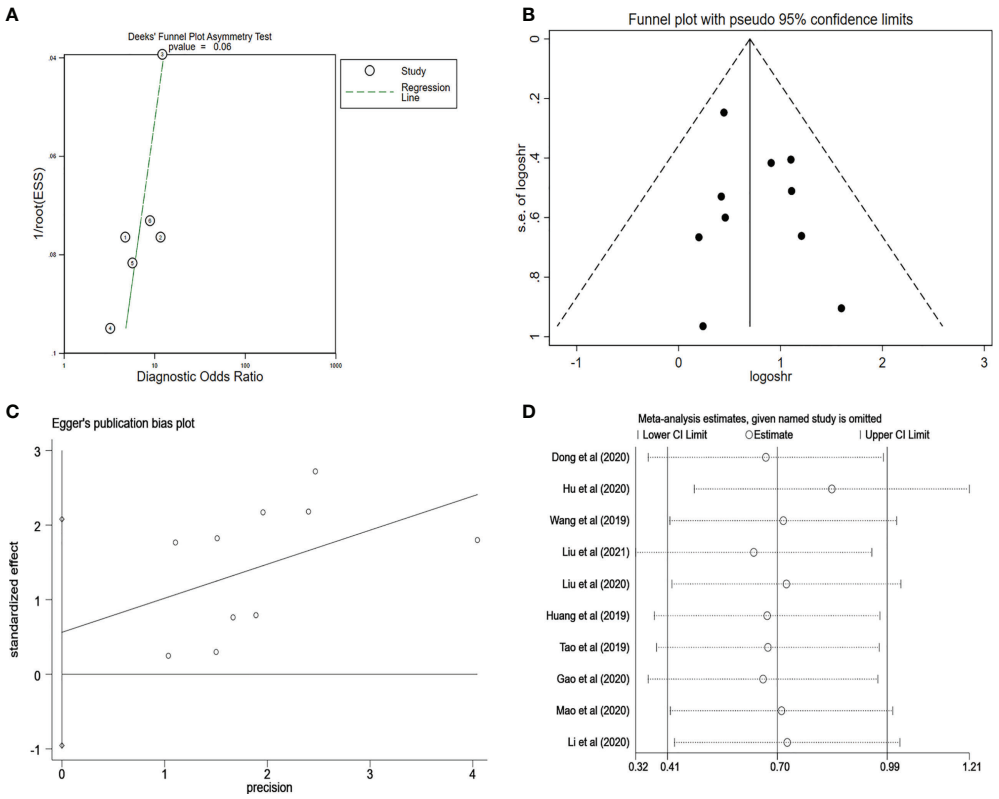


FIGURE 8
**(A)** Deeks' funnel plot for evaluating the publication bias of the included study on the diagnosis analysis. **(B)** Funnel plot for evaluating the publication bias of the included study on the prognosis analysis. **(C)** Egger's funnel plot for evaluating the publication bias of the included study on the prognosis analysis. **(D)** Sensitivity analysis for evaluating the influence of the omitted study on the pooled HR. PCa, prostate cancer; HR, hazard ratio.

previous meta-analyses, we excluded the effect of heterogeneity due to specimen source. Thus, our study is an update and addition to similar meta-analyses evaluating the role of circRNA in PCa and provides a grounding for related research.

There are still limitations in our current meta-analysis. First, we only included five papers in our diagnostic meta-analysis, and the small number of research has limited the popularization of circRNAs in clinical applications. Second, because of the restricted amount of data from included studies, we were unable to run a subgroup analysis to analyze the source of heterogeneity in the diagnostic meta-analysis. Furthermore, some research did not provide clear HR data. We retrieved necessary data from provided KM curves, which might have caused some bias. Finally, because the majority of the research examined was from China, the external application of our findings across diverse areas may be hampered. To ensure the accuracy and relevance of the findings, a comprehensive research is required.

## Conclusion

Together, our meta-analysis revealed that circRNAs have moderately high diagnostic accuracy for PCa. The results of the prognostic meta-analysis revealed that aberrant expression of circRNAs is significantly associated with poor prognostic outcomes and clinicopathological values. Therefore, circRNAs can be useful indicators for the diagnosis and prognosis of PCa. However, further studies with more multicenter data, as well as high-quality studies, are needed to demonstrate the role of circRNAs in PCa.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

## Author contributions

JX and HJ contributed to conceive this study, performed quality assessment of included studies, analyze the data, and write the manuscript. YZ analyzed the data and performed quality assessment of included studies. LZ and ZZ reviewed the manuscript. JL provided the financial support. All authors contributed to manuscript revision, read, and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.945143/full#supplementary-material

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# Robotic-assisted versus standard laparoscopic radical cystectomy in bladder cancer: A systematic review and meta-analysis

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**Background:** This study aimed to evaluate the efficacy and safety of robotic-assisted radical cystectomy (RARC) versus laparoscopic radical cystectomy (LRC) in the treatment of bladder cancer.

**Methods:** Two researchers independently searched PubMed, Embase, Cochrane, and CBM using the index words to identify the qualified studies which included randomized controlled trials (RCTs) and non-randomized controlled trials (prospective and retrospective studies), and the investigators scanned references of these articles to prevent missing articles. Differences in clinical outcomes between the two procedures were analyzed by calculating odds risk (OR) and mean difference (MD) with an associated 95% confidence interval (CI).

**Results:** Sixteen comparative studies were included in the meta-analysis with 1467 patients in the RARC group and 897 patients in the LRC group. The results indicated that RARC could significantly decrease blood loss ($P$ = 0.01; MD: -82.56, 95% CI: -145.04 to -20.08), and complications 90 days or more after surgery, regardless of whether patients were Grade ≤ II ($P$ = 0.0008; OR: 0.63, 95% CI: 0.48 to 0.82) or Grade ≥ III ($P$ = 0.006; OR: 0.59, 95% CI: 0.40 to 0.86), as well as overall complications ($P$: 0.01; OR = 0.52; 95% CI: 0.32 to 0.85). However, there was no statistical difference between the two groups at total operative time, intraoperative complications, transfusion rate, short-term recovery, hospital stay, complications within 30 days of surgery, and bladder cancer-related mortality.

**Conclusions:** The meta-analysis demonstrates that RARC is a safe and effective treatment for bladder cancer, like LRC, and patients with RARC benefit from less blood loss and fewer long-term complications related to surgery, and should be considered a viable alternative to LRC. There still need high-quality, larger sample, multi-centric, long-term follow-up RCTs to confirm our conclusion.

KEYWORDS

bladder cancer, laparoscopy, robotics, randomized controlled trials, meta-analysis

# Introduction

Bladder cancer is the 12th most common malignancy in the world, accounting for approximately 3.0% of all new cancer diagnoses and 2.1% of all cancer deaths in 2020, according to new reports (1). Alarmingly, the prevalence of bladder cancer has risen in many countries, especially in Europe (2). Open radical cystectomy (ORC) is the gold standard for the treatment of non-metastatic muscle-invasive and uncontrolled or high-risk superficial bladder cancer, which can effectively achieve local control of the tumor and long-term disease-free survival (3, 4). However, traditional ORC often has high surgical risks, many perioperative complications and high mortality, and previous research data show that the incidence of postoperative complications after ORC is as high as 30% to 60%, even if the surgeon knows enough about pelvic anatomy and the surgical technique is continuously improved (5). Therefore, minimally invasive surgery for bladder cancer is still necessary.

In the early 1990s, Parra et al. (6) and Sánchez de Badajoz et al. (7) reported the use of LRC for muscle-invasive bladder cancer, which had less intraoperative blood loss, less postoperative pain, faster postoperative bowel function recovery, and shorter hospital stay compared with ORC (8). After a decade, Menon et al. (9) reported the use of robotic-assisted radical cystectomy (RARC) in the treatment of bladder cancer, which was later adopted by many large medical units and proved to be feasibility. Later, a large number of studies compared RARC with ORC, the gold standard for the treatment of invasive bladder cancer, and also found that RARC can achieve the same radical effects as ORC in terms of lymph node count, positive surgical margins, and survival rate; but the RARC group had less intraoperative blood loss, lower blood transfusion rate, shorter postoperative exhaust time, and lower incidence of surgery-related complications, especially for elderly patients (10–13). More recently, the Catto et al. (14) study also demonstrated that, compared with ORC, RARC offered more days alive and out of the hospital within 90 days of surgery, less thrombo-embolic complications and wound complications, and a better quality of life at 5 weeks.

Currently, LRC and RARC appear to be safe and viable alternatives to ORC as they mature. Tang K et al. and Li K et al. performed meta-analysis of LRC and ORC, RARC and ORC respectively, the results of which show that the minimally invasive endoscopic technique (LRC and RARC) has reliable perioperative safety, and can achieve the same tumor resection effects and function of reconstruction bladder as ORC, meanwhile, has lower surgery-related complications than ORC (15, 16). However, there is a lack of evidence for the multicenter, large sample of controlled studies on which RARC and LRC are more advantageous in radical surgery for bladder cancer. Thus, the meta-analysis aimed to obtain a more powerful evaluation of the use of LRC versus RARC in the treatment of bladder cancer by incorporating more recent studies.

# Materials and methods

## Search strategy

The databases of PubMed, Embase, Cochrane, and CBM were searched to determine these qualified studies comparing the efficacy of RARC versus LRC in the treatment of bladder cancer. The mesh (cystectomy, laparoscopy, and robotic surgical procedures) and their corresponding keywords used for the searches, and the search strategy are detailed in the appendix. In addition, the investigators scanned other related articles and reference materials for these articles to prevent missing articles. The literature search was done independently by two investigators and was resolved by discussing with the third investigator when the search results were inconsistent.

## Inclusion and exclusion criteria

The study was included in our meta-analysis if it was: (1) English and Chinese articles; (2) the research subjects were patients with bladder cancer and no other serious cardiopulmonary vascular diseases; (3) the study included at least two groups (RARC group and LRC group); (4) report at least one result of interest to us; (5) no time limit for publication of included articles.

The study was excluded in our meta-analysis if it was: (1) a duplicate article; (2) the data had obvious mistakes; (3) the case report, theoretical research, conference report, systematic review, meta-analysis, expert comment, or economic analysis; (4) we went through various means but still could not get the full text of this study.

The screening process of the eligible studies was completed by two reviewers independently and was resolved by discussing with the third reviewer when there was a disagreement.

## Data extraction and quality assessment

The data, extracted from all included studies, consists of two parts: basic information and main results. The basic information includes the first author's name and publication time, country, study design, the sample size of interventions and control groups, matching/comparable variables, conversion (N), and follow-up time. The clinical outcomes excerpted were used for statistical analysis, including total operative time, blood loss, blood transfusion rate, length of hospital stay, days to oral intake and regular diet, complications, and oncologic outcomes. Two investigators independently assessed the methodological quality of randomized controlled trials (RCTs) using the Jadad scale, while the Methodological Index for Non-Randomized Studies (MINORS) tool was used to assess the methodological quality of the non-randomized controlled study (NRS) (17, 18). The Jadad scale focuses on randomization, blinded, and reported dropout, where the literature mentions the application of randomized methods and double-blind (+1) and correct methods (+2). The number of cases of withdrawal and loss of follow-up and the reasons for withdrawal were described in detail (+1) or not, and the total score > 2 were high-quality clinical trials. The MINORS tool contains a total of 12 items for the comparative studies, and each item is scored 0 to 2 points (0 = not reported; 1 = reported but insufficient information; 2 = reported and provided sufficient information), and the article was divided into low (> 17), moderate (≥ 10 and ≤ 17) and high bias risk (< 10) according to the methodological quality score (18). All of the above data extraction and quality evaluation processes were completed independently by two reviewers and disagreements between reviewers were resolved through discussion until a consensus was reached.

This meta-analysis does not require Institutional Review Board (IRB) approval.

## Statistical analysis

All statistical analyses in the meta-analysis were performed using the RevMan version 5.3 (The Cochrane Collaboration, Oxford, UK). The results of Chi-squared and $I^2$ tests were used to assess the heterogeneity and determine which analytical model (fixed-effect or random effect model) to use for data integration (19).

Assuming the chi-square test with a $P$ value of ≤ 0.05 and the $I^2$ test value > 50% were defined as the presence of greater heterogeneity and a random effects model was used for data analysis, meanwhile, we performed subgroup analysis or sensitivity analysis to find possible sources of heterogeneity and eliminate heterogeneity as much as possible. Conversely, if the Chi-squared $P$ value of > 0.05 and the $I^2$ test value ≤ 50%, the heterogeneity between the data was considered to be small, and the data analysis used a fixed-effect model. Continuous variables are expressed as the mean ± standard deviation (SD) and analyzed by mean difference (MD). In addition, clinical outcome measures were reported in the median and range or interquartile range in some studies. For ease of integration, mean and SD were generated by network calculators (http://www.comp.hkbu.edu.hk/~xwan/median2mean.html) based on the sample size, median, and range or interquartile range. Categorical data are presented as percentages and analyzed by odds risk (OR). Data associated with blood transfusion rate and complications and oncologic outcomes were analyzed by OR with 95%CI. MD along with 95% CI were used to analyze the data associated with total operative time, blood loss, length of hospital stay, days to oral intake, and regular diet.

# Results

## Characteristics of the included studies

A total of 851 articles were identified by searching, of which 97 articles are duplicates. 716 articles were excluded by reading the title or abstract of the studies, and 38 articles were left for further evaluation. After obtaining and reading the full text, 22 articles were further excluded, at last, 16 articles (four Chinese and twelve English) (20–35) were involved in the meta-analysis, which was performed with 1467 patients in the RARC group and 897 patients in the LRC group. The flow chart is presented in Figure 1. The basic information of the included studies is presented in Table 1. The risk assessment of the included studies is shown in Table 2. The only RCT of included studies had a Jadad scale score of 3, and the mean MINORS score is

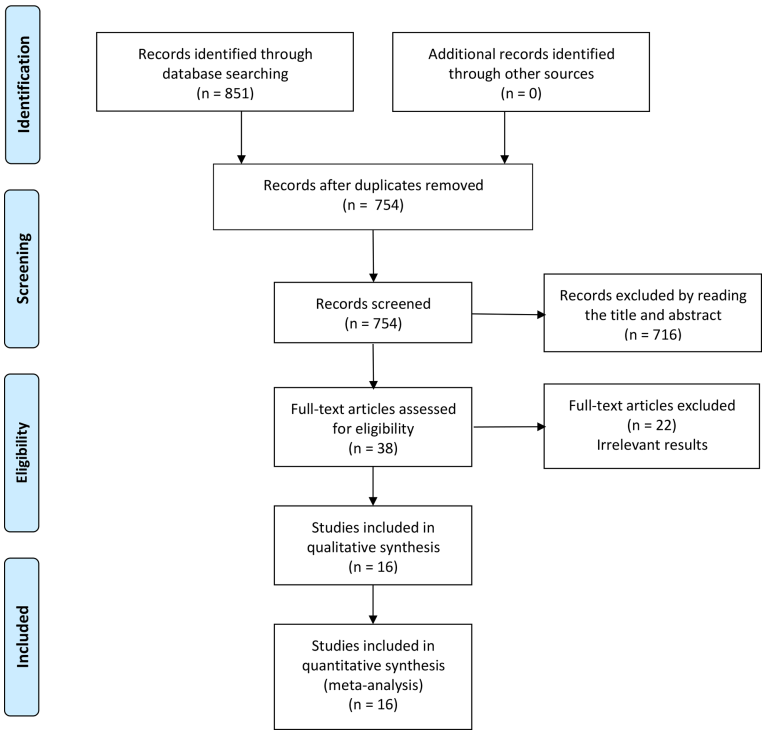


FIGURE 1
Flow diagram of the literature search and selection process.

16.69 ± 1.72, indicating that the quality of evidence from the included studies was moderate.

## Total operative time

Fifteen studies with 2353 patients (RARC group = 1461, LRC group = 892) reported the total operative time. Based on the Chi-squared ($P < 0.001$) and $I^2$ test ($I^2 = 99\%$), we used the random effect model to analyze the total operative time. The pooled results show there was no significant difference in the total operative time between the two groups ($P = 0.13$; MD: 17.43, 95% CI: -5.06 to 39.91, Figure 2A).

## Amount of blood loss

Fourteen studies with 2156 patients (RARC group = 1328, LRC group = 828) reported the amount of blood loss. Based on the Chi-squared ($P < 0.001$) and $I^2$ tests ($I^2 = 97\%$), we used the random effect model to analyze the amount of blood loss. Compared with LRC, the amount of blood loss during RARC was reduced at a statistically significant level ($P = 0.01$; MD: -82.56, 95% CI: -145.04 to -20.08, Figure 2B).

## Transfusion rate

Fourteen studies with 2262 patients (RARC group = 1441, LRC group = 821) reported the transfusion rate. Based on the Chi-squared ($P = 0.01$) and $I^2$ tests ($I^2 = 51\%$), we used the random effect model to analyze the transfusion rate. There was no significant difference in the transfusion rate between the two groups ($P = 0.18$; OR: 0.77, 95% CI: 0.53 to 1.13, Figure 2C).

## Hospital stay

Fourteen studies with 1939 patients (RARC group = 1093, LRC group = 846) reported the hospital stay. Based on the Chi-squared ($P < 0.001$) and $I^2$ tests ($I^2 = 99\%$), we used the random effect model to analyze the hospital stay. Compared with LRC, the hospital stay of RARC no significant difference ($P = 0.36$; MD: -0.66, 95% CI: -2.07 to 0.76, Figure 3A).

## Short-term recovery

For short-term recovery, we analyzed “day to oral intake” and “day to regular diet”, where day to oral intake included five studies with 470 patients (RARC group = 255, LRC group =

TABLE 1 The basic characteristics description of included studies.

| Study | Country | Study design | Patients (n): RARC/LRC | Matching/comparable variables | Conversion (N): RARC/LRC | Follow-up (months) |
|---|---|---|---|---|---|---|
| Abraham JB 2007 (6) | USA | prospective | 14/20 | 1,2,3,4,5,6,7 | 0/3 | NA |
| Gastecka, A 2018 (15) | Poland | retrospective | 52/37 | 1,2,3,6,7 | NA | 1/1 |
| Jia GZ 2017 (16) | China | retrospective | 38/61 | 1,2,3,4,5,6,7 | 0/0 | NA |
| Khan, M. S 2012 (17) | UK | prospective | 48/58 | 1,2,4,5,6,7 | 0/1 | 38.4/38.4 |
| Khan, M. S 2016 (18) | UK | RCT | 20/19 | 1,2,3,4,6,7 | 0/1 | 12/12 |
| Kim, T. H 2016 (19) | Korea | retrospective | 58/22 | 1,2,3,4,6,7 | NA | 32.0/28.8 |
| Matsumoto K 2019 (20) | Japan | prospective | 10/10 | 1,2,3,4,6,7 | NA | NA |
| Teishima, J 2014 (21) | Japan | prospective | 6/5 | 1,3,5,7 | 0/0 | 1/1 |
| Wei XS 2016 (22) | China | retrospective | 6/57 | 1,2,3,4,5,6,7 | 0/0 | NA |
| Bai, YC 2021 (23) | China | retrospective | 136/82 | 1,2,3,4,5 | NA | 33.0/33.0 |
| Arora, A 2020 (24) | France | retrospective | 188/112 | 1,2,3,4,5,7 | 5/5 | NA |
| Su SQ 2019 (25) | China | retrospective | 189/126 | 1,2,3,4,5,6,7 | NA | 34.2/34.2 |
| Zhang SW 2019 (26) | China | retrospective | 172/126 | 1,2,3,4,5,6 | 0/0 | NA |
| Porreca A 2022 (27) | Italy | prospective | 368/46 | 1,2,3,4,5,6,7 | 25/2 | 24/24 |
| Jiang S 2022 (28) | China | retrospective | 87/32 | 1,2,3,5,7 | 0/0 | NA |
| Huang XM 2019 (29) | China | retrospective | 75/84 | 1,2,3,5,6,7 | NA | NA |

NA, data not available; Matching/comparable variables: 1 = age, 2 = gender, 3 = BMI, 4 = ASA, 5 = Previous surgery history, 6 = Urinary diversion type, 7 = pathological stage.

215), and day to regular diet included five studies with 167 patients (RARC group = 56, LRC group = 111). The summary results showed no significant difference in the short-term recovery between the two groups, whether the day to oral intake ($P$ = 0.35; MD: -0.43, 95% CI: -1.33 to 0.48) or the day to regular diet ($P$ = 0.40; MD: 0.17, 95% CI: -0.22 to 0.55), as shown in Figures 3B, C.

## Oncologic outcomes

Mean lymph node yield and positive lymph node. Pooling data from six studies that counted lymph node yield in 832 patients (RARC group = 507, LRC group = 325) and seven studies including 819 patients (RARC group = 453, LRC group = 366) who reported positive lymph nodes, there was no significant difference between the two groups in terms of mean lymph nodes yield ($P$ = 0.19; MD: 1.40, 95% CI: -0.70 to 3.50) or positive lymph node ($P$ = 0.61; OR: 0.89, 95% CI: 0.57 to 1.39), as shown in Figures 4A, B.

Positive surgical margins. Pooling data of eight studies that reported positive surgical margins in 1103 patients (RARC group = 659, LRC group = 444) also showed that there was no significant difference between the two groups ($P$ = 0.49; OR: 1.23, 95% CI: 0.69 to 2.19, Figure 4C).

Cancer-related mortality. At the same time, we analyzed bladder cancer-related mortality, which was reported in a total of 442 patients (RARC group = 262, LRC group = 180), and the integration showed no significant difference between the two groups ($P$ = 0.71; OR: 0.75, 95% CI: 0.17 to 3.38, Figure 4D).

## Complications

The surgical-related complications were graded according to Clavien-Dindo (36) and were combined into two categories according to whether the complications required surgical intervention (Grade ≤ II and Grade ≥ III). According to the time of occurrence of surgery-related complications, we divided them into intraoperative complications, early complications 30 days after surgery, and long-term complications 90 days or more after surgery, and analyzed the total complication rates of the two groups.

