PERSONALIZED MEDICINE FOR UROLOGICAL CANCERS: TARGETING CANCER METABOLISM

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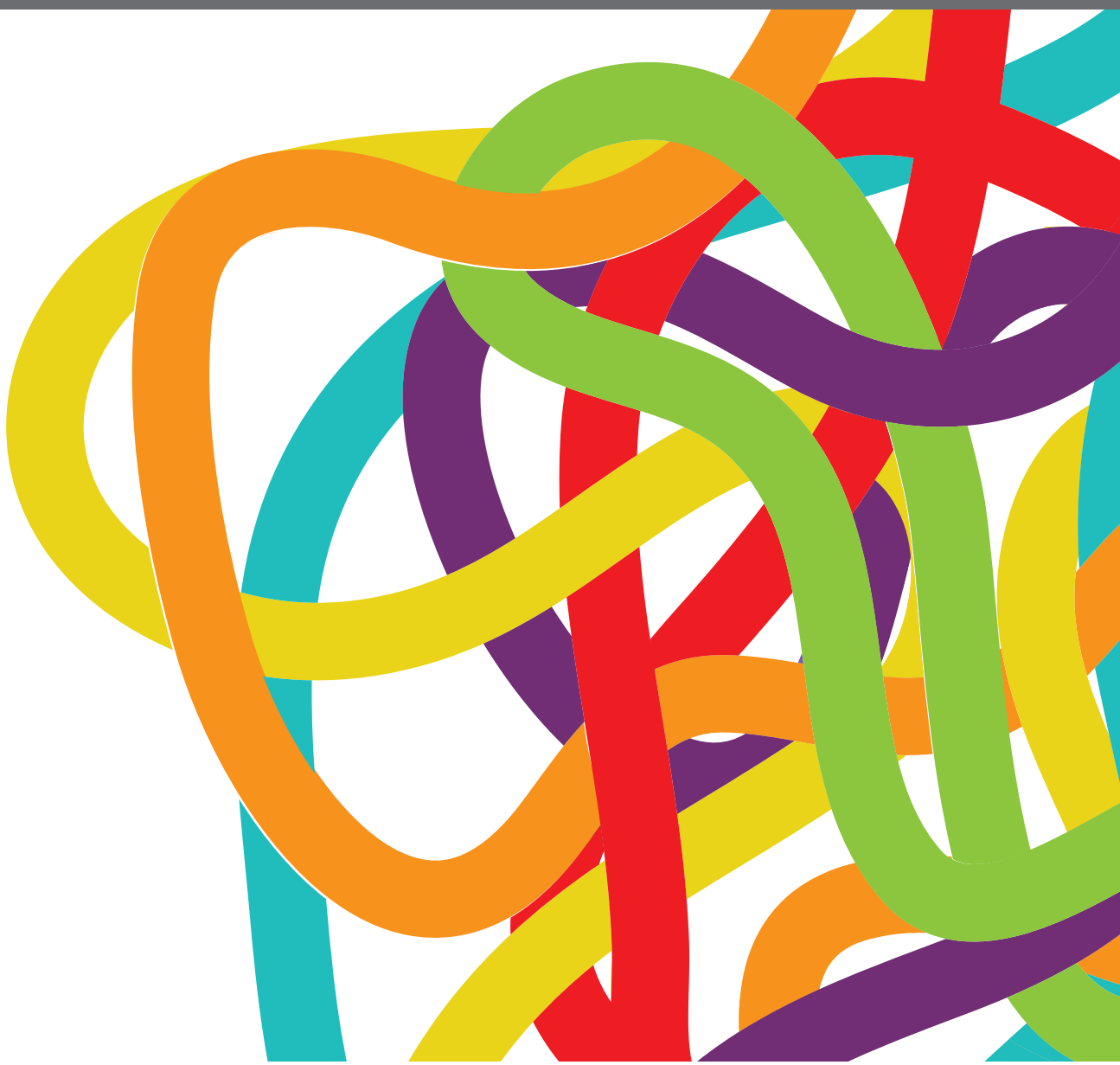

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PERSONALIZED MEDICINE FOR UROLOGICAL CANCERS: TARGETING CANCER METABOLISM

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EDITORIAL
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Editorial: Personalized Medicine for Urological Cancers: Targeting Cancer Metabolism

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Editorial on the Research Topic

Personalized Medicine for Urological Cancers: Targeting Cancer Metabolism

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The key concept of personalized medicine is to identify the best treatment possible for a selected patient, in order to maximize therapeutic efficacy, reduce side effects, and minimize the risk of drug resistance development. To achieve this, it is fundamental to define effective cancer classifiers, which would allow appropriate patient stratification, minimizing overtreatment of indolent disease and avoiding delay in therapeutic treatments.

Sample availability, intra-tumor heterogeneity and the lack of established models for disease progression, have been the main challenges in deciphering the functional impact of genomic alterations on urological cancers, both in their common or rare forms. However, it has been possible to subtype urological cancers, mainly prostate and bladder, based on genomic alterations, while there is still a lack of knowledge of the metabolome. With the advances in metabolomics, the evaluation of metabolites has emerged as a strategy to identify new biomarkers. As discussed in the review by Singh R. et al. (Singh and Mills), there is a strong contrast between the capacity to sequence at high scale and decode genomic data in contrast with the metabolites that can be currently identified by mass spectrometry or other methods. When the technology can overcome this technical challenge, we will be able to prove the crosstalk between metabolic pathways and other cancer drivers and identify the causative connection of this interplay during disease onset and progression.

The aim of this Research Topic is to illustrate examples of personalized medicine for urological cancers, where assessment of metabolism can be used as strategy to refine disease diagnosis and patient prognosis.

BLADDER CANCER

As recently discussed by Minoli et al. the clinical guidelines and standard operating procedures for BLCa have limitations and physicians are often met with treatment failure and/or reoccurrence, indicating that the management of BLCa is complex and current classification systems do not depict the heterogeneity of this disease (1). Transurethral resection of bladder tumor (TURBT) combined with individualized intravesical chemotherapy or immunotherapy is recommended as the routine treatment model by the major international guidelines (Yang et al.). En bloc resection of bladder tumor (ERBT) has served as a valuable alternative technique that has attracted increasing interest among urologists globally. However, there is no robust clinical evidence that ERBT performs better than conventional resection in terms of disease progression. ERBT has the advantage of an intact tumor specimen containing detrusor muscle (DM) that can be used for accurate histopathological assessment by pathologists (Yang et al.). Additionally, such intact specimens are extremely valuable for characterization of tumor heterogeneity from a metabolic point of view in the context of personalized medicine. Similarly, a characterization of the metabolic effects of novel drugs is needed, to understand whether modulation at metabolic levels could be used as combinatorial approach with therapeutic agents. An example of FGFR3 mutant metastatic bladder cancer successfully treated with a combination of Anlotinib (2), a novel multitarget tyrosine kinase inhibitor, and Sintilimab (Zhang et al.), an antitumor PD-1 antibody, is illustrated in the case report by Cao et al. Another report presents the first case of urachal carcinoma treated with PD-1 antibody (Zheng et al.). At present, the most prominent issues for such rare tumors are the difficulty of obtaining drugs and the lack of late-stage clinical trials to guide therapeutic decisions. Matrix-assisted laser desorption/ionization (MALDI)-Orbitrap-mass spectrometry imaging (MSI) was recently used in a proof of principle study to showcase that such new methods can be used to identify biomarkers in a scenario where reliable diagnostic standards are not available (3).

RENAL CELL CARCINOMA

The therapeutic scenario of metastatic renal cell cancer (mRCC) has noticeably increased in the last years, with the most recently introduced immunotherapies and tyrosine kinase inhibitors (TKI)-targeted therapies. Niu et al. have documented how the deletions in chromosomes 9 and 14, and the associated immunosuppressive microenvironment may indicate limited sensitivity to anti-PD-1/PD-L1 monotherapy for clear cell renal cell carcinoma patients with venous tumor thrombus. A new approach to predict clinical outcomes in mRCC patients treated with immune checkpoint inhibitors is the assessment of body composition, by measuring variables such as density of skeletal muscle (SM), subcutaneous fat, inter-muscular fat, and visceral fat. Martini et al. have proposed in this Research Topic that risk stratification using the body composition variables including total fat index may be prognostic and predictive of clinical outcomes in mRCC. The comprehensive review by Roberto et al. summarizes the latest clinical trials and provide guidance for overcoming the barriers to decision-making to offer a practical approach to the management of mRCC in daily clinical practice. The authors highlight the challenges to characterizing the renal neoplasia in all its complexity, as this would allow the lineage tracing of distant clones from a molecular point of view. Additionally, new challenges are still open such as patient stratification, which could also be achieved with metabolomics, treatment combination and timely intervention.

PROSTATE CANCER

Lipids and their metabolism have recently reached the spotlight with accumulating functional evidence for promoting PCa onset, progression, and metastasis. This led to the definition of lipogenic signatures where a variety of intermediate of lipid metabolism are represented, ranging from bigger lipids (4) to intermediates of beta oxidation (5). In their review, Scaglia et al. discuss extensively how the metabolic machinery of lipid metabolism impact the tumor microenvironment and the therapeutic implications of targeting the lipogenic hubs, including dietary modulation. The concept that PCa progression is "a matter of fats" is supported by the research work of Faviana et al., which shows the potential of evaluating the expression of gastrin-releasing peptide receptor (GRPR) in PCa biopsy to improve our ability to detect PCa with low grades at the earliest phases of development. These findings support previous research showing the involvement of GRPR in adipocyte differentiation (6) and reinforce the notion that understanding which fat is good and which fat is bad for the prostate is crucial to deepen our understanding of the disease. In addition to lipids, the role of alternative nutrients and interactions with the tumor microenvironment are discussed in a review by Fidelito et al. which examines the challenges to metabolic therapies in prostate cancer, which may be met with the emergence of patient-derived organoids and the growing biobanks of PDX collections.

In conclusion, when technology will allow the definition of genomic-equivalent signatures in the cancer metabolome, significant progress will be achieved in the identification of features that are associated between genomics, metabolomics and epigenomics. Significant progress is being made in developing single-cell spatial methods for the detection of well-known metabolites, and mass spectrometry imaging is showing promising results. To capture metabolic information in real time in an operating theatre, there is a need for new devices/biomedical engineering, as the signals are dynamic/unstable compared to sequencing data where DNA is stable and technology needs to address those differences. As pointed out in the review by Singh and Mills, we can anticipate a future in which spatial genomic and metabolomic data are aligned to radiomic features and imaging to risk stratify patients and simultaneously inform treatment selection.

AUTHOR CONTRIBUTIONS

EZ assembled the editorial team, coordinated the work and wrote the manuscript. JG and MK-D participated in the editorial team and proof read the manuscript. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Overnight Continuous Saline Bladder Irrigation After *En Bloc* Resection of Bladder Tumor Does Not Improve Oncological Outcomes in Patients Who Have Received Intravesical Chemotherapy

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Objective: To evaluate the safety and efficacy of overnight continuous saline bladder irrigation (CSBI) for patients who have received thulium laser *en bloc* resection of bladder tumor (TmLRBT) combined with immediate intravesical chemotherapy previously.

Methods: From October 2014 to June 2018, 235 patients with newly diagnosed non-muscle invasive bladder cancer (NMIBC) were included in this retrospective study. All patients received intravesical instillation of pirarubicin immediately after TmLRBT. The patients were divided into two groups according to the duration of postoperative bladder irrigation with normal saline. After immediate intravesical chemotherapy, patients in group 1 received overnight CSBI, while patients in group 2 did not receive overnight CSBI. Data on the time of initial tumor recurrence, recurrence-free survival (RFS) and progression-free survival (PFS) rates, and perioperative complications were collected and analyzed.

Results: Of 235 included patients (129 in group 1 and 106 in group 2), the median follow-up periods were 42 and 38 months, respectively. There were no significant differences in patients' baseline characteristics between the two groups. The RFS rates of patients in group 1 were 90.7, 82.7, and 76.8% at the end of the first, third, and fifth years, while the corresponding RFS rates of patients in group 2 were 87.7, 78.9, and 73.3%, respectively. Four patients in group 1 and five patients in group 2 experienced tumor progression. No significant differences between the two groups were observed in the time of initial tumor recurrence, RFS, and PFS rates. Only Grade I complications occurred in the two groups, and no significant difference was reached between the two groups.

Conclusions: For patients with NMIBC who have previously received TmLRBT combined with immediate intravesical chemotherapy, overnight CSBI may not improve oncological outcomes and reduce perioperative complications.

Keywords: non-muscle invasive bladder cancer, *en bloc* resection, continuous saline bladder irrigation, intravesical chemotherapy, recurrence

INTRODUCTION

Bladder cancer (BC) is the tenth most common cancer disease worldwide with 474,000 new incident cases and 197,000 deaths annually, and it is also the second most common malignant disease of the urinary system after prostate cancer (1). Approximately 75% of newly diagnosed BC presents as malignant lesions confined to the mucosa or submucosa, which are collectively referred to as non-muscle invasive bladder cancer (NMIBC) (2). For these patients, transurethral resection of bladder tumor (TURBT) combined with individualized intravesical chemotherapy or immunotherapy that is tailored to tumor risk stratification is recommended as the routine treatment model by the major international guidelines (2–5). However, piecemeal resection of tumor tissue in conventional TURBT results in exfoliated tumor cell dissemination and seeding, which goes against the recognized principle of oncological surgery and partly contributes to increasing the out of field recurrence (6, 7).

Initially, after transurethral tumor resection, continuous saline bladder irrigation (CSBI) was used to prevent the formation of blood clots and achieve excellent hemostasis. Meanwhile, in theory, CSBI can flush out exfoliated tumor cells effectively and prevent them from implanting in the bladder mucosa, thereby reducing the risk of tumor recurrence after conventional resection (8). However, CSBI has no therapeutic effect on the residual tumors at the initial resection site, so it is necessary to perform high-quality and complete tumor resection to make sure that the tumor specimens contain the lamina propria and superficial muscular layer (9).

In the past decade, *en bloc* resection of bladder tumor (ERBT) served as a valuable alternative technique that has obtained increasing interest among urologists worldwide (10). As a "no touch" surgical technique for the treatment of NMIBC, ERBT shows the potential to minimize the number of exfoliated tumor cells and reduce the risk of tumor cell reimplantation. The use of thulium laser as the energy source for ERBT does not generate high-frequency current and has excellent hemostatic effect. Therefore, the incidence of perioperative complications, such as obturator nerve reflex (ONR), bladder perforation (BP), and acute bleeding will be reduced (11). Then, we tested the hypothesis that overnight CSBI has little effect on improving oncological outcomes and reducing the incidence of perioperative complications for patients with NMIBC who have received thulium laser *en bloc* resection of bladder tumor (TmLRBT) combined with immediate intravesical chemotherapy.

Abbreviations: BC, Bladder cancer; NMIBC, Non-muscle invasive bladder cancer; TURBT, Transurethral resection of bladder tumor; CSBI, Continuous saline bladder irrigation; ERBT, *En bloc* resection of bladder tumor; ONR, Obturator nerve reflex; BP, Bladder perforation; TmLRBT, Thulium laser *en bloc* resection of bladder tumor; CIS, carcinoma *in situ*; EAU, European Association of Urology; WHO, World Health Organization; RFS, recurrence-free survival; PFS, progression-free survival; MMC: mitomycin C.

PATIENTS AND METHODS

Patients

From October 2014 to June 2018, all patients with newly diagnosed NMIBC, who underwent a TmLRBT combined with immediate intravesical instillation of pirarubicin were retrospectively included. Cystoscopy, ultrasonography, intravenous pyelography, and computed tomography were performed to select the appropriate patients before TmLRBT. The inclusion criteria were as follows: 1) patients who underwent TmLRBT successfully without switching to conventional TURBT; 2) patients were diagnosed with NMIBC for the first time; 3) detrusor muscle was contained in the tumor specimens. The exclusion criteria were as follows: 1) preoperative examinations revealed distant metastasis or pelvic lymph node metastasis; 2) carcinoma *in situ* (CIS) and upper urinary tract neoplasms were accompanied; 3) the intact tumor specimens were cut into two or three parts longitudinally in the bladder before being retrieved; 4) histopathological analysis of the tumor specimens showed that the muscle layer of the bladder was invaded. This study protocol was approved by the Ethics Committee of the First Hospital of Shanxi Medical University. All patients were informed and agreed to participate in the study.

Surgical Procedure

The surgical procedure of white light cystoscopy-assisted TmLRBT was performed by the same urologist with rich experience in endoscopy. All patients were placed in a lithotomy position under combined spinal and epidural anesthesia. Sterile normal saline was used for continuous bladder irrigation, and the Revolix^{TM} thulium laser system (LISA Laser Products, Lindau-Katlenburg, Germany) was used as the energy source during TmLRBT. Firstly, the urologist examined the entire bladder mucosa thoroughly and recorded the tumor location, number, size, and appearance. Then, a circular mucosal incision was made at a safe distance of 5–10 mm from the base of the tumor tissue, and the visible blood vessels near the tumor tissue were coagulated and blocked at the same time. At the circular incision line, the urologist first made a vertical incision from the bladder mucosa to the deep muscular layer and then removed the whole tumor tissue by vapor resection with the thulium laser and blunt dissection with the tip of the resectoscope. In order to minimize the cautery artifacts of the tumor specimens, laser vapor resection was mainly used to cut off the muscle fibers around the tumor. Finally, the intact tumor specimens were retrieved with an Ellick's evacuator *via* out sheath of the resectoscope. For the larger-size tumor specimens, an additional medical device, such as laparoscopic forceps, was required to complete the work.

Intravesical Chemotherapy

Immediate intravesical chemotherapy with 30 mg pirarubicin was administered within 6 h after TmLRBT. The duration of pirarubicin in the bladder was 1 h, and then patients in group 1 received overnight CSBI (2,000 ml/h for first 1 h, then 1,000 ml/h for 3 h, and then 250 ml/h for 12 to16 h) after intravesical chemotherapy, while patients in group 2 did not receive

overnight CSBI. After discharge from the hospital, patients with intermediate- and high-risk NMIBC received maintenance intravesical chemotherapy with pirarubicin for one year. The detailed scheme of intravesical instillation of pirarubicin was once a week for 8 weeks, followed by monthly intravesical chemotherapy to 12 months. Based on the European Association of Urology (EAU) guidelines, a second transurethral resection was performed in patients with T1 and/or high-grade bladder tumor (12).

Follow-Up Strategies

Patients with low-risk NMIBC were followed up every 3 months in the first year, every 6 months in the second year, and then once a year. For patients with intermediate- and high-risk NMIBC, the follow-up strategy was every 3 months for the first two years, then every 6 months until the fifth year, and then once a year. Routine examination items included ultrasonography, urine cytology, and cystoscopy.

The time of initial tumor recurrence was defined as the time interval between TmLRBT and the date of initial tumor recurrence. When cystoscopy showed space-occupying lesions in the bladder, tumor recurrence should be considered. Tumor progression was defined as an increase in pathological stage. All recurrent and progressive tumor lesions were further confirmed by histopathological assessment. Histological grade and pathological stage of the tumor specimens were evaluated according to the 2004 World Health Organization (WHO) grading system and the 2009 version of TNM staging system, respectively.

Statistical Analysis

Continuous data were expressed as median and tested using unpaired t-test. Qualitative data were described as numbers and percentages and compared through Chi-square test or Fisher's exact test. The patients' baseline characteristics and CSBI were included in multivariate Cox proportional hazard models to determine which variables correlate to recurrence-free survival (RFS). The RFS and progression-free survival (PFS) curves were estimated by the Kaplan–Meier method, and the difference between survival curves was analyzed by the log-rank test. Statistical analysis was performed using GraphPad Prism statistical software package, version 7.0 for Windows and IBM SPSS statistics, version 19.0 for Windows. All tests were two-sided, and statistical significance was reached when the P value <0.05.

RESULTS

The study population included 254 patients with newly diagnosed NMIBC who received TmLRBT. After reviewing the patients' clinical data, 19 cases were excluded due to the following reasons: six cases were accompanied with CIS, the intact tumor specimens of eight cases were cut into pieces in the bladder before being retrieved, and five cases switched to conventional TURBT during surgery. The remaining 235 patients were divided into two groups according to the duration of postoperative bladder irrigation with normal saline. Patients in group 1 (n = 129) received overnight CSBI after intravesical chemotherapy, while patients in group 2 (n = 106) did not receive overnight CSBI. The patients' baseline characteristics in the two groups were compared in terms of age, gender, smoking history, tumor diameter, number, grade and stage, and EAU risk stratification. The results showed that no significant differences existed between the two groups (**Table 1**).

Median follow-up period was 42 months (range 5–65 months) in group 1 and 38 months (range 5–63 months) in group 2. Twenty-six (20.2%) cases in group 1 and twenty-five (23.6%) cases in group 2 developed tumor recurrence during the follow-up period. In group 1, the RFS rates were 90.7, 82.7, and 76.8% at the end of the first, third, and fifth years. In group 2, the RFS rates were 87.7, 78.9, and 73.3% at the end of the first, third, and fifth years, respectively. The Kaplan–Meier curve of the RFS rate of all patients showed no significant difference between the two groups (log-rank test: $P = 0.51$) (**Figure 1A**). The RFS rates of patients with low-, intermediate- and high-risk NMIBC also did not reach a significant difference between the two groups (log-rank test: $P = 0.68$, 0.74 and 0.67, respectively) (**Figures 1B–D**). In multivariate analysis, overnight CSBI was not an independent predictor of RFS (HR 0.76, $P = 0.357$) (**Table 2**).

The median period of initial tumor recurrence was 13 months in group 1 and 12 months in group 2, and no significant difference was observed between the two groups (unpaired t-test: $P = 0.96$). Four (3.1%) cases in group 1 and five (4.7%) cases in group 2 developed tumor progression during the follow-up

TABLE 1 | Baseline characteristics of patients in the two groups.

Characteristics	Group 1 (n = 129)	Group 2 (n = 106)	P
Age (year)	66 (24–84)	65.5 (38–82)	0.62
Gender			
Male	103 (79.84%)	83 (78.30%)	0.87
Female	26 (20.16%)	23 (21.70%)	
Smoking history			0.86
Current	58 (44.96%)	44 (41.51%)	
Prior	36 (27.91%)	32 (30.19%)	
Never	35 (27.13%)	30 (28.30%)	
Tumor diameter (cm)			0.89
1.0–2.0 cm	90 (69.77%)	75 (70.75%)	
2.0–3.0 cm	39 (30.23%)	31 (29.25%)	
Tumor number			0.68
Single	84 (65.12%)	72 (67.92%)	
Multiple	45 (34.88%)	34 (32.08%)	
Grade (WHO 2004)			0.51
Low-grade	76 (58.91%)	57 (53.77%)	
High-grade	53 (41.09%)	49 (46.23%)	
T stage			0.87
Ta	106 (82.17%)	86 (81.13%)	
T1	23 (17.83%)	20 (18.87%)	
EAU risk stratification			0.93
Low-risk	37 (28.68%)	28 (26.42%)	
Intermediate-risk	62 (48.06%)	53 (50.00%)	
High-risk	30 (23.26%)	25 (23.58%)	

WHO, World Health Organization; EAU, European Association of Urology.

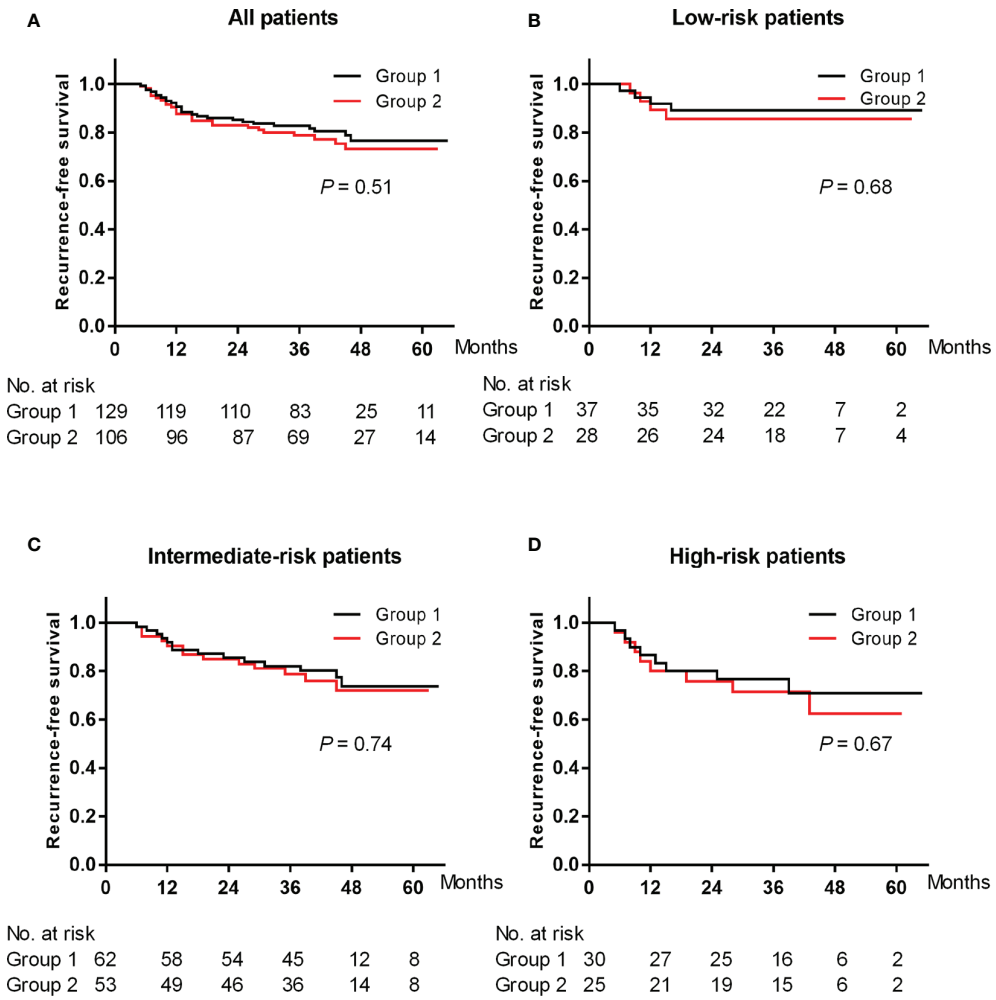

FIGURE 1 | Kaplan-Meier plot of the recurrence-free survival rates of all **(A)** and low-risk **(B)**, intermediate-risk **(C)** and high-risk **(D)** patients treated with overnight continuous saline bladder irrigation or not after thulium laser en bloc resection of bladder tumor combined with immediate intravesical chemotherapy.

TABLE 2 | Multivariate Cox proportional hazard analyses of recurrence-free survival in all patients.