Pooling data of three studies including 567 patients (RARC group = 339, LRC group = 228) who reported intraoperative complications, and five studies including 657 patients (RARC group = 402, LRC group = 255) described the occurrence of surgery-related complications within 30 days of surgery. Forest plot showing that there was no significant difference on intraoperative complications ($P$ = 0.22; OR: 0.64, 95% CI: 0.32

TABLE 2 Risk of bias for the involved studies.

**The quality of NRS was evaluated with the MINORS**

| Methodological Items for non-randomized studies | Abraham JB 2007 (6) | Gastecka, A 2018 (15) | Jia GZ 2017 (16) | Khan, M. S 2012 (17) | Kim, T. H 2016 (19) | Matsumoto K 2019 (20) | Teishima, J 2014 (21) | Wei XS 2016 (22) | Bai YC 2021 (23) | Arora, A 2020 (24) | SuSQ2019 (25) | ZhangSW 2019 (26) | Porreca, A2022 (27) | Jiang S 2020 (28) | Huang XM2019 (29) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Clearly Stated Aim | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Consecutive Patients | 2 | 1 | 1 | 1 | 2 | 2 | 0 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 1 |
| Prospective Data Collection | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Appropriate Endpoint | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Unbiased Endpoint Assessment | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Appropriate Follow-Up | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 |
| Loss to Follow-Up <5% | 2 | 0 | 0 | 2 | 0 | 2 | 2 | 0 | 1 | 0 | 2 | 0 | 2 | 0 | 0 |
| Prospective Study Size Calculation | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 |
| An adequate control group | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Contemporary groups | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Baseline equivalence of groups | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Adequate statistical analyses | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Score | 20 | 15 | 15 | 18 | 16 | 19 | 17 | 15 | 18 | 15 | 19 | 15 | 21 | 15 | 15 |
| The quality of remaining RCTs were assessed using the Jadad scale | | | | | | | | | | | | | | | |
| Khan, M. S 2016 (18) | 3 | | | | | | | | | | | | | | |



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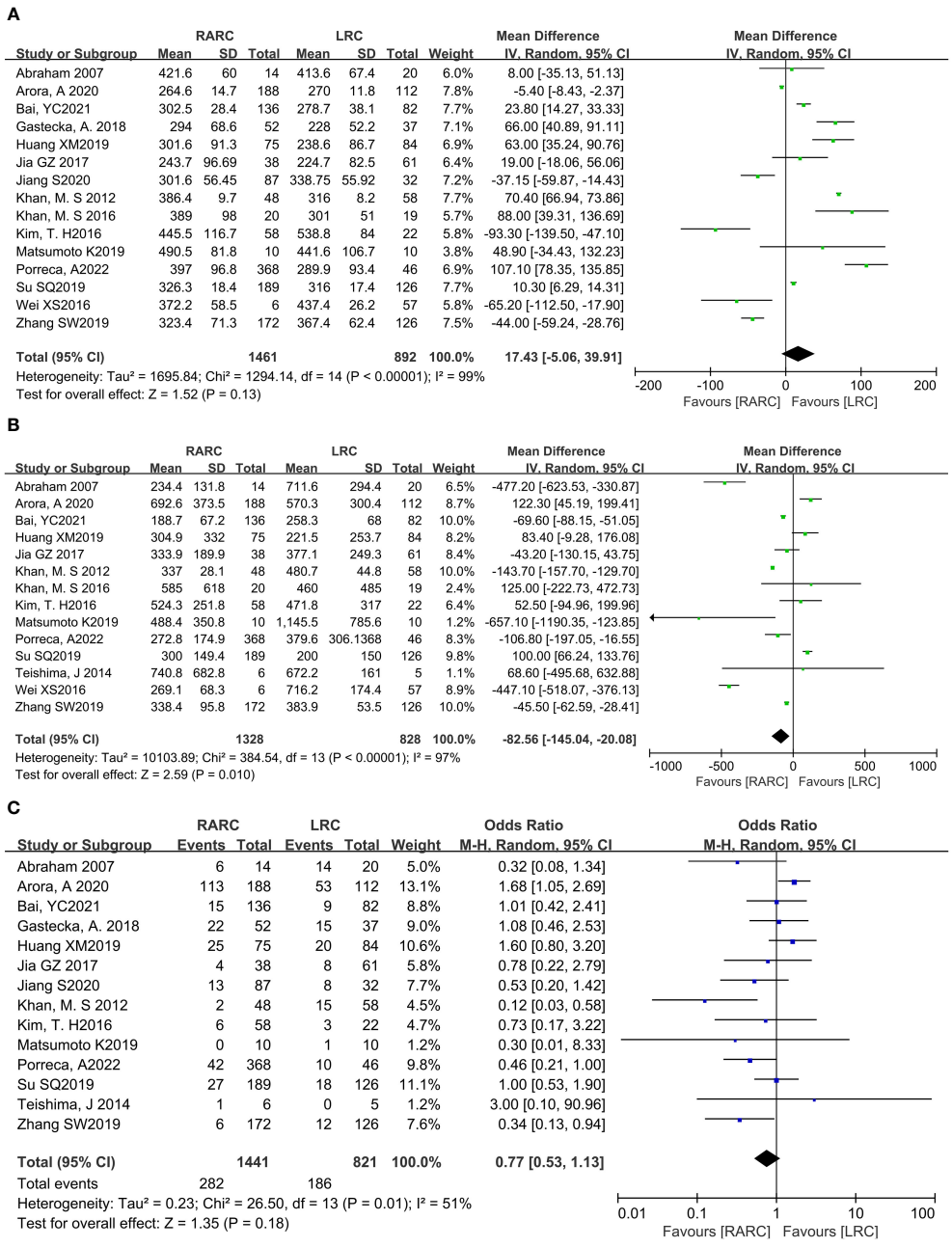


FIGURE 2
Forest plot of RARC versus LRC on **(A)** Total operative time, **(B)** Amount of blood loss, and **(C)** Transfusion rate.

to 1.30), and 30-day postoperative complications, whether it was Grade ≤ II ($P$ = 0.79; OR: 0.95, 95% CI: 0.68 to 1.34) or Grade ≥ III ($P$ = 0.8; OR: 0.95, 95% CI: 0.60 to 1.47), and total early surgery-related complication rates ($P$ = 0.66; OR: 0.85, 95% CI: 0.41 to 1.76) compared with LRC group, as shown in Figure 5.

Eight studies including 1158 patients (RARC group = 619, LRC group = 539) reported the long-term complications 90 days or more after surgery, and twelve studies including 1481 patients (RARC group = 801, LRC group = 680) described the occurrence of postoperative complications (short-term or long-term). Contrary to early complications at 30 days postoperatively, the RARC group had a significantly lower rate of long-term postoperative complications compared with the LRC group, regardless of Grade ≤ II ($P$ = 0.0008; OR: 0.63, 95% CI: 0.48 to

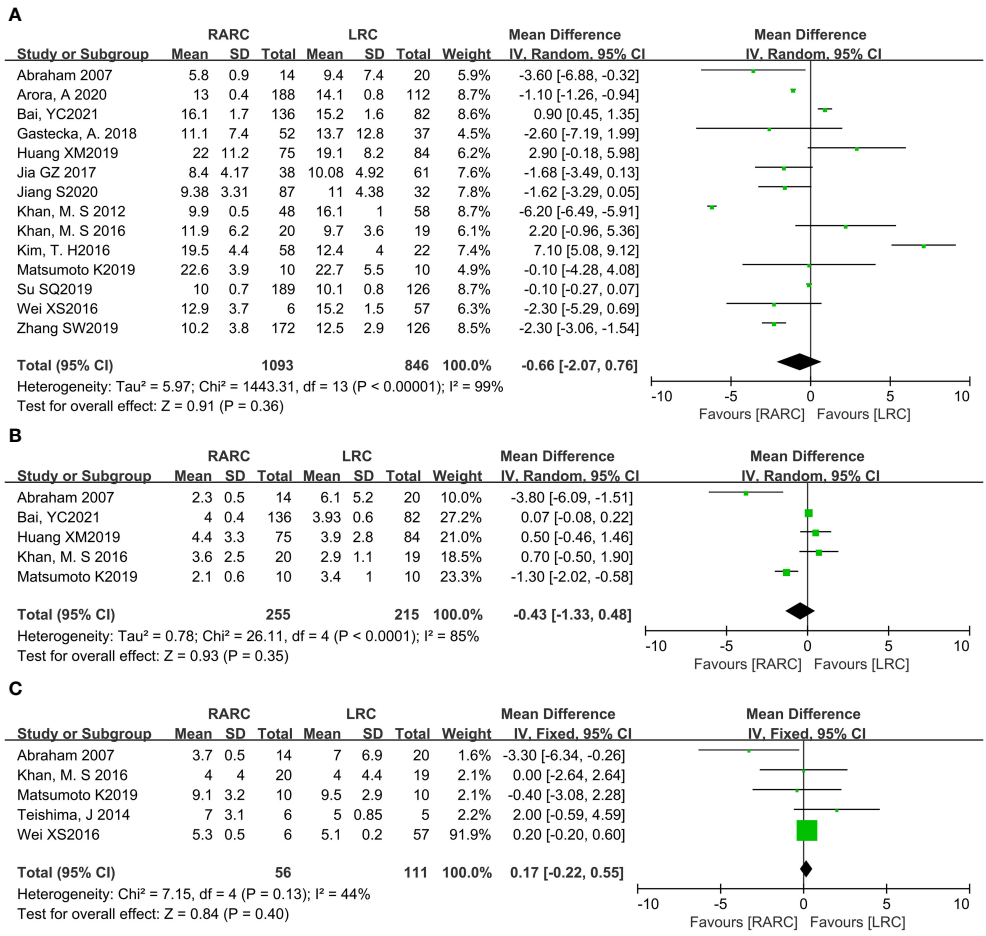


FIGURE 3
Forest plot of RARC versus LRC on **(A)** Hospital stay, **(B)** Days to oral intake, and **(C)** Days to regular diet.

0.82), or Grade ≥ III ($P$ = 0.06; OR: 0.59, 95% CI: 0.40 to 0.86), while the overall postoperative complication rate was still lower in the RARC group than in the LRC group ($P$ = 0.01; OR: 0.52, 95% CI: 0.32 to 0.85), as shown in Figure 6. All the data of the research project are summarized in Table 3.

## Sensitivity analysis and subgroup analysis

Sensitivity analysis and subgroup analysis were used to find sources of heterogeneity, and minimize the impact of heterogeneity on the stability of results. After removing one study, the heterogeneity of some indicators (transfusion rate and cancer-related death) were significantly reduced among other studies. The results of sensitivity or subgroup analysis, such as transfusion rate and total complication rate, were consistent with previous results (Table 4). As for cancer-related death, the results after sensitivity analysis contradicted the previous results, and the mortality was significantly lower in the RARC group than in the LRC group.

# Discussion

In this meta-analysis, 16 studies were included to determine the difference in efficacy between RARC and LRC in bladder cancer. The results showed that, compared with LRC, RARC significantly reduced surgical blood loss and reduced the incidence of postoperative complications, especially long-term complications of 90 days or longer. This may be related to the robotic arm of the robotic surgical system being very stable, avoiding the slight jitter of the human hand, and the robotic endoscope wrist is more flexible in the space that the human hand cannot reach, which is easier to protect the nerves and blood vessels, to achieve less trauma, less bleeding and fewer postoperative complications. In addition, the excellent image

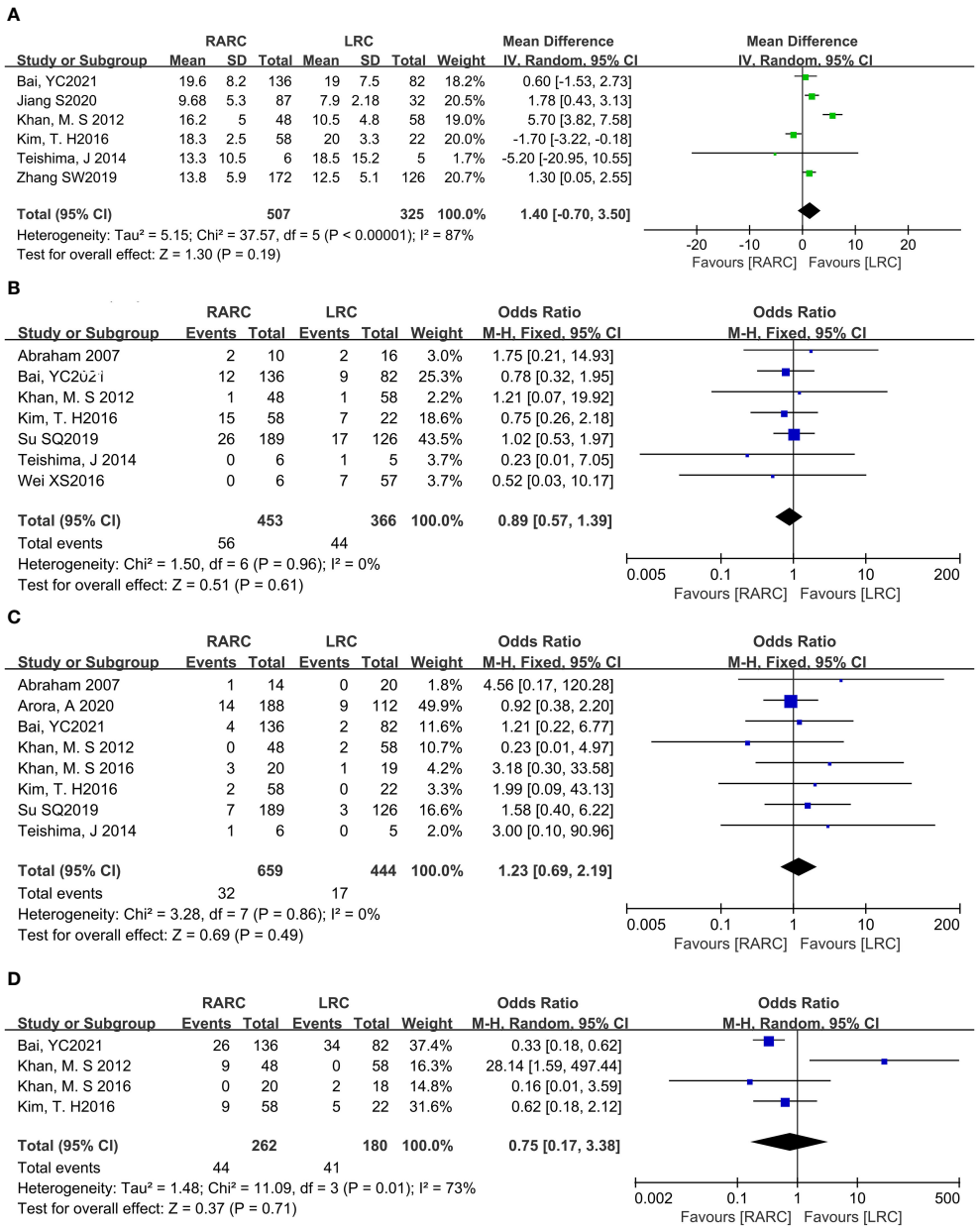


FIGURE 4
Forest plot of RARC versus LRC on the oncologic outcomes. **(A)** The mean lymph nodes, **(B)** The positive lymph nodes, **(C)** The positive surgical margins, and **(D)** Cancer-related mortality.

processing system of robot-assisted laparoscopic surgery makes the surgical field completely reach the real 3D stereoscopic effect, while the function of magnifying 10 times makes the operation more precise, and the anatomical level of blood vessels and nerves is clearer, which is more beneficial for retaining blood vessels and nerves (21, 37, 38).

Because invasive bladder cancer is a fatal disease, adequate marginal resection and pelvic lymph node dissection are important components of surgical treatment, and the quality of lymph node dissection is a key factor for the efficacy of radical cystectomy (21). When the positive surgical margin and positive lymph nodes are less during cystectomy, the survival rate is improved. Our meta-analysis indicated that there was no significant difference between RARC and LRC in the number of lymph node removals, positive lymph nodes, and positive surgical margins. Of course, whether RARC and LRC differ in the

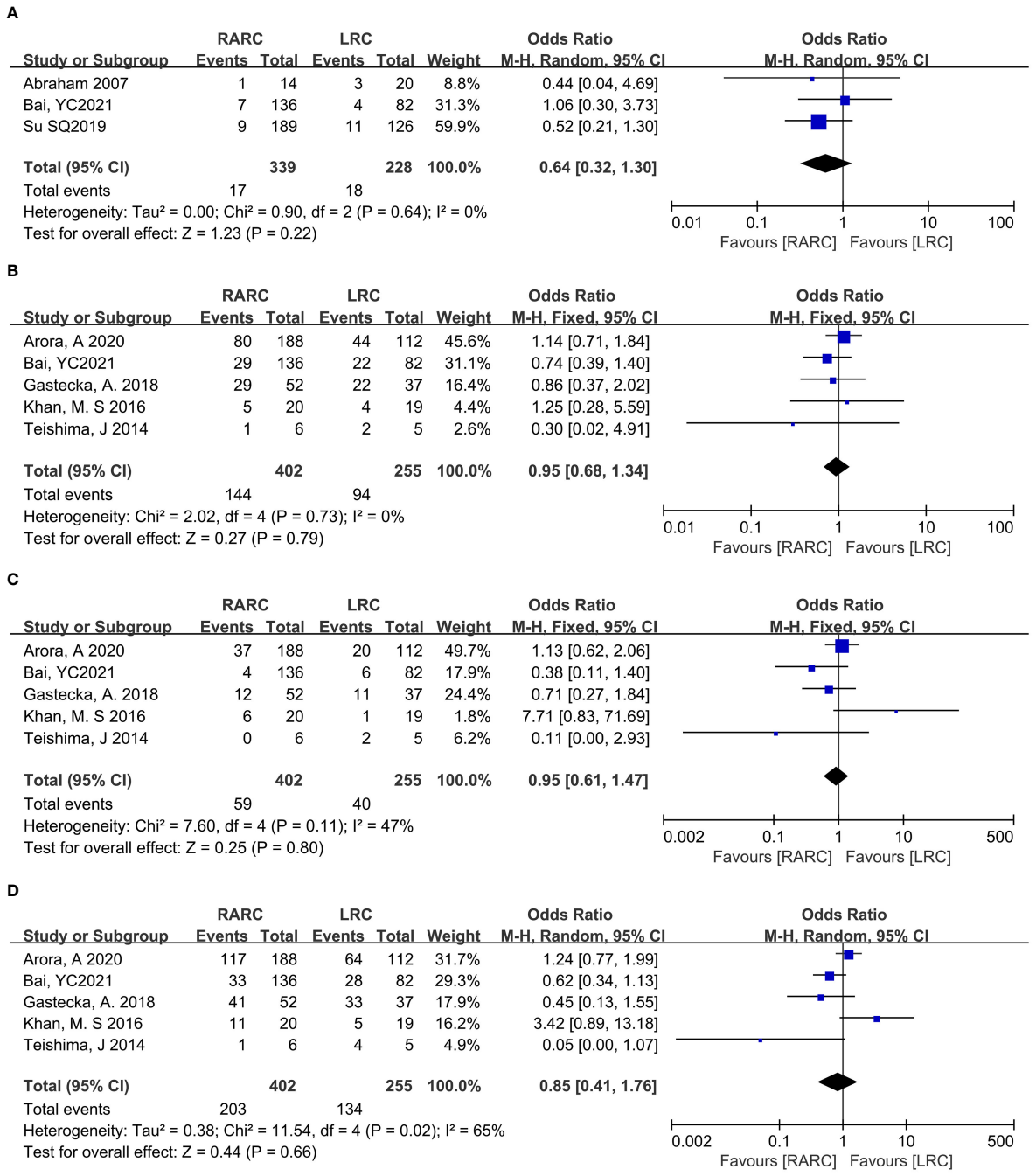


FIGURE 5
Forest plot of RARC versus LRC on **(A)** Intraoperative complication, **(B)** Postoperative complication Grade ≤ II within 30 days, **(C)** Postoperative complication Grade ≥ III within 30 days, and **(D)** Early complications within 30 days.

effectiveness of tumor surgery still requires long-term follow-up to determine. Other indicators, such as total operative time, short-term recovery, length of hospital stay, bladder cancer-related mortality (based on short-term follow-up results), intraoperative complications, and early complications within 30 days after surgery, there is no significant difference between the two groups. This is in agreement with the results of a network meta-analysis of open, laparoscopic, and robotic-assisted radical

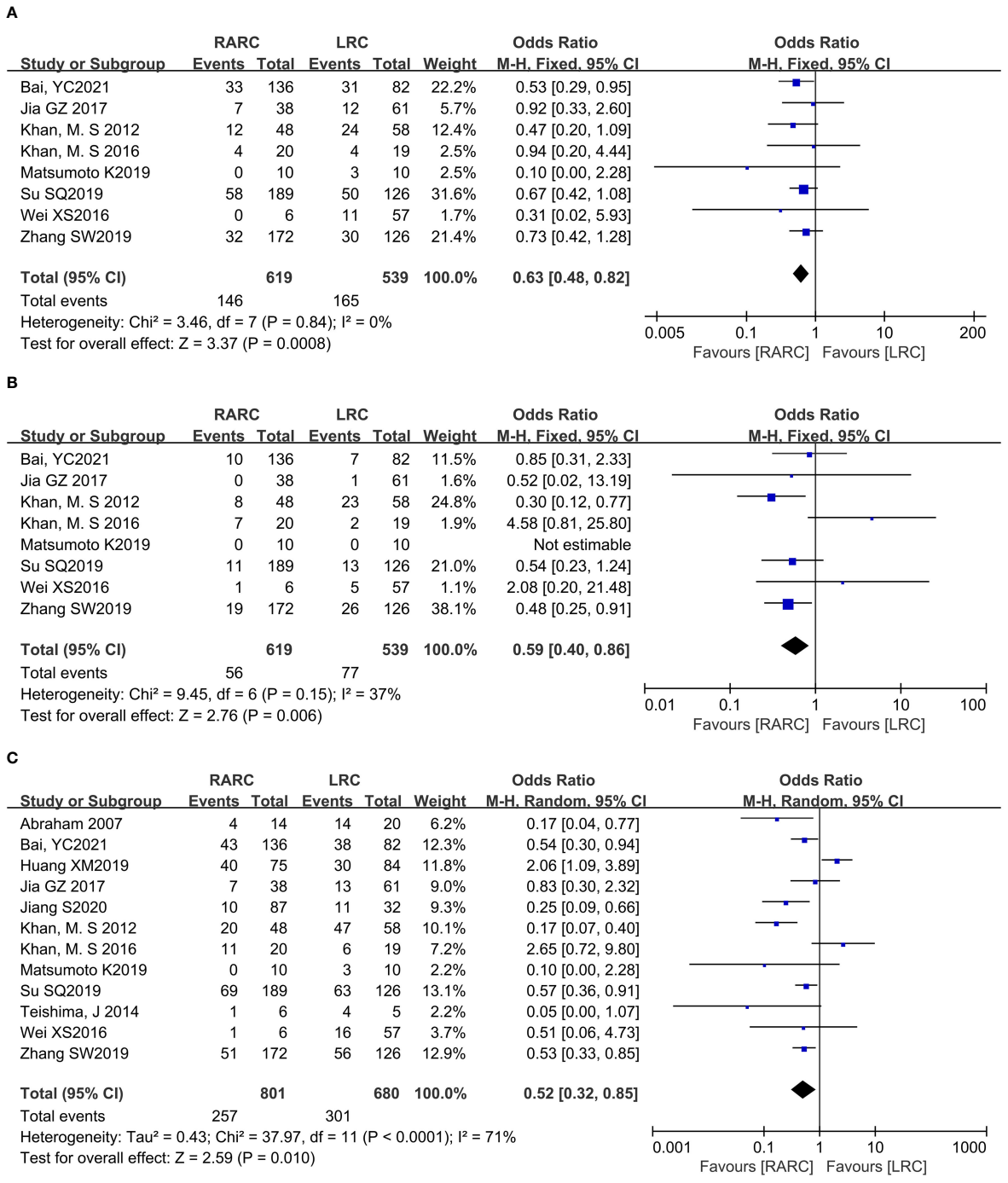


FIGURE 6
Forest plot of RARC versus LRC on **(A)** Complication Grade ≤ II postoperative ≥ 90 days, **(B)** Complication Grade ≥ III postoperative ≥ 90 days, and **(C)** Overall surgery-related complication rates.

cystectomy for bladder cancer performed by Feng D et al, with no significant difference in lymph node yield, positive surgical margins, operating time, transfusion rate, length of stay and days to regular diet between the two groups (39).

There is an interesting phenomenon in our meta-analysis. After we excluded the literature of Khan and M. S (24) in the sensitivity analysis, the disease-related mortality was significantly reduced ($P < 0.05$), which may be related to the

TABLE 3 Summary of research results.

| The research project | -OR/MD | 95% CI | P |
|---|---|---|---|
| Total operative time | 17.43 | [-5.06,39.91] | 0.13 |
| Amount of blood loss | -82.56 | [-145.04 ,-20.08] | 0.01 |
| Transfusion rate | 0.77 | [0.53 ,1.13] | 0.18 |
| Hospital stay | -0.66 | [-2.07 , 0.76] | 0.36 |
| Days to oral intake | -0.43 | [-1.33 , 0.48] | 0.35 |
| Days to regular diet | 0.17 | [-0.22 , 0.55] | 0.40 |
| Mean lymph-node yield | 1.40 | [-0.70 , 3.50] | 0.19 |
| Positive lymph nodes | 0.89 | [0.57, 1.39] | 0.61 |
| Positive surgical margins | 1.23 | [0.69 ,2.19] | 0.49 |
| Cancer-related mortality | 0.75 | [0.17 , 3.38] | 0.71 |
| Intraoperative complications | 0.64 | [0.32, 1.30] | 0.22 |
| Grade ≤ II within 30d | 0.95 | [0.68 , 1.34] | 0.79 |
| Grade ≥ III within 30d | 0.95 | [0.60 , 1.47] | 0.8 |
| Early complications within 30d | 0.85 | [0.41 ,1.76] | 0.66 |
| Grade ≤ II postoperative ≥ 90d | 0.63 | [0.48 , 0.82] | 0.0008 |
| Grade ≥ III postoperative ≥ 90d | 0.59 | [0.40 , 0.86] | 0.006 |
| Overall complication rate | 0.52 | [0.32, 0.85] | 0.01 |

small sample size or unexpected errors. In the future, we need larger randomized controlled studies to verify the potential association. And the efficacy of RARC vs LRC in the treatment of bladder cancer is shown in Table 5.

In addition, in order to evaluate the cost-effectiveness, we simply analyzed the cost of RARC and LRC, although the surgical outcome is crucial, after all, medical cost is also one of the main concerns of patients. Based on current evidence, RARC has a lower complication rate compared with LRC, but it is more costly. Based on the findings of Morii Y et al. (40), the operating cost of robotic surgery accounted for 63.1-70.5% of the total surgical cost. Interestingly, robotics-related costs accounted for a lower proportion of total surgical costs in institutions with more cases, and conversely, robotics-related costs accounted for a larger proportion of total surgical costs. Therefore, the most effective way to reduce the costs associated with robotic surgery is to shorten the operation time and increase the number of cases. But in addition to focusing on the cost of surgery, when studying the cost-effectiveness of surgery, quality measures such as quality of life and survival, and even costs related to the treatment of complications need to be considered. But there are also some European countries where RARC costs depend mainly on patient hospital stay and surgery time, rather than robotic instruments (41). Unfortunately, the studies we included did not report the cost-effectiveness-related indicators of the two types of surgery, and we were unable to draw conclusions by integrating them. In addition, the port site metastatic rate and intrabdominal seeding rate, which we were concerned about, were not mentioned in our included studies. And, these are the hotspots that need to be paid attention to in future RARC and LRC-related research.

TABLE 4 The results of sensitivity or subgroup analysis.

| Indicators | MD/OR | *p* | $I^2$ |
|---|---|---|---|
| Sensitivity analysis | | | |
| Transfusion rate | 0.70 [0.48,1,01] | 0.06 | 34% |
| Cancer-related death | 0.37 [0.21,0.93] | 0.0003 | 0 |
| Subgroup analysis | | | |
| Overall complication rate | | | |
| Mean age ≥ 70 | 0.14 [0.04,0.52] | 0.004 | 0 |
| Mean age < 70 | 0.60 [0.36,0.98] | 0.04 | 73% |

In this meta-analysis, we created a more precise classification of complications to compare the differences between the two groups, in order to more comprehensively and accurately evaluate the prognosis of the two surgical patients and provide more information for clinicians, which was not available in prior meta-analyses. In addition, new evidence on the efficacy and safety of RARC and LRC has been published in recent years, and some of the results are controversial. Our meta-analysis combined recent studies to clarify their pros and cons in bladder cancer treatment.

There are some limitations of this meta-analysis that should be noted: (1) there were differences in the inclusion and exclusion criteria for patients among the included studies; (2) the surgeons are different among the studies; (3) only studies that were published in English and Chinese were considered for inclusion, thus we may have missed some studies that satisfied the inclusion criteria; (4) there is large heterogeneity in partial analysis results that affects the stability of the results, and we tried to conduct sensitivity analysis and subgroup analysis but still cannot fully identify the source of heterogeneity.

TABLE 5 The summary of the efficacy of RARC vs LRC in the treatment of bladder cancer.

| RARC versus LRC in Bladder cancer | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Intraoperative Security | | | Postoperative recovery | | | Oncologic outcomes | | | | | | Complications | | | | |
| Total operative time | Amount of blood loss | Transfusion rate | Hospital stay | Day to oral intake | Day to regular diet | Mean lymph node yield | Positive lymph node | Positive surgical margins | Cancer-related mortality | Intraoperative complication | Postoperative within 30days complication Grade ≤ II | Postoperative within 30days complication Grade ≥ III | Early complications within 30d | Postoperative 90 days or longer complication Grade ≤ II | Postoperative 90 days or longer complication Grade ≥ III | Overall surgery-related complication |
| = | − | = | = | = | = | = | = | = | = | = | = | = | = | − | − | − |

− means RARC decreased the effect vs LRC group; = means that RARC has the same effect vs LRC group.
RARC, robotic assisted radical cystectomy; LRC, laparoscopic radical cystectomy.

In conclusion, our results suggest that RARC is a safe and effective treatment for bladder cancer patients as LRC. Patients with RARC might benefit from significantly less blood loss and fewer postoperative complications, especially long-term complications 90 days or more after surgery. However, despite the methodological review, due to the limitations associated with the included studies and our analysis, additional large sample-size, prospective, multi-center, long-term follow-up studies, and randomized control trials are needed to confirm this conclusion.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

## Author contributions

JL and LW wrote the main manuscript text, ND, XB, and SC prepared tables and figures, SS revised and polished the manuscript, HL and YL proposed research themes and coordinated work within the group. All authors reviewed the manuscript and agreed on the journal to which the article should be submitted.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1024739/full#supplementary-material

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# Impact of interaction networks of B cells with other cells on tumorigenesis, progression and response to immunotherapy of renal cell carcinoma: A review

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Ample evidence indicates that the development and progression of renal cell carcinoma (RCC) are complex pathological processes involving interactions between tumor cells, immune cells and stromal components. Tumor infiltrated immune cells determine whether tumor advancement is promoted or inhibited. Among them, infiltrated B lymphocytes are present in all stages of RCC, playing a major role in determining tumor formation and advancement, as an essential part in the tumor microenvironment (TME). Although the advent of targeted and immune therapies has remarkably improved the survival of patients with advanced RCC, few cases can achieve complete response due to drug resistance. In this review article, we intend to summary the recent studies that outline the interaction networks of B cells with other cells, discuss the role of B cells in RCC development and progression, and assess their impact on RCC immunotherapy.

KEYWORDS

**renal cell carcinoma, tumor microenvironment, B cells, immune cells, interaction network, immunotherapy**

## Introduction

Renal cell carcinoma (RCC) is one of the most prevalent malignant tumors in the human urinary system, accounting for 2-3% of all tumors worldwide (1). Although considerable progress has been made in targeted therapies in improving the 5-year survival rate, the overall clinical outcomes remain unsatisfied due to postoperative recurrence, metastasis or chemotherapy resistance (2, 3). Recently, analysis of the tumor microenvironment (TME) has emerged as a potential approach of RCC

treatment (4). Researchers have concentrated on tertiary lymphoid structures (TLS) and lymphoid aggregates in non-lymphoid tissues, where B cells as the principal components in surrounding T cell zones interact with other cells. TLS appear in various stages of maturity in different tumor types, culminating in germinal center (GC) formation (5). Tumor infiltrated B cells are mostly associated with TLS compared with other immune cells. Although B cells can also be recruited to the tumor bed directly rather *via* TLS, the density of B cells is relatively low under these circumstances. B cells and tumor-associated TLS can be found in the TME of most cancer types and are correlated with tumor immunotherapies (5).

The TME provides a complex tumor ecosystem composed of cancer cells and multiple non-cancerous cells, playing decisive roles in tumor initiation, progression and dissemination (6, 7). Cancer cells interact with stromal cells and immune cells, working together rather separately to form the principal structure of the TME. As one of the characteristics of cancer, prolonged inflammation initiates tumorigenesis or supports tumor progression, during which the entire immune landscape is altered drastically (8). To progress in the body, tumors have derived many mechanisms to escape immune surveillance by producing neoantigens to interfere with the immune system. Therapies targeting the TME have been studied and implemented from bench to bedside. It is described that the therapy of PD-1 and PD-L1 blockade has made therapeutic improvements about metastatic tumors in some reviews, but the objective response rates still remain unsatisfactory. Thus, it is worthwhile to investigate the multiple associations in TME to further explore B lymphocyte-targeted therapies of RCC.

In this review, we address questions regarding the interaction networks of B cells and other cell types by focusing on the association of infiltrated B cells with tumorigenesis, progression and response to immunotherapy of RCC. These cells are collaboratively engaged in aspects of the tumor process and immune TME. We will elucidate each part of B cell interaction that affects immune response in RCC from four specific sections, and review the advances of B cells and TLS with tumor immunotherapies (Figure 1).

## The interaction of B cells with T cells

B lymphocytes first differentiate from hematopoietic stem cells in the bone marrow, experiencing continuous development into immature B cells, and migration to second lymphoid tissues, where they mature (9). The process in the bone marrow involves immunoglobulin light chain gene rearrangement, and VDJ gene segment recombination. Upon stimulation by the antigen, B cells experience antibody class switching and somatic hypermutation in the GC, which has proved to be the mechanism for affinity maturation of antibodies (10, 11). The initiation of GC reaction involves several distinct cell types *via* a coordinated cascade, guiding antigen-engaged B cells into GC reaction. These processes along with GC formation are well assisted by other immune cells, especially T cells. Additionally, B cells are one of the critical components of the humoral immune system, regulated by numerous control mechanisms at both cellular and molecular levels. The induction of antibody response also requires the collaboration of T and B cells.

The function that T cells provide assistance for B cells has been recognized for decades, resulting in the demonstration of thymus-derived helper cells (12–14). This two-lymphocyte lineage model indicates issues of synergistic function and their cooperation. As the close partner, their history has been reviewed in detail (15). For various types of cancers, the associated T and B networks have already generated interests in immune therapy in many recent studies, and research highlighted the emerging roles of B cells in tumor immunity and the focus on T cell response. These findings could guide a protocol and provide potential therapeutic strategies for RCC patients.

### B cells with Tfh cells

T follicular helper (Tfh) cells are the crucial partner of infiltrated B cells, whose crosstalk within TLS in the TME has been verified to affect tumor immunotherapy (16). There exists a bidirectional interaction in the Tfh-B combination. B cells are essential for Tfh cell formation by mediating PI3K-dependent migration of CD4+T cells into follicles (17). In a murine model of lung adenocarcinoma, B cells were found to promote tumor-specific CD4+Tfh cell differentiation, which then produced IL-21 to enhance CD8+T cell responses to drive anti-tumor immunity (18). In turn, mature Tfh cells provide binding CD40 ligand (CD40L or CD154) and IL-21 for GC B cell differentiation into memory B cells and plasma cells (19–21). IL-21 also plays a pivotal role in Tfh cell formation, B cell growth and class switch recombination (CSR), and maintains the expression of Bcl-6 in GC B repertoires (20, 22, 23). These processes are modulated by other Tfh cell cytokines, including IL-4 and IL-10, which favor IgG and IgA production, respectively (17). In addition to offering help for T-dependent B cells, some specialized CXCL13-producing Tfh cells could guide CXCR5+ lymphocyte migration, promote local memory B cell differentiation, and behave as a potential surrogate marker for GC reaction and TLS formation (24–26). Studies suggested that anti-PD-1 therapy in cancer could strengthen B cell capacity by increasing circulating Tfh cells (27). An investigation of the clinical data of clear cell RCC (ccRCC) showed that immune-related prognostic gene signatures were differentially expressed in distinct lymphocyte clusters (28). In a study of metastatic

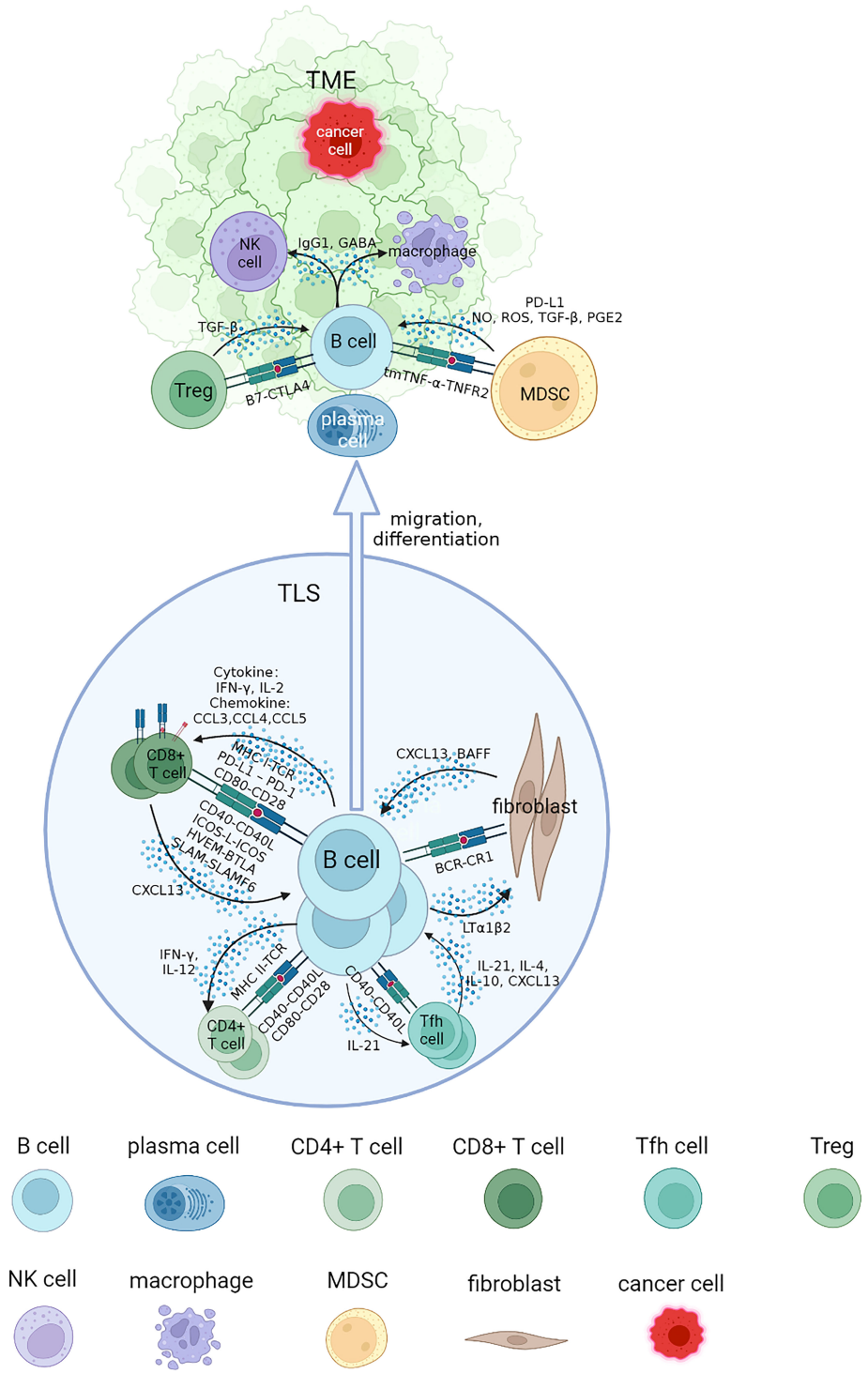


FIGURE 1
Interaction networks between B cells and other cells in tertiary lymphoid structure (TLS) and tumor microenvironment (TME). B cells cooperate with other cells to perform an immunomodulatory role on tumorigenesis and progression. B cells could be attracted to TLS by CXCL13, where they mature and interact with different types of T cells through specific co-stimulatory and co-inhibitory signal pairs such as CD40-CD40L, CD80-CD28. The class switch recombination and differentiation of B cells are promoted by cytokines including IL-4 and IL-10, and B cells receive IL-21 to develop into plasma cells and memory B cells, migrating to tumor bed. They cooperate with NK cells and macrophages to implement ADCC and ADCP process through antibodies including IgG1 in TME. Some inhibit regulatory effects of MDSCs and Tregs on B cells in TME could promote tumor progression. NK, natural killer; MDSC, myeloid-derived suppressor cell; NO, nitric oxide; ROS, reactive oxygen species; PGE2, prostaglandin E2.

ccRCC patients under ICB therapy, unswitched memory B cells correlated positively with Tfh cells, TLS and CD20+ B cells, associated with higher response rate and better overall survival (29). The related genes and the interaction mechanisms of B cells and Tfh cells may prove to be a biomarker for assessing prognosis of RCC and screening precise patients for specific immunotherapies.

## B cells with CD8+T cells

### Effects of B cells on CD8+T cells

Some evidence proves that B cells can exert a direct or indirect effect on CD8+T cells. B cells present tumor-specific antigens that they have captured by B-cell receptors (BCRs) to CD8+T cells (30), release cytokines, and participate in the formation of co-stimulatory and co-inhibitory receptors (31). Some cytokines such as IL-2 and IFN-γ secreted by B cells can bind to the receptors on CD8+T cells (31). The co-stimulatory and co-inhibitory signal pairs include CD80-CD28, CD40-CD40L, ICOS-L-ICOS; PD-L1-PD-1, HVEM-BTLA, SLAM-SLAMF6 (32). In addition, B cells can indirectly support CD8+T cells by interacting with Tfh cells through CD40-CD40L (32). *In vitro*, B cells were found differentiating into plasmablast-like cells and expressing T cell recruitment chemokines like CCL3, CCL4 and CCL5. Plasmablast-like cells increased the activation of PD-1+T cells *via* anti-PD-1 blockade, and their frequency could predict response and survival to immune checkpoint inhibitor (ICB) (33). In a metastatic RCC pre-surgical trial, B cell signatures were found enriched in tumors of responders of ICB treatment, which was also confirmed in another ICB-treated cohort of melanoma patients (34). Accordingly, we speculate that B cell subpopulations within the TLS could modulate T cell antitumor response and serve as a possible ICB response predictor of RCC.

### Effects of CD8+T cells on B cells

Current reports suggest that T cells may appear first in the tumor sites and then promote the recruitment of B cells. CD8+T cells activated by TGF-β will upregulate CD103 and release CXCL13, a potent B cell chemoattractant that binds to CXCR5 receptor of B cells, and then mediate B cell recruitment and TLS formation (35). Researchers observed similar phenomenon that infiltrated B cells were prone to colocalize with CD8+T cells (36), and the significant correlations between them imply their cooperation in a tumor-killing effect of several malignancies (31). A study on ccRCC demonstrated that the abundance of intratumoral CD8+T cells secreting CXCL13 was associated with increased TLS and immunoevasive TME, functioning as a potential immunotherapeutic marker for ccRCC treatment (37). Another study illustrated the prognostic value of CCL4, CCL5, CCL8, CCL19 and CXCL13 expression in ccRCC. Besides, DNA deletion of TLS gene signatures could greatly indicate poor outcome in ccRCC patients compared with wild-type gene signature (38). All these results provide insights into how B cells cooperate with CD8+T cells and their roles in ICB treatment, which may assist the development of RCC therapeutic targets.