Characteristics	RFS multivariate	
	HR (95% CI)	*P*
Age (year)	0.98 (0.96–1.01)	0.145
Gender (male)	1.29 (0.53–3.15)	0.580
Smoking (yes)	1.35 (0.65–2.80)	0.416
Diameter (2.0–3.0 cm)	1.69 (0.95–3.01)	0.077
Number (multiple)	2.55 (1.36–4.78)	0.003
Grade (high)	1.32 (0.73–2.39)	0.367
Stage (T1)	1.28 (0.54–3.03)	0.577
risk stratification (high)	3.85 (1.54–9.62)	0.004
CSBI (yes)	0.76 (0.43–1.36)	0.357

RFS, recurrence-free survival; CSBI, continuous saline bladder irrigation; HR, hazard ratio; CI, confidence interval.

period. There was no significant difference in PFS rates between the two groups (log-rank test: $P = 0.50$) (**Figure 2**).

Perioperative complications were recorded in all 235 patients. Based on the modified Clavien classification system for surgical complications (13), only Grade I complications happened in the two groups. The incidence of Grade I complications were 9.3% (12/129) and 12.3% (13/106) in group 1 and group 2, respectively. Although the incidence of complications in group 1 was less, there was no significant difference between the two groups (Fisher's exact test: $P = 0.53$). No patient experienced ONR and BP during operation (**Table 3**).

DISCUSSION

The well-known principle of oncological surgery is to resect the tumor tissue in one piece with negative surgical margins and prevent iatrogenic tumor cells scattering and local implantation. However, during conventional TURBT, the tumor tissue is resected piece by piece from the exophytic part of the tumor to

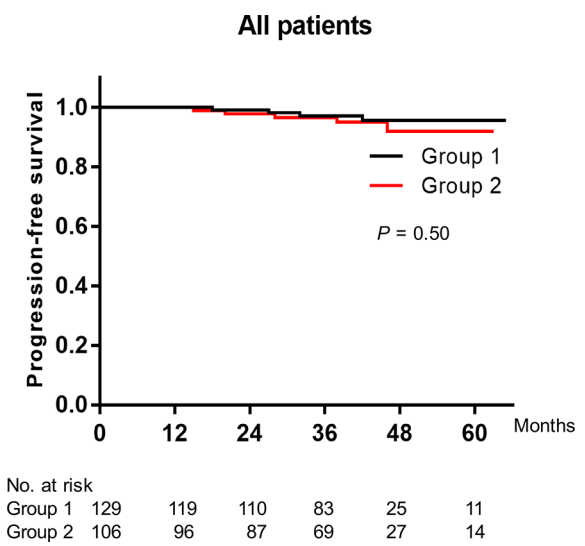

FIGURE 2 | Kaplan-Meier plot of the progression-free survival rates of all patients treated with overnight continuous saline bladder irrigation or not after thulium laser en bloc resection of bladder tumor combined with immediate intravesical chemotherapy.

TABLE 3 | Perioperative complications.

	Group 1 (n = 129)	Group 2 (n = 106)	*P*
Obturator nerve reflex	0	0	–
Bladder perforation	0	0	–
Complications			0.53
Grade I	12 (9.30%)	13 (12.26%)	
≥Grade II	0	0	

the superficial muscular layer by a wire loop. The integrity of the tumor tissue is destroyed, and tumor cells are dispersed, which may increase the risk of exfoliated tumor cell dissemination and implantation. Compared with conventional TURBT, ERBT adheres to the basic principle of cancer surgery and provides pathologists with an intact tumor specimen for accurate histopathological analysis (14, 15). Meanwhile, as a "no touch" technique for the treatment of NMIBC, ERBT shows the potential to minimize the number of exfoliated tumor cells, and then may reduce the risk of tumor cell reimplantation.

In several studies on ERBT, the duration of CSBI after operation was recorded. However, there were huge differences between the relevant data. Xu et al. analyzed the safety and efficacy of 1.9 μm Vela laser ERBT for the treatment of NMIBC in a retrospective study, and the mean duration of postoperative CSBI was 29.1 h (16). A European multicenter prospective study was conducted to compare the safety and efficacy of ERBT using different energy sources. After tumor resection with electrical current and laser energy, the mean periods of bladder irrigation were 0.76 and 0.63 days, respectively (17). Li et al. explored the safety and efficacy of TmLRBT for the treatment of NMIBC in a retrospective study, and the median duration of postoperative bladder irrigation with normal saline was 6.33 h (18). The monopolar current, initially used in conventional TURBT, was also applied as the energy source for *en bloc* resection. And the median time of bladder irrigation after ERBT was 1.16 days (19). While in a prospective study, no patient required bladder irrigation after 980 nm laser ERBT (20). Therefore, we question how to choose the best duration of bladder irrigation after ERBT. In this retrospective study, after TmLRBT combined with immediate intravesical chemotherapy, there were no significant differences in the time of initial tumor recurrence, RFS, and PFS rates between the patients who received overnight CSBI or not. Hence, when excellent hemostasis effect is obtained during ERBT, it is safe not to perform overnight CSBI after surgery.

The function of CSBI is to achieve excellent hemostasis and remove blood clots in the bladder. Meanwhile, continuous bladder irrigation after tumor resection can wash away exfoliated tumor cells and reduce the risk of tumor cell reimplantation in the injured bladder mucosa (21). However, CSBI has no therapeutic effect on the residual tumors at the initial resection site. Then, in order to achieve the effect of postoperative bladder irrigation to reduce tumor recurrence, the prerequisite is high quality and complete tumor resection. Unlike ERBT using electrical current as the energy source, no high-frequency current is generated during TmLRBT. Combined with more precise and controllable procedure of tumor resection, ONR and BP can be avoided (22). After surgery, patients can receive intravesical chemotherapy immediately without being restricted by perioperative complications, such as acute bleeding and BP. Thulium laser, as a diode pumped solid-state laser, works in continuous fashion at the wavelength of 2,013 nm and the penetration depth of 250 μm. Compared with pulsed holmium laser, ERBT using thulium laser as the energy source makes tumor resection more precise and controllable, and the hemostatic effect is excellent (23). Due to its excellent hemostatic effect, bladder irrigation may not be required after surgery to prevent the formation of blood clots in the bladder. In this trial, the results indicated that there were no significant differences in oncological outcomes and perioperative complications between patients who received overnight CSBI or not. Therefore, for well-selected patients with newly diagnosed NMIBC, TmLRBT combined with immediate intravesical chemotherapy can be performed in the day-surgery unit without the need of overnight CSBI. This is a better mode of allocating medical resources, which alleviates the logistical pressure caused by the expansion of the waiting number to a certain extent. It is also a process of reducing health-care costs and has a positive impact on medical expenses (24).

The main limitation of the trial is its retrospective design and relatively small patient population. However, we believe that our preliminary findings are very meaningful for urologists and patients with NMIBC. For patients with newly diagnosed NMIBC, urologists can choose to perform TmLRBT as day-surgery operation on them to shorten the waiting time outside the hospital, and then reduce the patient's nervousness related to waiting outside the hospital (25). Further prospective randomized controlled trials with more patients are needed to confirm our results. Second, thulium is a less commonly used laser energy source in transurethral tumor resection, and ERBT is less commonly

performed than conventional TURBT. Due to the excellent hemostatic effect of thulium and the theoretical benefits of ERBT, TmLRBT may gain more and more interest among urologists. Third, although pirarubicin is not as widely used in intravesical chemotherapy as gemcitabine or mitomycin C (MMC), in a systematic review, indirect comparisons could not detect any differences in efficacy between MMC and pirarubicin (26).

CONCLUSIONS

For patients with newly diagnosed NMIBC, after TmLRBT combined with immediate intravesical chemotherapy, overnight CSBI cannot improve oncological outcomes and reduce the incidence of perioperative complications. Therefore, TmLRBT may be performed as day-surgery operation for well-selected patients.

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.

ETHICS STATEMENT

The study was approved by the Ethics Committee of the First Affiliated Hospital of Shanxi Medical University (2011033/2011) and conducted according to the principles of the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

YY designed the clinical study, collected and analyzed the clinical data, and wrote the manuscript. CL analyzed and interpreted the patient data. XTY and JL collected and analyzed the clinical data. XFY designed the study, supervised the research, contributed to the experimental discussion, and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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En Bloc Tumor Resection, Optical Molecular Imaging, and the Potential Synergy of the Combination of the Two Techniques in Bladder Cancer

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Although transurethral resection of bladder tumor is the golden standard for the treatment of non-muscle invasive bladder cancer, this surgical procedure still has some serious drawbacks. For example, piecemeal resection of tumor tissue results in exfoliated tumor cells dissemination and implantation, and fragmented tumor specimens make it difficult for pathologists to accurately assess the pathological stage and histologic grade. En bloc tumor resection follows the basic principle of oncological surgery and provides an intact tumor specimen containing detrusor muscle for pathologists to make accurate histopathological assessment. However, there is no robust clinical evidence that en bloc tumor resection is superior to conventional resection in terms of oncological outcomes. Considering the high recurrence rate, small or occult tumor lesions may be overlooked and incomplete tumor resection may occur during white light cystoscopy-assisted transurethral resection. Molecular fluorescent tracers have the ability to bind tumor cells with high sensitivity and specificity. Optical molecular imaging mediated by it can detect small or occult malignant lesions while minimizing the occurrence of false-positive results. Meanwhile, optical molecular imaging can provide dynamic and real-time image guidance in the surgical procedure, which helps urologists to accurately determine the boundary and depth of tumor invasion, so as to perform complete and high-quality transurethral tumor resection. Integrating the advantages of these two technologies, optical molecular imaging-assisted en bloc tumor resection shows the potential to improve the positive detection rate of small or occult tumor lesions and the quality of transurethral resection, resulting in high recurrence-free and progression-free survival rates.

Keywords: bladder cancer, optical molecular imaging, *en bloc* tumor resection, detection, surgery

INTRODUCTION

Bladder cancer (BC) is the tenth most common cancer disease worldwide with 474 000 new incident cases and 197 000 deaths annually, and it is also the second most common malignant disease of the urinary system after prostate cancer (1). About 75% of newly diagnosed BC cases present as a lesion confined to the mucosa or submucosa, collectively referred to as non-muscle invasive bladder cancer (NMIBC) (2). For these patients, transurethral resection of bladder tumor (TURBT) combined with personal intravesical chemotherapy or immunotherapy that is tailored to tumor risk stratification is recommended as the routine treatment model by the major international guidelines (2–5). The quality of transurethral tumor resection plays an important role in histopathological assessment and treatment decision-making, which affects the prognosis of the disease. However, conventional TURBT has some serious drawbacks. First, piecemeal resection of tumor tissue leads to the dissemination and implantation of exfoliated tumor cells, which goes against the recognized principle of oncological surgery and contributes to increases in the rate of tumor recurrence (6–8). Second, fragmented tumor specimens make it difficult for pathologists to accurately assess the pathological stage and histologic grade (9). Finally, small or occult malignant lesions, particularly carcinoma *in situ* (CIS), are not easy to visualize and diagnose in the bladder wall during white light cystoscopy (WLC)-assisted transurethral resection (10). For NMIBC patients, accurate histopathological evaluation, especially of the boundary and depth of tumor invasion, is essential for selecting an optimal treatment strategy (11). Furthermore, the quality of the initial transurethral resection strongly determines overall medical cost of BC treatment (12). Therefore, there is an urgent need to improve the surgical procedure of transurethral tumor resection to solve the serious drawbacks of WLC-assisted conventional resection.

In the past decade, en bloc resection of bladder tumor (ERBT) has served as a valuable alternative technique that has attracted increasing interest among urologists globally (13). Compared with conventional resection, ERBT has three potential advantages with respect to tumor cell implantation, perioperative complications, and specimen quality. First, the technique follows the principle of cancer surgery and removes the tumor in one piece, thereby minimizing the number of exfoliated tumor cells and reducing the risk of cell implantation. Second, the more precise and controllable procedure of tumor resection may reduce the incidence of perioperative complications, such as blood loss, obturator nerve reflex (ONR), and bladder perforation (BP). Finally, an intact tumor specimen containing detrusor muscle (DM) can be collected after en bloc resection for accurate histopathological assessment by pathologists (14).

Optical molecular imaging is a novel molecular targeted imaging technology that can realize qualitative and quantitative analysis of pathological processes at the cellular and molecular levels prior to macrostructure changes of malignant tissue (15). The molecular fluorescent tracer specifically binds to the tumor site and then highlights the malignant lesions from normal mucosa tissue under a paired optical imaging device. Due to its high sensitivity and specificity, the positive detection rate of small or occult tumor lesions, especially CIS, can be improved (16–18). Meanwhile, optical molecular imaging can provide dynamic and real-time images during transurethral tumor resection, which is helpful for urologists to accurately determine the boundary and depth of tumor invasion.

Theoretically, integrating the advantages of the above two technologies, patients who receive optical molecular imaging-assisted en bloc resection may achieve high-quality and complete tumor resection. In this review, we focus on the evidence for the use of ERBT and optical molecular imaging in BC, and point out their potential clinical value in future applications.

CLINICAL BENEFITS OF EN BLOC RESECTION OF BLADDER TUMOR

The well-known principle of oncological surgery is to remove the tumor tissue in one piece with negative surgical margin and prevent iatrogenic tumor cells scattering and local implantation. Therefore, if a surgeon cuts the renal cell carcinoma tissue into pieces and disperses it throughout the surface of the remaining normal renal tissue when performing nephron-sparing surgery, he or she will be punished or even dismissed. However, for NMIBC, urologists do this every day during conventional resection without any restrictions. ERBT adheres to the principle of cancer surgery and provides pathologists with an intact tumor specimen containing DM (19–21). Meanwhile, through more controllable and precise surgical procedures of transurethral resection, the risk of perioperative complications in ERBT, such as blood loss, ONR, and BP, is reduced compared with conventional TURBT. As for oncological outcomes, there is still no robust clinical evidence that patients with NMIBC can benefit from ERBT (13, 14).

HISTOPATHOLOGICAL EVALUATION

The choice of treatment strategy for NMIBC depends on the clinicopathological characteristics of tumor lesions, such as stage, grade, number, diameter, prior recurrence rate, and coexistence of CIS (2). Tumor specimens collected after surgery should be sufficient for pathologists to evaluate the clinicopathological characteristics and perform risk

Abbreviations: BC, Bladder cancer; NMIBC, non-muscle invasive bladder cancer; TURBT, transurethral resection of bladder tumor; CIS, carcinoma in situ; WLC, white light cystoscopy; ERBT, en bloc resection of bladder tumor; ONR, obturator nerve reflex; BP, bladder perforation; DM, detrusor muscle; reTUR, repeat transurethral resection; RC, radical cystectomy; MM, muscularis mucosae; LVI, lymphovascular invasion; ICCR, International Collaboration on Cancer Reporting; EAU, European Association of Urology; PDD, photodynamic diagnosis; NBI, narrow-band imaging; CLE, Confocal laser endomicroscopy; OCT, optical coherence tomography; CAIX, Carbonic anhydrase IX; pHLIPs, pH low insertion peptides; ICG, indocyanine green; NIR, near-infrared; TBR, tumor-to-background ratio.

stratification of tumor tissue (22). Conventional TURBT ignores the principle of oncological surgery and removes tumor tissue piece by piece. Moreover, thermal damage, electrocautery artifacts and lack of spatial orientation of fragmented tumor specimens increase the difficulty of accurately assessing the pathological stage and histologic grade (9). The incidence of tumor upstaging found at repeat transurethral resection (reTUR) was 0%–8% (Ta to ≥T1) and 0%–32% (T1 to ≥T2) in patients with BC (23). Meanwhile, in a large retrospective cohort study including 18 277 patients diagnosed with T1 high-grade BC during the initial tumor resection, 41% of patients had tumor upstaging and 12.7% had positive nodes in the final histopathological analysis of specimens obtained from radical cystectomy (RC) (24).

The presence or absence of DM in tumor specimens is the most important marker for the quality and completeness of transurethral tumor resection (25). A high proportion of DM presence was found in the intact tumor specimens collected after en bloc resection (**Tables 1** and **2**). Bipolar electrodes have better hemostatic effects than monopolar electrodes. Therefore, under the guidance of clear operative vision, the surgical procedure of en bloc resection with a bipolar electrode as an energy source can be performed more controllably and precisely, making sure that the tumor specimens contain lamina propria and superficial muscle layers with minimal crush and cautery artifacts (38, 39). A European multicenter study was conducted to compare the safety and efficacy of ERBT using different energy sources. The results showed that the rate of DM presence in tumor specimens was high, and it was similar in electrical ERBT (96%) and laser ERBT (100%) (41).

In a recent meta-analysis, T1 substaging was shown to be closely related to the oncological outcomes of patients with NMIBC (42). A total of 601 patients with T1 BC were retrospectively followed up for 5.9 years. The results indicated that metric substaging was the best independent prognostic indicator of progression-free and cancer-specific survival rates (43). The substaging of pT1 BC depends on the depth of tumor invasion in muscularis mucosae (MM). Compared with conventional TURBT, the anatomical architecture and spatial orientation of tumor specimens are well preserved during ERBT, which helps to minimize inter-observer variability when pathologists identify pT1 BC substaging (44). Moreover, the spatial orientation of the biopsy sample is helpful in distinguishing MM from DM, thus allowing to accurately stage pT1 versus pT2 disease (45, 46). Lymphovascular invasion (LVI) is another important prognostic factor for the recurrence and progression of NMIBC (47). Unfortunately, no studies have explored whether en bloc resection is beneficial for diagnosis of LVI. According to the International Collaboration on Cancer Reporting (ICCR) guidelines, in addition to pathological stage and histologic grade, status of muscularis propria, histological variant and LVI should be included in pathology reports of tumor specimens obtained from biopsy or transurethral resection as required items, while T1 substaging is listed among the recommended items (48). However, it is almost impossible to collect all tumor fragments during conventional resection, so urologists prefer to select some representative samples and submit them to the pathology department. An intact and high-quality tumor specimen can be obtained through ERBT, which helps pathologists to make detailed pathological reports. Now, close cooperation and comprehensive information sharing between urologists and pathologists are advocated to comprehensively evaluate the clinicopathological characteristics and accurately stratify the risk of tumor tissue, and then formulate an optimal treatment strategy for NMIBC patients (49).

PERIOPERATIVE COMPLICATIONS

During conventional TURBT, the tumor tissue is resected piece by piece from an exophytic part of the tumor to the superficial muscular layers by a wire loop. From the perspective of equipment, monopolar energy generates high-frequency currents that flow through the resectoscope to the grounding pad adhered to the patient's lower limb, resulting in ONR and related BP due to muscle contraction and thermal damage to adjacent tissues. Moreover, the use of electrolyte-free solutions as intraoperative irrigation fluid increases the risk of TUR syndrome (50).

The general principle of ERBT is to make a circular mucosal incision at a safe distance from the tumor base and then remove the whole tumor tissue including superficial DM (51, 52). So far, the safety and efficacy of en bloc resection have been explored in several clinical trials, and the results showed that the perioperative complication rate was not higher than that of conventional resection (**Table 1**). The monopolar current originally used in conventional TURBT can also be applied as an energy source for en bloc resection. Although the occurrence of ONR is similar to that of conventional resection, BP can be controlled within an acceptable range through elaborate surgical procedures and less frequent cutting and coagulation (28, 29, 40). With the introduction of laser energy into en bloc resection, ONR could be avoided and BP occurred in only two patients in a series of studies involving 795 patients (20, 21, 26, 27, 30–36). However, the occurrence of ONR and BP was 12.0%–40.7% and 0%–11.1% in conventional TURBT, respectively (26, 27, 31–36). For the single-arm studies about en bloc resection, the incidence of perioperative complications was negligible (**Table 2**).

CAN *EN BLOC* RESECTION IMPROVE ONCOLOGICAL OUTCOMES?

In conventional resection, the integrity of tumor tissue is destroyed and tumor cells are dispersed, which increases the risk of dissemination and implantation of exfoliated tumor cells. Meanwhile, due to continuous bladder irrigation, the pressure in the bladder is higher than the venous pressure, causing exfoliated tumor cells to travel into the blood circulation *via* the vascular system (53, 54). En bloc resection, as a "no touch" technique for the treatment of NMIBC, shows the potential to minimize the number of exfoliated tumor cells and reduce the risk of tumor cells entering the blood circulation (55). Theoretically, ERBT can achieve the envisaged goal of decreasing the rate of tumor recurrence and progression.

TABLE 1 | Studies comparing perioperative complications, detrusor muscle, and oncological outcomes between transurethral resection of bladder tumor (TURBT) and *en bloc* resection of bladder tumor (ERBT) categorized on their level of evidence (LoE).

Study	Operation	Energy source	Patients	ONR	BP	Transfusion	Bladder irritation	Blood loss	Urethral stricture	DM	Follow-up	Recurrence	Progression
Randomized comparing studies (LoE 1b)													
Zhang et al. (26)	ERBT	Thulium laser	149	N.A.	0*	N.A.	N.A.	N.A.	N.A.	131†	At 36 mo	REC: 45.6%†	5.4% †
	TURBT	Bipolar	143		6					134		42.7%	7.7%
Chen et al. (27)	ERBT	Thulium laser	71	0*	0†	N.A.	N.A.	17.2ml†	N.A.	N.A.	At 18 mo	REC: 5.6%†	0†
	TURBT	Monopolar	71	18	0			14.5ml				9.9%	0
Non-randomized comparing studies (LoE 2a–2b)													
Bălan et al. (8)	ERBT	Bipolar	45	2*	N.A.	N.A.	N.A.	0.28 g/dL*	N.A.	N.A.	At 12 mo	REC: 17.1%*	N.A
	TURBT	Monopolar	45	5				0.76 g/dL				27.5%	
Hayashida et al. (19)	ERBT	Polypectomy snare	39	N.A.	N.A.	0†	N.A.	N.A.	N.A.	39	Mean 12 mo	REC: 15.4%†	N.A.
	TURBT	Monopolar	31			0				N.A.		19.4%	
Yang et al. (28)	ERBT	Monopolar	96	23†	2*	N.A.	N.A.	N.A.	N.A.	39†	At 24 mo	REC: 21.2%†	N.A.
	TURBT	Monopolar	87	21	9					66		27.5%	
Zhang et al. (29)	ERBT	Monopolar	40	9†	2†	N.A.	N.A.	N.A.	N.A.	40*	Median 10.8 mo	REC: 20.0%†	N.A.
	TURBT	Monopolar	50	12	4					27	Median 11.3 mo	24.0%	
Tao et al. (30)	ERBT	980nm laser	36	0*	0*	0†	N.A.	N.A.	N.A.	N.A.	At 12 mo	REC: 2.8%†	N.A.
	TURBT	Monopolar	48	6	3	0						4.2%	
Cheng et al. (31)	ERBT	KTP laser	34	0*	N.A.	N.A.	N.A.	5.0†	N.A.	33*	At 12 mo	REC: 8.8%*	0.0%*
	TURBT	Monopolar	30	10				7.5		24		33.3%	16.7%
Li et al. (32)	ERBT	Thulium laser	136	0*	0†	N.A.	N.A.	N.A.	N.A.	130*	Mean 41 mo	RFS: 31.8%†	N.A.
	TURBT	Bipolar	120	4	1					103	Mean 40.6 mo	27.9%	
D'souza et al. (33)	ERBT	Holmium laser	23	0*	0*	N.A.	5*	N.A.	2†	N.A.	At 36 mo	REC: 30.4%†	N.A.
	TURBT	Monopolar	27	11	3		14		2			37.0%	
Chen et al. (34)	ERBT	Green laser	83	0*	0†	N.A.	N.A.	0.87g/ml*	1†	N.A.	At 36 mo	N.A.	N.A.
	TURBT	Monopolar	75	9	2			1.00g/ml	3				
Xu et al. (35)	ERBT	Thulium laser	26	0*	0†	0†	N.A.	N.A.	N.A.	N.A.	12 to 24mo	REC: 15.4%†	N.A.
	TURBT	Monopolar	44	7	3	0						27.3%	
Migliariet al. (36)	ERBT	Thulium laser	58	0*	0†	0†	N.A.	N.A.	N.A.	58*	Mean 20 mo	REC: 20.6%†	0
	TURBT	Monopolar	61	8	3	2				53		N.A.	N.A.
Cheng et al. (37)	ERBT	HybridKnife	95	2†	0†	N.A.	N.A.	2.76 g/L†	0	N.A.	1 to 33mo	RFS: 95.5%*	N.A.
	TURBT	Monopolar	98	7	2			3.00 g/L	0			79.5%	

ONR, obturator nerve reflex; BP, bladder perforation; DM, detrusor muscle; N.A., not applicable; KTP, potassium-titanyl-phosphate; REC, recurrence rate; RFS, recurrence-free survival.

**Compared to TURBT, the rate was significantly decreased.*

†Compared to TURBT, the rate was not significantly decreased.

Table 1 lists the studies comparing the oncological outcomes of TURBT and ERBT, and **Table 2** lists the results of single-arm studies of en bloc resection. As shown in **Table 1**, there was a general downward trend in tumor recurrence and progression of the ERBT group. However, the data of oncological outcomes in clinical trials presented large variability across centers or urologists (30, 31), and not all studies have confirmed that en bloc resection was superior to conventional resection (26, 27). Meanwhile, different energy sources and no standardized postoperative intravesical instillation therapy have been used in different studies, resulting in non-comparable data on tumor recurrence and progression. In short, there is no robust clinical evidence that ERBT is superior to conventional resection with respect to oncological outcomes (13).

AVOIDING REPEAT TRANSURETHRAL RESECTION

According to the European Association of Urology (EAU) guidelines, reTUR is recommended for patients with incomplete initial tumor resection, no DM in Ta high-grade specimens, and all T1 tumors (2). The reTUR has two goals: to detect and remove residual tumors, and to reconfirm the pathological stage. However, reTUR is an invasive surgical procedure that may seriously affect the quality of patients' life and increase their negative emotions (56). Moreover, elderly patients with other serious diseases cannot tolerate this procedure under spinal or general anesthesia. Finally, the implementation of reTUR will increase overall health care costs and the economic burden of individual patients. Therefore, without compromising oncological outcomes, the selection of the right patients to avoid reTUR is particularly important.

A retrospective multicenter study collected 321 patients with T1 high-grade bladder tumor who underwent reTUR. The results showed that the presence of DM in the initial tumor specimens, the absence of concurrent CIS, and en bloc tumor resection were three independent predictors of the absence of residual tumor at reTUR (57). After thulium laser en bloc resection, the short-term oncological outcomes of recurrence and progression were not significantly different between patients who received reTUR and those who did not (58). With the use of new optical imaging technologies in clinical practice as adjunct to WLC, such as photodynamic diagnosis (PDD) and narrow-band imaging (NBI), the detection rate of BC has been improved (59). Based on this theoretical evidence, the new optical imaging technology-assisted ERBT is expected to achieve optimal initial transurethral resection and then reduce the need for reTUR in well-selected patients with NMIBC (60).

APPLICATION OF OPTICAL MOLECULAR IMAGING IN THE MANAGEMENT OF BLADDER CANCER

After WLC-assisted TURBT, around 15%–61% and 31%–78% of patients with NMIBC will develop tumor recurrence within one

TABLE 2 | Studies analyzing perioperative complications, detrusor muscle, and oncological outcomes of *en bloc* resection of bladder tumor (ERBT).