## Roles of Tregs on B cells and plasma cells

### Direct regulations

Regulatory T cells (Tregs) dampen B cell proliferation and plasma cell formation (39). Hyung et al. reported that Tregs could directly interact with B cells to suppress immunoglobin (Ig) response, production and CSR without the help of Tfh cells, and downregulate relative gene expressions of naïve B cells *via* contact-dependent mechanisms (40, 41). Interestingly, it was elucidated that Tregs reduced all other Ig production but induced B cells to produce IgG4 in a cell contact-, TGF-β- and IL-10-dependent manner (42, 43). Studies have demonstrated that Tregs are related to negative outcomes in RCC patients (44), and the expansion of Tregs could limit the function of IL-2 in RCC treatment (45).

### Indirect regulations

Tregs downregulate the function of T helper (Th) cells through various mechanisms, resulting in adverse effects on B cells (46). Tregs could also suppress *in vitro* B cell Ig production by inhibiting Th function *via* TGF-β (47). In addition, Tregs migrate to follicles in the GC and regulate GC B cell responses (48). A population of follicular regulatory T (Tfr) cells is also described, which share phenotypes of both Tregs and Tfh recruited during GC reaction, yet are distinct from both (49, 50). Tfr cells could balance the Tfh-mediated B cell responses through CTLA-4 expression (17), and it has been proved that a lack of Tfr expressing CXCR5 and Bcl-6 could lead to greater GC reaction, associated with GC B cells, affinity maturation of antibodies and plasma cells differentiation (51). Consequently, the regulation of B cells in the GC reaction is well counterbalanced by Tfh and Tfr cells. A recently published study indicated another population of Foxp3+T cells which existed in the end-stage of GC, displaying an immediate phenotype of Tfh and Tfr cells (52). Collectively, these suggest the indirect effects of Tregs on B and plasma cells, mainly through Tfh and Tfr cells in the GC.

## B cells cooperate with NK and macrophages

### ADCC and ADCP

The antitumor activity of NK cells depends on their antibody-dependent cell-mediated cytotoxicity (ADCC) when they encounter tumor-specific IgG1 antibodies secreted by plasma cells (30, 53). Unfortunately, some tumor-derived IgG may impede the ADCC process by binding with specific antigens lacking complement activation. To against high PD-L1-expressing tumors, NK cells combining with anti-PD-L1 antibodies helps promote ADCC activity (54). However, NK cells are generally scarce and become anergic in the TME (55). Tumor-associated macrophages (TAM) participate in the ADCC or phagocytosis process *via* antibody-dependent cell-mediated phagocytosis (ADCP). Intriguingly, Su et al. reported an unexpected finding that ADCP macrophages may inhibit ADCC and T cell-mediated cytotoxicity by upregulating immune checkpoint such as PD-L1 and indoleamine 2,3-dioxygenase to cause immunosuppression (56). Therefore, a combination of therapeutic antibodies and ICB potentially provide synergistic effects in RCC treatment.

### B cells with macrophages

Fc gamma receptor (FcRγ), a receptor binding to Fc region of an antibody, could modulate protumor bioactivities of macrophages. In the absence of B cells or FcRγ, macrophages were found reprogramming towards the M1-type inflammatory state (57). Specifically, B cells could foster tumor development through FcRγ by activating pro-angiogenesis and tissue remodeling of myeloid cells, especially macrophages and mast cells (57). Besides, the reprogramming of macrophages also regulates CD8+T cell recruitment. Researchers discovered that by using B cell-depleting αCD20 monoclonal antibody as an anticancer monotherapy in mice, the chemokine expression of macrophages altered, thus improving CD8+lymphocyte infiltration and chemotherapy response in squamous cell carcinoma (58). A study of pancreatic ductal adenocarcinoma showed that Bregs could induce polarization of M2 macrophages and aggregation of Tregs, thus resulting in T cell suppression (59). Likewise, in ccRCC cohorts, researchers observed that compared with early stage tumors, pro-inflammatory macrophages were reduced, while suppressive M2-like macrophages were elevated in advanced disease (60). In conclusion, immunosuppressive B cells and plasma cells tend to facilitate TAM conversion to protumoral M2 phenotype, while effector B cells could promote TAM conversion to tumoricidal M1 phenotype (30). In the study of ccRCC, researchers found that TAM-derived chemokine CCL5 displayed a correlation with increased B cells and CD8+T cells, and decreased CD4+T cells. Elevated CCL5+TAMs infiltration exhibited higher tumor-infiltrated lymphocytes, but reduced TLS, correlated with a distant prognosis of ccRCC patients (61).

Although interactions of lymphocytes are regulated with various cell-bound proteins, small metabolites, as essential intermediates in biochemical processes, could reflect neighboring cells when released into extracellular milieu (62, 63). Metabolite and neurotransmitter GABA, synthesized and secreted by activated B cells and plasma cells, could promote monocyte differentiation into macrophages, polarizing towards an anti-inflammatory phenotype. They function as protumor cells through releasing interleukin (IL)-10 and limiting CD8+T cell function (64). Furthermore, in RCC microenvironment where B cells and IgA+ plasma cells were highly infiltrated, GABA was almost exclusively detected. It may suggest that GABA could regulate T cells and monocytes in the TME of RCC (64). Further studies are needed to unravel the occasions of intracellular metabolites mediate interactions between cells to inhibit tumor growth or enhance B cell immunity to cancer.

## The interaction between fibroblasts, dendritic cells and B cells

Fibroblast reticular cells (FRCs) are considered immunologically specialized myofibroblasts originating from mesenchymal stem cells (65). Inside the encapsulated sponge-like network of FRCs in lymph node congregated B cells, T cells, plasma cells, dendritic cells (DCs) and macrophages. FRCs in B cell zone provide B cell growth factor—B cell-activating factor (BAFF) and transcribe B cell chemoattractant CXCL13 to maintain and attract B cells in support of B cell survival (66). During infection, FRCs rapidly upregulate the CXCL13 expression *via* crosstalk with B cells, and control the expansion of B cell follicle boundaries upon inflammation (67).

Of note, DCs are specialized non-hematopoietic stromal cells residing in lymphoid follicles and GC. They participate in optimal selection of B cells that secret antibodies (68). In addition, B cells populate in the network of follicular dendritic cells (FDCs). Mature TLS comprise a GC with CD23+FDCs, which could present antigens to B cell selection with high-affinity BCRs, and promote B cell activation and differentiation (69). FDCs present naïve antigens to GC B cells *via* complement receptors 1 (CR1) (70). In cohorts of prospective and retrospective lung cancers, a high density of mature DC is strongly linked with a substantial infiltration of T cells with effector-memory characteristics, T-cell activation gene expression and T-helper 1 phenotype (71). These results indicate that mature DCs may generate some specific immune contexture that influences infiltrated B cells and TLS in TME.

Researchers have demonstrated that fibroblasts could directly support TLS formation and development (72). In the settings of inflammation and persistent antigen presentation, TLS-associated fibroblasts differentiate from locally activated mesenchyme (73). Likewise, stromal cell priming and lymphocytes accumulation have close relationships and the former might occur before lymphocyte migration. This kind of cancer-associated fibroblasts (CAFs) play a pivotal role as lymphoid tissue organizer cells (LTo) and coordinate with multiple cell types that synergistically act as lymphoid tissue inducer cells (LTi) cells, such as Intratumoral CD8+T cells and B cells that drive TLS development. CAFs work as surrogate LTo and participate in TLS formation and orchestration. On the other hand, the reticular network of CAF is mediated by CD8+T cells, and its accumulation relies on the recruitment of B cells expressing lymphotoxin, namely intratumoral LTα1β2+B cells. Imaging analysis has confirmed an elevated density of B cells coexisting with a reticular network of LTo-like CAF (74). Analysis of TCGA data of RCC showed that CAF infiltration was higher in RCC, especially in RCC with advanced tumor pathological grades and stages, than that in normal tissues (75). In addition, studies of RCC have revealed that TLS are sites of *in situ* B cell maturation into plasma cells. The plasma cells formed in the TLS are embedded in the dense network of fibroblasts, and disseminate into the tumor beds along fibroblastic tracks (76).

## MDSC-mediated regulation of B cell response

Myeloid-derived suppressor cells (MDSCs) were first termed in 2007, representing a series of immunosuppressive macrophages, DCs and granulocyte precursor cells produced in response to tumor-derived cytokines (77). Since then, MDSCs have been considered a great obstacle for cancer immunotherapies because they have close relationships with other immune cells. Studies have identified that normal B cells could be transformed into a subtype of immune regulatory B cells (Bregs) inhibiting T cell response in the presence of MDSCs. Besides, some immune checkpoint molecules including PD-1 and PD-L1 might be changed and remolded predominantly. A novel MDSC-Induced B cell subset (PD-1-PD-L1+CD19+) has been demonstrated to inhibit T cell responses (78). Specifically, activation of the PI3K/AKT/NF-kB axis enhances the PD-1-PD-L1+ Breg protumor function (79). Another study on glioblastoma discovered that MDSCs delivered functional membrane-bound PD-L1 *via* microvesicles to Bregs, conferring the effector phenotype and function (80). Cell experiments have elucidated that 1) MDSCs suppress B cell proliferation by releasing suppressive mediators; 2) MDSCs induce decreased expression of B cell surface markers including IgM, HLA-DR, CD80 and CD86; 3) MDSCs induce specific B cell subset phenotypic alterations including antibody-secreting cells death; and 4) MDSCs elicit specific gene transcriptional changes which are associated with apoptosis, class-switch regulation and B cell differentiation and effector function (81).

In addition, some indirect regulatory effects of MDSCs on B cells have also been elaborated. MDSCs increase the number of FoxP3+ Treg cells, and facilitate the development of Tregs (82, 83). Tregs, along with Bregs, have similar suppressive characteristics and close relationships with B cells. FoxP3 +Treg cells inhibit antibody production, activation and differentiation of B cells. Besides, it is reported that MDSCs modulate B cells *via* different pathways, including TNF, STAT3 and TGF-signaling, and MDSC-derived nitric oxide (NO), reactive oxygen species (ROS), TGF-β, and prostaglandin E2 (PGE2) play roles in suppressing B cells (84). RCC is regarded as an immunogenic tumor, which elicits the influx of immune-inhibitory cells such as Tregs and MDSCs into the TME, resulting in immune dysfunction (85). Many possible mechanisms have been proposed to explain how MDSCs target immune suppression (86), and it has resulted in clinical response in some patients with RCC (85, 87). The regulation of B cells by MDSCs may be a prospective target for immunotherapy in RCC.

## Indications of TLS and immunotherapy

TLS have been identified in various types of tumors at every stage of disease, but their presence is in high heterogeneity between different cancer types and patients (88, 89). It is thought that TLS actively modulate antitumor immune activities rather than simply being a surrogate marker of rapid immune response (34). Mature TLS show indications of GC development. In colorectal cancer, TLS are associated with favorable outcomes and harbor prognostic information of disease recurrence (90). In lung squamous cell carcinoma, TLS are the independent prognostic marker and their development can be affected by chemotherapy and steroids (91). Interestingly, between different types of genitourinary cancer, one study showed that TLS displayed a distinct status in terms of the clinical outcome and immunogenomic profile (92). Researchers showed that the TLS detected in RCC cohort were less mature, contributing to poor outcomes, while in bladder cancer cohort, TLS were more mature with GC structures and associated with better outcomes (92). Another study certificated that in three gradual levels of immune infiltration of ccRCC clusters, higher abundance of T cells and TLS, suggesting an immune-enriched TME, was related to poor clinical outcome (93). These results

suggest the heterogeneity of TLS and indicate that comparison of the TLS characteristics may help show differences in the immune TME and prognostic effects in RCC.

## B cells and immunotherapy

Recent studies have contributed to an appreciation for B cells influencing immunotherapy outcome (30). Researchers have already reached consensus on the surface phenotype markers of various B subtypes except Bregs (94), and single-cell RNA sequencing (scRNA-seq) technique provides a broader perspective on cell markers and characteristics. scRNA-seq and cell-cell communication analysis in several recent studies have demonstrated that interactions of infiltrated B cells could influence tumor clinical outcomes (Table 1). The heterogeneity across B-cell subpopulations has been studied by single-cell techniques. Some single cell methods have helped dissect tumor heterogeneity and study the anti-tumor drug responses. However, the specific B-cell gene signatures between different cancer types still need more investigations. When initiating an antitumor response, tumor-infiltrated B cells (TIL-Bs) are first required to be recognized by tumor-specific neoantigens *via* BCRs. Studies have suggested that complementarity determining region-3 (CDR3), highly changeable regions in the BCR, have the potential to be prognostic biomarkers of different malignancies (104–106). Furthermore, the diversity of intratumoral BCR repertoires could reflect clonal expansion in response to tumor-associated antigens. Compared to scRNA-seq, repertoire studies may better characterize TIL-Bs including the investigation of B-cell phenotypes and BCR diversity within the RCC microenvironment (107). Some distinct RCC-associated gene mutations displayed by genomic techniques also have correlations with BCR repertories. Researchers found that among RCC patients with mutations in KDM5C, PBRM1, VHL and PTEN, BCR repertoire diversity was decreased (108). Intriguingly, PBRM1 mutation is pertinent with immune checkpoint inhibiters (ICI) response of RCC (109, 110). Some gene segments may be enriched in TIL-Bs with particular gene expression phenotypes. Besides, some BCR pathway molecules are upregulated and BCR-related kinases play a role in the TME of various tumors, suggesting an anticancer activity of targeting BCR-immune complex and BCR-related kinases (106, 111). Overall, this may provide a new insight of exploring B cell subpopulations most affected by molecular features of tumor and contribute to new targets of immunotherapy with the combination of scRNA-seq and BCR repertories.

Antibodies produced by B cells have associations with effective antitumor immune response. Researchers demonstrated a high level of plasmablasts in the blood of metastatic RCC patients who had not exhibited tumor progression for over a year, and reactive antibodies from B cell response are commonly detected, which exhibit a great level of somatic hypermutation (112). A study indicated that TIL-Bs had unique antibody repertoire characteristics, including elevated clonal polarization and high somatic hypermutation rates in treated-tumor-bearing mice (113). The signs of B cell activation and clonal expansion were similarly discovered in other human malignancies (111, 114). These results suggest a possible persistent B cell response targeting tumor antigens. The identification of antibody repertoire signatures could perform as markers to identify tumor-reactive B cells, and provide a new paradigm for discovering antitumor antibodies with RCC diagnosis and immunotherapy.

## Can B cells and TLS be predictive and prognostic factors in RCC?

We have reviewed the crosstalk between B cells and other cells in the TME, highly expecting that indications of B cells and TLS with immunotherapy could be the predictive and prognostic factor in RCC. Some current studies and clinical trials have confirmed the value for different B subsets in RCC. A clinical study on the plasma sample of RCC patients demonstrated that the blood concentration of unswitched memory B cells was correlated with the response condition to immune checkpoint blockade and survival rate in metastatic ccRCC patients (29). Similarly, researchers found that a subset of B cells with a memory phenotype was associated with positive outcomes in RCC patients treated with immune checkpoint inhibitors, and could predict response to checkpoint immunotherapy (115). Meylan et al. (76) used spatial transcriptomics to investigate B cell immunity within intratumoral TLS in RCC, and proved that TLS sustained B cell maturation and antibody generation, with response to immunotherapy possibly *via* direct antitumor effects (76). It has been proved that RCC exhibits distinct immune phenotypes and proteogenomic characteristics (116). Growing evidence indicates the diversity and heterogeneity of TME and tumor cells affect immunotherapy (117). Although no large comparative study has been reported to explore the specific effects and mechanisms of B cells on RCC patients in clinical trials, the clinically related outcomes of B cells and TLS with RCC occurred in previous studies. Therefore, further investigations are needed to confirm the predictive and prognostic value of B cells and TLS in RCC.

## Conclusion

Increasing evidence has demonstrated the important role of B cells in tumor immunotherapy, and some innovative techniques including scRNA-seq and BCR repertoires have provided intensive insights into B cells and TME. In this review, we focus on the interaction networks of B cells with other cells in RCC microenvironment. Some subtypes of T cells including CD8+T cells and Tfh cells contribute to the recruitment, maturation and differentiation of B cells. Besides, B cells also act as a provider of

TABLE 1 Current research about the interactions of B cells influencing tumor clinical outcomes, using scRNA-seq or cell-cell communication analysis.

| Tumor type | Interactions | Outcomes | Reference |
|---|---|---|---|
| **Non-small cell lung cancer (NSCLC), malignant pleural effusion (MPE)** | In MPE, Bregs interact primarily with CD4+ T cells (including Th1/17, Treg and Tfh), but not CD8+ T cells. | Bregs in MPE show great cell proliferation signaling and are related to poor clinical outcomes. | (95) |
| **Colorectal Cancer (CRC)** | Compared to non-tumor tissues<br>- Enhanced interactions between non-immune cells (including MKI67+ goblet cells, DEFA5+DEFA6+ metaplastic paneth cells, colonocytes, and fibroblasts) and immune cells (including B cells, T cells and myeloid cells) in tumor tissues.<br>- Altered interaction pathways between B cells and T cells. | - B, plasma and non-immune cells in tumor tissues exert important roles in shaping tumor microenvironment.<br>- Proliferative B-cell signatures are enriched in tumors that respond to immunotherapy. | (96) |
| **CRC** | Compared to non-tumor tissues<br>- Enhanced interactions between myeloid cells and lymphocytes (including B cells, plasma cells and T cells) in tumor tissue.<br>- Enhanced interactions between B cells and T cells through CD48-CD244.<br>- B cells tend to interact with SIGLEC10+ T cells and inhibit T cell activation.<br>- Enhanced interactions between IgA+ IGLC2+ plasma cells and multiple types of T cells.<br>- CCL8+ IGLC2+ plasma cells and cycling B cells interacte with CCR5+ T cells in CRC and recruit CCR5+ T cells to the tumor foci.<br>- Attenuated interactions between epithelial cells and B cells, but the SIRPA-CD47 and NRG1-ERBB3 pathways are enhanced. These are associated with immune escape and epithelial-mesenchymal transition (EMT)-associated metastasis.<br>Compared to early stage tumor tissue<br>- Enhanced interaction of B cells with other immune cells in advanced tumor tissue.<br>- IgA+IGLC2+ plasma cells, which are associated with poor prognosis, have significant interactions with myeloid cells and cytotoxic T cells. | - B cells and myeloid cells are predominantly responsible for immunoregulatory functions in CRC rather than CD4+ regulatory T cells.<br>- B cells in early CRC tumors exhibit pre-B like tumor suppressors, while B cells in advanced CRC tumors tend to develop into plasma cells.<br>- B cells in CRC may contribute to tumor progression. | (97) |
| **CRC** | B cells, MKI67+ T cells and dysfunctional T cells, interact with tumor-associated macrophages (TAMs), which are enriched by preoperative chemotherapy, through HLA-F-LILRB2 and HLA-DPB1-NRG1 pathways in the cell niche of primary tumors. | - Less-activated B cells are more prevalent in the tumor microenvironment of treatment-naïve tumors.<br>- B cell activation is observed in tumors treated with preoperative chemotherapy. | (98) |
| **Nasopharyngeal Carcinoma (NPC)** | - The three exhausted T cell populations in TME (HAVCR2+, TOX+, and LAG3+ T cells) preferentially interact with memory B cells, innate-like B cells, unactivated B cells, and IFN-induced B cells, but not with plasma cells, naive B cells, and double-negative B cells.<br>- Among them, B cells interact with HAVCR2+ and TOX+ exhausted T cell populations mainly through the CXCL13-CXCR5 axis. | - A higher abundance of B cells is correlated with a better clinical prognosis in NPC patients.<br>- A higher percentage of double-negative B cells is predictive of worse survival rate in NPC patients. | (99) |
| **Esophageal squamous cell carcinoma (ESCC)** | Attenuated interactions between tumor cells and B cells compared to interactions between tumor cells and other immune cells in TME. | The specific cellular communication potentially shapes the unique TME in ESCC. | (100) |
| **Ovarian Cancer** | - Tumor-infiltrating B cells (B-TILs) interact with CD4+CXCL13 T cells as well as dysfunctional CD8+GZMB T cells through CXCR5-CXCL13, which is a possible mechanism for recruiting B cells into the tumor microenvironment.<br>- B-TILs interact with endothelial cells *via* CCR7-CCL21, suggesting another possible mechanism for recruiting B cells into the tumor microenvironment. | Stromal cells and T cells participate in the recruitment of B cells in tumor and stromal compartments of ovarian cancer. | (101) |
| **Lymphoma** | - Malignant B cells receive suppressive signals from all four major T cell subsets (T helper, T toxic, Tfh, Treg) through CD80/CD86-CD28 and CD80/CD86-CTLA.<br>- Malignant B cells interact with T helper cells and Tregs through BCMA-BAFF, BAFF-R-BAFF, and CD40-CD40LG.<br>- Malignant B cells interact with Tfh cells through IL4-IL4R, IL4-IL13RA1 and IL21-IL21R. | - B cells modulate the frequency of various lymphoma-infiltrated T cell subsets to shape the microenvironment.<br>- Malignant B cells are heterogenous among lymphoma patients with different proliferative activity. This is associated with lymphoma-specific transcriptional features. | (102) |
| **Head and neck squamous cell carcinoma (HNSCC), caused by environmental carcinogens or human papilloma virus (HPV)** | - B cells interact with CD4+ T cells in both HPV- and HPV+ TME. Besides, the spatial distance between B cells and CD4+ T cells is closer, which reflects the probability of interaction.<br>- Interactions between GC B cells and TFH are only in HPV+ TME. | B cells in germinal center are observed in HPV+ tumor microenvironment, and correlate positively with the overall survival in HNSCC. | (103) |

antigen presenting cells and release cytokines to help T cells perform their duties in tumor sites. On the contrary, subsets of Tregs and MDSCs suppress the performance of B cells. In addition, NK cells and macrophages perform ADCC and ADCP function through using antibodies, and fibroblasts perform an essential role in the maturation of B cells in TLS. We speculate that the study of the interaction networks of immune cells can provide valuable information for RCC treatment, and how to improve the response rate to immunotherapy of RCC is an important issue that needs to be considered seriously.

## Author contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the literature. All authors were involved in critical revision of the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Role of tumor-derived exosomes in metastasis, drug resistance and diagnosis of clear cell renal cell carcinoma

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Renal cancer is one of the most extensively studied human tumors today, with clear cell renal cell carcinoma accounting for approximately 80% of all cases. Despite recent advances in research on clear cell renal cell carcinoma, advanced distant metastasis of the disease, delay in diagnosis, as well as drug resistance remain major problems. In recent years, as an important mediator of material and information exchange between cells in the tumor microenvironment, exosomes have attracted widespread attention for their role in tumor development. It has been reported that tumor-derived exosomes may act as regulators and have an important effect on the metastasis, drug resistance formation, and providing targets for early diagnosis of clear cell renal cell carcinoma. Therefore, the extensive study of tumour-derived exosomes will provide a meaningful reference for the development of the diagnostic and therapeutic field of clear cell renal cell carcinoma. This article reviews the biological role and research progress of tumor-derived exosomes in different aspects of premetastatic niche formation, tumor angiogenesis, and epithelial-mesenchymal transition during the progression of clear cell renal cell carcinoma. In addition, the role of tumor-derived exosomes in the development of drug resistance in clear cell renal cell carcinoma is also addressed in this review. Furthermore, recent studies have found that cargoes of exosomes in serum and urine, for example, a series of miRNAs, have the potential to be biological markers of clear cell renal cell carcinoma and provide meaningful targets for early diagnosis and monitoring of tumors, which is also covered in this article.

KEYWORDS

**clear cell renal cell carcinoma, tumor-derived exosomes, metastasis, diagnosis, drug resistance**

# 1 Introduction

Kidney cancer is the third most common cancer of the urinary system. According to the World Health Organization, renal cancer ranked 16th among all cancers worldwide in terms of new cases and deaths in 2020 (1). Clear cell renal cell carcinoma (ccRCC), a renal cortical tumor characterized by malignant epithelial cells, is the most common type of kidney cancer, accounting for 80% of total cases, and other subtypes are mainly papillary renal cell carcinoma, suspicious cell carcinoma, clear cell papillary renal cell carcinoma, and MIT family translocation-associated renal cell carcinoma (2). At present, the global morbidity and mortality of ccRCC continue to rise, and although recent progress has been made in the study of ccRCC, its prognosis is still poor. The diagnosis and treatment of ccRCC also involves many social factors, such as racial imbalances, like the racial disparities that exist among patients undergoing robotic radical nephrectomy (3). In fact, more than 60% of ccRCCs are found incidentally. Despite improvements in imaging techniques, more than 30% of patients have already been found to have tumor metastases, especially bone metastases, lung metastases, accounting for approximately 15% of total cases at the time of diagnosis (4–6). At the same time, the drug resistance of ccRCC is also one of the main reasons for their clinical treatment failure, especially metastatic ccRCC. For example, vascular endothelial growth factor (VEGF), mammalian target of rapamycin (mTOR) inhibitors, and RAF kinase have been used to treat ccRCC (7), but these drugs often encounter the problem of drug resistance in clinical application. Therefore, if ccRCC can be diagnosed and monitored early, it is beneficial to improve the clinical management of ccRCC, perform medical or surgical intervention before tumor cell metastasis, and prolong the overall survival of ccRCC patients. There are no specific molecular markers for ccRCC for clinical use (8). However, it has been found that tumor-released extracellular vesicles, for example, exosomes, may represent a new class of biomarkers for liquid biopsy, providing a meaningful target for the early diagnosis and monitoring of ccRCC (9).

Extracellular vesicles (EVs) are a collective term for various vesicular structures with membrane structures released by cells. According to their different diameters, they can be divided into three types: exosomes, microvesicles, and apoptotic bodies (10). Among them, exosomes are goblet extracellular vesicles with a diameter of 40-100 nm, surrounded by a lipid bilayer membrane, which is currently a research hotspot. Exosomes were found in nearly all body fluids, including serum, urine, cerebrospinal fluid, saliva, and tears. Human normal cells as well as tumor cells can secrete exosomes, which play an important role in intercellular communication, and the cargo transported by them includes proteins, mRNAs, miRNAs and signaling molecules. Exosomes regulate the physiological state of cells by carrying and transmitting signaling molecules and are involved in antigen presentation, cell differentiation, growth, tumor immune response, and migration and invasion of tumor cells (11). Many researches have shown that tumor-derived exosomes play an important role in the occurrence, progression and metastasis, immune escape, drug resistance and early diagnosis of ccRCC by transporting different cargoes and mediating related signaling pathways (12–16), and these findings provide us with meaningful targets for further study of ccRCC (shown in Figure 1).

# 2 Tumor-derived exosomes promote metastasis of ccRCC

## 2.1 Involved in the formation of premetastatic niches

Paget first proposed the'seed and soil' theory in 1889, which likens metastatic tumor cells as seeds and potential metastatic sites as soil to explain that tumor metastasis has organ tropism (17). There is increasing evidence that primary tumors can remotely regulate the formation of the tumor microenvironment in secondary organs by secreting a variety of cytokines, allowing tumor cells to colonize the circulation. This local tumor microenvironment is known as the premetastatic niche (PMN) (18). The establishment of PMN is closely related to the release of tumor-secreted factor (TSF) and tumor-secreted exosome (TSE) from the primary tumor, and TSE is considered to be the main driver of PMN formation. A key step in the process of promoting the dissemination of tumor cells from the primary site to the metastatic site is the enhancement of vascular permeability. A study by Zeng showed that exosomes secreted by colorectal cancer (CRC) cells are rich in miR-25-3p, which can disrupt the tight junctions of vascular endothelial cells by targeting the transcription factors KLF2 and KLF4, leading to tumor angiogenesis increased permeability, thereby promoting tumor metastasis (19).

In ccRCC, the ability to promote PMN formation mainly comes from EVs secreted by cancer stem cells (CSC). Renal CSC

**Abbreviations:** ccRCC, Clear cell renal cell carcinoma; VEGF, Vascular endothelial growth factor; mTOR, Mammalian target of rapamycin; EVs, Extracellular vesicles; PMN, premetastatic niche; TSF, Tumor-secreted factor; TSE, Tumor-secreted exosome; CRC, Colorectal cancer; CSC, Cancer stem cells; CAFS, Cancer-associated fibroblasts; SFRP1, Secreted frizzled-related protein 1; TNF-α, Tumor necrosis factor-alpha; ApoC1, Apolipoprotein C1; CA9, Carbonic anhydrase 9; TKI, Tyrosine kinase inhibitor; HIF1α, Hypoxia-inducible factor 1 alpha; EMT, Epithelial-mesenchymal transition; GGT, γ-glutamyltransferase; HSPA5, Heat shock protein 5; PTRF, Polymerase I and transcription release factor; EGFR, Epidermal growth factor receptor; PDGFR-β, PDGF-β-receptor; Flt-3, FMS-like tyrosine kinase 3; KTZ, Ketoconazole; MCF-7/ADM, Human breast cancer enamycin-resistant cells.

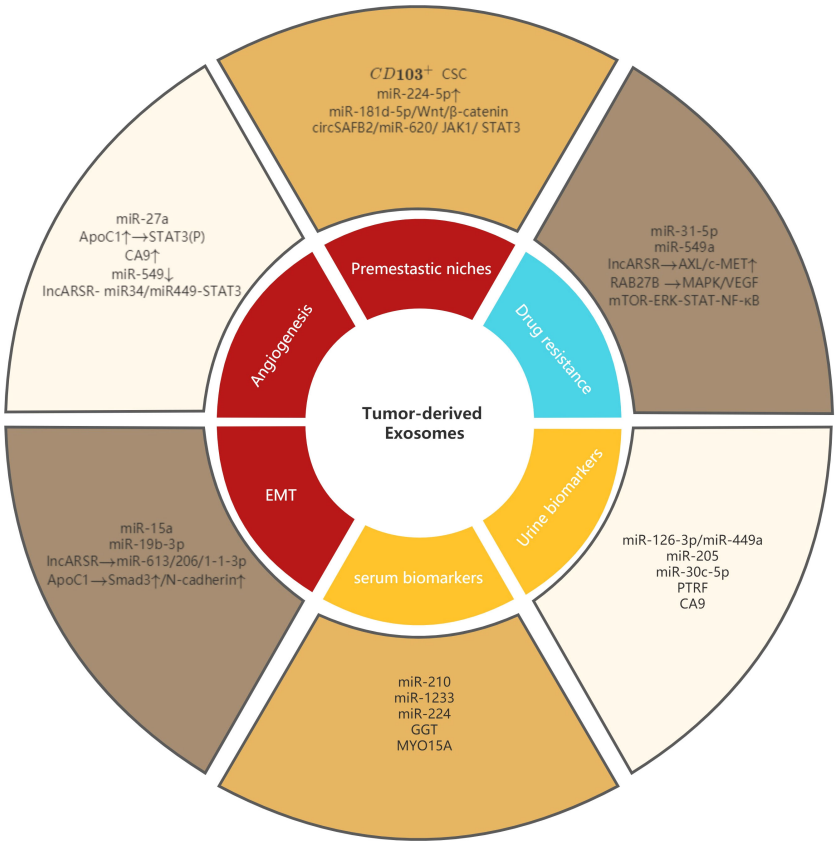


FIGURE 1
Role of tumor-derived exosomes in metastasis, diagnosis and drug resistance of ccRCC. Tumor-derived exosomes involved in premetastatic niches, angiogenesis, EMT and drug resistance of ccRCC. Tumor-derived exosomes can also be used as serum biomarkers and urine biomarkers in ccRCC.

EVs contain a variety of miRNAs, which may be involved in biological pathways related to cell growth and cell matrix adhesion (20). Studies have shown that CSC exosomes from metastatic ccRCC patients accelerate ccRCC cell proliferation and lung metastasis. Especially CSC exosomes expressing integrin CD103. Wang et al. found that the proportion of exosomes in the blood of metastatic ccRCC patients to total exosomes was significantly higher than that of non-metastatic ccRCC patients. CSC exosomes are more organotropic than CSC exosomes and play an important role in targeting distant organs and forming PMN (13).

Furthermore, in the PMN of ccRCC, besides tumor cells, there are a large number of stromal cells and immune cells. Among them, cancer-associated fibroblasts (CAFS) are the main stromal components in the ccRCC tumor microenvironment and are closely related to tumor progression (21). Liu et al. found that miR-224-5p was enriched in CAFS-derived exosomes in PMN, and miR-224-5p could be transferred from CAFS-derived exosomes into ccRCC cells, and then further secreted in the form of exosomes. After upregulation of miR-224-5p, the number of ccRCC cells undergoing migration and invasion was significantly increased (22). Fu also found that in the process of ccRCC metastasis, CAFS-derived exosomes can enter ccRCC cells through internalization, inhibit tumor cell apoptosis and regulate cell cycle, and promote the progression of ccRCC (12). A new study found that CAFS-derived exosomes directly inhibit ring finger protein 43 (RNF43) expression and activate the Wnt/β-catenin signaling pathway by delivering miR-181d-5p, thereby promoting RCC stemness and progression (23). Additionally, Huang et al. found that circSAFB2 delivered by ccRCC-derived exosomes mediates the polarization of M2 macrophages *via* the miR-620/JAK1/STAT3 axis, thereby remodeling the tumor microenvironment and ultimately promoting ccRCC metastasis (24).

In conclusion, tumor-associated exosomes are involved in the formation of PMN, providing suitable environmental conditions for the metastasis of ccRCC.

## 2.2 Involved in angiogenesis of ccRCC

It is well known that tumor angiogenesis is an important step in the metastatic progression of malignant tumors. Relevant studies have shown that tumor-derived exosomes play an important role in tumor angiogenesis. For example, Yang et al. demonstrated that exosomal miR-130a promoted gastric cancer angiogenesis by targeting C-MYB in vascular endothelial cells, thereby promoting tumor metastasis (25). Huang et al. found that exosomes derived from liver cancer cells participate in the angiogenesis of liver cancer by carrying circRNA-100,338, and then promote the lung metastasis of liver cancer (26). In addition, exosomal miR-27a from pancreatic cancer cells can participate in the angiogenesis of human microvascular endothelial cells by targeting BTG2 (27).

In ccRCC, tumor-derived exosomes can also participate in tumor angiogenesis by delivering a variety of miRNA and protein molecules. A recent study found that gene secreted frizzled-related protein 1 (SFRP1) was underexpressed and miR-27a was overexpressed in ccRCC cells. Grange et al. have proved that miR-27a acts as an oncomiR. CcRCC cell-derived exosomes accelerate tumor growth and angiogenesis *in vivo* by delivering miR-27a, downregulating the expression of the tumor suppressor gene SFRP1, while increasing the expression levels of vascular endothelial growth factor and tumor necrosis factor-alpha (TNF-α) (28). According to the Oncomine database, we found that among all cancer types, the greatest upregulation of apolipoprotein C1 (ApoC1) was observed in kidney cancer samples. Li et al. demonstrated that ccRCC-derived exosomes can mediate the transfer of ApoC1 from ccRCC cells to tumor vascular endothelial cells, which in turn promotes angiogenesis by activating the transcription factor STAT3, and promotes the metastasis of ccRCC cells (29). In addition, studies have also shown that carbonic anhydrase 9 (CA9) in exosomes released from hypoxic ccRCC cells can enhance angiogenesis in the tumor microenvironment, thereby promoting cancer progression (30). Interestingly, miRNAs delivered by tumor-derived exosomes may also play a role in inhibiting ccRCC angiogenesis. Xuan et al. found that miR-549a expression was lower in tyrosine kinase inhibitor (TKI)-resistant ccRCC cells and their derived exosomes. They demonstrated that exosomal miR-549a could reduce the expression level of hypoxia-inducible factor 1 alpha subunit (HIF1α) by binding to the 3'-UTR region of HIF1α mRNA, which in turn attenuate tumor angiogenesis and endothelial cell migration in ccRCC (31). A new study found that ccRCC-derived exosomes can activate the miR34/miR449-STAT3 signaling pathway by delivering lncARSR, which in turn promotes the transformation of M1 macrophages to M2 and enhances the phagocytic ability of macrophages, which in turn induces angiogenesis and ultimately promotes the development and progression of tumors (32). In summary, ccRCC cell-derived exosomes participate in tumor angiogenesis by delivering a variety of miRNA and protein molecules, thereby affecting the metastasis process of ccRCC.

## 2.3 Involved in EMT of ccRCC

Epithelial-mesenchymal transition (EMT) is the process by which epithelial cells (E cells) lose their polarity and transform into mesenchymal cells (M cells). EMT is widely recognized as a key event in tumor invasion and distant metastasis. In part, this process occurs through the degradation of cell adhesion junctions and changes in gene expression, resulting in an enhanced ability of tumor cells to invade tissues and metastasize (33). At present, a large number of studies have shown that tumor-derived exosomes can mediate EMT of tumor cells by delivering a variety of miRNA and protein molecules, and promote the ability of tumor cells to invade and migrate. For example, He et al. found that exosomes derived from non-small cell lung cancer cells, targeting the mTOR pathway by delivering miR-499a-5p, could promote the EMT process of lung adenocarcinoma (34). You et al. also demonstrated that exosomes derived from cervical cancer cells, stimulated by TGF-β1, can deliver miR-663b targeting MGAT3 to promote EMT and metastasis of cervical cancer (35).

In ccRCC, the relationship between tumor-derived exosomes and EMT has also received attention. Li et al. demonstrated that miR-15a was upregulated in exosomes derived from ccRCC cells. Furthermore, exosomal miR-15a can enhance EMT activity in ccRCC by downregulating BTG2 gene and promoting PI3K/AKT signaling pathway activity (36). Wang et al. demonstrated that in metastatic ccRCC patients, CSC exosomes induced EMT by transporting miR-19b-3p to cancer cells and inhibiting the expression of PTEN gene (13). Hu et al. found that lncHILAR was abundant in the cytoplasm of ccRCC cells, and exosomes were its main carrier. At the same time, they found that exosomal lncHILAR was associated with EMT gene bank markers through gene enrichment analysis. Under normoxia and hypoxia, knockdown of lncHILAR could reverse EMT, indicating that exosomal lncHILAR could promote ccRCC metastasis by inducing EMT (37). Li et al. also found that the overexpression of exosomal ApoC1 increased the mRNA levels of Smad3 and N-cadherin on ccRCC cells, which in turn promoted the EMT process and increased the migration and invasion abilities of ccRCC (29). In conclusion, ccRCC cell-derived exosomes can promote the EMT process of ccRCC by delivering various cargos, especially miRNA, and then promote the metastasis of ccRCC.

# 3 Tumor-derived exosomes promote drug resistance in ccRCC

As mentioned above, tumor-derived exosomes do play a non-negligible role in the metastasis of ccRCC. Moreover, tumor-derived exosomes can further cause drug resistance and immune escape of tumor cells on this basis. The development of drug resistance is one of the main causes of clinical treatment failure in tumors. At present, renal cancer is not sensitive to radiotherapy and chemotherapy, and patients with advanced

renal cancer treated with targeted agents have a low rate of complete remission. In most patients with advanced or metastatic ccRCC, systemic first-line therapy mainly includes immune checkpoint inhibitors, TKIs, and mTOR inhibitors (38). Among them, TKI is one of the first-line therapies for advanced ccRCC, and most patients will eventually develop TKI-resistant ccRCC and then develop metastasis after 6-15 months (39). Numerous studies have shown that drug-resistant tumor cells secrete exosomes containing genetic information of multiple drug-resistance-related proteins, which in turn leads to the acquisition of drug resistance by other drug-sensitive tumor cells (40). In addition, tumor-specific exosomes have also been found to play an important role in immunosuppression (41, 42). For example, exosomes secreted by kidney cancer cells can induce immune responses in T cells to trigger apoptosis of activated T lymphocytes by activating the caspase pathway (16). In ccRCC patients, tumor-derived exosomes play an important role in the formation of drug resistance in ccRCC, and exosomes can mediate drug resistance by delivering miRNA, lncRNA, and protein molecules. The role of tumor-derived exosomes in drug resistance in ccRCC is specifically addressed in the following section.

## 3.1 Exosomal miRNAs mediate ccRCC drug resistance

Sorafenib is an oral multikinase inhibitor that targets growth signaling and angiogenesis in ccRCC by blocking VEGF-2-receptor (VEGFR-2), VEGF-3-receptor (VEGFR-3), PDGF-β-receptor (PDGFR-β), RAF- 1, c-Kit protein (c-Kit), and FMS-like tyrosine kinase 3 (Flt-3) (43). He et al. found that tumor-derived exosomes transferred drug resistance information from sorafenib-resistant ccRCC cells to non-resistant ccRCC cells by delivering miR-31-5p (44). At the same time, they further demonstrated that miR-31-5p promotes sorafenib resistance by targeting the 3'-UTR region of the MLH1 gene. But interestingly, Xuan et al. found that miR-549a expression was lower in TKI-resistant ccRCC cells and their derived exosomes compared with TKI-sensitive ccRCC, and they suggested that delivery of miR-549a to TKI-resistant renal cancer cells may reverse their own TKI resistance (31).

## 3.2 Exosomal lncRNA mediate ccRCC drug resistance

LncRNAs are noncoding RNAs greater than 200 nucleotides in length that have been found to be aberrantly expressed in various human cancer types and play important roles in tumorigenesis, metastasis, and drug resistance (45–47). Sunitinib, an oral multi-targeted TKI with a strong anti-angiogenic effect, is the first-line treatment for advanced ccRCC, but it is ineffective in a quarter of ccRCC patients, and most patients will relapse after 1 year of treatment (48, 49). Qu et al. found that an uncharacterized lncRNA was abundant in sunitinib-resistant ccRCC cell lines and their exosomes, and named it lncARSR. LncARSR can be secreted from resistant cells through exosomes to convert sunitinib-sensitive cells into resistant cells, thereby spreading drug resistance (50). Furthermore, they proved that lncARSR promotes sunitinib resistance by competitively binding to miR-34 and miR-449, leading to the upregulation of AXL/c-MET and activation of STAT3, AKT and ERK signaling pathways. In turn, activated AKT promotes lncARSR expression by inhibiting transcription factors FOXO1 and FOXO3a, forming a positive feedback loop. Greenberg et al. demonstrated that ketoconazole (KTZ) can reduce tumor-specific exosomes by inhibiting the protein expression of Alix, nSMase and Rab27a related to exosome biogenesis and secretion in ccRCC cells,reducing the delivery of substances from exosomes, which in turn enhances the efficacy of sunitinib and reduces the development of drug resistance (51).

## 3.3 Exosomal protein molecule mediate ccRCC drug resistance

It has been found that tumor-derived exosomes can mediate targeted drug resistance by delivering related protein molecules. Studies have shown that in breast cancer, exosomes derived from human breast cancer enamycin-resistant cells (MCF-7/ADM) are rich in P-gp and UCH-L1 proteins. MCF-7/ADM-derived exosomes can induce drug resistance in drug sensitive cells by internalizing and delivering P-gp and UCH-L1 to drug sensitive cells (52, 53). In ccRCC patients, Tsuruda et al. found that the protein expression level of RAB27B was significantly increased in sunitinib-resistant ccRCC cell lines. RAB27B is one of the main proteins involved in exosome secretion, and RAB27B may be involved in the formation of drug resistance through MAPK and VEGF signaling pathways (54). Wang et al. found that ccRCC cell-derived exosomes can help tumor cells evade immune killing and develop drug resistance through the mTOR-ERK-STAT-NF-κB protein signaling pathway (14).