Study	Energy	Patients	ONR	BP	Transfusion	Bladder irritation	Blood loss	Bladder bleeding	Urethral stricture	DM	Follow-up	Recurrence	Progression
Zhang et al. (38)	Bipolar	82	0	0	N.A.	N.A.	N.A.	0	N.A.	100%	At 18 mo	RFS: Ta: 88.5% T1: 74.5%	N.A.
Abotaleb et al. (39)	Bipolar	46	0	0	1	0	1.3g/dL	3	N.A.	100%	At 12 mo	REC: 15.2%	N.A.
Zhang et al. (20)	Vela laser	38	0	0	0	0	N.A.	0	0	100%	At 12 mo	REC: 21.6%	N.A.
Hurle et al. (40)	Monopolar	74	N.A.	1	N.A.	N.A.	N.A.	0	N.A.	100%	At 24 mo	REC: 17.6%	N.A.
Xu et al. (21)	Thulium laser	141	N.A.	2	N.A.	N.A.	N.A.	N.A.	N.A.	100%	At 36 mo	RFS: 68.3%	0

ONR, obturator nerve reflex; BP, bladder perforation; DM, detrusor muscle; N.A., not applicable; RFS, recurrence-free survival; REC, recurrence rate.

and five years, respectively (2). The high recurrence rate may be attributed to tumor multicentricity, incomplete tumor resection, implantation of exfoliated tumor cells, and neogenetic tumor formation. WLC, a traditional optical imaging system for the diagnosis and treatment of suspected BC, has several drawbacks. First, CIS is a high-risk kind of NMIBC confined to the mucosa, which can easily be confused with inflammatory lesions due to its similar structural appearance under WLC. A recent systematic review was conducted to analyze the results of random bladder biopsy in >10 000 patients with NMIBC, and the total incidence of CIS was 17.35% (61). Moreover, the rate of concurrent CIS reach up to 50% in patients with high-grade or sessile tumors (61, 62). Second, small or occult tumors, some of which may be high-grade or invasive lesions, are difficulty to detect (63). Finally, the boundary and depth of tumor invasion judged by the two-dimensional images of cystoscopy and operator's clinical experience are often subjective and inaccurate, even among senior urologists (64). Imprecise estimation of tumor tissue depth and demarcation impedes the completeness of tumor resection. Although no solid data have indicated that ERBT is superior to conventional TURBT with respect to oncological outcomes, it should be noted that 77% of tumor recurrence was located away from the original surgical site after en bloc resection (65), while most residual tumors (36%-86%) found at reTUR were located at the initial resection site after conventional resection (23). Thus, in order to detect more small or occult tumor lesions, especially CIS, during transurethral resection, new adjunctive optical imaging technologies for WLC are urgently needed.

According to the scope of imaging field, optical imaging technologies can be grossly divided into two groups: macroscopic and microscopic models. Macroscopic imaging modalities, such as PDD and NBI, provide a wide field of view of bladder wall in a similar manner to WLC and rely on additional contrast enhancement to highlight uncertain malignant lesions (37). Compared with WLC-assisted TURBT, the blood loss, ONR, and BP are significantly reduced, and the recurrence-free survival rate is significantly improved in patients treated with NBI-assisted en bloc resection (66). Even though PDD and NBI have the ability to improve the detection rate of papillary lesions and CIS, the incidence of false-positive results in prior intravesical therapy, inflammation, and intraoperative acute hemorrhage was significantly increased due to non-tumor specificity (67). Optical coherence tomography (OCT) and confocal laser endomicroscopy (CLE) belong to microscopic imaging technologies that produce high-resolution images of abnormal bladder mucosa to provide real-time pathological information about the changes in tissue microstructure and cell morphology (68, 69). However, OCT and CLE can only provide a limited view of bladder tissue during the examination. These technologies need to be combined with another macroscopic imaging modality (such as WLC, PDD, or NBI) to delimit the boundaries of tumor tissue (70).

Optical molecular imaging is a new molecular-targeted imaging technology that can qualitatively and quantitatively analyze the pathological process at the cellular and molecular levels prior to macrostructural changes of malignant tissue (15). The urinary bladder is a highly compliant hollow organ that can provide perfect closed operating dark environment for optical molecular imaging without interference from external stray light sources. Moreover, intravesical instillation of fluorescent targeted tracers through the urethra is convenient and simple before surgery. Molecular targeted tracers have the ability to bind tumor cells with high sensitivity and specificity. Optical molecular imaging mediated by it can detect small or occult tumor lesions while minimizing the occurrence of false-positive results.

BLADDER CANCER DETECTION

CD47, a member of the immunoglobulin superfamily, is overexpressed on more than 80% of BC cell membranes but not on normal urinary tract epithelium (71). When CD47 binds to signal regulatory protein α on phagocytes, it can inhibit the phagocytosis of tumor cells by macrophages to promote tumor proliferation (72). After RC, twenty-one fresh intact bladder specimens were collected and incubated with anti-CD47-Qdot625, and then detected under blue light. Finally, a total of 119 suspicious bladder tissues were examined, and the sensitivity and specificity were 82.9% and 90.5%, respectively (16). Carbonic anhydrase IX (CAIX), as a member of the carbonic anhydrase family, participates in intracellular pH modulation under hypoxic conditions, thus changing the biological features of tumor in terms of proliferation, adhesion, and progression (73). Using a similar experimental strategy, after incubating with anti-CAIX-Qdot625, the entire mucosa of the fresh intact bladder specimen was carefully examined under WLC, and then detected under blue light cystoscopy. The overall sensitivity and specificity for BC detection under WLC were 76.0% and 90.5%, while a high detection accuracy was achieved and the sensitivity and specificity rose to 88.00% and 93.75% under CAIX targeted optical molecular imaging (17).

Unlike monoclonal antibodies, peptides are synthesized by phage-display peptide libraries or the one-bead one-compound combinatorial chemistry approach, with the smallest molecular weight (0.5–2.0 kDa) among molecular tracers (74). The CSNRDARRC peptide, which specifically binds to human bladder tumor HT-1376 cells, is the first targeting peptide selected by phage-display libraries. In the N-butyl-N-(4-hydroxybutyl) nitrosamine-induced BC model, the fluorescein-conjugated CSNRDARRC peptide actively targets the luminal epithelium of malignant lesions, but not to normal bladder regions after intravesical instillation of bladder (75). The CSSPIGRHC peptide (NYZL1), which binds to human bladder tumor BIU-87 cells, was also selected by phage-display technology. After intravenous administration of fluorescein-labeled NYZL1 peptide, the tracer specifically binds to malignant tissue in a nude mouse model of human bladder tumor xenografts (76). The CSDRIMRGC peptide is another bladder tumor-specific peptide, named PLSWT7. A preclinical trial was conducted to explored the diagnostic accuracy of the corresponding molecular fluorescent tracer in the diagnosis of

malignant lesions in eight fresh intact bladder specimens. The specimens were incubated with PLSWT7-IRDye800CW, and the sensitivity and specificity of optical molecular imaging-assisted BC detection were 84.0% and 86.7%, respectively (77). Unlike the previous peptides, the CQDGRMGFC peptide (PLZ4) is synthesized by the combinatorial chemical method and can specifically bind to bladder tumor cells. An *in vivo* research showed that after intravenous administration, PLZ4-Cy5.5 selectively labeled the tumor tissue in the patient-derived xenograft mouse model (78).

Tumor heterogeneity can occur between different patients, or even in the same patient, which greatly impairs the diagnostic accuracy of molecular targeted tracers mediated optical molecular imaging (79). Many researchers have found that malignant cells, including BC cells, have increased glycolytic activity, resulting in an acidic tumor microenvironment (80, 81). The pH low insertion peptides (pHLIPs) target the acidity on the surface of tumor cells, and then penetrate the cancer cell membrane. The acidic tumor microenvironment contributes to this pathological process (82, 83). Twenty-two fresh intact bladder specimens were incubated with indocyanine green (ICG)-labeled pHLIP, and then the bladder mucosa was examined using paired optical imaging equipment. Regardless of the pathological stage, the sensitivity and specificity of malignant tissue detection were 97% and 100%. However, when including necrotic and previously treated tissues with intravesical chemotherapy, the total number of false-positive results increased and the specificity decreased to 80% (18).

A variety of molecular fluorescent agents have been explored in optical molecular imaging of bladder tumor and have shown the ability to improve the detection rate of BC lesions in urothelial cancer animal models and patient's tumor specimens. Tumor heterogeneity and different protein expression patterns between patients complicate the selection of molecular fluorescent tracers (84). Then the following question arises: How can urologists choose the best molecular targeted agent to perform optical molecular imaging-assisted en bloc resection for a specific patient with NMIBC. Fortunately, according to the EAU guidelines, cystoscopy-guided biopsy is strongly recommended for patients with suspected malignant lesions, followed by histopathological evaluation of the tissue samples as the initial diagnostic procedure (2). Immunohistochemical analysis and next-generation sequencing of tumor tissue can reveal biomarkers for molecular imaging, thereby helping urologists choose the most appropriate molecular fluorescent tracer for candidates who may benefit from optical molecular imaging (85).

REAL-TIME GUIDANCE IN BLADDER CANCER SURGERY

During WLC-assisted TURBT, urologists rely on their own clinical experience and indirect visual feedback to determine the location of the tumor lesion and the boundary and depth of tumor invasion. Unfortunately, at the time of reTUR, residual tumors occur in 51% of patients with T1 bladder tumors, which reflects the inaccuracy of initial intraoperative evaluation (2). For NMIBC patients, complete resection of all visible malignant lesions and accurate histopathological evaluation of tumor specimens are the keys to prolonging recurrence-free and progression-free survival rates. Meanwhile, in order to avoid unnecessary deepening and expanding resection to preserve more adjacent normal tissues, urologists need extra intraoperative optical imaging guidance to perform complete and high-quality tumor resection, regardless of the surgeon's clinical experience in urological endoscopic surgery. Optical molecular imaging is a promising adjunctive imaging mode for WLC in BC surgery. In a preclinical trial, bladder tumor xenograft mouse models were randomly subdivided into two groups, and tumor resection was operated under the sunlight condition (control group, n = 20) and with optical molecular imaging guidance (experimental group, n = 20). A week later, the recurrence rates of the control group and experimental group were 95% and 5%. At 30 days after operation, the overall survival rates of the two groups were 0% and 90%, respectively (77).

The fresh intact bladder tumor specimens collected after ERBT can help pathologists make accurate histopathological assessments and assess the status of tumor surgical margin. After the intact tumor specimen was incubated with anti-CD47-Alexa Fluor 790 and imaged under a near-infrared (NIR) imaging device, the mean fluorescence intensity of tumor tissue was significantly higher compared with adjacent normal background tissue, which might further assist pathologists in selecting the best pathological material to find the positive surgical margin (86). Therefore, on the one hand, en bloc tumor resection can ensure accurate pathological evaluation of the resected specimens through better protecting the spatial orientation and architecture of the tumor tissues during surgery (87). For another, optical molecular imaging shows the potential to improve the positive detection rate of small or occult tumor lesions. Meanwhile, based on the difference in the fluorescence signal between tumor tissue and adjacent normal tissue, urologists can objectively judge the depth and boundary of tumor invasion during transurethral resection. Therefore, optical molecular imaging-assisted en bloc tumor resection could help urologists perform high-quality and complete tumor resection. After surgery, an intact tumor specimen containing DM can be collected for pathologists for histopathological assessment (**Figure 1**). Then the risk stratification of NMIBC and the depth of tumor invasion can be assessed accurately and objectively. Based on the above information, urologists can select the most appropriate treatment strategy for patients with NMIBC to improve the oncological outcomes (**Figure 2**).

CHALLENGES AND FUTURE PERSPECTIVES

The major challenge in performing ERBT is the size of bladder tumor (13). In the ERBT group, most clinical trials excluded

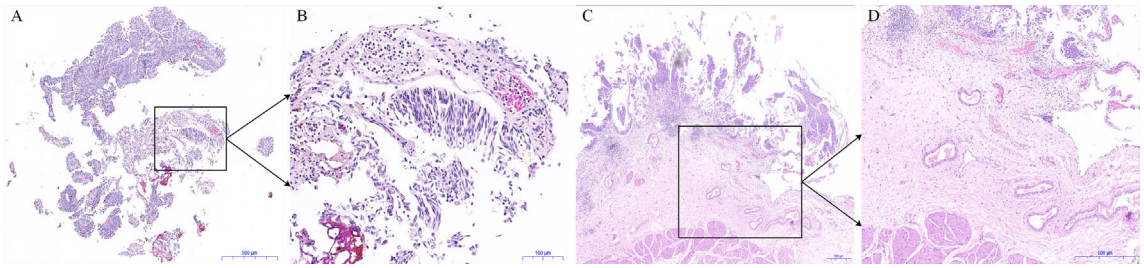

FIGURE 1 | Histological pictures of the tumor specimens obtained from conventional transurethral resection of bladder tumor (TURBT) and en bloc resection of bladder tumor (ERBT). **(A)** The hematoxylin and eosin (HE) staining image of tumor specimen obtained from conventional TURBT. **(B)** The enlarged image in the black frame in **(A)**. **(C)** The HE staining image of tumor specimen obtained from ERBT. **(D)** The enlarged image in the black frame **(C)**.

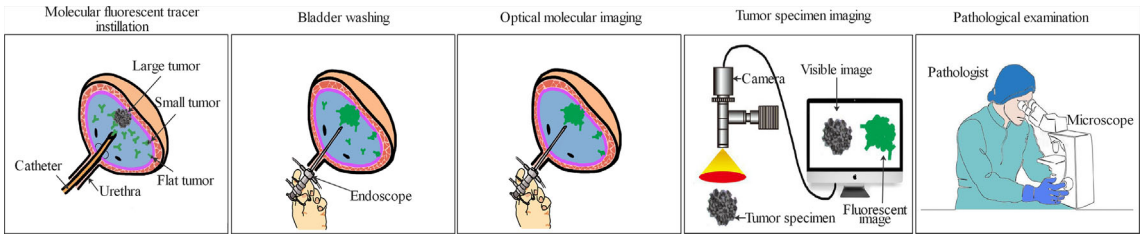

FIGURE 2 | Principle of optical molecular imaging-assisted en bloc resection for the treatment of bladder cancer. After instillation of the molecular targeted tracer (from half an hour to a few days before operation relying on the pharmacokinetics and distribution of the molecular targeted tracer in vivo) into the bladder, the bladder is flushed to drain the uncombined molecular targeted tracer. Then, a paired imaging device is used for optical molecular imaging to detect the entire bladder mucosa. In addition to large tumors, small or occult tumor lesions can be detected under optical molecular imaging-assisted en bloc resection. The intact tumor specimens collected after the operation is immediately sent to the pathology department and examined using fluorescence microscope. Pathologists can accurately assess the status of the tumor surgical margin and the depth of tumor invasion, which will help urologist choose the most appropriate treatment strategy for patients with non-muscle invasive bladder cancer to improve the oncological outcomes.

patients with tumor diameter larger than 3 cm (8, 19, 31). Although a previous study reported that HybridKnife-assisted en bloc resection is suitable for larger bladder tumors with diameters up to 7.5 cm, the intact tumor specimen retrieval remains a significant challenge (88). Without the help of additional surgical instruments, it is impossible to extract a large intact tumor specimen *via* the outer sheath of the resectoscope (89). Given the high medical costs of NMIBC (90), some medical equipment manufacturers have designed new medical devices to improve the quality of surgery. Meanwhile, urologists should work closely with equipment manufacturers to improve resection instruments and extraction devices to ensure that transurethral tumor resection follows the basic principle of oncological surgery. In addition, all patients have a strong desire to receive high-quality tumor resection in order to avoid further reTUR, especially among the high-risk NMIBC patients (91).

Optical molecular imaging is a new and promising visualization system for tumor diseases. However, the available evidence is limited to preclinical trials. There are some technical and scientific issues that urgently need to be resolved before patients and urologists can reap the benefits of high diagnostic accuracy associated with it. For instance, to achieve a balance between drug safety and fluorescence image quality, it is essential to carry out dose optimization experiments. Meanwhile, the use of bright fluorescent targeted molecules and sensitive optical imaging equipments guarantees high-resolution images. Up to now, the only NIR imaging device approved for clinical application by the US Food and Drug Administration is designed and developed based on the photophysical properties of ICG (92). However, for some newly synthesized fluorophores, due to their unique photophysical characteristics, they might not be well compatible with the existing imaging devices, so a customized optical imaging equipment may be required to present high-resolution images. Compared with WLC, extra optical imaging device and molecular targeted tracers are required to perform optical molecular imaging. With respect to the cost-effectiveness of this new optical imaging technology, the costs of fluorescent targeted molecules, amortization of imaging devices, and the benefits obtained from the improvement of oncological outcomes should be taken into account. Similarly, PDD and NBI, as enhanced imaging systems, have been recommended by the EAU guidelines for transurethral tumor resection, which also requires extra optical imaging devices (2).

Due to the reduced recurrence rate and the associated reoperation rate, PDD-assisted TURBT can save €168 per patient per year (93), and obtain the greatest economic benefits among moderate-risk NMIBC patients (94). For patients with NMIBC receiving NBI-assisted TURBT, compared with WLC-assisted TURBT, each patient can save $230 to $500 per year (95).

Molecular targeted tracers and optical imaging equipments are two basic essentials for optical molecular imaging. However, there is no expert consensus on the standardized evaluation of the performance of molecular targeted tracers, which makes it difficult to organize prospective multicenter studies and analyze the experimental data from different scientific research institutions. The fluorescence intensity of the tissue to be examined and the tumor-to-background ratio (TBR) are two important references for evaluating the clinical application value of molecular targeted tracers. However, the fluorescence signal of the detection area is affected not only by the drug dosage, pharmacokinetics, and the duration between administration of molecular targeted tracers and imaging examination, but also by the manufacturer, technical parameters, and performance of the optical imaging device used (96). In addition, even if a satisfactory TBR could be observed during the inspection, the data collection might be affected by the region of interest selected by different operators.

The ultimate goal of related research is to introduce optical molecular imaging technology into the clinical application of en bloc tumor resection. Therefore, a promising fluorescent targeted tracer for molecular imaging must pass the drug security analysis before being explored in clinical studies. But unfortunately, the drug toxicity analysis and good production process are expensive and time-consuming, which makes them the major obstacles to the design and development of new fluorophores and molecular tracers. Ideally, molecular targeted tracers should have the following characteristics, such as good drug safety, high tumor tissue specificity, appropriate pharmacokinetics and chemical stability in the human body.

CONCLUSIONS

ERBT is a safe and feasible technique for the treatment of NMIBC, and it provides an intact tumor specimen containing DM for pathologists for accurate histopathological assessment. Indeed, there is no robust clinical evidence that ERBT performs better than conventional resection in terms of oncological outcomes. Optical molecular imaging shows the potential to improve the detection rate of malignant lesions and highlight the boundary and depth of tumor invasion. In theory, optical molecular imaging-assisted en bloc tumor resection integrates the advantages of both and is expected to improve the quality and completeness of transurethral tumor resection, and ultimately improve the oncological outcomes of patients with NMIBC.

AUTHOR CONTRIBUTIONS

YY designed the study, collected and analyzed clinical data, and wrote the manuscript. CL, XtY, and JL collected the related literature. XfY designed the study, supervised the research, and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Gastrin-Releasing Peptide Receptor in Low Grade Prostate Cancer: Can It Be a Better Predictor Than Prostate-Specific Membrane Antigen?

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The aim of the present study was to evaluate whether prostate cancer (PC) patients can be accurately classified on the bases of tissue expression of gastrin-releasing peptide receptor (GRPR) and prostate-specific membrane antigen (PSMA). This retrospective study included 28 patients with PC. Formalin-fixed paraffin-embedded samples were used for diagnosis. Immunohistochemistry staining techniques were used to evaluate PSMA and GRPR expression (both number of cells expressed and % of area stained). To assess the independent associations among selected variables, a multi-dimensional scaling (MDS) analysis was used. It was found that the PSMA expression was inversely correlated with GRPR expression. Only the number of cells expressing GRPR was significantly related to the Gleason score. Both the percentage of area expressing GRPR and the number of cells expressing PSMA were close to reaching significance at the 0.05 level. MDS provided a map of the overall, independent association confirming that GRPR and PSMA represent inversely correlated measures of the same dimension. In conclusion, our data showed that GRPR expression should be evaluated in prostate biopsy specimens to improve our ability to detect PC with low grades at the earliest phases of development. Considering that GRPRs appear to be directly involved in the mechanisms of tumor proliferation, advancements in nuclear medicine radiotherapy can focus on this receptor to improve the therapeutic approach to PC. Further studies in our laboratory will investigate the molecular mechanisms of activation based on GRPR.

Keywords: gastrin-releasing peptide receptor (GRPR), prostate-specific membrane antigen (PSMA), prostate cancer, multi-dimensional scaling (MDS), Gleason score

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INTRODUCTION

Prostate cancer (PC) has one of the highest incidence rates and represents the sixth leading causes of cancer death among men worldwide (1). Unfortunately, the worldwide PC incidence is expected to increase nearly two-fold by 2040, simply due to the growth and aging of the population. To prevent this global crisis, accurate diagnosis and staging of PC are of paramount importance. Effective

therapies can dramatically increase the probability to survive this disease, especially when it is identified at its early stages. The Gleason score (GS) is among the most used instrument for PC grading (2). The International Society for Urological Pathology (ISUP) has published guidelines for the classification and outcome of PC. This grading system divides PCs into 5 prognostic grade groups. Group 1 (I/V) includes GS 6 (3 + 3), grade 2 (II/V) corresponds to GS 7 (3 + 4), while grade 3 (III/V) identifies prostate tumors with GS 7 (4 + 3). Grade groups 4 (IV/V) and 5 (V/V) correspond to GS 8 and GS 9 or 10 respectively. The division of risk classes and TNM staging are aimed at the correct planning of the diagnosis and therapy of patients with prostate cancer.

Prostate tissue is made up of three types of epithelial cells: basal cells, secretory cells and neuroendocrine cells (NE). The function of NE cells in the prostate is still unknown, but an active role in the regulation of growth, differentiation, and development of prostate gland is assumed (3). This assumption is based on three factors: the morphology of NE cells, the action of hormones produced by NE cells, and the analogy with the function performed by NE cells in peripheral system (4). NE cells are able to secrete growth factors and hormones that can support the growth of the surrounding tumor *via* paracrine signaling (5). Among these growth factors, bombesin has a mitogenic function on the PC cells, which it exerts by activating the transcription factor Elk-1 (6). In addition, bombesin is involved in the expression of some metalloproteases (such as MMP-9), which stimulate the remodeling of the extracellular matrix and thus tumor invasion, angiogenesis and the formation of metastases (7).

Gastrin releasing peptide (GRP) is a neuropeptide that induces gastrin secretion in the stomach (8). GRP acts through attachment to the gastrin-releasing peptide receptor (GRPR or BB2), a member of the G protein coupled receptor (GPCR) superfamily expressed in the gastric, respiratory and nervous system as well as endocrine glands and muscles (9). GRP mediates gastrointestinal motility and hormone and neurotransmitter release in the intestine, colon and other organs (10). GRP can act centrally in the nervous system, and it has been linked with a variety of essential homeostatic and behavioral regulations, including daily cycle, fear, anxiety, stress and modulation of memory (11–13). It is overexpressed in cancer cells and the GRP production along with GRPR overexpression leads to growth autocrine stimulation. GRPR overexpression gradually increases in prostatic carcinogenesis reaching from low-grade prostatic intraepithelial neoplasia (PIN) over high-grade PIN to low grade PC, whereas GRPR shows only little expression in normal prostate tissue in benign prostate hyperplasia and high grade PC (14).

Prostate-specific membrane antigen (PSMA), also known as glutamate carboxypeptidase II (GCP II), N-acetyl-L-aspartyl-L-glutamate peptidase I (NAALDase I) or N-acetyl-aspartyl-glutamate (NAAG) peptidase, is a transmembrane glycoprotein encoded by the FOLH1 gene (15). PSMA is expressed in tumor cells of almost all prostate cancers, and its increased expression is associated with tumor aggressiveness, metastasis and recurrence. The cellular localization of PSMA is cytoplasmic and/or membranous (16).

The aim of the present study was to evaluate whether PC patients can be accurately classified on the bases of tissue expression of GRPR and PSMA. Previous studies have found a significant inverse correlation between GRPR and several indices of tumor growth (GS, PSA and tumor size), on the other hand, we focused on low grade PC with neuroendocrine differentiation as indicated by the relationship between GRPR and PSMA. On the basis of preliminary results, it was hypothesized that their expression would be inversely related to each other. As a consequence, it was also hypothesized that low GRPR expression was associated with low GS values at TNM. A secondary aim was to investigate if PSMA and GRPR expression could be used to correctly classify each of the individual investigated on their PC cancer risk. Increasing the spectrum of markers available to diagnosis cancer risk would be extremely important to improve imaging and therapeutic target.

MATERIALS AND METHODS

Subjects

This retrospective study included 28 patients with PC followed at the Departments of Surgical, Medical, Molecular Pathology and Critical Area and Department of Translational Research and Advanced Technologies in Medicine, University of Pisa. Samples were randomly selected from a limited database of patients who undergo a specific set of diagnoses including both PSMA and choline tumor imaging PETs. This study has been conducted in accordance with the principles embodied in the World Medical Association Declaration of Helsinki. All samples were acquired with informed consent.

The mean age was 64 years (range 58 to 75 years). Formalin-fixed paraffin-embedded samples were used for diagnosis. The histology and classification of prostatic lesions have been established by experienced pathologists in accordance with the Consensus of the International Society of Urological Pathology (ISUP).

PSMA and GRPR

All immunohistochemistry staining slides were assessed with a light microscope (Nikon, Eclipse Ci) using a double-blinded procedure to avoid estimation biases. Quantitative measures of PSMA and GRPR (number of cells and % of area expressed) were obtained using the software Nis-Elements 5.01 (Laboratory Imaging Nikon).

After deparaffinization, antigen retrieval was performed by heating microwave (700 W) for 20 min in a 10 mM citrate buffer at pH 6.0, with a cool down period of 20 min afterward. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 20 min. Slides were than incubated with the primary anti-human-PSMA/GRPR mouse monoclonal antibodies, YPSMA-1 (Dako, Santa Clara, CA, USA; Thermo Fisher, Waltham, MA, USA), both diluted at 1:100 in 1% bovine serum albumin/phosphate-buffered saline

(1% BSA/PBS) for 1 h at room temperature. The secondary step consisted of incubation with rabbit anti-mouse antibody conjugated to polymer-horseradish peroxidase, diluted at 1:100 in 1% BSA/PBS with 1% AB serum. For the tertiary step, goat anti-rabbit. antibody conjugated to polymer-horseradish peroxidase was used, diluted at 1:100 in 1% BSA/PBS with 1% AB serum. Both the secondary and tertiary step required incubation for 30 min at room temperature. Next, the slides were immersed for 10 min in a solution of 0.05% 3,3'-diaminobenzidine (Roche Diagnostic, Corporation, Indianapolis, IN, USA) and 0.03% hydrogen peroxide in PBS for the visualization of the signal as brown staining. After washing with demineralized water, the slides were slightly counterstained with hematoxylin, dehydrated and mounted with Eukitt mounting medium (Roche Diagnostic, Corporation, Indianapolis, IN, USA).