# 4 Exosomes offer potential targets for early diagnosis of ccRCC

As the study of tumor-derived exosomes in ccRCC progresses, the value of tumor-derived exosomes as potential targets in the diagnosis of ccRCC is becoming increasingly evident. With the widespread application of imaging techniques, advances in surgical techniques, and improvements in pathological examinations, the early diagnosis of ccRCC has increased, and the prognosis of ccRCC has also improved (55). Although new or

recurrent tumors can be diagnosed clinically by abdominal and chest CT, 30% of patients with ccRCC, when detected, already develop metastases (4–6). Once metastasis occurs, the cure rate of ccRCC will be greatly reduced, which is one of the important reasons hindering the further improvement of the cure rate of ccRCC. Therefore, there is a great need to have specific biomarkers suitable for ccRCC screening and monitoring as a supplement to imaging diagnosis. From a clinical standpoint, liquid biopsies are preferred over tissue biopsies because they are less invasive. Therefore, screening candidate biomarkers from body fluids such as serum or urine is crucial. A previous study reported that VEGF, VEGFR2, and CA9 regulated by HIF-1α were important biomarkers in liquid biopsies of ccRCC (56). In recent years, exosomes have become a new source of non-invasive tumor biomarkers. The bilayer membrane structure of the exosome is able to resist exogenous RNases and proteases, which produce more stable mRNAs, miRNAs, and intracellular functional proteins, making the exosome a sensitive marker for disease diagnosis (57). Additionally, exosomes derived from tumor cells are abundant in blood, urine and other body fluids, and have the advantages of easy access, non-invasive examination and tumor specificity (58). Studies have demonstrated the potential of exosomes as diagnostic markers for bladder cancer, pancreatic cancer, liver cancer and other tumors (59–61). In the serum and urine of ccRCC patients, the cargo of tumor-derived exosomes, for example, a series of miRNAs, can also be used as biological markers of ccRCC to provide meaningful targets for the early diagnosis and monitoring of ccRCC (Table 1).

## 4.1 Exosomal biomarkers in serum

Exosomes in the serum of ccRCC patients can serve as a new source of ccRCC biomarkers. Zhang et al. found that ccRCC patients with different TNM stages had significantly higher expression levels of exosomal miR-210 and miR-1233 in their serum than healthy controls, and significantly lower expression levels of exosomal miR-210 and miR-1233 after surgery. They demonstrated that exosomal miR-210 and miR-1233 in serum may be used for liquid biopsy and contribute to the diagnosis and monitoring of ccRCC patients (15). Wang et al. also demonstrated that serum exosomal miR-210 was upregulated in ccRCC, especially in patients with advanced tumor stage, high Fuhrman grade, and metastases. Patients with ccRCC overexpressing miR-210 have a shorter chance of disease recurrence and survival time. Meanwhile, they found that exosomal miR-210 was superior to serum miR-210 in detecting ccRCC and was a good prognostic biomarker (62). Fujii et al. found that high levels of exosomal miR-224 in the serum of ccRCC patients were associated with poor patient prognosis (63). They demonstrated that exosomal miR-224 in the serum of ccRCC patients may be a promising prognostic biomarker for detecting microinvasion or tumor

TABLE 1 List of some original articles cited in this section, with the main results summarized.

| Reference | Evaluationmethods | Number of patient samples | Results |
|---|---|---|---|
| **Zhang et al.** (15) | qRT-PCR | Serum of 82 ccRCC patients and 80 healthy controls | Exosomal miR-210 and exosomal miR-1233 significantly higher in each stage of ccRCC compared to controls |
| **Wang et al.** (62) | qRT-PCR | Serum of 45 ccRCC patients (including 5 patients with lung metastases) and 30 healthy controls | Exosomal miR-210 is upregulated in ccRCC, especially in patients with advanced tumor stage, high Fuhrman grade and metastases. |
| **Fujii et al.** (63) | qRT-PCR | Serum of 108 ccRCC patients | Exosomal miR-224 is highly expressed in ccRCC and is associated with poor prognosis. |
| **Horie et al.** (64). | Western Blot | Serum of 28 RCC patients | Serum exosomal GGT activity was significantly increased in patients with advanced RCC and in patients with microvascular invasion. |
| **Butz et al.** (65) | RT-PCR | Urine of 81 ccRCC patients, 24 patients with benign lesions, and 33 healthy controls | Combination of exosomal miR-126-3p and miR-449a in urine can discriminate healthy and ccRCC patients with high sensitivity. |
| **Song et al.** (66) | qRT-PCR | Urine of 70 early-stage (T1aN0M0) ccRCC patients and 30 healthy controls | The expression level of miR-30c-5p in urinary exosomes of ccRCC patients was significantly lower than that of controls, and the overexpression of miR-30c-5p inhibited ccRCC progression *in vitro* and *in vivo*. |
| **Zhao et al.** (67) | qRT-PCR/Western Blot | Gene expression samples were obtained from 533 ccRCC samples of The Cancer Genome Atlas (TCGA). Blood, urine and tumor tissue samples were collected from 4 ccRCC patients. | PTRF could be detected in the exosomes of the urine samples of ccRCC patients, which was significantly higher than that of the normal control group, and the expression of PTRF was significantly decreased after surgery. PTRF is regulated by the SHC1 gene through the EGFR pathway. |

metastasis after surgery in ccRCC patients. In addition, studies have found that γ-glutamyltransferase (GGT) activity in serum exosomes is significantly elevated in patients with advanced RCC and distant metastases, as well as in patients with microvascular invasion (64). Exosomal GGT activity in serum may be a clinically useful marker for advanced ccRCC patients, and its combined use with conventional diagnostic modalities may improve the diagnosis of ccRCC patients. In a recent study, exosomal MYO15A was found to be significantly elevated in the serum of ccRCC patients, associated with a poorer prognosis, and may be a diagnostic target for ccRCC (68).

## 4.2 Exosomal biomarkers in urine

Exosomes in the urine of ccRCC patients can also serve as a new source of ccRCC biomarkers. A urine sample is preferable to a blood sample because it is anatomically close to the kidney and is one of the most readily available body fluids. Butz et al. found that the combination of exosomal miR-126-3p and miR-449a in urine can discriminate healthy people from ccRCC patients with high sensitivity. They demonstrated the potential utility of urinary exosomal miRNAs as a potential diagnostic tool for ccRCC, especially for small renal masses (65). Some studies have found that in ccRCC patients, urinalysis of exosomal miRNAs may be more suitable for selecting down-regulated miRNAs as biomarkers. Many of these down-regulated miRNAs, such as miR-205, may have large differences in expression levels between tumor and non-tumor cells, which helps make ccRCC easier to detect (69). Song et al. found that the expression level of miR-30c-5p in urinary exosomes of ccRCC patients was significantly lower than that in healthy controls. Furthermore, they discovered that miR-30c-5p could inhibit the progression of ccRCC by targeting heat shock protein 5 (HSPA5). Their study further demonstrated that urinary exosomal miR-30c-5p could be used as a highly specific and sensitive biomarker for the diagnosis and monitoring of ccRCC progression (66). Zhao et al. found that polymerase I and transcription release factor (PTRF) in urinary exosomes may also be potential biomarkers for ccRCC (67). PTRF could be detected in exosomes in urine samples of ccRCC patients, and was significantly higher than that in normal individuals. At the same time, the expression of PTRF was significantly decreased after operation. They demonstrated that PTRF is regulated by the SHC1 gene through the epidermal growth factor receptor (EGFR) pathway. By high-throughput sequencing, MB et al. found five novel mRNAs specific for stage I ccRCC in urinary exosomes, namely NME2, AAMP, CAPNS1, VAMP8, and MYL12B (70). In addition, a pilot study on the development of a reliable technique to detect exosomal CA9 in the urine of CCRCC patients for molecular diagnosis of CCRCC is underway in France (71).

# 5 Conclusion and prospect

Tumor-derived exosomes have received increasing attention in tumor research due to their role in intracellular communication during tumor progression. But compared with other cancer types, ccRCC has been relatively neglected in this research hotspot. In this review, we introduced the biological roles and research progress of tumor-derived exosomes in different aspects such as PMN formation, tumor angiogenesis, and EMT in the progression of ccRCC metastasis. Meanwhile, tumor-derived exosomes also play an important role in the development of ccRCC drug resistance. In ccRCC patients, tumor-derived exosomes can mediate drug resistance by delivering miRNA, lncRNA, and protein molecules. However, it is worth noting that the detailed mechanism of action of tumor-derived exosomes in ccRCC metastasis and drug resistance development remains to be further elucidated. In addition, the application of tumor-derived exosomes in ccRCC liquid biopsy holds great promise. Due to the encapsulation of the lipid bilayer membrane, the bioactive molecules within exosomes are not degraded by exogenous nucleases or proteases and are stable in biological fluids (72). A series of cargoes delivered by exosomes, especially a series of miRNAs, can be used as biomarkers of ccRCC, providing meaningful targets for early diagnosis and monitoring of ccRCC. Of course, the molecular characterization of each specific subtype of diagnostic markers is relevant and should be acknowledged like it has be done for urothelial malignancies (73). Meanwhile, miRNAs with high specificity and sensitivity in urinary exosomes need to be further discovered and identified, and simple biological tests for the diagnosis and monitoring of ccRCC need to be developed. At present, there are few studies on tumor-derived exosomes in the early monitoring and drug resistance development of ccRCC. In the future, more and more studies will focus on the application of tumor-derived exosomes in ccRCC liquid biopsy and treatment. In conclusion, a lot of work needs to be done to better understand the role of tumor-derived exosomes in ccRCC metastasis, drug resistance and diagnosis, and to make the potential clinical utility of tumor-derived exosomes in ccRCC a reality.

# Author contributions

All authors conceived and refined the idea. TJ conducted the literature searches and drafted the paper. ZZ, JZ refined the details of the article. MC and SC provided expert advice and

support throughout. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Safety and efficacy of the pan-FGFR inhibitor erdafitinib in advanced urothelial carcinoma and other solid tumors: A systematic review and meta-analysis

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**Objective:** This review aimed to comprehensively analyze the safety and efficacy of erdafitinib in treating advanced and metastatic urothelial carcinoma and other solid tumors.

**Methods:** PubMed, Embase, and ClinicalTrials.gov were searched until 10 February 2022. The safety outcome as adverse events and efficacy outcomes, including objective response rate, stable disease rates, and progressive disease rates, were selected and analyzed by comprehensive meta-analysis version 3.0 and STATA 15.0.

**Results:** The most common all-grade adverse events were hyperphosphatemia, dry mouth, stomatitis, diarrhea, and dysgeusia. The occurrence of ≥3 adverse events was relatively low, and stomatitis and hyponatremia were the most common. Moreover, eye disorders could not be ignored. Efficacy in urothelial carcinoma patients was obviously better than in other solid tumor patients, with a higher objective response rate (0.38 versus 0.10) and lower progressive disease rate (0.26 versus 0.68). All responses occurred in patients with fibroblast growth factor receptor (FGFR) alteration. In those patients, a specific FGFR alteration (*FGFR3-TACC3*) was observed to have a maximum response.

**Conclusion:** Erdafitinib has satisfactory clinical activity for metastatic urothelial carcinoma and other solid tumors, while the toxicity is acceptable. With more RCTs and combination therapy trials published, erdafitinib will be applied widely.

KEYWORDS

**erdafitinib, urothelial carcinoma, FGFR, hyperphosphatemia, central serous chorioretinopathy**

# 1 Introduction

Urothelial carcinoma (UC) refers to a transitional urothelial tumor of the urinary tract. It can be divided into upper and lower urothelial carcinoma according to the diseased region. Bladder cancer is the most common type of lower urothelial carcinoma, accounting for 90% of the total. Other types of urothelial carcinoma, such as renal pelvis and urethral carcinoma, are scarce. Generally, UC is the 10th most commonly diagnosed cancer worldwide, with approximately 573,000 new cases and 213,000 deaths in 2020 (1, 2). It has a predominance of male patients, with respective incidence and mortality rates of 9.5 and 3.3 per 100,000 among men, which are approximately four times those among women globally. For non-muscle-invasive bladder cancer, the present treatment is transurethral resection of bladder tumor (TURBT), intravesical chemotherapy, and intravesical BCG immunotherapy (3, 4). For unresectable or metastatic bladder tumor, platinum-based combination chemotherapies are the major therapy (5, 6). However, the efficacy of platinum-based drugs is not satisfactory, with a median survival of only 7.4 months. Since 2019, the application of FGFR inhibitors has innovated treatment options for advanced and metastatic UC, increasing the median survival by 3 months (7).

Erdafitinib is an ATP-competitive inhibitor of FGFR1–4. It is a small molecule inhibitor (SMi) that reversibly inhibits FGFR kinase autophosphorylation and decreases resultant downstream signaling (8). Under physiological conditions, fibroblast growth factor receptor 1–4 (FGFR1–4) bind to fibroblast growth factors (FGFs) to exert tyrosine kinase regulatory effects (9), which play a vital role in angiogenesis and damage repair. The FGFR molecule includes three extracellular immunoglobulin domains, one transmembrane domain, and one intracellular domain. The intracellular domain can activate the RAS-MAPK-ERK and PI3K-AKT pathways (10–14). However, gene amplification, mutation, rearrangement, or translocations occur and alter the signaling pathway, which leads to cell proliferation or migration (15–19).

The mechanism of erdafitinib inhibits these pathways from upstream, which can impede the growth of tumors. In a study, BLC2001(NCT02365597), those patients who had not responded to PD-1 treatment achieved an objective response with erdafitinib treatment. In other clinical trials (NCT01703481 and NCT01962532), erdafitinib resulted in prolonged progression-free survival and median duration of response. Therefore, the FDA granted approval to erdafitinib for metastatic urothelial carcinoma in patients with susceptible alterations in FGFR2 or FGFR3 who have progressed platinum-containing chemotherapy, including within neoadjuvant or adjuvant platinum-containing chemotherapy. It was also the first FGFR kinase inhibitor approved by FDA for urothelial carcinoma. However, erdafitinib causes adverse effects, such as increased serum phosphate, stomatitis, and central serous chorioretinopathy. These adverse events (AEs) may reduce medication compliance, which leads to reduced efficacy.

To our knowledge, there have not been any meta-analyses about the safety and efficacy of erdafitinib. To offer evidence-based references for physicians, we conducted this study to determine the most meaningful AEs and efficacy outcomes of erdafitinib.

**Abbreviations:** UC, Urothelial carcinoma; SMi, Small molecule inhibitor; CRR, Complete response rate; PRR, Partial response rate; ORR, Objective response rate; SD, Stable disease; HR, Hazard ratio; RR, Risk ratio; PFS, Progression-free survival; AE, Adverse events; CMA, Comprehensive meta-analysis; ROBINS-I, Risk of bias in nonrandomized studies of interventions; pCSC, Pseudo-central serous chorioretinopathy; CSC, Central serous chorioretinopathy; RPE, Retinal pigment epithelium.

# 2 Methods

## 2.1 Literature search

PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) guidelines were followed to complete this meta-analysis. PubMed, Embase, ClinicalTrials.gov, Cochrane Library, and China National Knowledge Infrastructure (CNKI) were searched for clinical trials and related articles until 10 February 2022. In addition, the references of reviews or trials related to erdafitinib were screened to avoid the omission of valuable articles. There was no restriction to language. The following words were used for searching: “erdafitinib” or “JNJ-42756493” or “Balversa.”

## 2.2 Study selection

Two reviewers independently selected the search results according to PRISMA flow diagrams. Discrepancies were resolved by the third author. The inclusion criteria were as follows: (1) patients were confirmed to have carcinoma by pathology; (2) the gene alteration of patients was included in fusion, mutation, and amplification; (3) the interventions of studies included erdafitinib singly or combined with other drugs; and (4) relevant data of efficacy and safety were reported. Unrelated articles, case reports, retrospective studies, reviews, and studies that lacked necessary data or full text were excluded.

## 2.3 Data extraction

Data from the included articles were extracted independently by two reviewers while discussing disagreements with the third reviewers. Basic information, such as the first author’s name, publication year, clinical trial sequence number, study phase, study design, sample size, median age, median follow-up, carcinoma histology, and treatment regimens, was extracted.

The efficacy indicators included the complete response rate (CRR), partial response rate (PRR), objective response rate (ORR, which referred to the presence of at least a confirmed complete response or confirmed partial response), stable disease (SD) rate, progressive disease rate (defined as >20% increase in the longest diameters of target lesions or the appearance of a new lesion), hazard ratio (HR), and risk ratio (RR). The data used for safety analyses were collected from all-grade and grade ≥3 AEs.

## 2.4 Statistical analysis

STATA was used to count the standard error of CR, PR, and ORR. We conducted a single-rate meta-analysis to draw a forest plot. Meanwhile, the odds ratio was calculated to compare erdafitinib with other treatments. Comprehensive Meta-Analysis (CMA) Version 3.0 was used to analyze all-grade and grade ≥3 AEs to calculate the event rate and 95% CI. STATA and CMA were used to analyze heterogeneity. $I^2 > 50\%$ and $p < 0.05$ were considered as high heterogeneity. A fixed-effects model was used for $I^2$ <50%; random-effects model analysis was used for $I^2$ > 50%.

If we included 10 more studies, STATA 15.0 was used to analyze the heterogeneity of the included literature. If there was high heterogeneity, the METAREG command was used for meta-regression analysis. We discussed the sources of heterogeneity. At the same time, if 10 more articles were included, Begg's and Egger's funnel plots were conducted to investigate publication bias.

## 2.5 Risk of bias and study quality

The risk of bias of randomized controlled trials was obtained by RevMan 5.4 (The Cochrane Collaboration). The articles were evaluated in the following processes: sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and others. For the nonrandomized studies, the bias was assessed by the risk of bias in nonrandomized studies of interventions (ROBINS-I) tool (20, 21). ROBINS-I included seven domains: allocation bias, selection bias, observer bias, performance bias, attrition bias, detection bias, and analysis reporting bias. Meanwhile, ROBINS-I was used to assess the quality of non-randomized studies.

# 3 Results

## 3.1 Study selection

A total of 968 articles were produced through the search strategy. Five articles were searched through the references of previous reviews. After removing duplications, 557 were screened based on the title and abstract, and 546 unrelated articles were excluded. Eleven studies were selected, but five articles lacked the necessary data. Finally, six trials were included. The study selection procedure is shown in Figure 1.

## 3.2 Characteristics of studies

The included studies were published from 2015 to 2022. There were three phase I and three phase II clinical trials, and all were nonrandomized. In phase I trials, patients with various kinds of solid tumors were included, in which some patients with UC were not being classified between other solid tumors. Meanwhile, every patient in phase II was suffering from urothelial carcinoma. Most of the included trials described FGFR alterations in patients except NCT01703481 (Tabernero,2015) and NCT01962532 (Nishina,2017). Mutations and fusions were the major gene alterations of FGFR. In NCT02365597 (Loriot,2019), the proportion of FGFR3 mutations was 74/99, while that of FGFR3 fusions was 25/99. In Bahleda's research, the scale of FGFR mutations or fusions [mutation (+)/fusion (−) OR mutation (−)/fusion (+)] was 58/187, that of the amplifications was 45/187, and the ratio of co-alteration [mutation (+)/fusion (+)] was 5/187. In addition, all 12 patients in Monterio's trial were found to have FGFR3 alterations. In NCT02365597 (Siefker-Radtke,2022), 70/101 were FGFR mutation (+)/fusion (−), 25/101 were mutation (−)/fusion (+), 6/101 were FGFR mutation (+)/fusion (+), and 5/101 were FGFR mutation/fusion co-alterations.

Erdafitinib was singly used for all six articles with constant, escalation, or intermittent doses. Most of the persistent doses ranged from 6 to 9 mg, while the intermittent dose was 10 or 12 mg. Notably, we found that in the latest trials, a constant dose of 8–9 mg was used more frequently, which might be related to the recommendation by the FDA. All the included articles used RECIST 1.1 (Response Evaluation Criteria in Solid Tumors) to assess the efficacy, while CTCAE 4.0–5.0 (Common Terminology Criteria for Adverse Events) was used for safety assessment. More basic information is displayed in Table 1.

## 3.3 Safety of erdafitinib

The rates of all-grade and grade ≥3 AEs were pooled from single-arm studies. The all-grade AEs are shown in Table 2 and Supplementary Material 1. Among them, the top five most frequent AEs were dry mouth (42.4%, 95% CI 38.0%–46.9%), dysgeusia (30.8%, 95% CI 26.3%–35.7%), dry skin (30.6%, 95% CI 26.5%–34.9%), abnormal hepatic function (21.5%, 95% CI 13.2%–33.2%), and nausea (20.5%, 95% CI 17.1%–24.5%) in the fixed-effects model. In the random-effects model, hyperphosphatemia ranked first in incidence (68.2%, 95% CI

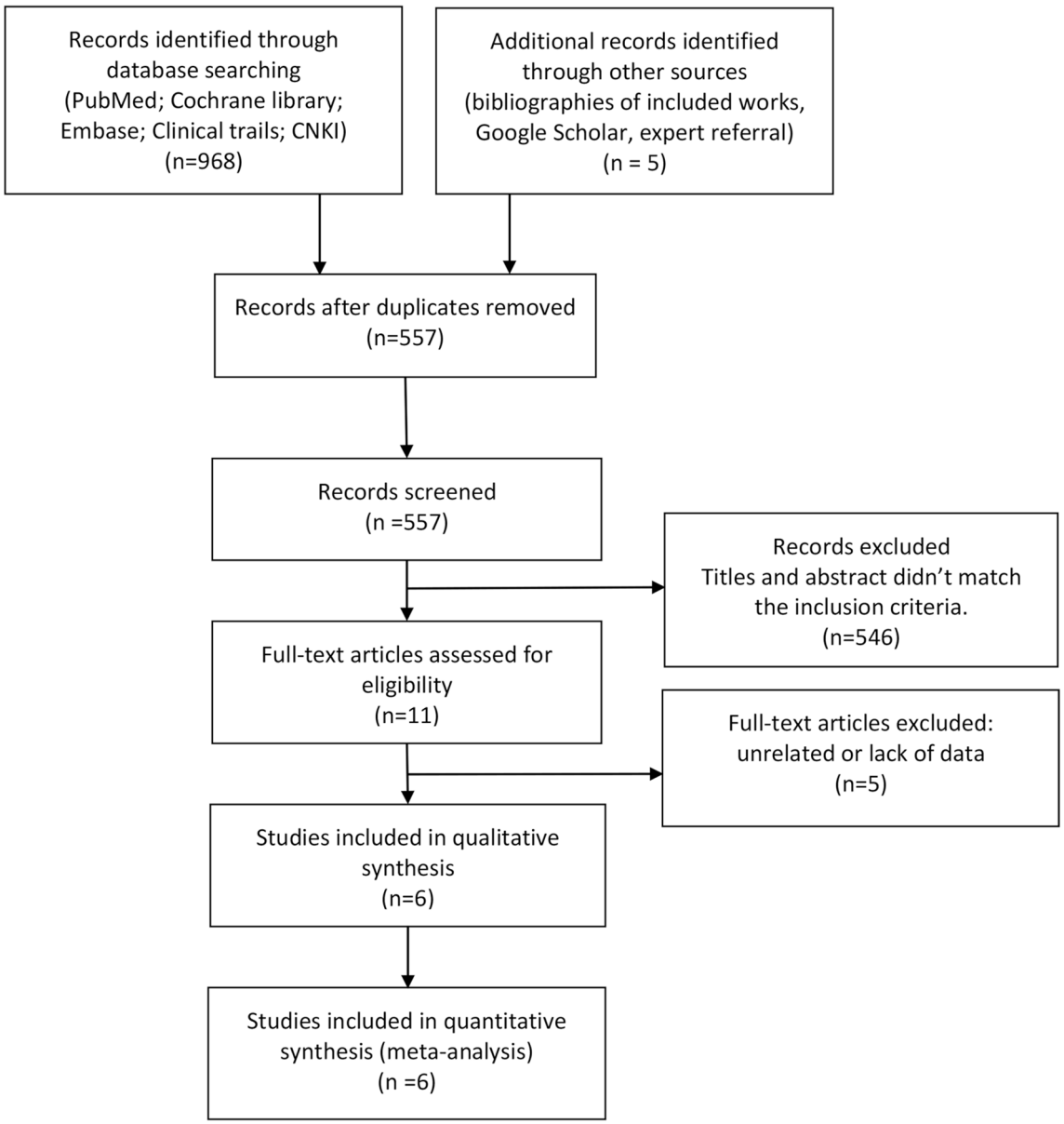


FIGURE 1
Flow diagram of literature selection for systemic reviews and meta-analyses (PRISMA).