Statistical Analysis

Correlation among the PSMA and GRPR scores were calculated with Pearson's r. General Linear Models (GLM), including ANOVAs, were used to determine the changes in PSMA and GRPR scores across Gleason score and T stage. All significance values were calculated at the α level of 0.05. Bonferroni corrections were used to prevent inflated Type II errors on repeated calculations using the same data. Power analysis revealed that all analyses, including both GRPR and PSMA, were above the 80% threshold.

To assess the independent associations among selected variables (clinical-pathologic variables T, N, and GS, plus the % and number of positive cells for both GRPR and PSMA), a multi-dimensional scaling (MDS) analysis was used. MDS is a data reduction technique used to reveal the similarities among variables and individual cases in a set of data (17). Distances between variables were calculated looking at partial correlations (i.e., proximities) among variables, which were subsequently used to create a matrix of distances that could be displayed graphically. The closer two or more variables are on the map, the more highly correlated they are, while the farther apart they are, the less correlated they are. In order to arrange the variable into a map sensitive to each individual contribution, a limited lack of fit between the data and the model is inevitable. This lack of fit is known as the s-stress. The values of s-stress range from 0 (perfect fit) to 1 (worst possible fit). Thus, the aim of MDS is to find a map of the variables that minimizes the s-stress. The number of dimensions in a map is linked to the number of latent underlying factors in the dataset, similarly to other procedures like factor analysis. As a consequence, the optimal number of dimensions to represent the data is dependent on several factors: the number of variables in the model; the lack of fit (s-stress value), given the number of dimensions; an index of fit of the model (R^2-value); and interpretability of the dimensions (18). Typically, R^2-values of 0.8 or higher are considered acceptable.

To evaluate the overall, independent effects of the overall Gleason score as the grouping variable on PSMA and GRPR (as predictors) we ran a series of models using a stepwise Discriminant Analysis (DA). DA is a cluster analysis often used to classify cases into groups on the bases of various response variables. It offers information as to which characteristics discriminate best between groups and analyzes the precision of these characteristics for group classification. Three stepwise DA were conducted on each of the Gleason score grade (high and low) using PSMA and GRPR expression as predictors. A total of 4 predictors were initially entered in each model.

All analyses were performed using SPSS 27.0 (IBM, Armonk, NY).

RESULTS

All the 28 samples were available for immunohistochemistry. PSMA expression was seen intracellular, mainly cytoplasmic, GRPR was concentrated at the luminal side of the cell membrane. Representative cases for positive and negative expression of both GRPR and PSMA are shown in **Figure 1**.

It was found that the PSMA expression was inversely correlated with GRPR expression (number of cells: r= -0.723, p<0.001– **Figure 2A**; percentage of area: r=-0.412, p<0.029 – **Figure 2B**).

The 28 samples were classified on the bases of Gleason scores: 25% (7/28) were classified as Grade Group 1, 46% (13/28) as Grade Group 2, and 29% (8/28) as Grade Group 3. Only the number of cells expressing GRPR was significantly related to the Gleason score ($F_{2,25} = 10.71$, p<0.001 – **Figure 3**). Tukey post-hoc testes revealed that the number of GRPR cells expressed was significantly different in all 3 grade groups (Grade 1 vs. Grade 2: p=0.018; Grade 1 vs. Grade 3: p<0.001; Grade 2 vs. Grade 3: p=0.013). Both the percentage of area expressing GRPR and the number of cells expressing PSMA were close to reaching significance at the 0.05 level (GRPR area: p=0.087; PSMA count: p=0.081 – all p-values > 0.20). Indeed, Tukey post-hoc tests revealed that the difference between Grade 1 and 3 was significant for both GRPR area (p=0.029) and PSMA counts (p=0.027).

Samples were also classified in risk groups accordingly to their T stage: 25% (7/28) were classified as T stage < T2c; 54% (15/28) were classified as T stage ≥ T2c; the remaining 21% (6/28) as T stage ≥ T3. No significant relationships between T stage and expression of GRPR and PSMA were found (all p-values > 0.20).

Multi-Dimensional Scaling (MDS) provided a map of the overall, independent association of multiple classification groups and the two measures of GRPR and PSMA expression (cell count and % area - **Figure 4**). The model parameters indicated that the map was very reliable (R^2 = 0.99; S-stress=0.031). The map confirmed that GRPR and PSMA represent inversely correlated measures of the same dimension. The overall Gleason score was positively associated with PSMA measures and inversely with GRPR measures. T was also positively associated with PSMA and

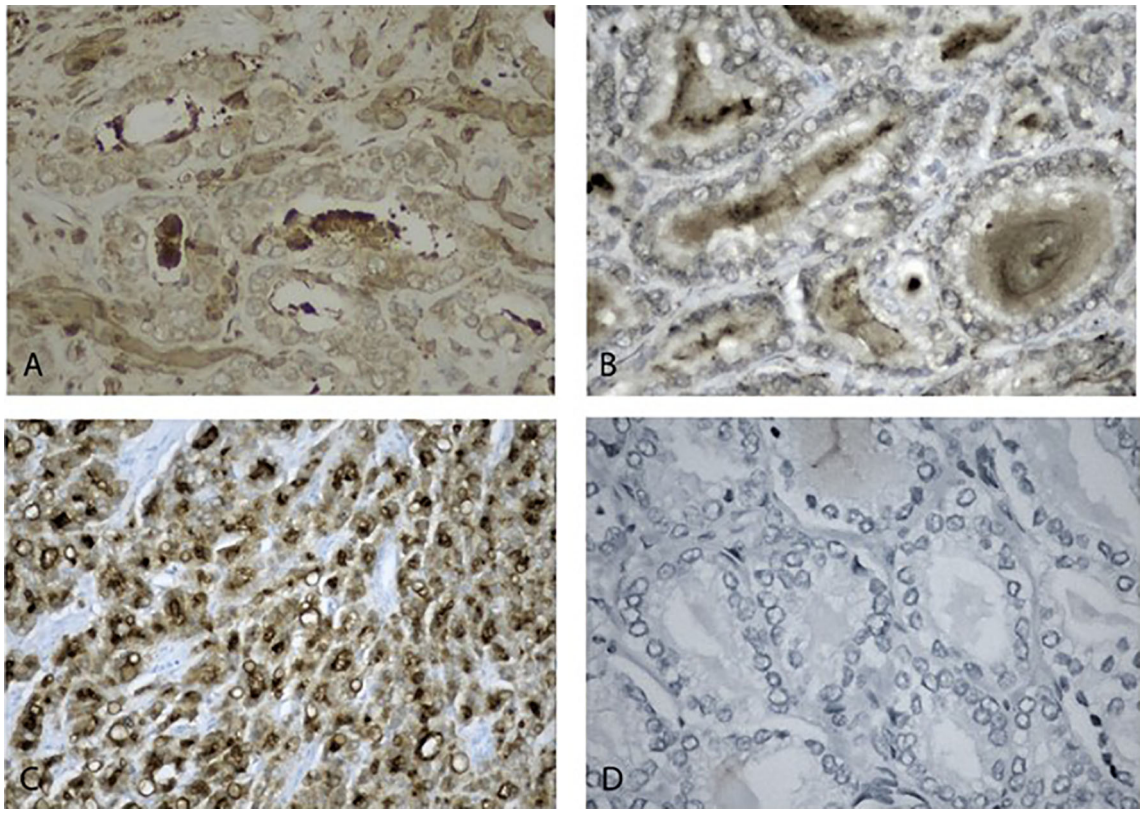

FIGURE 1 | Immunohistochemistry staining of positive and negative expression of biomarkers. **(A)** positive GRPR low-grade prostate neoplasms 40x. **(B)** Negative high-grade prostate neoplasms, 40x. **(C)** Positive PSMA high-grade prostate neoplasms, 20x. **(D)** negative PSMA low-grade prostate neoplasms, 20x.

inversely related to GRPR. N was not associated with any other measures.

A stepwise Discriminant Analysis (DA), with the overall Gleason score as the grouping variable and PSMA and GRPR measures as predictors, was used to identify the most useful parameter to classify the patients on the bases of the measures taken. The best model (Wilk's λ=0.539, $p<0.001$; $R^2 = 0.49$, % of correct cases classified=100%) included only the number cells expressing GRPR.

DISCUSSION

In the present study it was found that the expression of PSMA and GRPR can accurately predict the grade and stage of PC. More interestingly, multi-dimensional scaling revealed that PSMA and GRPR are inversely related, confirming that both measures can be used as a reliable indicator of the size of the tumor tissue. In terms of diagnostic capabilities, discriminant analysis further showed that the best predictor of grade and stage was the number of GRPR cells immunoreactive. Consequently, this study presented supporting evidence to include GRPR expression in routine diagnosis to improve early detection of PC.

The significance of GRPR expression in PC is still unclear. GRPR is absent or is expressed at very low levels in the normal prostate glands, but it increases significantly in tumors (19–23). However, the influence of this overexpression on the degree and stage of cancer is controversial. Nagasaki et al (19). found out that the expression of GRPR was correlated with high Gleason scores, but a subsequent study with a very large sample (n=530) found that the expression of GRPR was inversely correlated with the Gleason score, as well as with preoperative PSA tumor concentration and with the size of the neoplastic tissues (21). In the current study we found that the inverse relations between GRPR and PSMA can accurately identify low grade PC with neuroendocrine differentiation. Because NE cells differentiation has been positively correlated with tumor grade, and considering that GRPRs are profusely expressed in NE cells in advanced state of cancer, this would explain why identifying their expression in the prostate can represent a useful and innovative diagnostic tool (24–28). Moreover, NE cells differentiation and the production of neuroendocrine peptides, such as GRPR, are thought to be important mechanisms in the development of castration resistance in prostate cancer (29) thus making the study of NE cells differentiation even more important in the diagnosis and therapeutic approach to PC.

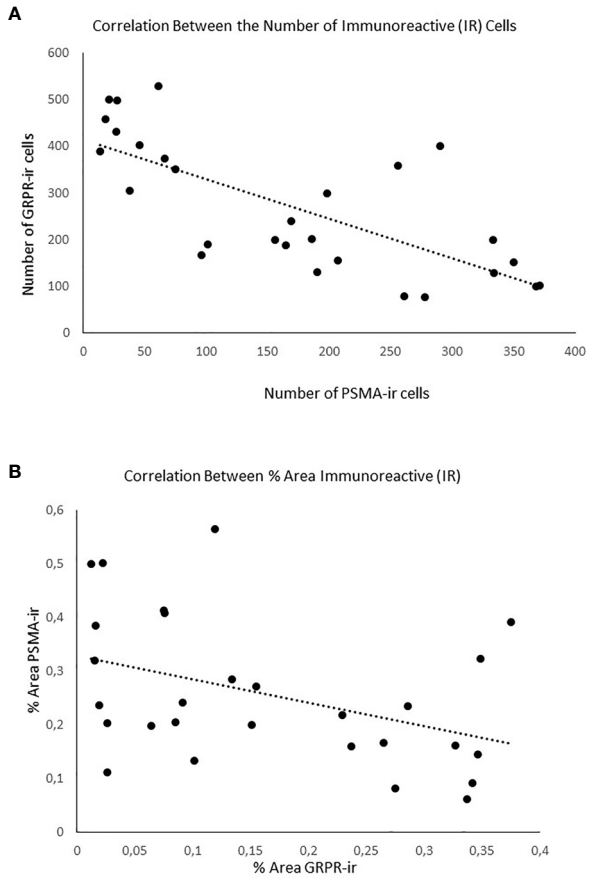

FIGURE 2 | Negative correlation between the expression of the markers PSMA and GRPR. **(A)** Correlation between the numbers of cells expressed (immunoreactive); **(B)** Correlation between the % of area expressed.

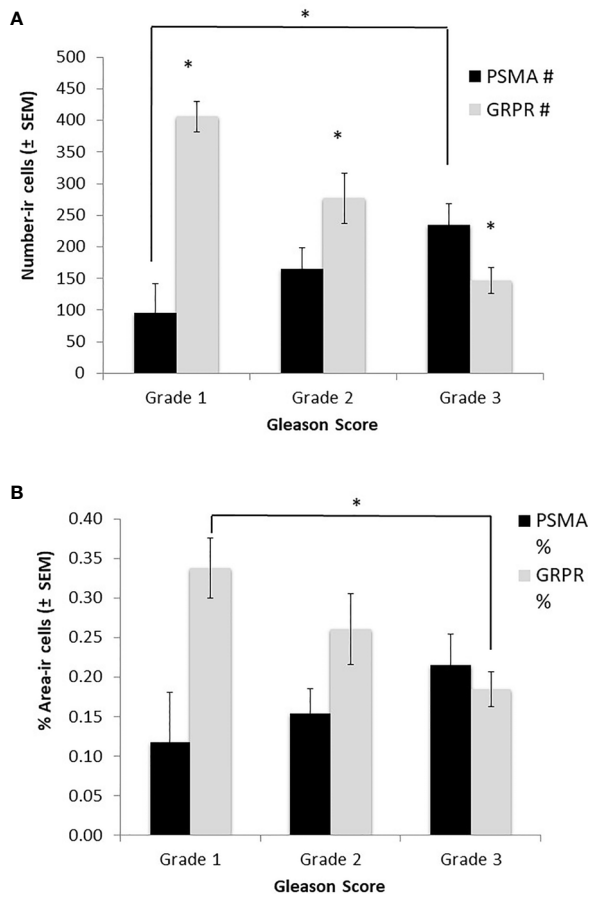

FIGURE 3 | Relationship between tumor grade, using Gleason scores, and expression of PSMA and GRPR. **(A)** the number of GRPR cells expressed (immunoreactive) was significantly higher in low grade tumors; **(B)** The % of area expressed was not significantly different by Grade for both markers. *$p < 0.05$.

Considering that different oncotypes, such as epithelial and neuroendocrine, can coexist within a neoplasm, the evaluation of the 'neoplastic immunohistochemical status' becomes a crucial and fundamental parameter not only from a diagnostic point of view, but above all as a prognostic tool. Morpho-functional modifications of the receptor structure can make the prostatic neoplastic cells resistant to treatments and therefore the neoplastic progression often cannot be controlled. This would be extremely dangerous for the patient, especially considering that this therapy can also be used as a "reductive" therapy neo-adjuvant. After the radiolabeled somatostatin peptide analogues have being used successfully in neuroendocrine tumors for nuclear imaging and therapy (30, 31), GRPR radioprotections (GRPR radiolagind5,6) have been synthesized and used in preclinical and clinical studies, currently including the prostate (32, 33). The use of these radiotracers in the prostate indicating GRPR could help identify probably preneoplastic lesions (high grade PIN) and low grade prostate tumors with NED in cancer patients. These observations opened an intriguing field of application of GRPR-specific radiopharmaceuticals for prostate cancer imaging and therapy, using a theranostic approach. In particular, understanding the features of GRPR signal in prostate cancer tissue seems critical for improving cancer care, as supported by very preliminary data. Our sample was quite small due to a limited database, and thus this is a limitation of the study that will require further investigations in a more general population. Another limitation is the lack of survival data on the patients, which will be the focus of a future investigation.

In conclusion, our data showed that GRPR expression should be evaluated in prostate biopsy specimens to improve our ability to detect PC with low grades at the earliest phases of development. Considering that GRPRs appear to be directly involved in the mechanisms of tumor proliferation, advancements in nuclear medicine radiotherapy can focus on this receptor to improve the therapeutic approach to PC. Further studies in our laboratory will investigate the molecular mechanisms of activation based on GRPR.

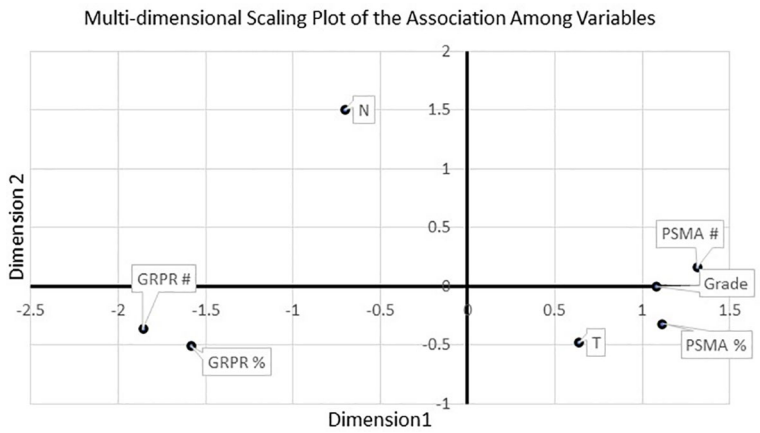

FIGURE 4 | MDS revealed a significant inverse relationship between GRPR markers and tumor grade, as well as a positive association with PSMA markers. T stage was also related to PSMA markers.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Universita' di Pisa. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Writing-original draft preparation, PF. Methodology, IS, CG. Supervision, MB. Investigation, PE, FM, RB, and LG. Writing—review and editing, PF, LB, and MB. All authors contributed to the article and approved the submitted version.

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REVIEW
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Metastatic Renal Cell Carcinoma Management: From Molecular Mechanism to Clinical Practice

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The therapeutic sc"enario of metastatic renal cell cancer (mRCC) has noticeably increased, ranging from the most studied molecular target therapies to those most recently introduced, up to immune checkpoint inhibitors (ICIs). The most recent clinical trials with an ICI-based combination of molecular targeted agents and ICI show how, by restoring an efficient immune response against cancer cells and by establishing an immunological memory, it is possible to obtain not only a better radiological response but also a longer progression-free and overall survival. However, the role of tyrosine kinase inhibitors (TKIs) remains of fundamental importance, especially in patients who, for clinical characteristics, tumor burden and comorbidity, could have greater benefit from the use of TKIs in monotherapy rather than in combination with other therapies. However, to use these novel options in the best possible way, knowledge is required not only of the data from the large clinical trials but also of the biological mechanisms, molecular pathways, immunological mechanisms, and methodological issues related to both new response criteria and endpoints. In this complex scenario, we review the latest results of the latest clinical trials and provide guidance for overcoming the barriers to decision-making to offer a practical approach to the management of mRCC in daily clinical practice. Moreover, based on recent literature, we discuss the most innovative combination strategies that would allow us to achieve the best clinical therapeutic results.

Keywords: renal cancer carcinoma, targeted therapy, tyrosine kinase inhibitor (TKI), immune checkpoints inhibitor, new biomarkers

INTRODUCTION

Renal cancer is the 10th most common cancer in Italy, with approximately 13,400 new cases per year (1), 70–80% have clear cell histology, while papillary, medullary, chromophobe, and other forms classified as non-clear cell histology are rare. Approximately 25% of patients present with the advanced-stage disease since their diagnosis, and among those undergoing nephrectomy, about one-third experience a distant recurrence during the rest of their lives and are initiated to systemic treatment.

Despite the significant therapeutic improvements, the 5-year survival rate of patients with metastatic renal cell cancer (mRCC) remains poor, especially in patients with unfavorable prognostic factors (2). The two validated prognostic models for the classification of patients with mRCC within clinical trials are the Memorial Sloan Kettering Cancer Centre (MSKCC) model (3) and the International mRCC Database Consortium (IMDC) that date back to 2005 and 2009, respectively (4). Although more than 10 years have elapsed, and in the meantime, drug molecules with new mechanisms of action have been developed, clinical trials still stratify patients into those with favorable (with 0 poor prognostic factors), intermediate (with 1–2 poor prognostic factors), or poor risk in the presence of at least three of the following prognostic factors: less than 1 year from diagnosis to treatment time, a Karnofsky PS score of <80 at the start of treatment, anemia, neutrophil or platelet count greater than the normal upper limit, or hypercalcemia (corrected Ca >10 mg/dl or >2.5 mmol/L).

The therapeutic scenario of mRCC has undergone incredible enrichment in recent years, ranging from the most studied tyrosine kinase inhibitor (TKI)-targeted therapies (anti-vascular endothelial growth factor (VEGF) and anti-mTOR) to those most recently introduced (anti-MET, anti-RET, and anti-FGFR) up to immunotherapy (IO) (anti-PD-1, anti-PD-L1, and anti-CTLA-4). Literature data on new therapeutic indications with cabozantinib in both the first and second lines (5, 6), nivolumab after anti-VEGF TKI progression (7), nivolumab combined with ipilimumab in naive patients with poor prognostic factors, and pembrolizumab combined with axitinib in all prognostic subgroups (8), have modified the prognosis of patients with mRCC. Especially, patients classified as 'intermediate' risk pass from a historical median survival of approximately 20 months to 3 years in the front line, which is almost equal to that of patients with favorable prognosis. On the one hand, we have seen a considerable improvement in the therapeutic algorithm of mRCC [clinical guidelines reported different therapeutic options only in patients with intermediate prognosis (1)]. On the other hand, the rate of development in the identification of new prognostic and predictive factors has not been the same throughout. Therefore, MSKCC/IMDC remains the standard prognostic classification criteria. However, in light of the complex mechanisms of action of the new TKI molecules, such as cabozantinib or combinations of TKIs and IO, or even more combinations of different immune checkpoint inhibitors (ICIs), are we sure that these 'old' criteria are sufficient?

In the era of precision medicine, in which knowledge of the molecular and genomic aspects of renal cancer has become ever wider, how can we think that criteria based on obvious clinical considerations (poor performance status and a short progression-free interval) and hematochemical parameters are sufficient to determine a therapeutic choice? To make the best use of new drugs and associations and to propose new therapeutic sequences, better knowledge is required not only of the data derived from the large clinical trials but also of the basic biology, the complexity of involved molecular pathways, the immunology of tumours, and methodological problems related to both new response criteria and new endpoints. In this complex scenario, this review aims to provide a practical approach to the management of advanced renal cancer, framing the new results in daily clinical practice and providing points for reflections to overcome decision-making barriers based on physician therapeutic choice.

THE HETEROGENEITY OF RENAL TUMOR

RCC includes a heterogeneous group of tumors that are characterized by different clinical and genomic factors and are increasingly well defined in both syndromic and sporadic settings (9). These tumor types originate from different cells; for example, clear cell and papillary carcinomas arise from the proximal or parietal kidney cells, whereas chromophobe carcinomas arise from the intercalated cells (10) and are characterized by different genomic drivers that lead to tumorigenesis. In more than 90% of clear cell RCC cases, large-scale genomic sequencing has identified chromothripsis of chromosome 3p, typically with a concurrent gain of 5q (>67%) and loss of 15q (45%) (9). In particular, the loss of 3p results in the inactivation of Von Hippel–Lindau disease tumor suppressor protein (VHL). Mutations in genes encoding other components of the VHL complex [such as TCEB1 (also known as ELOC)] also lead to VHL inactivation (11–13). pVHL is part of a multiprotein complex with ubiquitin ligase activity. Within this complex, pVHL is the subunit that recognizes protein substrates, stimulating their ubiquitination and proteasome-dependent degradation. The main target of this complex is the transcription factor hypoxia-inducible factor 1 (HIF-1α), which plays a key role in the cellular response to hypoxic conditions. It stimulates the transcription of genes involved in promoting angiogenesis and invasive growth. In renal cancer cells, this complex does not function; therefore, HIF-1α accumulates in cells and activates a cascade of other genes that encode factors that induce hypoxia, including VEGF or those involved in alternative pathways to VEGF, such as fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptors (PDGFRs), AXL, and c-MET, all of which are involved in angiogenesis, tumor growth, and survival (14).

Zinc-finger and homeobox protein 2 (ZHX2) is a VHL target. VHL loss-of-function mutations usually result in an increased abundance and nuclear localization of ZHX2. Loss of ZHX2 inhibits signaling through the transcription factor NF-κB, and ZHX2 binds to many NF-κB target genes, revealing that ZHX2 is a potential therapeutic target for RCC (15).

VHL inactivation alone is insufficient for RCC tumorigenesis, and several gene mutations contribute to tumor heterogeneity that characterizes RCC. Intratumoral heterogeneity, defined as the presence of genetically different clones in different subpopulations of the same tumor, is a typical renal tumor condition (16). Accordingly, phylogenetic studies show how the tumorigenesis in the RCC follows an evolutionary model, 'tree-like': in the trunk lies the main mutation (*e.g.* the *VHL* gene in the clear cell tumor) that paves the way for tumorigenesis, and from the trunk, different subclonal mutations branch out, which contribute to tumor growth and progression. Data from the TRACERx renal study have identified secondary mutations and chromosomal changes involved in tumor evolution (17).

Excluding hereditary forms, which cover only 4% of cases, for sporadic forms, The Cancer Genome Atlas (TCGA) has identified 19 genes involved in addition to *VHL*, including *BAP1, PBRM1, SETD2, KDM5C, KDM6A, mTOR, PTEN, PIK3CA*, and *p53* (18). The constitutive activation of the mTOR cascade plays an equally important role in renal tumorigenesis through the loss of p53 expression or mutation of genes such as *PI3K* and *PTEN*. Therefore, TKI therapies directed against one or more of these factors will always be a therapeutic weapon of fundamental importance, as these are precisely targeted against the genetic mechanisms based on tumorigenesis and proliferation of renal cancer cells (**Figure 1**).

In addition to proper genetic damage, we must consider the variations induced by the environment (epigenetics), alterations in receptor expression, and all the complexity that revolves around the tumor microenvironment.

Systemic inflammation is frequently observed in advanced RCC (19). Nevertheless, the functional correlation between inflammation and RCC metastasis remains unclear. Recent data have demonstrated that cancer cells can secrete cytokines and chemokines through a process known as cancer-cell-intrinsic inflammation, altering the immune landscape (20–22). Cancer-cell-intrinsic inflammation contributes to cancer metastasis and the initial progression of cancers. The driver gene mutations responsible for the inflammation in different tumors are *TP53* and *KRAS* mutations (23–26). These mutations lead to increased cytokine release, which recruits myeloid cells in the primary tumor microenvironment or (pre-) metastatic sites.

It has been demonstrated that epigenetic remodeling determines the massive expression of inflammation-related genes in RCC. Synchronous inhibition of the bromodomain and extra-terminal motif suppressed C-X-C-type chemokines in clear cell RCC cells and decreased neutrophil-dependent lung metastasis, suggesting a potential therapeutic strategy (27).

The cells of the immune system (T cells, B cells, and natural killer cells), which represent the targets of known ICIs, such as anti-CTLA4, anti-PD-1, or anti-PD-L1, are found within the tumor microenvironment. In addition to playing a key role in the carcinogenesis process, some parameters such as the expression of PD-L1 have been associated with a worse prognosis (28) as well as a higher degree of tumor aggressiveness (29). Thus, the use of ICIs that block PD-1/PD-L1 binding or amplify the overall immune response finds in this biological rationale its high activity in patients with mRCC (**Figure 1**).

It remains evident that the intratumoral heterogeneity problem is responsible for the difficulty in identifying a single driver mutation and for overcoming mechanisms of clonal selection during targeted treatment (30). To make things

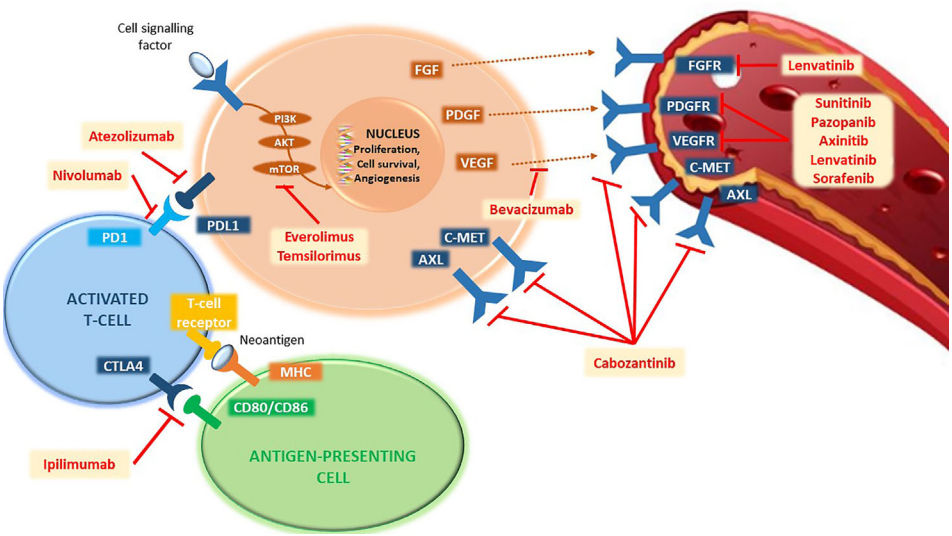

FIGURE 1 | Representation of the main pathways involved in the mechanisms of tumorigenesis and proliferation of renal cancer cells and their targeted agents. PD1, programmed cell death-1 receptor; PD-L1, programmed death-ligand 1; CTLA4, cytotoxic T-lymphocyte-associated protein 4; CD80, cluster of differentiation 80; CD86, cluster of differentiation 86; MHC, major histocompatibility complex; PI3K, phosphatidylinositol-3-kinase; AKT, serine/threonine kinase 1; mTOR, mechanistic target of rapamycin; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; cMET, mesenchymal epithelial transition factor; AXL, AXL receptor tyrosine kinase; FGFR, fibroblast growth factor receptor; PDGFR, platelet-derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor.

worse, a microenvironment response exists: tumors treated with anti-angiogenic agents present an inflammatory infiltrate consisting mainly of regulatory T cells ($CD4^+FOXP3^+$) and express high levels of PD-L1, thus demonstrating the conditions associated with a worse prognosis (31). These findings suggest that the immunosuppressive phenotype found in metastatic sites, for example, is the result of close communication between the occurrence of anti-angiogenic treatment-resistant subclones and the enrichment of inflammatory infiltration with Treg cells to evade the anti-tumor immune response. Given the above-mentioned data, the rationale for combining TKIs with ICIs has become increasingly clear.

THE LATEST APPROVED THERAPEUTIC STRATEGIES IN MRCC

Cabozantinib

Cabozantinib is a multi-targeting TKI directed against the receptors of factors involved in tumor growth, angiogenesis, pathological bone remodeling, chemoresistance, and metastatic progression of cancer, such as VEGF, MET, GAS6(AXL), RET, ROS1, TYRO3, MER, KIT (stem cell factor), TRKB, Fms-like tyrosine kinase-3 (FLT3), and TIE-2 (32). Based on its broad mechanism of action, it is believed to overcome resistance to anti-VEGF agents, such as sunitinib and pazopanib; thus, it was first tested as a second-line therapy in patients previously treated with anti-VEGF therapy (5) and subsequently as first-line therapy in patients with intermediate–poor-risk prognosis (6).

In the phase III METEOR trial, 658 patients with mRCC, who had previously been treated with at least one VEGF tyrosine kinase receptor inhibitor (VEGFR-TKI), were randomized 1:1 to receive cabozantinib (n = 330) or everolimus (n = 328), including those who may have previously been treated with other therapies, including cytokines and antibodies directed against VEGF, the PD-1 receptor, or other ligands. Additionally, patients with treated brain metastases were included. The primary endpoint of the study was progression-free survival (PFS). Secondary endpoints were objective response rate (ORR) and overall survival (OS). Most patients were males (75%), with a median age of 62 years. Seventy-one percent of patients had previously been treated with only one VEGFR-TKI. In 41% of patients, sunitinib was the single VEGFR-TKI previously received. According to the MSCKK criteria for the prognostic risk category, in 46% of patients, the prognosis was favorable; in 42%, it was intermediate (one risk factor); and in 13%, it was poor (two or three risk factors). In 54% of patients, three or more organs, including the lungs (63%), lymph nodes (62%), liver (29%), and bones (22%), had metastatic disease. The median duration of treatment was 7.6 months (range 0.3–20.5) for patients who received cabozantinib and 4.4 months (range 0.2–18.9) for patients who received everolimus. A statistically significant improvement has been demonstrated in PFS for cabozantinib compared to everolimus (7.4 months compared to 3.9 months, hazard ratio [HR] = 0.51 [0.41–0.62], p = 0.0001). In a subsequent interim analysis, a statistically significant improvement was also demonstrated in terms of OS [320 events, median value of 21.4 months compared to 16.5 months; HR = 0.66 (0.53, 0.83), p = 0.0003]. Comparable OS results were observed with a follow-up analysis (descriptive) at 430 events. Exploratory analyses of PFS and OS in the intent-to-treat population also showed consistent results in favor of cabozantinib compared to everolimus in different subgroups defined by age (<65 years compared to ≥65 years), sex, risk group, ECOG status (0 compared to 1), time from diagnosis to randomisation (<1 year compared to ≥1 year), tumor expression of MET (high compared to low compared to unknown), bone metastasis, visceral metastasis, number of VEGFR-TKIs previously received (one *vs* two), and duration of first treatment with VEGFR-TKI (≤6 months *vs* >6 months). Dose reductions were more frequent with cabozantinib than with everolimus, but no statistically significant difference in terms of discontinuation of severe adverse events was reported (5, 33).

The safety and efficacy of the first-line cabozantinib were evaluated in the CABOSUN study, a randomized, open-label, controlled *vs* sunitinib phase II study, which enrolled 157 mRCC patients, classified as intermediate or poor risk according to IMDC criteria. The patients (n = 157) were randomized 1:1 to receive cabozantinib (n = 79) or sunitinib with a schedule of 4 weeks on/2 weeks off (n = 78). The patients were stratified according to the IMDC risk category (81% intermediate and 19% poor) and the presence or absence of bone metastases. Approximately 75% of patients underwent nephrectomy before the start of treatment. The primary endpoint was the PFS, and the secondary endpoints were ORR and OS. Most patients were males (78%) with a median age of 62 years. Most patients (87%) had an ECOG performance status of 0 or 1; 13% had an ECOG performance status of 2. Thirty-six percent of the patients had bone metastases. The study has reached the primary endpoint of statistically significant improvement of the PFS for cabozantinib compared to sunitinib [8.6 months regarding 5.3 months; HR = 0.48 (0.32–0.73), p = 0.0005]. Patients showed a favorable effect with cabozantinib compared to sunitinib irrespective of MET status (positive or negative); however, cabozantinib demonstrated greater activity in patients with positive MET status than that in patients with negative MET status [HR = 0.32 (0.16 and 0.63) *vs* 0.67 (0.37 and 1.23)]. In addition, compared to the treatment with sunitinib, treatment with cabozantinib has been associated with a trend of longer OS (30.3 months compared to 21.0 months; HR 0.74 [0.47–1.14]) (6).

In the two aforementioned studies, the most frequently reported serious adverse events with cabozantinib were hypocalcemia, hypokalemia, thrombocytopenia, hypertension, palm-plantar erythrodysesthesia syndrome, proteinuria, and gastrointestinal events (abdominal pain, inflammation of the mucous membranes, constipation, diarrhea, and vomiting) and were generally found during the first 8 weeks of treatment. In the METEOR study, dosing reductions and dosing interruptions of 59.8 and 70%, respectively, occurred in relation to an adverse event caused by cabozantinib. In CABOSUN, where patients were naïve to treatment, the percentages of reduction and

treatment interruption were quite similar (46 and 73% of patients, respectively). Therefore, it does not seem to be a condition of drug toxicity. However, hypertension has been observed more frequently in the population of naïve patients (67%) than in patients included in the METEOR trial who had been previously treated with anti-VEGF targets (37%).

Nivolumab: Monotherapy and ICI Combination Therapy

Nivolumab was the first anti-PD-1 ICI approved for the treatment of mRCC, first as monotherapy in patients previously exposed to a VEGFR-TKI and then in combination with ipilimumab as the first-line treatment in patients with intermediate- and poor-risk prognosis. According to data from the Phase III Checkmate 025, patients who progressed during or after 1–2 previous anti-angiogenic regimens were eligible for treatment with nivolumab monotherapy (34). This study included patients regardless of tumor PD-L1 status and with a 70% Karnofsky performance status (KPS). Patients with a history of brain metastasis or concomitant brain metastasis, previously treated with an mTOR inhibitor, affected with an autoimmune disease in the active phase, or with medical conditions requiring systemic immunosuppression were excluded from the study. A total of 821 patients were randomized to receive nivolumab (n = 410) or everolimus (n = 411). The study reached the primary endpoint of efficacy (median OS equal to 25 months with nivolumab compared to 19.6 months with everolimus, HR = 0.73 [0.7–0.93], p = 0.0018). Secondary endpoints included ORR and PFS, as evaluated by the investigator. In this study, nivolumab was shown to be better than everolimus in pre-treated patients in terms of ORR (25 *vs* 5%, p < 0.001, HR for OS = 0.73; 95% confidence interval (CI) = 0.57–0.93). However, no significant advantages in terms of PFS have been reported.

Nivolumab in combination with ipilimumab proved to be superior to sunitinib as the first-line therapy in the Phase III study Checkmate 214 (8). The study included patients with mRCC, with clear cell components that were not previously treated. The primary efficacy population included patients at intermediate/poor-risk according to the IMDC criteria. A total of 1,096 patients were enrolled, of which 847 at intermediate/poor-risk were randomized to nivolumab in combination with ipilimumab (n = 425) for four cycles followed by nivolumab monotherapy or sunitinib (n = 422). The primary endpoints were the OS, ORR, and PFS. Patients with mRCC with intermediate/poor prognosis according to IMDC reported a statistically significant benefit in terms of both OS and ORR (HR for OS = 0.63, 95% CI = 0.44–0.89; ORR 42 *vs* 27%, p < 0.001), regardless of the expression level of PD-L1, although in the PD-L1 >1% group, the advantage was even more significant (HR = 0.52; 95% CI = 0.34–0.78). The PFS was not significantly different between the two groups (HR = 0.82; 95% CI = 0.64–1.05). In addition, in the 249 patients at favorable risk, nivolumab plus ipilimumab was detrimental in terms of OS compared to sunitinib (HR = 1.13 [0.64–1.99] p = 0.6710). In terms of tolerability, the combination of ipilimumab and nivolumab was burdened with a higher toxicity than sunitinib (22 *vs* 12% of patients, respectively, discontinued treatment for toxicity) (8) and compared to IO with a single agent, resulting in a more severe immune-related toxicity percentage (35). However, a more recent report on the Checkmate 214 study demonstrated that patient-reported outcomes were more favorable with nivolumab plus ipilimumab than sunitinib in patients at intermediate or poor risk, leading to fewer symptoms and better health-related quality of life (36). Moreover, to better characterize the association between outcomes and IMDC risk in CheckMate 214, a *post-hoc* analysis (n = 1051) of efficacy by the number of IMDC risk factors was completed. ORR with nivolumab plus ipilimumab was consistent across zero to six IMDC risk factors, whereas with sunitinib, it decreased with an increasing number of risk factors. The benefits of nivolumab plus ipilimumab over sunitinib in terms of ORR (40–44% *vs* 16–38%), OS (HR = 0.50–0.72), and PFS (HR = 0.44–0.86) were consistently observed in subgroups with one, two, three, or four to six IMDC risk factors. These results demonstrate the benefit of first-line nivolumab plus ipilimumab over sunitinib across all intermediate- and poor-risk groups, regardless of the number of IMDC risk factors (37).

Thanks to the data reported, the combination of nivolumab and ipilimumab was approved by ESMO guidelines in intermediate- and poor-risk prognostic subgroups of mRCC.

Moreover, a *post-hoc* analysis of nivolumab plus ipilimumab or sunitinib in IMDC intermediate/poor-risk patients with previously untreated mRCC with sarcomatoid features showed an ORR of 56.7% (CI = 43.2–69.4, p < 0.001) in the combination arm against 19.2% (9.6–32.5) of standard treatment and a rate of complete response (CR) of 18.3% in the experimental group, whereas no CR was observed in the sunitinib arm (38).

Elderly patients with pre-treated mRCC may benefit from therapy with nivolumab or nivolumab plus ipilimumab as a first-line option (7, 39), and salvage-line cabozantinib may offer the best survival outcomes, although evidence suggests that the majority of first-line treatments have worse efficacy in older patients than in younger patients (40, 41).

Despite the undeniable benefits of ICIs in the treatment of mRCC, some aspects must be considered: i) only a subset of patients achieves objective responses, ii) some patients have a delayed response, and iii) a significant number of patients do not benefit even clinically. In detail, although the so-called 'combo' IO is particularly active as the upfront treatment in patients with intermediate/poor prognosis, it cannot be a universal choice for all patients, but only for those patients 'fit' for a more intensive combined treatment. Moreover, the ipilimumab–nivolumab combination was less effective than sunitinib in patients over 75 years of age, who represent most of those we met in clinical practice. Therefore, IO is an important strategy both as first- and second-line treatment in patients with mRCC, but TKI agents remain the central focus of mRCC treatment in all therapeutic lines. Several hypotheses have been formulated regarding the lack of efficacy of ICIs in all patients, and among these, tumor heterogeneity and the dynamism of the tumor microenvironment typical of renal cancer cells seem to be the main conditions (29, 42).

The Combination of VEGF-Targeting Agents With ICIs

The upfront combination of VEGF-targeting agents with ICIs is emerging as a therapeutic alternative that could overcome the limitations of IO alone as well as target both the cascade of angiogenesis and the tumor microenvironment (**Figure 1**). Anti-VEGFR inhibitors, in addition to their intrinsic anti-angiogenic effect, showed immunomodulatory effects: unlocking the inhibitory brake of VEGF, promoting infiltration and activation of effector cells, and inhibiting immunosuppressive cells (43). Although the initial studies of sunitinib or pazopanib associated with nivolumab had negative results for the high rates of liver and gastrointestinal toxicity (44), new combinations are proving to be active and well tolerated (45–47).

In the IMmotion151 study, the anti-PD-L1 atezolizumab combined with the anti-VEGF bevacizumab performed better than sunitinib monotherapy in patients with PD-L1-positive tumors (HR = 0.74 [95% CI = 0.57–0.96]; p = 0.02]; however, in the intention-to-treat (ITT) population, the median OS was 33.6 months in the combination arm *vs* 34.9 months in the sunitinib arm, and the results (HR = 0.93) had not yet crossed the significance boundary (45). A pre-specified subgroup analysis of IMmotion151 demonstrated a significant benefit in terms of PFS in patients with mRCC with sarcomatoid features in the bevacizumab plus atezolizumab treatment arm when compared with the sunitinib treatment arm (48).

Other promising combinations always used as first-line treatment are axitinib plus avelumab and axitinib plus pembrolizumab, tested in the Javelin Renal 101 (46) and the Keynote-426 (47) trials, respectively.

The Javelin Renal 101 randomized 442 and 444 patients to the avelumab plus axitinib and sunitinib arms, respectively, and showed that the combination treatment was higher than sunitinib monotherapy in terms of PFS and ORR, regardless of the PD-L1 status and prognostic risk category (46). The last update of the study confirmed previous results; in particular, in the overall population, the median PFS was 13.3 (95% CI = 11.1e15.3) *vs* 8.0 months (95% CI = 6.7e9.8), HR = 0.69 [95% CI = 0.574–0.825]; p < 0.0001); moreover, the combination prolonged PFS2 compared with sunitinib. However, OS data (primary endpoint of both studies) are still immature (49).

The Keynote 426 study is a phase 3 trial that randomly assigned 861 patients with previously untreated advanced RCC to receive pembrolizumab plus axitinib or sunitinib. The primary endpoints were the OS and PFS in the ITT population. The key secondary endpoint was ORR. After a median follow-up of 12.8 months, this study observed a significant benefit in terms of PFS (15.1 *vs* 11.1%, HR = 0.69; 95% CI = 0.57–0.84; p = 0.001) and ORR (59.3 *vs* 35.7%, p = 0.001) in favor of the combined treatment arm, disregarding the status of PD-L1 and the prognostic risk category (47).

The results of the extended follow-up of the randomized phase III study KEYNOTE-426 (median follow-up 30.6 months) confirmed the benefit for the experimental arm, which was proven statistically significant in terms of median OS [not reached with pembrolizumab and axitinib *vs* 35.7 months (95% CI = 33.3–not reached) with sunitinib; HR = 0.68 (95% CI = 0.55–0.85), p = 0.0003], median PFS [15.4 months with pembrolizumab and axitinib (12.7–18.9) *vs* 11.1 months for sunitinib (95% CI = 9.1–12.5); HR = 0.71 (95% CI = 0.60–0.84), p = 0.0001], and ORR (60% in the combo arm *vs* 40% in the sunitinib group). Although the trial was not designed to observe differences between risk categories, it should be noted that the benefit in terms of OS was particularly evident in the population at intermediate and unfavorable risk [pembrolizumab plus axitinib *vs* sunitinib: HR = 0.63 (95% CI = 0.50–0.81)], while it was not significant in the favorable risk group [HR = 1.06; (95% CI = 0.60–1.86)]. Moreover, in terms of toxicity, no significant news emerged with the continued follow-up of patients in the study. The most frequent treatment-related grade 3 or higher adverse events (10% of patients in both groups) were hypertension [95 (22%) of 429 patients in the pembrolizumab group plus axitinib *vs* 84 (20%) of 425 patients in the sunitinib group], increased alanine aminotransferase levels [54 (13%) *vs* 11 (3%)], and diarrhea [46 (11%) *vs* 23 (5%)] (50).

The fact that the advantage in OS for the combination, already known from the first analysis, is maintained over time, although half of the patients randomized to only sunitinib had then received progression IO (*vs*. 8% in the experimental arm), suggests the synergistic activity of the combination of pembrolizumab plus axitinib, which may therefore not be reproducible by their use in sequence. With regard to drug synergy, the role of a single agent in the overall result may also be different: while axitinib is more responsible for shrinkage, pembrolizumab could then be more decisive in maintaining the volumetric reduction effect over time (51).

Furthermore, although all the front-line combination trials enrolled patients with clear cell RCC, exploratory *post-hoc* analyses from these studies demonstrated that patients with sarcomatoid differentiation, which has historically been associated with worse prognosis, derive marked benefits from ICI-based therapy. Based on these data, the Food and Drug Administration (FDA) and EMA in 2019 approved the axitinib–pembrolizumab combination as the first-line treatment for patients with clear cell mRCC in the all-risk category.

CheckMate-9ER is a randomized controlled trial comparing the combination of nivolumab and cabozantinib *vs* sunitinib as a first-line treatment for mRCC with a clear cell component and any IMDC risk group. In the first analysis of the study, the superiority of the combination arm over standard treatment was shown to meet all three efficacy endpoints, with a 40% reduced risk of death [HR = 0.60 (98.89% CI = 0.40–0.89); p = 0.0010; median OS not reached in both arms]. In patients treated with the combination cabozantinib and nivolumab, the median PFS, the primary endpoint of the study, is doubled compared to patients who received only sunitinib: 16.6 months compared to 8.3 months [HR = 0.51 (95% CI = 0.41–0.64), p = 0.0001]. In addition, cabozantinib in combination with nivolumab showed a higher ORR (56 *vs* 27%) and 8% of patients compared to 5% who achieved a complete response. Moreover, the combination treatment was associated with a longer response duration compared to sunitinib, with a median duration of 20.2 months

compared to 11.5 months. In addition, patients treated with the combination showed a lower rate of discontinuation of treatment than those treated with sunitinib (44.4 *vs* 71.3%) and a significantly lower rate of discontinuation for disease progression than sunitinib (27.8 *vs* 48.1%). All these key efficacy results are consistent in pre-specified subgroups and all risk categories according to the IMDC and PD-L1 expression (52, 53). Based on this study, the ESMO guidelines proposed the combination of nivolumab and cabozantinib as a valid first-line therapy in all prognostic subgroups (52).

Unlike the KEYNOTE 426 trial, no significant results were obtained in terms of OS when the experimental arm was compared with the standard treatment in the other phase III studies. In fact, in the Javelin Renal 101 study, OS data were immature in the 2019 publication, while in the IMmotion-151 trial, OS was not met.

Among the new drug combinations tested in mRCC, there is also the one examined in the phase Ib/II TiNivo study, which evaluated the efficacy and safety of combination therapy with nivolumab plus tivozanib, a highly potent and selective VEGFR-TKI approved by the European Medicines Agency (EMA) for first-line treatment of patients with mRCC (54), and showed a generally tolerable profile and promising anti-tumor efficacy (55).

HOW THERAPEUTIC ALGORITHM HAS CHANGED IN MRCC TREATMENT WITH THE APPROVAL OF COMBO?

For a decade, it has been wondered what the best sequence treatment between TKI–mTORi–TKI *vs* TKI–TKI–mTORi is. However, the next future question will be much more complex since there are no comparative studies, clear prognostic factors, or predictive markers, thus making a weighted choice between the various options available in the first- and second-line very difficult.

The new treatment strategies range from molecular targeted agents such as cabozantinib, able to overcome some anti-angiogenic mechanisms of resistance, through ICIs, such as nivolumab, as a single agent, up to the combinations of ICIs (nivolumab + ipilimumab), or between ICIs with VEGF-targeting agents (atezolizumab + bevacizumab, pembrolizumab + axitinib, avelumab + axitinib, cabozantinib + nivolumab, and others under investigation).

The paradigm of first-line treatment in advanced RCC, firmly occupied for more than 10 years by monotherapy with anti-angiogenic TKIs, such as sunitinib or pazopanib, has changed, and combinations of ICIs, either with each other or with TKIs, have shown efficacy compared to monotherapy with TKIs. In light of the results of recent combinations, except for comorbidity and clinical contraindications, in the first-line, the therapeutic proposal is to administer all prognostic classes the combination TKI/IO (axitinib plus pembrolizumab/nivolumab plus cabozantinib) or IO/IO (nivolumab plus ipilimumab), and considering the combination IO/IO for patients with sarcomatoid components, whereas all other cases remain valid for TKI monotherapy, in particular, cabozantinib in the intermediate- and high-risk subgroups unfit for combo treatment, and pazopanib or sunitinib in the good risk unfit for combo (56).

Whether the objective is to achieve a complete response (CR) as well as a long survival (or possible cure), ipilimumab plus nivolumab or nivolumab plus cabozantinib would be the treatment of choice. In fact, the CR rates in CheckMate 214, CheckMate9ER, Keynote426, and Javeline Renal 101 were 9, 8, 5.8, and 3.8%, respectively. In contrast, we should also consider that a higher rate of progressive disease (PD) was observed as the best response to treatment in the CheckMate 214 trial, while the lowest was observed in CheckMate9ER. The toxicity profile is a further discriminant in the choice of combination treatment. In fact, for the IO–IO combination, the major toxicities are limited to the induction phase with ipilimumab, while for the combination of an ICI and a VEGFR-TKI, safety issues tend to persist over time due to the prolonged administration of both agents.

As the field stands now, the immuno-target combination could represent a particularly valid opportunity, especially in patients with a 'cold' phenotype, whose tumor is characterized by poor immune infiltration and are considered less likely to respond to ICI-based treatment alone.

Given the lack of head-to-head comparative studies, both experience and common sense must guide the choice of a physician according to the following considerations: i) patient characteristics, comorbidities, drug interactions with concurrent therapy, occupation, preferences of patient, and side effects that can affect the quality of life; ii) neoplasia features, its histology, if it has a representative sarcomatoid components, the genetic structure, the burden of cancer disease, and the location of the metastases and their related symptoms; iii) balancing the risk and benefit of treatment itself: for safety, we should consider that the trade-off between efficacy and safety that a first-line patient is willing to accept is usually unbalanced in favor of efficacy; iv) biological aggressiveness of the tumor: in the case of an aggressive disease, the combo IO/TKI seems a very reasonable choice to control disease growth while waiting for the tail effect of IO; otherwise, one could head for the long-term benefit of the IO/IO combo, as well as for complete response, trying to spare the additional toxicity derived from the continuous use of the VEGFR-TKI.

Moreover, a recent meta-analysis network on the choice of the first-line showed that cabozantinib is the best molecular targeted agent for the advantage in terms of PFS in patients at intermediate/poor risk compared to sunitinib, with a 91% chance of giving the best benefit in PFS (57). Therefore, the choice will be conditioned by our primary endpoints, even if they do not always coincide with those of large clinical trials.