59.4%–75.8%), followed by stomatitis (37.4%, 95% CI 27.0%–49.0%), diarrhea (30.9%, 95% CI 18.8%–46.4%), decreased appetite (30.6%, 95% CI 22.3%–40.4%), and asthenia (27.7%, 95% CI 15.4%–44.7%). Eye disorders that might be caused by central serous chorioretinopathy cannot be ignored.

When fixed-effects models were applied to grade ≥ 3 AEs analysis, hyponatremia was found to be the most common AE (8.3%, 95% CI 5.6%–12.2%), while abnormal hepatic function (7.7%, 95% CI 3.2%–17.2%), anemia (7.0%, 95% CI 4.9%–9.8%), asthenia (6.6%, 95% CI 4.5%–9.5%), and nail dystrophy (6.0%, 95% CI 3.4%–10.3%) were other major AEs. Regarding random-effects models, stomatitis (9.7%, 95% CI 6.1%–15.1%) and general physical health deterioration (7.0%, 95% CI 2.8%–16.5%) commonly occurred in grade ≥3 AEs, and these consequences are listed in Table 3 and Supplementary Material 2.

## 3.4 Efficacy of erdafitinib

For the ORR, stable disease rate, and progressive disease rate, STATA 15.0 was used to conduct a single-rate analysis. For solid tumors and urothelial carcinoma, we calculated their response

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TABLE 1 Basic characteristics of the included trials.

| Author/ Year | Clinical trials information | Study design | Study phase | Sample size | Median age (years) | Treatment | Treatment regimens | Median follow-up | Histology |
|---|---|---|---|---|---|---|---|---|---|
| Josep Tabernero, 2015 | NCT01703481 | Open-label, multicenter (escalating multiple dose cohorts) | I | 65 | 59 (27–75) | Erdafitinib | 0.5/2/4/6/9 mg qd==21 days OR 10/12 mg 7 days on+7 days off==28 days | 8–16 weeks | Solid tumor (advance) |
| Tomohiro Nishina 2017 | NCT01962532 | Open-label, multicenter, single-arm, dose escalation | I | 19 | 62.1 | Erdafitinib | 2/4/6 mg qd OR 10/12 mg qd [7 days on/off] | 12 weeks | Advanced or refractory solid tumors |
| Rastislav Bahleda 2019 | NCT01703481 | Multicenter, escalating multiple-dose cohorts | I | 187 | 60 (21–84) | Erdafitinib | 9 mg qd==21days OR 10/12 mg qd (7 days on +7 days off==28 days) | 24 weeks | Advanced or refractory solid tumors |
| Y. Loriot 2019 | NCT02365597 | Open-label | II | 99 | 68 (36–87) | Erdafitinib | 8–9 mg qd | 24 months (IQR = 0.7–17.4) | Locally advanced and unresectable or metastatic urothelial carcinoma |
| Fernando Sabino M. Monteiro 2021 | NA | Single-arm trial | II | 12 | 76 | Erdafitinib | 8 mg qd | 16.2 months | Metastatic urothelial carcinoma (mUC) |
| Arlene O Siefker-Radtke 2022 | NCT02365597 | Open-label, non-comparator | II | 101 | 67 (61–73) | Erdafitinib | 8–9 mg qd | 24 months (IQR = 22.7–26.6) | Locally advanced and unresectable or metastatic urothelial carcinoma |

rates separately, as shown in Figures 2–4. In the study of urothelial carcinoma, a random-effects model was used to analyze stable disease and progressive disease rates, which were 0.36 (0.26–0.46) and 0.26 (0.04–0.48), respectively. For urothelial carcinoma, the ORR was 0.38 (0.31–0.44) for the fixed model. Similarly, the ORR of solid tumors was 0.10 (0.07–0.14), while the overall stable disease and progressive disease rates of solid tumors were 0.16 (0.06–0.26) and 0.68 (0.41–0.95), respectively. Other details are shown in Table 4.

Only three trials provided data on the median duration of response and median progression-free survival (PFS). The most prolonged duration occurred in studies published in 2022, which had a median duration of response of 6.0 months, while the median PFS was 5.5 months. A continuous dose of 8 mg or 8 to 9 mg was used for treatment.

In Siefker-Radtke's trial, the median PFS of FGFR mutation was longer than that of FGFR fusion. For the patients who presented both FGFR mutation and fusion, the median PFS was 6.9 months, while mutation (−)/fusion (+) was 2.8 months, and mutation (+)/fusion (−) was 5.6 months.

## 3.5 Assessment for risk of bias and publication bias

RevMan 5.4 was used to assess the risk of bias. However, they were all single-arm studies. In Loriot's study, except for performance bias, which was assessed as high risk because of open label, the other aspects were assessed as low risk. Five nonrandomized studies were evaluated as low to moderate risk in the ROBINS-I assessment. Overall, the quality of the studies was satisfactory. Because there were fewer than 10 included articles, the meta-regression and funnel plot were not made.

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TABLE 2 The all-grade adverse events classified by CACTE 5.0 and the details.

| Adverse events | No. of studies | No. of AE | No. of patients | Event rate with 95% CI | *p*-value | Model |
|---|---|---|---|---|---|---|
| Blood and lymphatic system disorders | | | | | | |
| Anemia | 4 | 56 | 284 | 0.199[0.156, 0.250] | 0.000 | Fixed |
| Eye disorders | | | | | | |
| Blurred vision | 5 | 54 | 418 | 0.137[0.106, 0.175] | 0.000 | Fixed |
| Cataract | 1 | 6 | 99 | 0.061[0.027, 0.128] | 0.000 | Fixed |
| Dry eye | 4 | 103 | 452 | 0.220[0.165, 0.286] | 0.000 | Random |
| Keratitis | 1 | 5 | 99 | 0.051[0.021, 0.116] | 0.000 | Fixed |
| Blepharitis (eye disorders, other) | 1 | 1 | 19 | 0.053[0.007, 0.294] | 0.005 | Fixed |
| Gastrointestinal disorders | | | | | | |
| Abdominal pain | 3 | 18 | 149 | 0.125[0.069, 0.218] | 0.000 | Fixed |
| Abdominal pain upper (abdominal pain) | 1 | 8 | | 0.123[0.063, 0.227] | 0.000 | Fixed |
| Angular cheilitis (cheilitis) | 1 | 1 | 19 | 0.053[0.007, 0.294] | 0.005 | Fixed |
| Colitis | 1 | 5 | | 0.051[0.021, 0.116] | 0.000 | Fixed |
| Constipation | 5 | 105 | 471 | 0.231[0.152, 0.335] | 0.000 | Random |
| Diarrhea # | 6 | 160 | 483 | 0.309[0.188, 0.464] | 0.017 | Random |
| Dry mouth * | 6 | 203 | 483 | 0.424[0.380, 0.469] | 0.001 | Fixed |
| Gastritis | 1 | 1 | 19 | 0.053[0.007, 0.294] | 0.005 | Fixed |
| Gingivitis (periodontal disease) | 1 | 1 | 19 | 0.053[0.007, 0.294] | 0.005 | Fixed |
| Stomatitis (mucositis oral) # | 6 | 195 | 483 | 0.374[0.270, 0.490] | 0.034 | Random |
| Aphthous ulcer (mucositis oral) | 1 | 4 | 99 | 0.040[0.015, 0.103] | 0.000 | Fixed |
| Nausea * | 5 | 95 | 471 | 0.205[0.171, 0.245] | 0.000 | Fixed |
| Toothache | 1 | 1 | 19 | 0.053[0.007, 0.294] | 0.005 | Fixed |
| Vomiting | 4 | 52 | 370 | 0.154[0.093, 0.244] | 0.000 | Random |
| General disorders and administration site conditions | | | | | | |
| Fatigue | 5 | 92 | 418 | 0.222[0.134, 0.344] | 0.000 | Random |
| General physical health deterioration (general disorders and administration site conditions, other) | 1 | 5 | 99 | 0.051[0.021, 0.116] | 0.000 | Fixed |
| Asthenia (fatigue) # | 4 | 123 | 452 | 0.277[0.154, 0.447] | 0.012 | Random |
| Pyrexia (fever) | 1 | 1 | 19 | 0.053[0.007, 0.294] | 0.005 | Fixed |
| Malaise | 1 | 1 | 19 | 0.053[0.007, 0.294] | 0.005 | Fixed |
| Hepatobiliary disorders | | | | | | |
| Hepatic function abnormal (hepatic failure) * | 1 | 14 | 65 | 0.215[0.132, 0.332] | 0.000 | Fixed |
| Infections and infestations | | | | | | |
| Paronychia | 3 | 35 | 219 | 0.161[0.118, 0.216] | 0.000 | Fixed |
| Herpes zoster (shingles) | 1 | 1 | 19 | 0.053[0.007, 0.294] | 0.005 | Fixed |
| Upper respiratory tract infection (upper respiratory infection) | 1 | 3 | 19 | 0.158[0.052, 0.392] | 0.008 | Fixed |
| Urinary tract infection | 3 | 38 | 265 | 0.144[0.107, 0.192] | 0.000 | Fixed |

*(Continued)*

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TABLE 2 Continued

| Adverse events | No. of studies | No. of AE | No. of patients | Event rate with 95% CI | *p*-value | Model |
|---|---|---|---|---|---|---|
| Urosepsis (infections and infestations, other) | 1 | 3 | 99 | 0.030[0.010, 0.090] | 0.000 | Fixed |
| Injury, poisoning, and procedural complications | | | | | | |
| Contusion (bruising) | 1 | 1 | 19 | 0.053[0.007, 0.294] | 0.005 | Fixed |
| Investigations | | | | | | |
| Alanine aminotransferase increased | 2 | 20 | 120 | 0.167[0.110, 0.244] | 0.000 | Fixed |
| AST increased (aspartate aminotransferase increased) | 1 | 3 | 19 | 0.158[0.052, 0.392] | 0.008 | Fixed |
| Increase in γ-glutamyltransferase (GGT increased) | 1 | 3 | 99 | 0.030[0.010, 0.090] | 0.000 | Fixed |
| Weight decreased (weight loss) | 1 | 17 | 101 | 0.168[0.107, 0.254] | 0.000 | Fixed |
| Metabolism and nutrition disorders | | | | | | |
| Decreased appetite (anorexia) # | 5 | 143 | 471 | 0.306[0.223, 0.404] | 0.000 | Random |
| Hyperphosphatemia # | 6 | 331 | 483 | 0.682[0.594, 0.758] | 0.000 | Random |
| Hyponatremia | 2 | 13 | 118 | 0.113[0.067, 0.186] | 0.000 | Fixed |
| Musculoskeletal and connective tissue disorders | | | | | | |
| Arthralgia | 1 | 7 | 65 | 0.108[0.052, 0.209] | 0.000 | Fixed |
| Back pain | 1 | 8 | 65 | 0.123[0.063, 0.227] | 0.000 | Fixed |
| Muscle spasms (muscle cramp) | 1 | 7 | 65 | 0.108[0.052, 0.209] | 0.000 | Fixed |
| Nervous system disorders | | | | | | |
| Dysgeusia * | 4 | 113 | 370 | 0.308[0.263, 0.357] | 0.000 | Fixed |
| Psychiatric disorders | | | | | | |
| Insomnia | 1 | 2 | 19 | 0.105[0.026, 0.337] | 0.004 | Fixed |
| Psychiatric disorders | 1 | 2 | 19 | 0.105[0.026, 0.337] | 0.004 | Fixed |
| Renal and urinary disorders | | | | | | |
| Acute kidney injury | 1 | 6 | 99 | 0.061[0.027, 0.128] | 0.000 | Fixed |
| Hematuria | 1 | 10 | 99 | 0.101[0.055, 0.178] | 0.000 | Fixed |
| Cystitis (Renal and urinary disorders, other) | 1 | 1 | 19 | 0.053[0.007, 0.294] | 0.005 | Fixed |
| Respiratory, thoracic, and mediastinal disorders | | | | | | |
| Rhinitis allergic (allergic rhinitis) | 1 | 2 | 19 | 0.105[0.026, 0.337] | 0.004 | Fixed |
| Cough | 1 | 2 | 19 | 0.105[0.026, 0.337] | 0.004 | Fixed |
| Dyspnea | 4 | 57 | 284 | 0.155[0.055, 0.364] | 0.003 | Random |
| Oropharyngeal pain | 1 | 1 | 19 | 0.053[0.007, 0.294] | 0.005 | Fixed |
| Laryngeal pain (respiratory, thoracic and mediastinal disorders, other) | 1 | 1 | 19 | 0.053[0.007, 0.294] | 0.005 | Fixed |
| Skin and subcutaneous tissue disorders | | | | | | |
| Alopecia | 5 | 109 | 471 | 0.229[0.158, 0.321] | 0.000 | Fixed |
| Nail disorder (skin and subcutaneous tissue disorders, other) | 2 | 10 | 111 | 0.093[0.051, 0.164] | 0.000 | Fixed |
| Nail dystrophy (skin and subcutaneous tissue disorders, other) | 3 | 43 | 387 | 0.115[0.086, 0.151] | 0.000 | Fixed |

*(Continued)*

TABLE 2 Continued

| Adverse events | No. of studies | No. of AE | No. of patients | Event rate with 95% CI | *p*-value | Model |
|---|---|---|---|---|---|---|
| Onycholysis (skin and subcutaneous tissue disorders, other) | 3 | 55 | 387 | 0.146[0.114, 0.185] | 0.000 | Fixed |
| Onychalgia (skin and subcutaneous tissue disorders, other) | 1 | 1 | 19 | 0.053[0.007, 0.294] | 0.005 | Fixed |
| Dry skin * | 5 | 141 | 471 | 0.306[0.265, 0.349] | 0.000 | Fixed |
| Dermatitis (skin and subcutaneous tissue disorders, other) | 1 | 1 | 19 | 0.053[0.007, 0.294] | 0.005 | Fixed |
| Nail discoloration | 1 | 1 | 19 | 0.053[0.007, 0.294] | 0.005 | Fixed |
| Palmar-plantar erythrodysesthesia syndrome | 2 | 27 | 166 | 0.156[0.085, 0.269] | 0.000 | Random |
| Hand-foot syndrome (palmar–plantar erythrodysesthesia syndrome) | 2 | 43 | 286 | 0.160[0.071, 0.321] | 0.000 | Random |
| Pruritus | 1 | 2 | 19 | 0.105[0.026, 0.337] | 0.004 | Fixed |
| Rash maculo-papular | 1 | 2 | 19 | 0.105[0.026, 0.337] | 0.004 | Fixed |

* The top 5 adverse events with highest occurrence rate analyzed by the fixed model.
# The top 5 adverse events with highest occurrence rate analyzed by the random model.

TABLE 3 The grade ≥3 adverse events classified by CAC Table 5.0 and the details.

| Adverse events | No. of studies | No. of AE | No. of patients | Event rate with 95% CI | *p*-value | Model |
|---|---|---|---|---|---|---|
| Blood and lymphatic system disorders | | | | | | |
| Anemia * | 4 | 30 | 452 | 0.070[0.049, 0.098] | 0.000 | Fixed |
| Eye disorders | | | | | | |
| Cataract | 1 | 2 | 99 | 0.020[0.005, 0.077] | 0.000 | Fixed |
| Dry eye | 2 | 2 | 200 | 0.010[0.003, 0.039] | 0.000 | Fixed |
| Keratitis | 1 | 3 | 99 | 0.030[0.010, 0.090] | 0.000 | Fixed |
| Gastrointestinal disorders | | | | | | |
| Abdominal pain | 2 | 10 | 252 | 0.040[0.022, 0.072] | 0.000 | Fixed |
| Colitis | 1 | 2 | 99 | 0.020[0.005, 0.077] | 0.000 | Fixed |
| Constipation | 2 | 2 | 200 | 0.010[0.003, 0.039] | 0.000 | Fixed |
| Diarrhea | 3 | 10 | 212 | 0.052[0.028, 0.095] | 0.000 | Fixed |
| Dry mouth | 1 | 1 | 99 | 0.010[0.001, 0.067] | 0.000 | Fixed |
| Stomatitis (mucositis oral) # | 3 | 36 | 387 | 0.097[0.061, 0.151] | 0.000 | Random |
| Aphthous ulcer (mucositis oral) | 1 | 2 | 99 | 0.020[0.005, 0.077] | 0.000 | Fixed |
| Nausea | 2 | 2 | 200 | 0.010[0.003, 0.039] | 0.000 | Fixed |
| Intestinal obstruction (small intestinal obstruction) | 1 | 7 | 187 | 0.037[0.018, 0.076] | 0.000 | Fixed |
| Vomiting | 1 | 2 | 99 | 0.020[0.005, 0.077] | 0.000 | Fixed |
| General disorders and administration site conditions | | | | | | |
| Fatigue | 3 | 6 | 212 | 0.041[0.010, 0.153] | 0.000 | Random |
| General physical health deterioration (general disorders and administration site conditions, other) # | 3 | 32 | 351 | 0.070[0.028, 0.165] | 0.000 | Random |
| Asthenia (fatigue) * | 3 | 25 | 387 | 0.066[0.045, 0.095] | 0.000 | Fixed |

*(Continued)*

TABLE 3 Continued

| Adverse events | No. of studies | No. of AE | No. of patients | Event rate with 95% CI | *p*-value | Model |
|---|---|---|---|---|---|---|
| Hepatobiliary disorders | | | | | | |
| Hepatic function abnormal (hepatic failure) * | 1 | 5 | 65 | 0.077[0.032, 0.172] | 0.000 | Fixed |
| Infections and infestations | | | | | | |
| Paronychia | 2 | 6 | 200 | 0.030[0.014, 0.065] | 0.000 | Fixed |
| Urinary tract infection | 3 | 11 | 265 | 0.045[0.025, 0.079] | 0.000 | Fixed |
| Urosepsis (infections and infestations, other) | 1 | 3 | 99 | 0.030[0.010, 0.090] | 0.000 | Fixed |
| Investigations | | | | | | |
| Alanine aminotransferase increased | 2 | 4 | 200 | 0.020[0.008, 0.052] | 0.000 | Fixed |
| AST increased (aspartate aminotransferase increased) | 1 | 10 | 187 | 0.053[0.029, 0.097] | 0.000 | Fixed |
| Increase in γ-glutamyltransferase (GGT increased) | 1 | 2 | 99 | 0.020[0.005, 0.077] | 0.000 | Fixed |
| Weight decreased (weight loss) | 1 | 1 | 101 | 0.010[0.001, 0.067] | 0.000 | Fixed |
| Metabolism and nutrition disorders | | | | | | |
| Decreased appetite (anorexia) | 2 | 2 | 166 | 0.012[0.003, 0.048] | 0.000 | Fixed |
| Hyperphosphatemia | 4 | 6 | 399 | 0.020[0.009, 0.044] | 0.000 | Fixed |
| Hyponatremia * | 2 | 23 | 286 | 0.083[0.056, 0.122] | 0.000 | Fixed |
| Nervous system disorders | | | | | | |
| Dysgeusia | 2 | 5 | 164 | 0.031[0.005, 0.158] | 0.000 | Random |
| Renal and urinary disorders | | | | | | |
| Acute renal failure (acute kidney injury) | 1 | 2 | 65 | 0.031[0.008, 0.115] | 0.000 | Fixed |
| Acute kidney injury | 1 | 2 | 99 | 0.020[0.005, 0.077] | 0.000 | Fixed |
| Hematuria | 1 | 2 | 99 | 0.020[0.005, 0.077] | 0.000 | Fixed |
| Respiratory, thoracic and mediastinal disorders | | | | | | |
| Dyspnea | 3 | 11 | 387 | 0.030[0.017, 0.053] | 0.000 | Fixed |
| Skin and subcutaneous tissue disorders | | | | | | |
| Nail disorder (skin and subcutaneous tissue disorders, other) | 2 | 4 | 111 | 0.039[0.015, 0.099] | 0.000 | Fixed |
| Nail dystrophy (skin and subcutaneous tissue disorders, other) * | 2 | 12 | 200 | 0.060[0.034, 0.103] | 0.000 | Fixed |
| Onycholysis (skin and subcutaneous tissue disorders, other) | 3 | 7 | 265 | 0.029[0.014, 0.059] | 0.000 | Fixed |
| Palmar–plantar erythrodysesthesia syndrome | 2 | 8 | 166 | 0.048[0.024, 0.093] | 0.000 | Fixed |
| Hand-foot syndrome (palmar–plantar erythrodysesthesia syndrome) | 1 | 5 | 99 | 0.051[0.021, 0.116] | 0.000 | Fixed |

* The top 5 adverse events with highest occurrence rate analyzed by the fixed model.
# The top 2 adverse events with highest occurrence rate analyzed by the random model.