Taking into account that the IMDC prognostic model was developed at the time of a first-line anti-VEGF-based therapy (58) and that neither validated prognostic models in first-line with ICIs or with the immuno-target combo nor data on the second-lines are available, the therapeutic algorithm of mRCC could be revised in the following way (**Figure 2**): i) for the first-line, to assess whether the patient is considered 'fit' for a

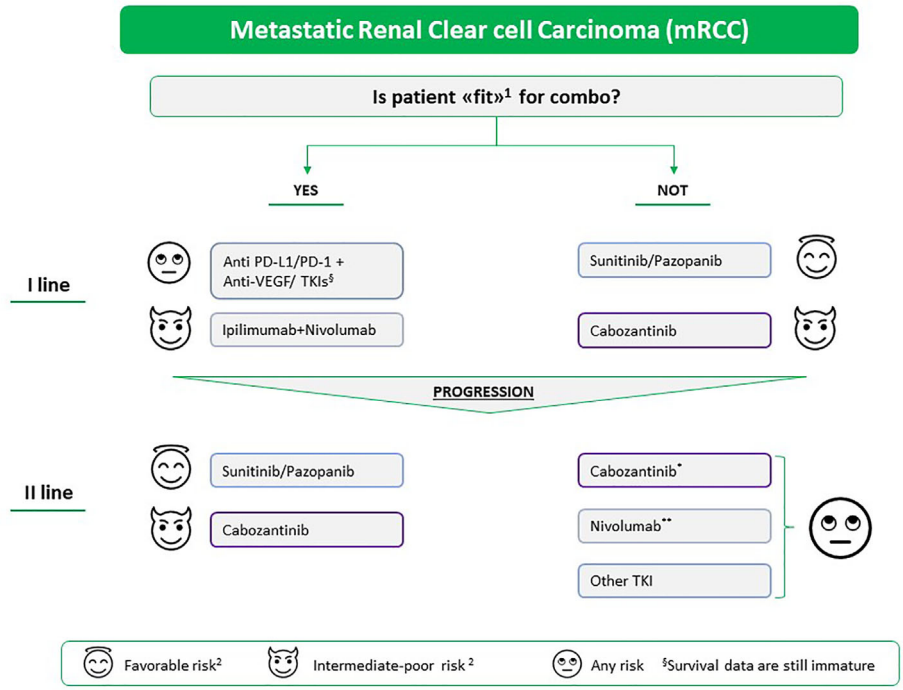

FIGURE 2 | Proposed therapeutic algorithm for the treatment of mRCC in and beyond the first-line setting. The choice of treatment is based on (1) i) patient characteristics: comorbidities, potential drug interactions with the concomitant therapy, occupation, patient preferences and the side effects that can affect the quality of life; ii) and tumor characteristics: histology, if it has a representative sarcomatoid component, the genetic structure, the tumor burden, site of metastases, and related symptoms. (2) MSKCC/IMDC prognostic classification; *if not previously carried out, **if the patient does not have autoimmune disease in the active phase, solid organ transplant, or interstitial pneumopathy or if the patient requires high doses of corticosteroids. TKI, tyrosine kinase inhibitor Pazopanib and cabozantinib are still not indicated as second-line treatment after immunotherapy; however, real-world analysis of patients treated with cabozantinib after anti-PD-1 treatment reported promising results.

combination strategy; ii) for subsequent lines, taking into account what has been done previously (in immuno-naive patients, the choice could fall on nivolumab or another TKI such as cabozantinib, while in TKI-naive patients, the choice could fall on an anti-VEGF TKI such as sunitinib, pazopanib, or cabozantinib). Nevertheless, data on pazopanib or cabozantinib as second-line treatment after ICI-based treatment are not available. However, cabozantinib demonstrated impressive PFS and OS when administered post-IO in patients with mRCC, according to findings from the METEOR sub-analysis[33] and recent retrospective real-world studies (59, 60). After disease progression to first-line TKI-based monotherapy, the factors that could guide the choice towards a second-line of treatment in favor of another TKI are low or intermediate risk, long duration of first-line treatment with VEGFR-TKI, good tolerability to previous treatment lines, low tumor burden, slow progression, revascularization of pre-existing lesions, and high probability of receiving further treatment lines. In favor of IO-based second-line treatment, we have considered the following factors: high risk, short duration of first-line treatment with VEGFR-TKI, poor tolerability to TKI, dose reductions and interruptions, high tumor burden, rapid progression, progression not guided by angiogenesis, and low probability of receiving further therapy.

Currently, there are no data comparing the available strategies that combine two IO agents or TKI plus IO, but increasing evidence suggests that some biomarkers and genetic features could guide optimal treatment options for patients.

WHAT TO EXPECT FROM DIAGNOSTIC IMAGING?

The complex therapeutic scenario described above makes imaging evaluation extremely challenging, both at the time of diagnosis and in assessing the response to treatment. At the time of diagnosis, owing to high intratumoral heterogeneity and heterogeneity between the gene expression profiles of primary cancer and its metastases, tumor genomic characterization is necessary. Considering the technical difficulties and morbidity in performing multiple renal biopsies (61), a solution may be represented by radiogenomics. Radiogenomics, a result of advances in both computational hardware and machine-learning algorithms, is an emerging field in which quantitative information is extracted from radiological images (radiomics) and is correlated with tumor genomic profiling (62). Although

studies are still preliminary (63, 64), it is expected that quantitative imaging data might become a useful biomarker for assessing tumor prognosis, treatment selection, and prediction of treatment response.

With the advent of anti-VEGF and TKIs and then ICIs, the evaluation of response to therapy made it necessary to introduce new objective response criteria [*i.e.* modified Choi (65), SACT (66), iRECIST (67)], since conventional RECIST (66) is not adequate for categorizing patient response. However, there are still open issues regarding the assessment of pseudo-progression and dissociated response (68, 69), both of which are strongly associated with the clinical benefit of ICIs and hyper-progression. Further challenges will await radiology with the advent of combined treatments. The solution will probably be found in the integrated analysis of imaging data (from different sources, including CT, MRI, and PET, combining morphological and functional studies, targeting tumor perfusion and cellularity), tumor mutational burden, and biological markers. Once collected, this large amount of data will be processed by high-speed processors driven by artificial intelligence.

POSSIBLE FUTURE PREDICTIVE AND PROGNOSTIC BIOMARKERS

PD-L1 Expression

Several studies have demonstrated the negative prognostic role of the expression of PD-L1 in the setting of mRCC (70–72). The expression of PD-L1 on tumor cells was associated with a higher tumor stage and a worse response to TKI therapy in two *post-hoc* analyses of the COMPARZ study and the METEOR and CABOSUN trials (28, 72–74). In addition, a meta-analysis including more than 1,300 patients showed that higher PD-L1 expression correlated with an approximately doubling risk of death (75). In contrast, the predictive role of PD-L1 in response to IO is still controversial, and the results obtained in the exploratory analyses of clinical trials investigating ICIs are inconclusive (7, 8, 45–47). In the CheckMate 025 trial, PD-L1 expression was associated with poor survival independent of the treatment received, but not with response to nivolumab (7). The CheckMate 214 trial showed a higher PFS in the ipilimumab plus nivolumab arm than in the sunitinib arm for IMDC intermediate–poor-risk patients, with PD-L1 expression in 1% or greater of cells (median PFS 22.8 *vs* 5.9 months), but this advantage was not observed when PD-L1 was less than 1% (median PFS, 11 *vs* 10.4 months). Conversely, a better ORR and OS for IO over an anti-vascular agent was reported regardless of tumor PD-L1 expression level (8). In the IMmotion 151 trial, the magnitude of benefit derived from the combination therapy with atezolizumab plus bevacizumab increased in patients with PD-L1 expression by more than 1% of tumor-infiltrating lymphocytes compared with the ITT population (45). In the JAVELIN Renal 101 and KEYNOTE-426 trials, the combination therapy showed a benefit over sunitinib irrespective of PD-L1 expression (49, 50).

The above-mentioned results suggest that the expression of PD-L1 in mRCC cannot completely predict the responsiveness of tumors to ICIs. Its role remains controversial and warrants further investigation. Moreover, the assessment method and tumor heterogeneity are the major limitations of the evaluation of PD-L1 (76). The technique used for the IHC analysis has an elevated variability among the different methods available, and the scoring systems are not concordant for the target cells evaluated, whether tumor cells, immune cells infiltrating the neoplastic stroma, or a combination of both; additionally, there is no validated positivity cut-off (77, 78). Furthermore, PD-1 and PD-L2 evaluation should be considered, and their role should be clarified (79). Finally, the expression of PD-L1 is dynamic, changing depending on the history of the disease and the treatments received. In addition, intratumoral variability and a different expression in the primitive tumor and metastases, which would explain the high response rates obtained despite the negativity of PD-L1 in the primitive lesion, should be considered when the expression of this biomarker is examined (80).

Tumor Mutational Burden

TMB is defined as the total number of mutations per coding area of the tumor genome, measured as mutations per megabase (mutations/Mb) (81). In tumors with high TMB, there is an increased production of surface neoantigens that stimulate the anti-tumor immune system response, which could explain the potential association between TMB and response to ICIs (82). In the setting of mRCC, TMB is variable, ranging from a very low level in chromophobe type to a higher value in clear cell and papillary tumors and is not concordant with the clinically defined prognostic groups according to IMDC and MSKCC (83). Regarding its prognostic role, the data in the literature are discordant, since some studies observed a correlation between improved survival and increased TMB, while others demonstrated a negative prognostic role (84, 85). Concerning the predictive significance of TMB, no association between TMB and survival, PD-L1 expression on tumor cells, or clinical benefit was observed (86).

Microenvironment

RCC is characterized by a heterogeneous population of tumor-infiltrating immune cells; however, conflicting data have been obtained to date. Infiltration of effector T cells, such as $CD8^+$ lymphocytes, and M1 macrophages may be associated with a better prognosis, whereas infiltration of regulatory T cells, such as Tregs and M2 macrophages, have a poorer outcome (87–90). In contrast, high intra- and peri-tumoral $CD8^+$ cell density was also correlated with poor prognosis (91). It was demonstrated that PD-L1 expression on tumor cells could lead to higher $CD8^+$ T cell infiltration, distinguishing two groups of tumors with $CD8^+$ infiltrate, and the group with low expression of immune checkpoints and localization of mature dendritic cells was associated with a good prognosis (92).

Concerning the predictive role of the microenvironment, a comprehensive analysis of patients enrolled in four clinical trials on nivolumab demonstrated a poorer and greater response in

correlation to the overexpression of genes involved in metabolic functions (*e.g. UGT1A*) and the increased expression of immune markers (*e.g.* BACH2 and CCL3), respectively (93). Moreover, an exploratory analysis of the IMmotion150 trial reported that a T-effector immune gene in association with the expression of PD-L1 and the infiltration of T $CD8^+$ cells correlates with a higher ORR and prolonged PFS in the atezolizumab arm (45). In particular, it was observed that VEGF blockade could promote the infiltration of T cells into the tumor microenvironment, thus potentiating the mechanism of action of ICIs (94).

Circulating Tumor Markers

Circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) are peripherally detectable tumor-derived materials. These markers could detect primary and metastatic sites non-invasively and evaluate the response to therapy (95–97). Variable frequencies of genomic alterations were detected in the front-line and second-line treatment settings, showing an increased incidence of genomic alterations, particularly those affecting TP53 and MTOR, after first-line treatment with VEGFR-TKI therapy (96). These differences could reflect treatment-selective pressures and the effect of front-line therapy on ctDNA load, but might also simply depend on the technical limitations of ctDNA assessment in this disease (98).

Other circulating protein and lipid markers have demonstrated predictive and prognostic value in advanced disease. Based on 52 circulating markers, a cohort of 69 patients treated with first-line sorafenib was grouped by either an angiogenic or an inflammatory signature, with correlations to PFS (HR = 0.2 *vs* 2.25; p = 0.0002) (99). Additional markers in serum have been investigated, such as soluble VEGF, circulating microRNAs, carbonic anhydrase 9, and inflammatory markers, such as IL-6 and IL-8, but most of these studies were conducted in the era of targeted therapies (99–103), and new dedicated investigations are required to address the dramatic changes in treatment paradigms brought about by the advent of ICIs.

Genomic and Transcriptomic Environments

There are three possible treatment strategies in the therapeutic landscape of mRCC: angiogenic inhibitors at one end, IO at the other, and combinations of the two classes in the middle. The challenge, however, is identifying the subset of patients who could benefit from one therapeutic class alone to avoid the unnecessary toxicity of combination approaches.

Using RNA-based analyses, four distinct molecular subgroups associated with different responses and survival were defined: Cluster 3 had the best prognosis with high angiogenic gene expression and was associated with a better outcome under anti-angiogenic therapy (*PBRM1* mutation was frequently associated) (104); Cluster 4, with upregulation of the immune pathway, had a worse prognosis, with a frequent sarcomatoid differentiation and expression of PD-L1 (105); and Clusters 1 and 2 were intermediate clusters with a lower expression of angiogenic and immune genes. These results may have the potential to inform treatment personalization in patients with mRCC (106).

The phase 2 IMmotion150 trial investigated the efficacy, as measured by PFS, of atezolizumab with or without bevacizumab against sunitinib in patients with untreated mRCC and correlated differential gene expression signatures (angiogenesis, T-effector, and myeloid) with therapeutic response. Highly angiogenic tumors, which coincided with tumors exhibiting *PBRM1* mutations, seemed to benefit more from sunitinib, but not from atezolizumab either alone or in combination with bevacizumab. The combination treatment improved clinical benefits compared with sunitinib in T-effector high tumours. Tumors with T-effector high and lower myeloid inflammation-associated gene expression benefited from atezolizumab monotherapy. Instead, in T-effector high tumors, a concomitant high myeloid inflammation predicted a worse response to IO alone. Myeloid inflammation is associated with high expression of IL-6, prostaglandins, and the CXCL8 family of chemokines, which suppress the anti-tumor immunity. The improved clinical outcome associated with atezolizumab + bevacizumab compared with atezolizumab monotherapy in this subgroup suggests that the addition of bevacizumab to atezolizumab may overcome innate inflammation-mediated resistance in these tumors (104).

Based on the analysis of the angiogenic profile in comparison with the immunological profile of the study IMmotion151, it is possible to define subgroups of tumors that benefit from different treatment strategies. Given the new associations, it would be interesting to evaluate these aspects in other combinations of IO/TKI and see if, for example, the addition of TKI modifies the immunogenicity of these tumors.

CONCLUSIONS

Considering the continuously evolving scenario in the treatment of patients with mRCC, the future goal will be to better characterize renal neoplasia in all its complexity, from the trunk to the last of its branches. However, to outline the most appropriate treatment path for each patient, we cannot deny that only clinical criteria are very likely to understand the needs of patients. Given the significant improvement in therapeutic options, prospective studies are needed that would elucidate: what will be the most effective therapeutic algorithm and how patients will be selected to hit more targets; will it be more effective to use therapeutic agents in sequence or by focusing completely on the first therapeutic line; will it be more effective to use a combination strategy from the beginning of mRCC treatment? Further studies are required to answer these questions.

AUTHOR CONTRIBUTIONS

All authors have read and agreed to the published version of the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CASE REPORT
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Case Report: Anlotinib Combined With Sintilimab as Third-Line Treatment in a Metastatic Urothelial Bladder Carcinoma Patient With FGFR3 Mutation

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We report on a case of metastatic urothelial bladder carcinoma (mUBC) treated with anlotinib combined with sintilimab. A 69-year-old male was diagnosed with non-muscle invasive bladder cancer (NMIBC). He received transurethral resection of bladder tumor (TURBT) and intravesical gemcitabine chemotherapy. After the patients' cancer progressed to mUBC, cisplatin-based chemotherapy (gemcitabine combined with cisplatin, GC) was performed to this patient as first line therapy for four cycles. However, the disease progressed again within 6 months. Local radiotherapy was performed on the metastatic lesions, and after radiotherapy, the patient received anti-PD-1 antibody (sintilimab 200 mg, q3w)combined with Albumin-bound (Nab)-paclitaxel (100 mg, qw) as the second-line therapy, but the patient's cancer was still observed to be progressing. Molecular characterization confirmed the presence of FGFR3 mutations in the patient. Anlotinib was recommended to this patient. After the patient was fully informed and he was aware of off-label use of the drug, then, Nab-paclitaxel was replaced by anlotinib (10 mg D1–14, q3w) and sintilimab infusions were maintained for every 3 weeks. Partial response (PR) was observed through imaging examinations and stable disease (SD) was observed for more than 11 months; the patient's quality of life also improved. This case suggested that anlotinib combined with sintilimab may be a safe and effective choice in the treatment of mUBC in patients with FGFR3 mutations.

Keywords: anlotinib, sintilimab, immunotherapy, targeted therapy, metastatic urothelial bladder carcinoma

BACKGROUND

The prognosis of patients with mUBC is very poor if the disease progresses after platinum-based chemotherapy (1). The immune checkpoint inhibitors programmed cell death ligand-1 (PD-L1)/ programmed death-1 (PD-1) are clinically active reagents for mUBC. Both of them are FDA-approved as second-line treatments (2). However, the objective response rate (ORR) was achieved in only 17% to 24% of these patients (3–7).

Fibroblast growth factor receptors (FGFRs) induce signaling through networks that regulate cell proliferation, survival, migration, and differentiation (8). FGFR3 is one of the most frequently mutated genes and is a promising target in urothelial carcinoma (UC) (9). FGFR3 is altered in 50% to 80% of low-grade and low-stage UC, particularly in the luminal I subtype. Conversely, FGFR3 mutations are less common (20%) in mUC (9, 10). FGFR inhibitors such as erdafitinib, when used to treat patients who had locally advanced or unresectable mUC with FGFR alterations, were shown to have an objective tumor response of 40% (11). The FDA has approved the use of erdafitinib for patients with locally advanced or mUC that has progressed during or after platinum-based chemotherapy and whose tumors have susceptible FGFR3 or FGFR2 genetic alterations (12).

Anlotinib is a novel multitarget tyrosine kinase inhibitor. It was originally designed to inhibit VEGFR2/3, FGFR1–4 with high affinity (13, 14). Clinical trials have indicated that anlotinib significantly prolongs the progression-free survival (PFS) of patients with non-small cell lung cancer (NSCLC) (13, 15, 16). medullary thyroid carcinoma (MTC) (13) and metastatic renal cell carcinoma (mRCC) (17). Sintilimab is an IgG4 monoclonal PD-1 antibody that was derived from humans, and it blocks the binding of PD-1 to PD-L1 or PD-L2 (18). It has been shown excellent clinical benefits in the treatment of relapsed or refractory Hodgkin's lymphoma (19, 20) and NSCLC (21, 22).

Here we report on a 69-year-old male mUBC patient who had the FGFR3 mutations and was successfully treated with sintilimab combined with anlotinib as the third-line treatment, following the progression of cancer after first-line platinum-based chemotherapy and second-line Nab-paclitaxel plus sintilimab treatment.

CASE PRESENTATION

A 67-year-old man with no family and psychosocial history presented with hematuria in 2016 and was diagnosed with bladder carcinoma using cystoscopy. TURBT pathology indicated that the patient has stage-TaG1 UBC (**Figure 1A**). Single-dose intravesical gemcitabine chemotherapy within 24 hours after receiving TURBT was recommended for the patient. However, 2 months after TURBT was performed, cystoscopy revealed bladder carcinoma recurrence. Between July 2016 and May 2018, TURBT was repeated five times due to cancer recurrence and progression (**Figures 1B–F**). After the fifth TURBT was performed, abdominal computed tomography (CT) (**Figure 2**-1) revealed bladder carcinoma recurrence and pelvic metastasis. Further evaluation of the pelvis using magnetic resonance imaging (MRI) suggested multiple metastatic foci of the pelvic muscle 、 bone and lymph nodes. Pelvic bone metastases were also found using emission computed tomography (ECT). The patient was diagnosed as mUBC on December 13, 2018. At that time, the ECOG score of this patient was 0 and the glomerular filtration rate (GFR) was 69 ml/min. He was treated with first-line cisplatin-based chemotherapy (gemcitabine combined with cisplatin, GC) for four cycles. The disease obtained an objective response and was stable for 6 months, after which imaging suggested that the disease

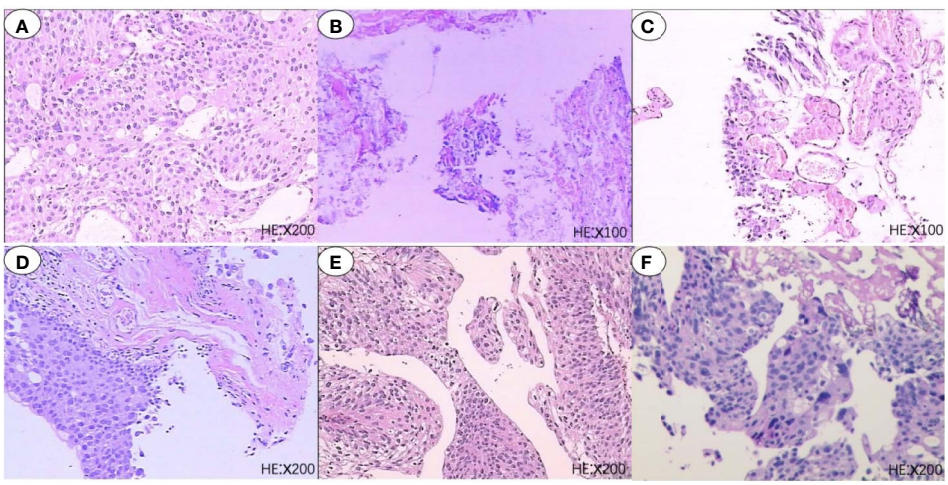

FIGURE 1 | Six pathological reports of TURBT. **(A)** 2016-07-07, Papillary urothelial carcinoma, low-grade; **(B)** 2016-09-27, Necrotic tissue; **(C)** 2017-06-15, Chronic inflammation of the bladder mucosa with atypical hyperlasia; **(D)** 2017-12-14, Chronic inflammation of the bladder mucosa with atypical hyperlasia; **(E)** 2018-05-10, Papillary urothelial carcinoma, low-grade; **(F)** 2018-11-23, Papillary urothelial carcinoma, high-grade.

progressed again. As the patient responded to GC chemotherapy previously, the patient received another two cycles of GC chemotherapy. Unexpectedly, the patient experienced severe pain and could not tolerate the toxicity of chemotherapy. Next, the patient received radiotherapy to the metastatic lesions from July to August 2019, with a total dose of 55 Gy, 2.2 Gy daily, up to 25 fractions to control pain and improve local cancer control. Then, anti-PD-1 antibody (sintilimab 200 mg, q3w) combined with Nab-paclitaxel (100mg, qw) was given to this patient as the second-line therapy on November 28, 2019. Unfortunately, after four cycles treatment, the patient's abdominal CT (**Figure 2**–6) showed that the treatment had failed and the patients' condition continued to deteriorate. He experienced severe bladder irritation due to tumor progression and radiation cystitis, the patient required 120 mg of Morphine Sulfate sustained-release tablets to control local pain.

To provide further treatment to this patient, we performed genetic sequencing and molecular characterization confirmed the presence of FGFR3, PIK3CA, and TP53 mutations in the patient (**Figure 3**). Both erdafitinib and rogaratinib have been tested in clinical trial for FGFR mutated mUBC (23), however, both drugs are not affordable in China. We encouraged the patient to enrollment a clinical trial (NCT03390504) sponsored by Johnson & Johnson (24), which treated mUBC patients with FGFR mutation by erdafitinib. However, the patient did not have strong intention to enroll into this trial.

Thus, after careful consideration, anlotinib, a multitarget tyrosine kinase inhibitor designed to inhibit VEGFR2/3, FGFR1–4 with high affinity, was recommended to this patient. The patient was fully informed and he was aware of off-label use of the drug. With the patient's consent, Nab-paclitaxel was changed to anlotinib (10 mg D1-14 q3w) on March 9, 2020, and the infusion of sintilimab was maintained. After treatment with sintilimab combined with anlotinib, the patient's symptoms improved within 2 weeks and the dose of Morphine Sulfate sustained-release tablets was decreased and eventually stopped. After three cycles, the disease was evaluated by abdominal CT (**Figure 2**) and PR was observed. Currently, the cancer has been stable for over 11 months and the patient is still being followed up. Except for a mild Hand-foot syndrome, no serious adverse events(AE) occurred while receiving sintilimab combined with anlotinib. The patient has not significant decreased in quality of life during treatment and he is quite satisfied with the outcome. The timeline of the patients' treatment is described in **Figure 4**.

DISCUSSION

Platinum-based chemotherapy can prolong overall survival (OS) in patients with mUBC, but cancer progression is almost inevitable (25). Nab-paclitaxel as a second-line treatment has demonstrated some positive preliminary activity in patients with mUBC (26). Yoo-Joung Ko reported on a single-group phase II trial which investigated the activity of Nab-paclitaxel 260 mg/m^2 every 3 weeks as a second-line therapy for mUBC (26, 27). The results suggested that Nab-paclitaxel was well tolerated, but the clinical effect of Nab-paclitaxel monotherapy was limited (27). Recently, PD-1/PD-L1 inhibitors have been demonstrated to be relatively safe and have shown positive clinical activity in patients with mUBC (2, 6, 28), however, the ORR of single PD1/PD-L1 treatment was only 17% to 24% (3–7). To further improve PD1/PD-L1 treatment efficacy, PD-1 was combined with Nab-paclitaxel as a second-line therapy for mUBC. The open-label, single-arm, phase II PEANUT study found that the Pembrolizumab and Nab-paclitaxel salvage therapy for platinum-treated failed mUBC had a favorable safety profile, the PFS was 5 months, and the clinical ORR was 44.4% (29). Thus, when the patient was found that the disease progressed quickly after first line GC therapy, we tried to combine Nab-paclitaxel and PD-1/PD-L1 to control the disease.

Although five kinds of PD-1/PD-L1 are FDA approved for second-line treatments of mUBC (12), only Pembrolizumab and Nivolumab were available in China when the patient decided to receive PD-1/PD-L1 therapy. However, both Pembrolizumab and Nivolumab did not get formal permission for indication of

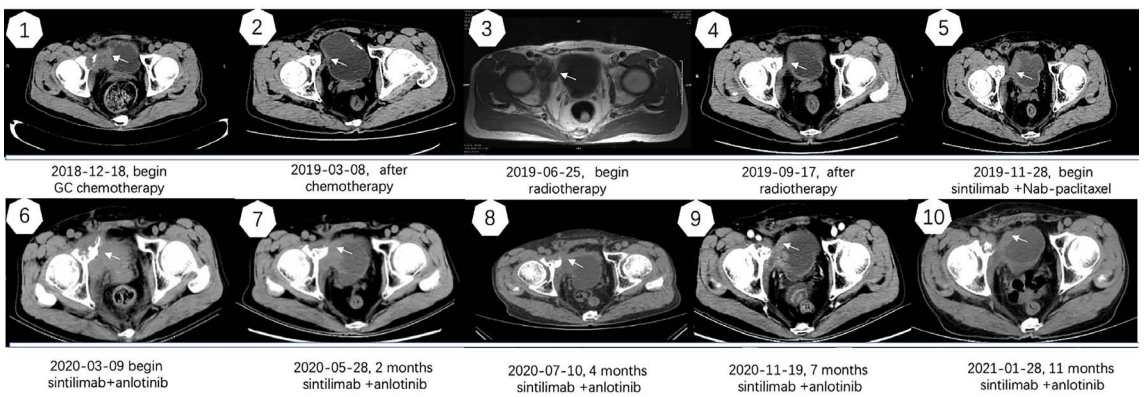

FIGURE 2 | Imaging manifestations of treatment.

Detection of type	Results	
Somatic mutation	There are 7 somatic mutations, 3 of which have clinical significance	
Germline mutation	None	
Mutations with clinical significance	FGFR3	p.Y373.C
	PIK3CA	p.E542K
	TP53	p.R213*
TMB	4.99	
MSI	MSS	

FIGURE 3 | The molecular characterization.

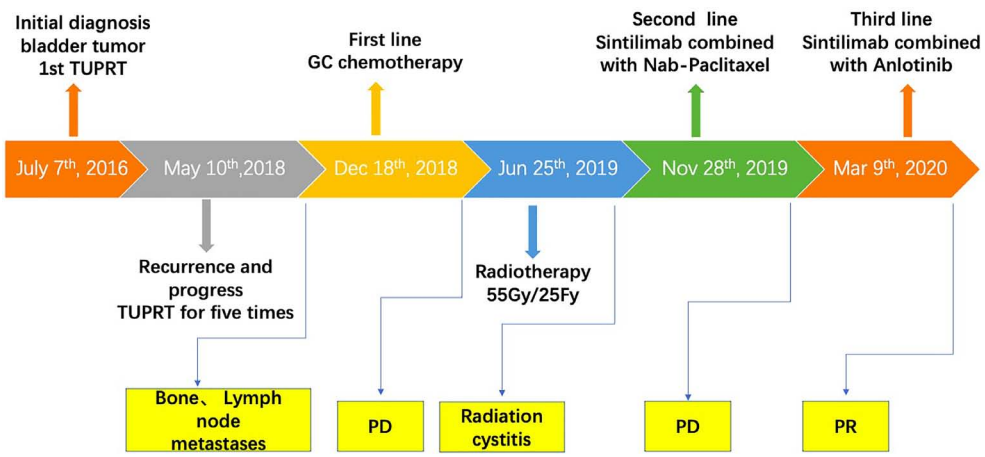

FIGURE 4 | The timeline of treatment.

mUBC treatment in China. Moreover, both drugs were too expensive to use. Thus, after careful discussion with the patient, we decided to choose another cheaper PD-1 antibody to substitute Pembrolizumab or Nivolumab.

According to the pre-clinical data, sintilimab binds to human PD-1 with a greater affinity than nivolumab and pembrolizumab (18). The high binding affinity and unique PD-1 epitopes bound by sintilimab might be responsible for its superior clinical effectiveness (18). Sintilimab has shown excellent clinical benefits in the treatment of relapsed or refractory Hodgkin's lymphoma (19, 20) and NSCLC (21, 22). Theoretically, sintilimab combined with Nab-paclitaxel may produce favorable results for this patient, however, in this case, the combination was unsuccessful.

Genetic testing confirmed the presence of FGFR3, PIK3CA, and TP53 mutations in the patient. FGFR3 mutations are associated with a lower response to platinum-based chemotherapy and a shorter recurrence time in patients with

mUBC (30, 31), which was consistent with this patient who received six cycles of platinum-based chemotherapy. Erdafitinib, a pan-FGFR inhibitor, has been granted accelerated approval by the FDA for platinum-pretreated mUBC with susceptible to FGFR3 or FGFR2 genetic alterations (32), but Erdafitinib is unaffordable in China.

Anlotinib was originally designed to inhibit VEGFR2/3, FGFR1–4 with high affinity (13, 14). Anlotinib also suppresses the activity of PDGFRα/β, c-Kit, Ret, Aurora-B, c-FMS, proving that it has broad inhibitory effects on tumor proliferation, vasculature, and tumor microenvironment (13, 14). In clinical trials, anlotinib showed broad antitumor activity against a variety of tumors. In advanced refractory solid tumors, anlotinib displayed manageable toxicity, and broad-spectrum antitumor potential (13). In locally advanced or metastatic MTC, 56.9% of patients experienced a PR after anlotinib treatment. PFS rate at 48 weeks was 85.5% (33). In a phase II trial on 166 patients with refractory metastatic soft-tissue sarcoma, the median progression-free survival and OS were 5.6 and 12 months, respectively (14). Similarly, in advanced NSCLC, Anlotinib appeared to lead to prolonged OS and PFS. OS was significantly longer in the anlotinib group than the placebo group (9.6 months *vs* 6.3 months) (16). Furthermore, anlotinib demonstrated that it had a better prognosis compared to sunitinib as the first-line treatment for patients with mRCC in a randomized phase II trial (17). In this trial, Anlotinib's safety profile was excellent, especially in terms of hematological toxicities (17).

In addition to anti-tumor efficacy, anlotinib has the potential to modulate the tumor microenvironment and improve immunotherapy. A lung cancer mouse model showed that anlotinib could increase infiltration of innate immune cells such as natural killer (NK) cells and antigen-presenting cells (APC) into tumor microenvironment (15). Subsequently, when combined with PD-1/PD-L1 blockade, anlotinib provided significantly synergistic therapeutic benefits (15). A retrospective study further demonstrated the efficacy and safety of anlotinib with immunotherapy in advanced NSCLS as a third-line therapy (34). Based on these studies, we attempted to treat this patient by anlotinib combined with sintilimab, and favorable results were obtained.

To our knowledge, this is the first report that used anlotinib combined with sintilimab as the third-line treatment in an mUBC patient with FGFR3 mutation, who obtained a PR and was stable for more than 11 months. This case indicates that mUBC patients with FGFR3 mutations whose disease progresses after platinum-based chemotherapy may be able to use anlotinib combined with sintilimab as a new potential treatment choice, but we have only one case and further studies should be conducted to evaluate the efficacy and safety of this combination.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethical review committee of Ningbo First Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conception/design: QM. Provision of study materials or patients: QM, WW, and J-HJ. Collection and/or assembly of data: QM, J-ZC, J-FP, WW, and H-WW. Data analysis and interpretation: QM and J-ZC. Manuscript writing: J-ZC and QM. Final approval of manuscript: All authors. All authors contributed to the article and approved the submitted version.

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Genomic Landscape of Chinese Clear Cell Renal Cell Carcinoma Patients With Venous Tumor Thrombus Identifies Chromosome 9 and 14 Deletions and Related Immunosuppressive Microenvironment

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Background: Clear cell renal cell carcinoma (ccRCC) with venous tumor thrombus (VTT) is associated with a poor clinical outcome. Although several studies have examined the genomic features of ccRCC, the genetic profile of VTT along with its matched primary tumor has not been fully elucidated.

Materials and methods: Samples of VTT tissues and matched primary tumor tissues from ccRCC patients (n = 25), as well as primary tumor tissues from patients without VTT (n = 25) were collected and analyzed using whole-exome sequencing. Four additional ccRCC patients who were unfit for surgery were treated with an anti-programmed death receptor-1 (PD-1) monoclonal antibody (Toripalimab, 240 mg, Q3W, IV).

Results: By comparing the primary kidney tumors from ccRCC patients with or without VTT, a relatively higher prevalence of *BAP1 and KDM5C* alterations were found in ccRCC patients with VTT, and these alterations were associated with worse overall survival in the kidney renal clear cell carcinoma (KIRC) database. Based on subclone analysis, VTT was predicted to primarily originate directly from the primary renal mass. A significantly higher prevalence of *CELSR2* and *TET2* alterations were identified in the VTTs compared with the matched primary tumors. An increased prevalence of DNA damage repair genes, especially those involved in homologous recombination repair and non-homologous end joining, was found in ccRCC patients with VTT. Notably, VTT was characterized by the increase incidence of copy number loss in the whole exome ($p < 0.05$), particularly in the chromosome 9 and 14 regions. Deletion of chromosome 9 and 14 was associated with worse survival, unfavorable clinical features, and the presence of an immunosuppressive microenvironment, which was characterized by higher infiltration of

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regulatory T cells, follicular helper T cells, and resting mast cells, but lower counts of resting CD4 memory T cells and CD8 positive T cells. A significantly lower count of CD4+ and CD8+ tumor-infiltrated lymphocytes was identified in the VTT samples comparing with matched primary tumor. Of note, three out of the four ccRCC patients with VTT in our cohort who were treated with the anti-PD-1 therapy exhibited remarkable remission in the renal mass but no notable shrinkage in the VTT mass.

Conclusion: Our study revealed the genetic profile of Chinese ccRCC patients with VTT, and identified multiple features associated with known poor outcomes, including gene alterations and copy number loss. The deletions in chromosomes 9 and 14, and the associated immunosuppressive microenvironment may indicate limited sensitivity to anti-PD-1/PD-L1 monotherapy in VTT.

Keywords: venous tumor thrombus, ccRCC, genomic feature, copy number variant, immune microenvironment

INTRODUCTION

Renal cell carcinoma (RCC) is the second most common genitourinary malignancy in China, with an estimated 66,800 new cases and 23,400 deaths in 2015 (1). Notably, 4–10% of locally advanced RCC patients develop venous tumor thrombus (VTT), and their overall 5-year cancer-specific survival (CSS) is only 40–65% (2). The median survival of untreated RCC patients with VTT is only 5 months, and the 1-year survival rate is less than 30% (3). To date, radical nephrectomy with thrombectomy remains the best therapeutic choice for RCC patients with VTT, and notably, a higher VTT level under the Mayo classification system is associated with worse CSS, increased rates of complications, and increased mortality following surgery (4, 5). To improve the safety and therapeutic effects of surgery, neoadjuvant therapies have been developed to shrink both the primary lesion and the VTT. Although previous studies have reported the use of anti-angiogenesis drugs preoperatively, including sorafenib and sunitinib, the clinical benefits are limited (6). Therefore, it is essential to understand the molecular mechanisms underlying the formation and development of VTT.

A few previous studies have reported the genomic features of RCC with VTT. As depicted by the TRACERx Renal project, most genetic alterations in the VTT tissues were also present in their matched primary tumor tissue; however, the primary tumor tissues also possessed more recently developed driver mutations that were absent from the VTT tissue (7). Similarly, another study also suggested that the VTT originated from the primary tumor, as all subclones in the VTT were also shown to match the primary renal tumors (8). In the same study, a homologous recombination deficiency feature, described as the BRCAness mutation signature, was also identified in a subset of RCC tumors with VTT and samples from The Cancer Genome Atlas (TCGA), indicating a DNA damage repair (DDR) deficiency and potential sensitivity to poly(ADP-ribose) polymerase inhibitors or platinum-based therapy in these patients (8). Nevertheless, most research on the genomic landscape of RCC was performed in Caucasians, and the results may differ for Chinese RCC patients with VTT, particularly considering the different genetic backgrounds and the exposure to aristolochic acid in traditional Chinese medicines.

In this study, we applied whole-exome sequencing to study the genomic features of VTT in Chinese patients with clear cell (cc)RCC with the matched primary tumors, and these were also compared with primary tumors from RCC patients without VTT to elucidate the genomic features and their potential clinical significance in ccRCC with VTT.

MATERIAL AND METHOD

Sample Source and Ethic Data

We prospectively enrolled 50 ccRCC patients at the Third Medical Centre of Chinese PLA (People's Liberation Army) General Hospital from 2018 to 2019. Twenty-five were ccRCC with VTT, and twenty-five were ccRCC without VTT. All patients had undergone radical nephrectomy with or without thrombectomy and provided written informed consent. This study was approved by the ethics committee of the First Medical Center of PLA General Hospital (S2017-100-01) and conducted under the principles of the Declaration of Helsinki and the Good Clinical Practice guidelines. Patient characteristics were listed in **Table 1**. All samples were collected for DNA isolation after pathological evaluation, but two VTT samples did not yield enough DNA for further testing. Finally, 23 VTT samples (V group), 25 primary tumor samples (VP group), and 25 renal tumor samples without VTT (NP group) were analyzed by whole-exome sequencing (WES), using the DNA from peripheral blood as germline control. In addition, another four ccRCC patients with VTT were evaluated as unfit for surgery and were treated with anti-programmed death receptor-1 (PD-1) monoclonal antibody (Toripalimab, 240 mg, Q3W, IV).

DNA Isolation

DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Inc.) under the manufacturer's instructions. The purified gDNA was quantified using Qubit 3.0 Fluorometer (Life Technologies, Inc.)

TABLE 1 | Clinical Characteristics of 50 RCC Patients.

Characteristics		With VTT n = 25	Non-VTT n = 25
Median age, year (range)		52 (25–84)	55 (34–86)
Sex			
	Male	21	20
	Female	4	5
ISPU Grade			
	1	0	1
	1-2	0	1
	2	12	12
	2-3	6	7
	3	5	3
	3-4	2	1
	4	0	0
Histological subtype	Clear cell RCC	25	25
IVC wall invasion	Yes	19	–
	No	6	–

and StepOnePlus System (Life Technologies, Inc.). For tumor and non-tumor samples, 100 ng of DNA was sheared with a Covaris E210 system (Covaris, Inc.) to generate fragments with a length of 200 bp.

WES

The Accel-NGS 2S HYB DNA LIBRARY KIT (Swift Biosciences, 23096) and HotStart ReadyMix (KAPA, KK2612) for library preparation and amplification were used, respectively. The amplified libraries were purified by using SPRISELECT (Beckman, B23319) and further captured with xGen Exome Research Panel v2 (IDT), whose target region was 33 Mb. Finally, samples underwent paired-end sequencing on a Novaseq 6000 platform (Illumina) with a 150 bp read length. The mean depth for the tumor, VTT and non-tumor tissues was 500×, 500×, and 100×, respectively.

Data Analysis

Raw sequencing data were aligned to the reference human genome (UCSC hg19) through Burrows-Wheeler Aligner (9). After deduplication and local realignments, Genome Analysis Toolkit (GATK) was used for calling of single nucleotide variation (SNV) and small insertion and deletion (indel) (10). Somatic variants that were present only in tumor or VTT were identified by removing the germline alterations identified in the matched non-tumor samples. Variants were annotated by using the ANNOVAR software (11). CNVkit was used to determine the copy number variations (CNVs) (https://github.com/etal/cnvkit).

Tumor Mutation Burden

The tumor mutation burden of each sample was calculated according to the widely used method described by Chalmers, Z.R, et al. (12).

Mutational Signatures

Mutational signatures were analyzed by R package YAPSA with supervision. A linear combination decomposition of the mutational catalog with known and predefined signatures was computed by non-negative least squares (NNLS). By comparing the whole genome to WES capture regions, mutational catalog correction was performed to account for the differences in the occurrence of triplet motifs. A set of 30 publicly available mutational signatures AC1-AC30 (AC standing for Alexandrov COSMIC) were analyzed.

Homologous Recombination Deficiency

The homologous recombination deficiency, also called genomic scar scores was determined by counting the number of loss of heterozygosity (LOH), large scale transitions (LSTs), and telomeric allelic imbalances (TAIs). WES sequencing data analysis was performed by using the method described by Zsofia Sztupinszki et al. (13), which showed a good correlation (r = 0.87) between SNP array-based and WES sequencing-based HRD analysis.

Gene Expression Signature Analysis

Gene expression signature of kidney renal clear cell carcinoma (KIRC) in TCGA was analyzed based on the RNA-seq data (downloaded for cbioportal, https://www.cbioportal.org). Signatures were classified into angiogenesis, immune and antigen presentation, myeloid inflammation according to the IMmotion 150 trial (14).

Tumor-Infiltrating Immune Cell Analysis

Tumor-infiltrating immune cell counts were analyzed based on RNA-seq data from the KIRC in TCGA by using a CIBERSORT R package (15).

Immunohistochemical Detection of CD3, CD4, and CD8

Immunohistochemistry (IHC) analysis on the archived tumor samples was applied to compare the expression level of CD3, CD4, and CD8 in tumor infiltrated lymphocytes. Anti-CD8 (EP1150Y, ab93278), anti-CD4 (EPR6855, ab133616), and anti-CD3 (SP7, ab16669) antibody were purchased from Abcam

(Cambridge, MA). All experiments were undergone following the instructor's protocol. Two pathologists independently interpreted IHC staining by assessing background staining, positive and negative controls, and localization and amount of biomarker staining in all specimens. Percent positive cells = (number of positive lymphocytes/tumor area occupied by tumor cells, associated intratumoral, and contiguous peritumoral stroma) × 100.

Statistical Analysis

Gene prevalence between different groups was analyzed by Chi-Square test or Fisher exact test. A two-sided P value of less than 0.05 was considered to be statistically significant. All analyses were performed using SPSS 25.0 software.

RESULTS

Genomic Landscape of the ccRCC Patients in our Cohort

Overall, we identified 9,879 non-synonymous somatic alterations in the 73 samples. The mean and median numbers of somatic alterations per sample were 135.33 and 107, respectively. The most frequently altered gene was *VHL*, with an approximately 60% alteration rate in all three groups. In the V group, the other frequently altered genes included *TTN* (43%), *BAP1* (30%), *CELSR2* (30%), *PBRM1* (22%), *KDM5C* (22%), and *MUC16* (22%), respectively (**Figure 1A**). All samples in the three groups were microsatellite instability stable (MSS), and there was no significant difference in the median tumor mutation burden (**Figure 1B**).

To identify the different genomic features between Chinese and Western cohorts, we compared the prevalence of alterations in known driver genes in ccRCC, including *VHL*, *KDM5C*, *BAP1*, *PBRM1*, *SETD2*, *MTOR, TP53*, and *PTEN* between our cohort and KIRC database from TCGA. The prevalence of *VHL* alterations was similar between our cohort and data obtained from TCGA, whereas the prevalence of *KDM5C*, *BAP1*, *TP53*, and *PTEN* alterations were different (**Figure 1C**, $p > 0.05$). Notably, a high prevalence of *KDM5C* and *BAP1 alterations* was identified in both the primary tumor and VTT tissues from ccRCC patients with VTT in our cohort (*KDM5C*: 21.74, 24.00, 4.00, and 7.00%; *BAP1* 30.43, 24.00, 4.00, and 10.00% for V, VP, NP, and TCGA, respectively). No pathogenic or likely pathogenic germline variants in ccRCC-related susceptible genes were identified in all three groups of our cohort.

Comparison of the Somatic Alterations Between ccRCC With or Without VTT

First, we compared the prevalence of altered genes in the primary tumors (VP *vs*. NP). Of the 4,931 mutated genes, only 651 genes were shared by the two groups (**Figure 2A**). The majority of the mutated genes had a similar prevalence in those two groups, whereas the genes with a different altered frequency were mainly involved in the following pathways: glucose transport, apoptotic cleavage of cellular protein, cell cycle, laminin interactions, and regulation of glucokinase. No gene with a significantly higher prevalence was found in the VP group.

Comparison of the Somatic Alterations Between VTT and Matched Primary Tumors

Next, we compared the altered genes between VTT tissues and matched primary tumors (V *vs*. VP). Compared with the NP group, more genes were co-mutated in the V and VP groups. The genes with a different prevalence in VTT were primarily involved in vesicle-mediated transport, interleukin-6 (IL-6) family, transcriptional regulation by *TP53*, and the regulation of p53 activity through acetylation and Chromatin modifying enzymes (**Figure 2B**). We also identified two genes with significantly higher prevalence in the VTT tissues compared with the matched primary tumor tissues, including *CELSR2* (30.43 *vs*. 4.00%, $p < 0.05$) and *TET2* (17.39 *vs*. 0, $p < 0.05$, **Figure 2C**). Interestingly, the prevalence of *CELSR2* was also higher in the V group compared with the NP group (30.43 *vs*. 4.00%, $p < 0.05$).

Analysis of the Subclone Phylogeny Between the VTT and Matched Primary Tumors

Pyclone was used to reconstruct the clonal population for each VTT and its matched primary tumor sample (16). As shown in **Figure 3**, the bars with distinct colors represented different subclones in paired samples. The majority (15/23) of the paired samples shared the same subclones. However, eight VTT samples had notably distinct subclones from their matched primary tumors, suggesting the existence of genomic heterogeneity between the VTT tissue and its primary tumor.

DNA Damage Repair Gene Alterations and Related Signatures

Given the potential association between the DNA damage repair (DDR) pathway and VTT reported by previous studies, we investigated the prevalence of gene alterations involved in specific DDR pathways (**Figure 4A**). The most frequently mutated DDR pathway was the homologous recombination repair (HR) pathway in all three groups, whereas no alteration in the base excision repair (BER) genes was identified. A trend of increase prevalence of alterations in DDR genes, especially homologous recombination repair and non-homologous end joining genes, was found in both the V and VP groups. The median homology recombination deficiency scores of V, VP, and NP groups that were 22.5, 15.5, and 19, respectively, did not differ significantly between the groups (**Figure 4B**). Similarly, no difference in the microhomology deletions, a signature of microhomology-mediated end-joining, was found among the three groups (**Figure 4C**). Next, we analyzed the mutation signature in these groups. Known signatures, including AC1 (related to spontaneous deamination), AC3 (associated with defects in DNA double-strand break repair by homologous recombination), AC4 (associated with smoking), AC6 (associated with defective DNA mismatch repair), AC22 (resulting from exposure to aristolochic acid), AC24 (resulting

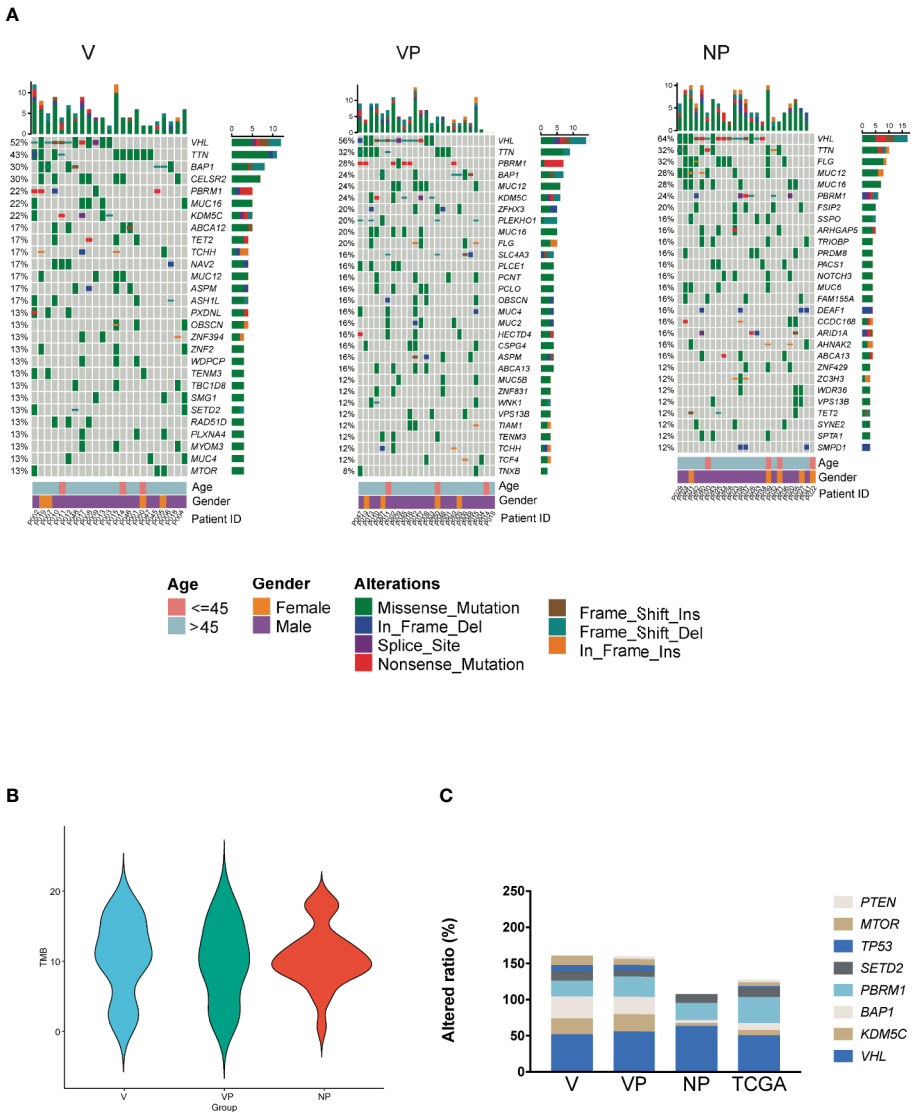

FIGURE 1 | Somatic mutation landscape of ccRCC with and without VTT. **(A)** Mutation landscape of the NP (n = 25), VP (n = 25), and V (n = 23) samples. The vertical histogram shows the number of different mutational types in each sample. The heat map shows the distribution of top genes across samples. The horizontal histogram shows the number of different mutational types in each gene. **(B)** The tumor mutation burden of the V, VP, and NP groups. **(C)** The frequency of mutations in driver genes of ccRCC in the three groups (V, VP, and NP) and in data obtained from TCGA. TCGA, The Cancer Genome Atlas; VTT, venous tumor thrombus; ccRCC, clear cell renal cell carcinoma; V, VTT; VP, matched primary tumor; NP, normal primary tumors without VTT.

from exposures to aflatoxin), and AC29 (related to habit of chewing tobacco), were identified in all three groups (**Figure 4D**). Although a trend of a higher portion of AC3 was found in V and VP groups (*versus* NP group), it did not reach statistical significance.

Copy Number Variation in ccRCC With and Without VTT

CNV features were analyzed in all three groups (**Figures 5A–C**). Notably, the VTT samples had significantly more deletions compared with the VP (p = 0.011) and NP (p = 0.013) groups (**Figure 5D**). By contrast, no differences were found in the levels of copy number gain among the three groups. We also identified a significantly higher prevalence of chromosome 9 deletion in the VTT samples (**Figure 5E**). Compared with the NP group, a significantly higher prevalence of chromosome 14 deletion was found in the V group (**Figure 5E**).