## 4 Discussion

Based on the present findings, we conducted a meta-analysis to summarize six published clinical trials (22–27), comprehensively investigating the safety and efficacy of erdafitinib. Our review analyzed the ORR, stable disease rate, and progressive disease rate of UC and other solid tumors separately. As mentioned in the *Characteristics of studies* section, some articles reported the outcomes regardless of UC and other solid tumors. Thus, we calculated the efficacy of UC and solid tumors separately by the

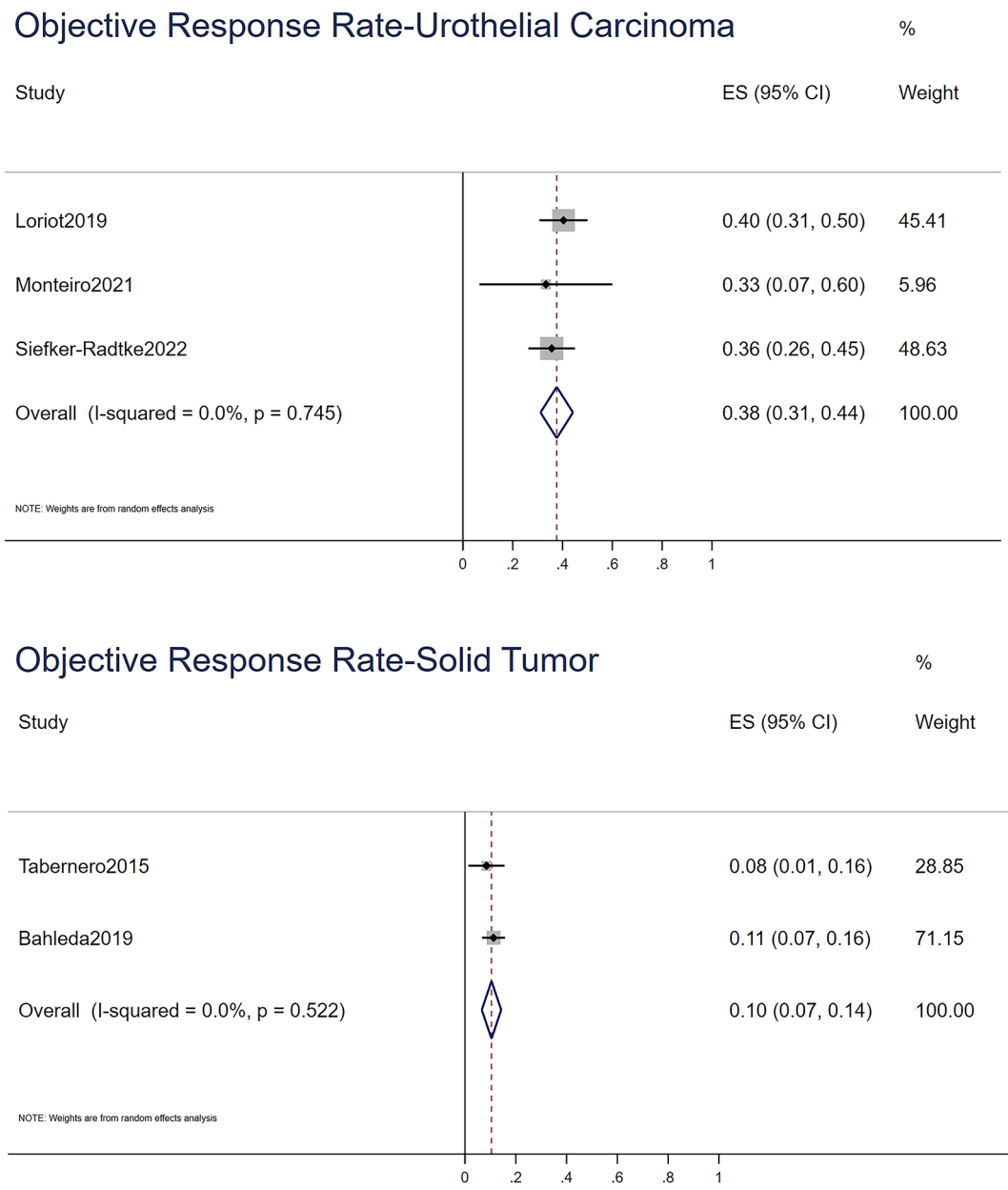


FIGURE 2
The objective response rate of urothelial carcinoma and solid tumor.

trials that were specific to UC and the rest. Finally, we indicated that erdafitinib had a more satisfactory effect in UC than in solid tumors, with a higher ORR and lower progression rate.

Based on the development of next-generation DNA sequencing, it is now easy to determine the genetic alteration type of tumors. The effect of erdafitinib is surprising in some specific categories of FGFR gene alteration like *FGFR3-TACC3*. In Loriot's trial, 4 of 11 patients responded to erdafitinib. All four patients had *FGFR3:TACC3v1* gene alteration (a specific kind of gene fusion). In Tabernero's article, patients with *FGFR3-TACC3* tended to have greater response or tumor shrinkage than patients with other gene alterations. This phenomenon can be explained by a fusion of FGFR3 and TACC3, which contributed to constitutive tyrosine kinase activation and disruption of mitotic activity (28). Nevertheless, in addition to mutation and fusion, amplification may also occur. The trial (25) indicated that patients with FGFR mutations/fusion/co-alterations had significantly higher ORRs (12/27) than those with FGFR amplification (only 2/23 patients responded). Previous reviews have also found that for FGFR inhibitors, qualitative FGFR1–3 alterations such as mutation and

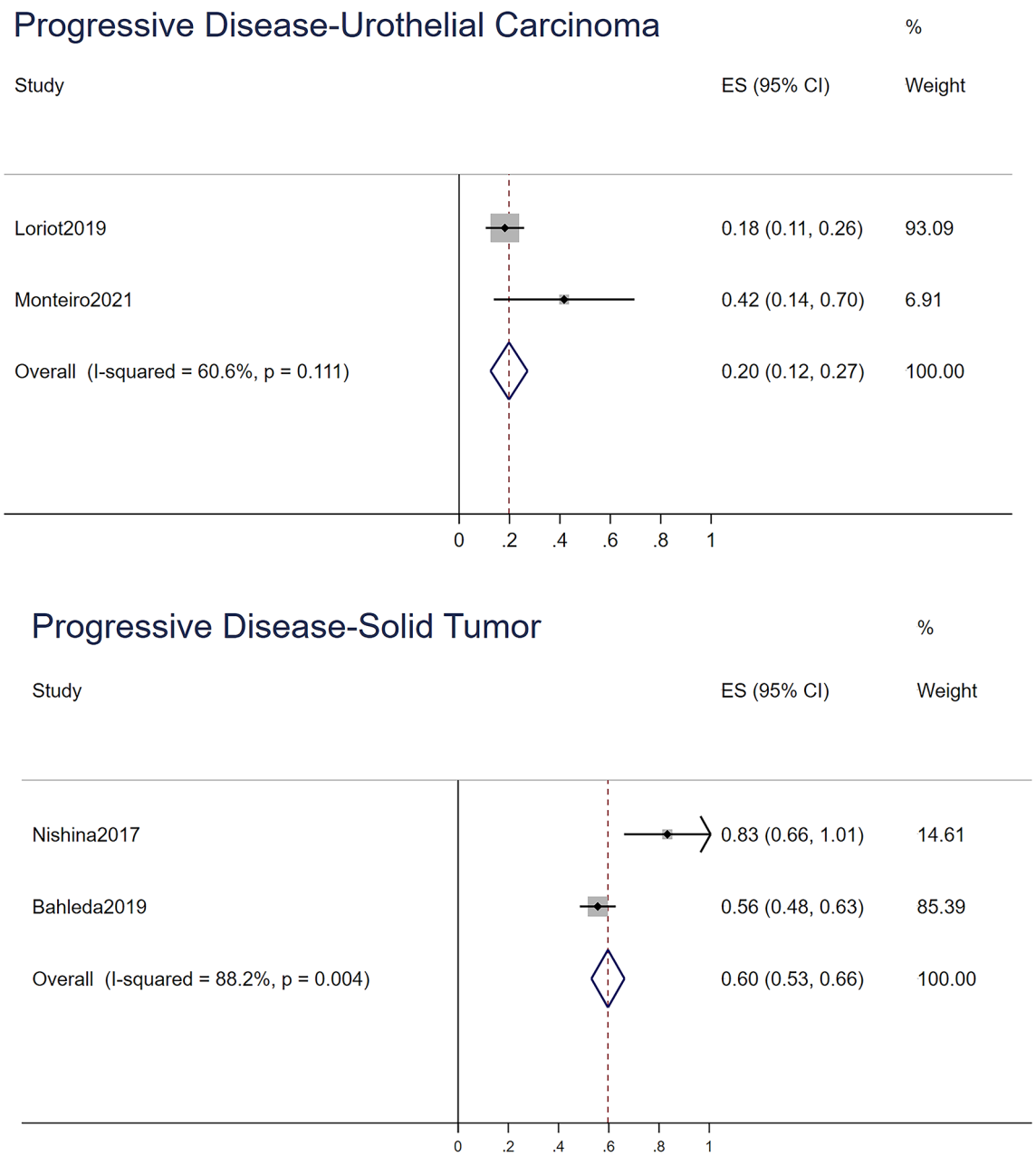


FIGURE 3
The progressive disease of urothelial carcinoma and solid tumor.

rearrangement are more sensitive to drugs, and quantitative alterations like gene amplification rarely exhibit clinical activity (29). This might be because amplification leads to oncogene redundancy, which can lead to the overexpression of related proteins and initiate downstream signaling that promotes carcinoma proliferation and survival. Some studies have demonstrated that redundant oncogenes were associated with immune escape, which reduced or nullified the effect of FGFR SMi (30, 31).

In the study of AEs, we indicated that the most common all-grade AEs were hyperphosphatemia, stomatitis, dry mouth, dysgeusia, and diarrhea. Hyperphosphatemia occurred in more than half of the patients; however, all of them were grade 1–2. The most common grade ≥3 AEs were stomatitis, hyponatremia, and abnormal hepatic function. Generally, severe AEs were relatively rare.

For gastrointestinal AEs such as diarrhea, stomatitis, dry mouth, decreased appetite, nausea, vomiting, and abdominal

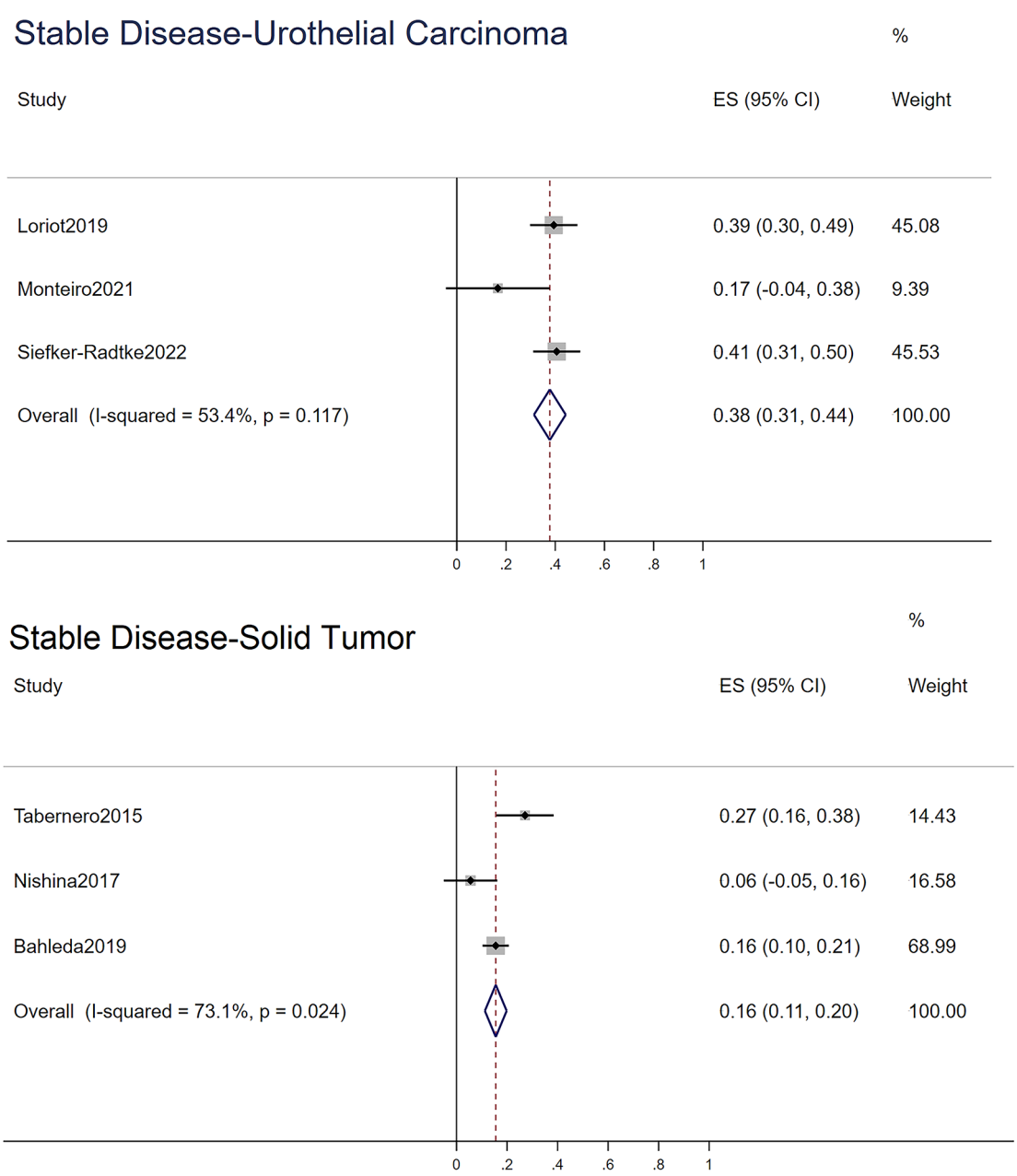


FIGURE 4
The stable disease of urothelial carcinoma and solid tumor.

pain, most can be controlled with symptomatic treatments (32). There have been no trials that reported drug withdrawal because of gastrointestinal AEs.

In the remaining AEs, hyperphosphatemia needed to be noted for its high occurrence and the possibility of causing reduction or withdrawal of erdafitinib. Hyperphosphatemia is the most common all-grade AE, occurring at 68.2%, related to fibroblast growth factor 23 (FGF-23) in bone metabolism (33, 34). FGF23 is a bone-derived mediator that maintains phosphate homeostasis, which inhibits the synthesis of 1,25-dihydroxyvitamin D3. Meanwhile, FGF-23 interacts with Klotho (which is the main structure of FGF receptor complex) to suppress renal phosphate reabsorption by decreasing the expression of the sodium-phosphate cotransporters NPT2A and NPT2C in the brush-border membrane of proximal tubule epithelial cells (35–37). FGFR1 co-expresses with Klotho, which increases the affinity of FGF23 for FGFR1 (38). A previous study suggested that Klotho was regulated by

TABLE 4 Efficacy profile of erdafitinib.

| STUDY | Complete response (CR) | Partial response (PR) | Stable disease (SD) | Objective response rate (ORR) | Progressive disease (PD) | Median duration of response | Median progression-free survival (PFS) |
|---|---|---|---|---|---|---|---|
| Josep Tabernero, 2015 | 0 | 5/59 | 16/59 | 5/59 | NA | NA | NA |
| Tomohiro Nishina, 2017 | 0 | 0 | 1/18 | 0 | 15/18 | NA | NA |
| Bahleda, 2019 | 0 | 21/187 | 29/187 | 21/187 | 104/187 | 9.0 months | 2.3 months |
| Y. Loriot, 2019 | 3/99 | 37/99 | 39/99 | 40/99 | 18/99 | 5.6 months (95% CI = 4.2–7.2) | NA |
| Monteiro, 2021 | 0 | 4/12 | 2/12 | 4/12 | 5/12 | NA | NA |
| Arlene O Siefker-Radtke, 2022 | 0 | 36/101 | 41/101 | 36/101 | NA | 6.0 months (95% CI = 4.2–7.5 | 5.5 months (95% CI = 4.2–6.0 |

phosphaturia and that FGFR1 expression was modulated by FGF23. In a trial with a pan-FGFR inhibitor (PD173074), researchers found that the biologic activity of FGF-23 was counteracted, leading to hyperphosphatemia and high 1,25 (OH)2 D3 (39). Therefore, the decrease in FGF-23 contributed to hyperphosphatemia and increased the production of calcitriol. In two phase I trials (NCT0103481 and NCT01962532), hyperphosphatemia appeared with 4 mg of erdafitinib. However, they both noted no dose-related changes in FGF23 values and vitamin D. Since no raw data on FGF23 content were provided, we did not perform a comprehensive analysis of FGF23. We suspected that the inconspicuous changes in FGF23 may be due to the following reasons. First, the sample size was small (the total number of patients was 82), and second, in NCT0103481, some patients reduced the dose of medication while others were given intermittent administration (27). These therapies may alleviate the inhibition of FGF23 by FGFR inhibitors. Therefore, FGFR23 did not show significant changes (40). Thus, the mechanism of erdafitinib-induced hyperphosphatemia needs further study. In patients with hyperphosphatemia, phosphate binders like sevelamer, acetazolamide, and sevelamer carbonate can be taken (25). Satisfactorily, the use of sevelamer has no significant effect on the pharmacokinetic parameters of erdafitinib.

In addition to hyperphosphatemia, central serous chorioretinopathy (CSC) was another AE mentioned in *FDA NEWS RELEASE: FDA approves first targeted therapy for metastatic bladder cancer*. In BLC2001, CSC occurred in 27 of 101 patients. A case report noticed that patients' visual acuity changed from 20/25 OD and 20/15 OD to 20/20 OU after using erdafitinib (41). Meanwhile, in Tabernero's study, one patient reported visual spots. As Jung et al. mentioned, this symptom might be caused by drug-induced pseudo-central serous chorioretinopathy (pCSC). However, it is worth noting though that primary CSC and paraneoplastic retinopathy (PNR, a retinopathy that occurs in patients with carcinomas) have the same symptoms (42). Therefore, a differential diagnosis is necessary for targeted treatment and appropriate prognosis prediction. For pCSC, retinopathy is often self-limited. The symptom disappears simultaneously or shortly after discontinuation of therapy, which is the most significant feature. The main difference of pathology between true CSC and PNR is that the former has typical features for lipofuscin irregularities, and the latter has progressive lesion (42). The included trials in this review showed that the dose of erdafitinib had no noticeable difference in the occurrence rate of retinopathy disease, with rates of 15/60 (25%) in the 8-mg QD group and 12/41 (29%) in the 9-mg QD group. After dose interruption, reduction, or shutoff, 17 of 27 patients were solved. After resolving detachment of retinal pigment epithelium (RPE), a grade 3 retinopathy patient recurred as grade 2. A similar phenomenon also occurred in Bahelda's article, which indicated that after dose interruption, the pathological changes of the retina reversed except for the patients who had grade 1–2 retinopathy. However, grade 1–2 retinopathy events have not been solved in some patients. We have no accurate conclusions about why mild retinal damage still exists.

We suspected that this is related to other pathways downstream of FGFR, like MAPK. Some studies have indicated that MEK inhibitors have a toxic effect on RPE (43, 44), which leads to retinal-related AEs. Other studies have formulated some hypotheses. For instance, the Wnt/β-catenin signaling pathway can promote the proliferation of RPE and the accumulation of extracellular matrix. If the signaling pathway is impacted, RPE will become pathological (45). The pathological contraction and traction of the fibrocellular membranes cause

retinal detachment (46). Another hypothesis states that FGFR-1 and FGFR-2 increase L-type Ca2+ channel activity in retinal pigment epithelial cells, and consequently promote the secretion of vascular endothelial growth factor A (VEGF-A), which plays a critical role in neovascularization. Decreasing visual acuity might cause that (47).

Nevertheless, this hypothesis for mild retinal damage requires further evidentiary support. Because pCSCs are self-limited, we do not suggest physicians use additional drugs other than closely observing and reducing the dose accordingly.

Presently, erdafitinib is being used with other drugs for clinical treatments, for example, combined with the PD-1 inhibitor cetrelimab (NCT03473743). More RCTs comparing erdafitinib to intravesical chemotherapy in non-muscle-invasive bladder cancer are ongoing. The unsatisfactory effect caused by FGFR gene amplification might be solved during new therapy, while AEs are alleviated by adjusting erdafitinib dosing.

There were some limitations in our article. For instance, the patients' characteristics, the dose of erdafitinib, and FGFR gene alterations differ, which result in unavoidable heterogeneity. Moreover, restricted to the small number of included studies, we did not conduct meta-regression and funnel plots to assess the publication bias. Last but not least, all of the included articles are single-arm trials and lacked comparisons to other therapies.

As the first FDA-approved FGFR inhibitor to treat urothelial cancer, erdafitinib has a more satisfactory effect than traditional therapy. The most common AE is hyperphosphatemia, which occurs in grade 1–2 and can be controlled with sevelamer. Another AE worth discussing is pCSC. pCSC is caused by inhibiting the MAPK pathway, which needs to be distinguished from true CSC and PNR. Moreover, erdafitinib has rare severe AEs. In efficacy analysis, erdafitinib can increase the PFS significantly, among which, patients with FGFR mutations have a better response than those with fusions, while in FGFR gene fusion, FGFR3-TACC3 is the most sensitive gene alteration. Further studies on single-use and combined therapy of erdafitinib are ongoing, such as the phase III PROOF 302 trial (NCT04197986), which evaluates the efficacy of the FGFR1–3 inhibitor infigratinib in invasive urothelial carcinoma, which provides evidence for FGFR inhibitors in clinical decisions (48). After more clinical trials are published, the discoveries will be further improved.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

## Author contributions

XYZ, HW, and JD wrote the original manuscript, and XLM designed and supervised this study. MHY made the major contribution to the data extracted. XHZ and FZ edited the language and improved figures and tables. All authors analyzed the data and edited the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.907377/full#supplementary-material

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