Clinical Features and the Tumor Immune Microenvironment in ccRCC Patients With Chromosome 9 and/or 14 Deletion

To investigate the clinical features in ccRCC patients with chromosome 9 and/or 14 deletion, we analyzed the clinical data and tumor immune microenvironment in the KIRC

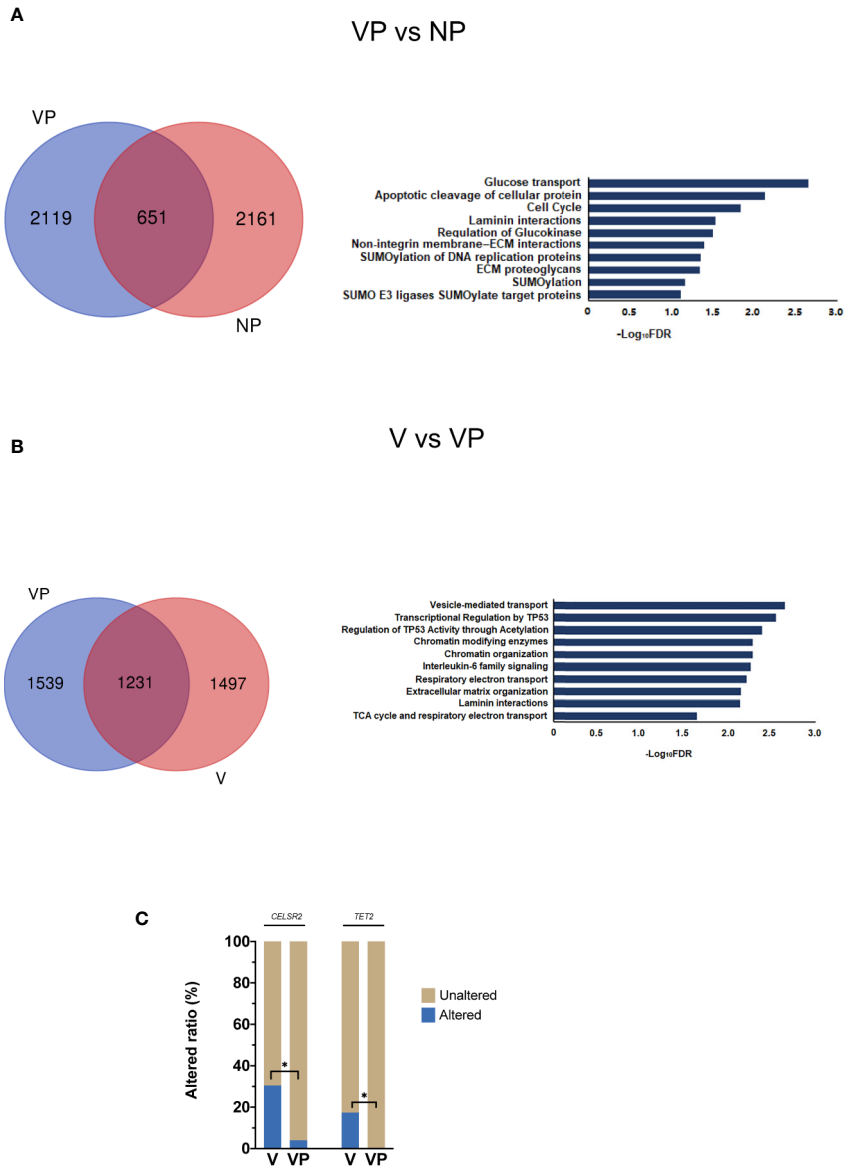

FIGURE 2 | Analysis of differences in mutant gene prevalence. **(A)** Venn diagram and reactome pathway analysis of the differentially mutated genes in the VP and NP groups. **(B)** Venn diagram and reactome pathway analysis of the differentially mutated genes in the V and VP groups. **(C)** Differences in *CELSR2* and *TET2* mutational prevalence between the V and VP groups. *$p < 0.05$. VTT, venous tumor thrombus; V, VTT; VP, matched primary tumor; NP, normal primary tumors without VTT.

database. In total, 32.68% (134/410) and 43.90% (180/410) of the ccRCC patients in KIRC had chromosome 9 or 14 deletion, respectively, which was associated with an increased incidence of distant metastasis (26.1 *vs* 11.2% and 22.8 *vs* 10.9%, respectively, $p < 0.01$) and neoplasm disease stage of stage 4 (26.3 *vs* 11.6% and 22.2 *vs* 11.7%, respectively, $p < 0.01$) in comparison with the cases with neither chromosome 9 nor chromosome 14 deletions (**Figure 6A**). In agreement with the unfavorable clinical features, chromosome 9 and chromosome 14 deletions were also significantly associated with worse overall survival, particularly for patients with both chromosome 9 and chromosome 14 deletions (**Figure 6B**).

The heat map of the expression levels of genes involved in angiogenesis, immune and antigen presentation, and myeloid inflammation was shown in **Figure 6C**. There was a relatively lower expression of genes associated with angiogenesis in samples with chromosome 4 deletion, but a higher expression of genes involved in myeloid inflammation in ccRCC patients with chromosome 9 deletion in the KIRC database. Furthermore, there was a significantly higher accumulation of follicular helper

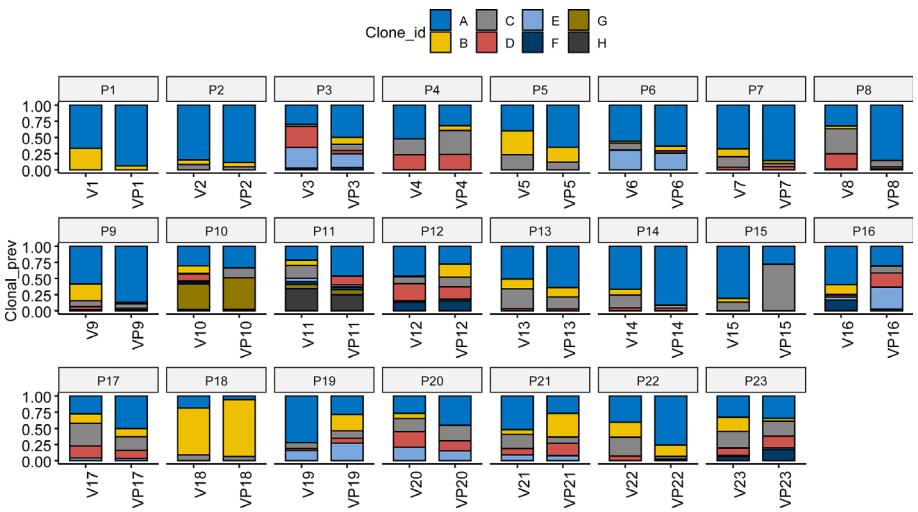

FIGURE 3 | Analysis of clonal phylogeny between the VTT and matched primary tumor. The constitution of subclones in each VTT and its matched primary tumor. V, VTT; VP, matched primary tumor.

T cells, regulatory T cells (Tregs) and macrophage M0 cells, but a significantly lower number of CD4 memory resting cells, resting mast cells were identified in ccRCC patients with chr9 and/or chr14 deletion in KIRC (**Figure 6D**). Furthermore, a significantly lower content of CD8 positive cells was identified in ccRCC patients with chromosome 9 deletion in KIRC (**Figure 6E**). Notably, by immunochemistry analysis, there was a significantly lower count of CD4+ and CD8+ tumor-infiltrated lymphocytes in the VTT samples compared with their primary tumor tissues from our cohort (**Figures 6F, G**).

The Response to Immune Checkpoint Inhibitors Differs Between VTT Tissues and the Renal Mass

A total of four ccRCC patients with VTT in our center who were unfit for surgery in the initial evaluation were treated with anti-programmed death receptor-1 (PD-1) monoclonal antibody treatment (Toripalimab, 240 mg Q3W IV) (**Supplementary Table 1**). One patient stopped treatment after only three cycles of Toripalimab due to personal reasons. The other three finished the six cycles of therapy, and all three exhibited significant reduction of the renal mass after six cycles of immune checkpoint inhibitor (ICI) treatment. However, the VTT did not regress in two of the three ccRCC patients with VTT (**Figure 7**). Patient A's renal mass decreased from 9.56 to 4.31 cm in diameter after immunotherapy; however, his thrombus did not show significant regression (19.31 to 18.52 cm in diameter); Patient B also had a notable response in the renal mass (6.45 to 2.84 cm), but not in the VTT lesion (13.14 to 13.03 cm).

DISCUSSION

The 5-year survival rate of RCC has been strikingly improved to 74% over the past decades, and this was mainly attributed to the successful advances of targeted drugs and ICI (17). However, for ccRCC patients with VTT, the efficacy of neoadjuvant and adjuvant treatments remains contested (18). A comprehensive understanding of the genomic feature of VTT and its differences from the primary tumor may assist clinicians with regard to therapeutic decisions.

Consistent with the genetic profile of ccRCC patients in the KIRC and literature, the most common genetic alterations we identified in our patients, either with or without VTT, were *VHL*, *PBRM1*, *BAP1*, and *KDM5C*, which are all known driver genes in ccRCC (19). Compared with ccRCC patients without VTT and the corresponding data in the KIRC, we found a trend of an increased prevalence of *BAP1* alterations in both VTT and their matched primary tumor tissues, and alterations of *BAP1* have been widely regarded as an unfavorable prognosis biomarker in ccRCC (20). Limited by the sample sizes, the prevalence of the majority of mutated genes did not differ significantly between the primary tumor tissues from ccRCC with or without VTT.

As for the genetic difference between VTT and the respective primary tumor, no studies have reported a difference in the prevalence of mutated genes, to the best of our knowledge. In this study, we found a significantly higher incidence of *CELSR2* and *TET2* alterations in the VTT compared with the matched primary tumors. *CELSR2*, which encodes a family member of the cadherin EGF LAG seven-pass G-type receptors, has been identified in 1.8% of ccRCC patients in the KIRC database, which is not statistically different from the frequency in our cohort (4.00% in the VP group). Notably, *CELSR2* was observed in

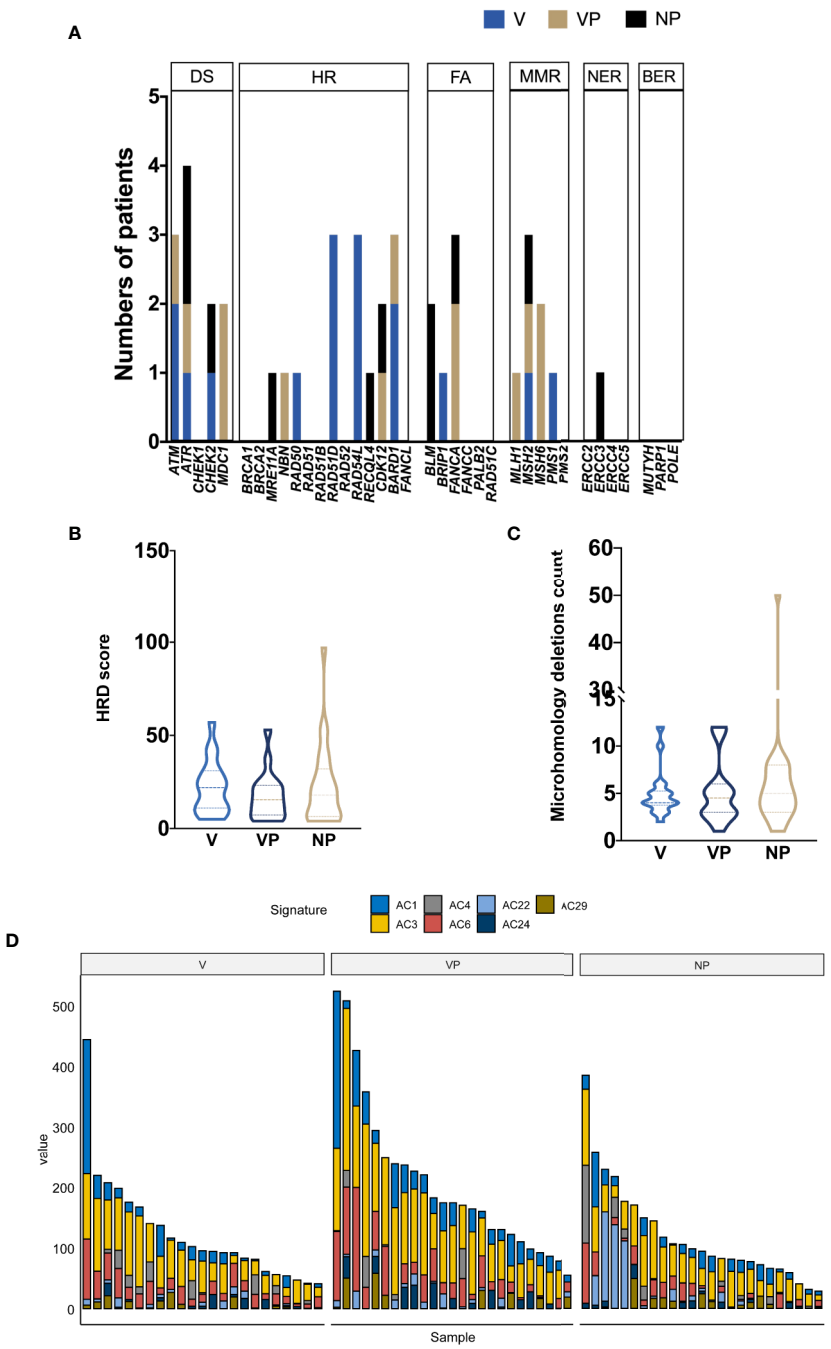

FIGURE 4 | Mutational signature profile and DNA damage repair pathway analysis. **(A)** The distribution of DDR somatic mutations in each group (V, VP, and NP). The prevalence of HRD score **(B)** and microhomology deletions **(C)**. **(D)** Mutational signature in each group (NP, V, and VP). AC, Alexandrov COSMIC; DS, damage sensor; HR, homologous recombination repair; DDR, DNA damage repair; FA, Fanconi anemia; MMR, mismatch repair; NER, nucleotide excision repair; BER, base excision repair; VTT, venous tumor thrombus; V, VTT; VP, matched primary tumor; NP, normal primary tumors without VTT; HRD, homology recombination deficiency.

30.43% of the V group. Though it has been found to be involved in the contact-mediated intercellular communication and was suggested to participate in kidney development and physiology, the specific function of *CELSR2* in the ccRCC has not been clarified (21). *TET2* encodes a methylcytosine dioxygenase that was shown to be involved in DNA demethylation, and it is frequently mutated in myeloid malignancies and other disorders (22). *TET2* activation, which can be induced by Ascorbic acid, may lead to the loss of hydroxymethylcytosine, which is associated with a more adverse prognosis in ccRCC (23). The

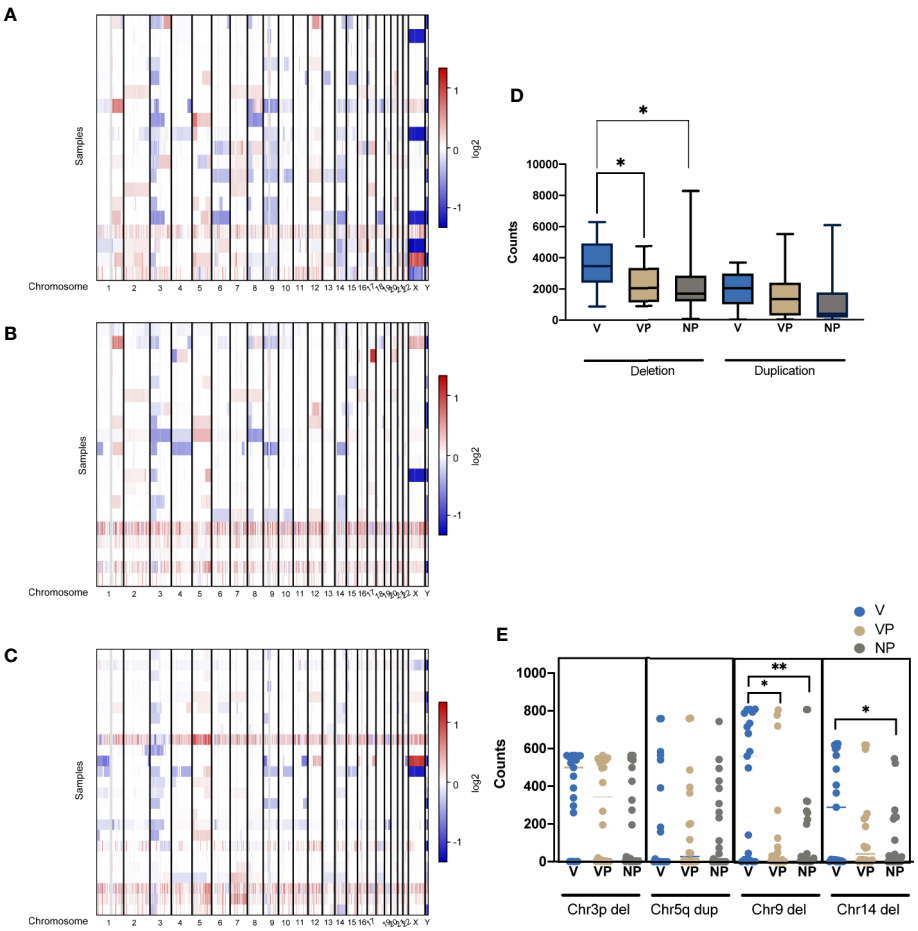

FIGURE 5 | CNVs of tumors were plotted by chromosomal location (vertical axis) of the entire dataset in the V **(A)**, VP **(B)**, and NP groups **(C)**. **(D)** Differences in the copy number deletions and duplications in the three groups. **(E)** The counts of deletion of chromosome 3p (Chr3p Del), duplication of chromosome 5q (Chr5q Dup), deletions of chromosome 9 (Chr9 Del) and chromosome 14 in the three groups. *$p < 0.05$; **$p < 0.01$. CNV, copy number variation; VTT, venous tumor thrombus; V, VTT; VP, VTT matched primary tumor; NP, normal primary tumors without VTT.

presence of frequent *CELSR2* and TET2 mutation in VTT suggested that these genes may be involved in the metastasis of the ccRCC cells, or alternatively, they may contribute to cell survival and/or proliferation in the thrombus, which is a novel environment distinct from the kidney. Further studies are required to reveal the cellular and molecular functions of these genes.

A previous study by Gregor Warsow et al. reported the presence of signature AC3 in VTT, an indicator for homology recombination deficiency (8). However, in our cohort, AC3 was not significantly more prevalent in the VTT tissues (**Figure 4D**). The difference in the results may be due to the widely spread genomic instability in our ccRCC cohort, regardless of the presence of VTT or not. A retrospective study in MSKCC found that 17% of metastatic ccRCC patients possessed alterations in DDR genes, and this was associated with better overall survival in immunotherapy-treated cohort, but not in the tyrosine kinase inhibitor-treated cohort (24). A previous study also found an unfavorable prognostic role of homologous recombination deficiency in ccRCC based on the data from TCGA (25). In the present study, a relatively higher incidence of DDR gene alterations was found in VTT and their matched primary samples compared with the NP group, suggesting the presence of genomic instability and metastasis-prone features in VTT and its primary tumor.

Notably, VTT samples possessed an increased number of CNV alterations; in particular, a higher number of CNV loss than the respective primary lesions, as well as when compared with ccRCC without VTT. We found a significantly higher prevalence of deletions in chromosome 9 and other chromosomes in the VTT group, which contained multiple tumor suppressor genes (including *PTPRD*, *CDKN2A*, *CDKN2B*, *BNC2*, *FANCC*, *TGFBR1*, *TLE4*, *TLE1*, *TSC1*, *PTCH1*, *KLF4*, *ROBO1*, *RAD51B*, and *MAX*), suggesting that the deletion of these tumor suppressor genes may contribute to either the metastasis and or thrombosis process. Deletions in the

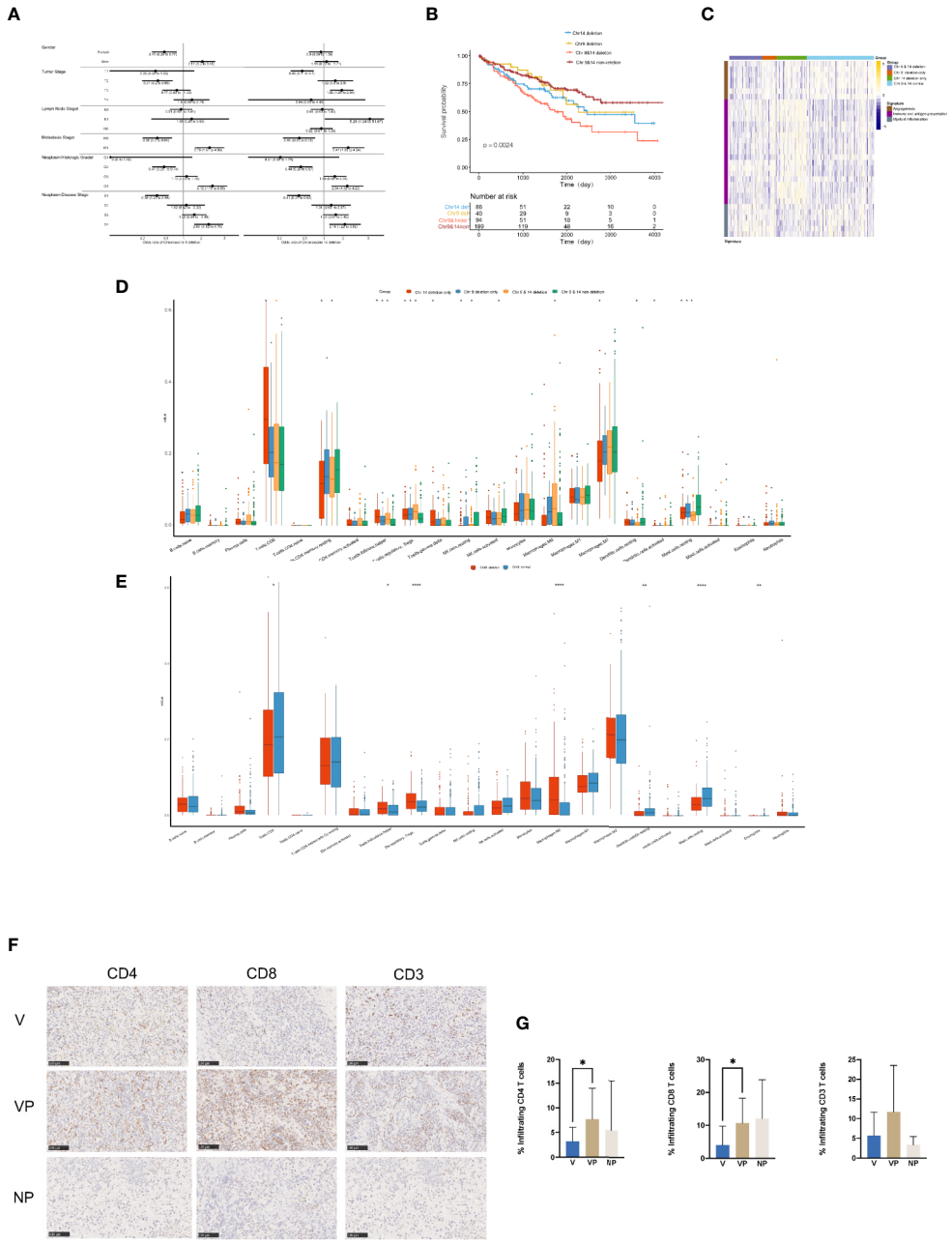

FIGURE 6 | Clinical features and tumor microenvironment of the ccRCC patients with deletions in chromosomes 9 and 14 based on the KIRC database. **(A)** Clinical features of the ccRCC patients in the KIRC database with or without deletions in chromosome 9 (left) or 14 (right). **(B)** Kaplan–Meier curves for OS of ccRCC patients in the KIRC database with or without chromosome 9 and/or 14 deletions. **(C)** Heat map of gene expression signatures in angiogenesis, immune and antigen presentation and myeloid inflammation of ccRCC patients in the KIRC database with or without chromosome 9 and/or 14 deletion. **(D)** Analysis of tumor-infiltrating immune cell types in ccRCC patients in the KIRC database with or without chromosome 9 and/or 14 deletion. **(E)** Analysis of tumor-infiltrating immune cell types in ccRCC patients in the KIRC database with or without deletions in chromosome 9. Immunostaining **(F)** and analysis **(G)** for CD4, CD8 and CD3 in the V, VP, and NP groups. **(G)** $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. ccRCC, clear cell renal cell carcinoma; KIRC, kidney renal clear cell carcinoma; OS, overall survival; V, VTT; VP, VTT matched primary tumor; NP, normal primary tumors without VTT.

chromosome 9 have been found to be associated with a more aggressive clinicopathological feature in ccRCC, including more advanced stage diseases, larger tumor volume and notably, increased renal vein invasion, and may thus serve as an independent predictor of recurrence and survival following surgery (26). Additionally, deletions of regions in chromosome 9 were predictive of a worse prognosis based on the KIRC database. Moreover, our study identified a higher prevalence of

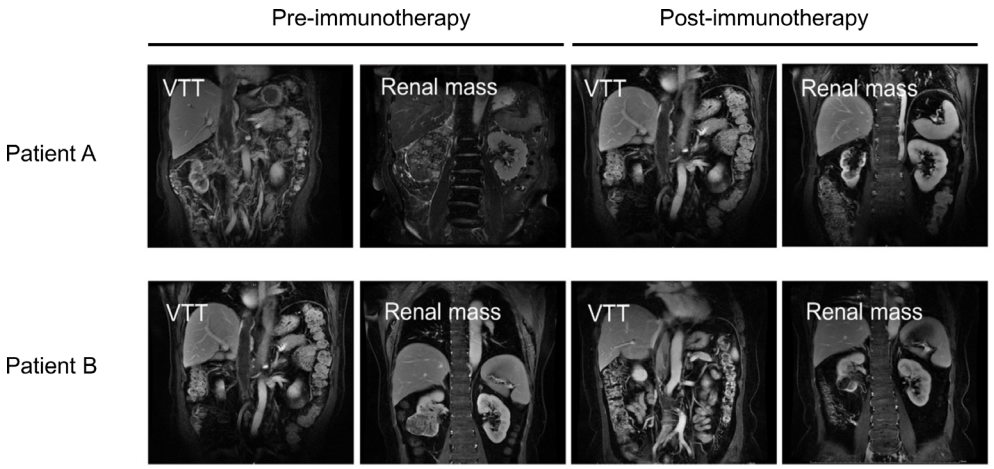

FIGURE 7 | Images of VTT and the renal mass pre- (left) or post-immunotherapy (right) of two ccRCC patients with VTT. VTT, venous tumor thrombus; ccRCC, clear cell renal cell carcinoma.

chromosome 14 deletion in the VTT tissues, which contained a pivotal regulator in ccRCC, hypoxia-inducible factor 1α (HIF1α) (27). Previous studies have demonstrated that chromosome 14 deletion is associated with lower HIF1α levels and poor prognosis in ccRCC patients (28). It may be of value for examining the role of t chromosome 9 loss, as well as HIF1α deletion in the formation and development of VTT in future.

In addition to the tumor suppressor genes, we also found a unique tumor microenvironment feature in ccRCC patients with chromosome 9 deletions. Tumor-infiltrating immune cells in the microenvironment serve an essential function in tumor development, metastasis, and response to ICIs (29, 30). Recently, biomarker analysis of the JAVELIN Renal 101 trial showed that ccRCC patients with different gene expression signatures of immune and angiogenesis functions possessed distinct responses to avelumab plus axitinib or axtinib monotherapy (31). Interestingly, we found both a relatively lower accumulation of the angiogenesis signature and a higher accumulation of the myeloid inflammation signature in ccRCC patients with chromosome 9 deletions in the KIRC database, which may be related to the lower response rate to either the anti-angiogenesis or ICI monotherapy in ccRCC patients. The potential immunosuppressive feature of ccRCC with chromosome 9 deletions was further shown by the analysis of immune cell features, which showed a notably lower count of CD8 positive T cells, but higher levels of Treg cells. Treg cells act as a negative regulator of anti-tumor immunity by inhibiting the activation and differentiation of CD4 and CD8 positive T cells (32). Additionally, a higher level of tumor associated macrophages and/or a reduced level of CD8 positive T cells has also been correlated with a lower response rates to ICI monotherapy (33). Notably, for the three patients who received ICI therapy, ICI had potent beneficial effects on the primary renal mass, but not on the VTT, which may be due to the differences in the immune microenvironments present between VTT and the primary kidney lesions. Unfortunately, these patients were unfit for surgery, and we could not obtain the primary tumor and VTT tissues for genetic testing, which precluded the direct analysis of the correlation between the efficacy of immunotherapy and the genomic features. Nevertheless, this finding may serve as a clue for further clinical research on the immune microenvironment of VTT and immunotherapy.

In summary, a unique genomic feature, including chromosome 9 and 14 deletions was identified in this study, which may be associated with the development and/or maintenance of VTT. The deletions of chromosome 9 and 14 (particularly chromosome 9) may be associated with a suppressive immune microenvironment, suggesting a poor response to ICI monotherapy in the VTT of the ccRCC patients.

LIMITATIONS

This study was limited by the sample size to draw any conclusions and vulnerable to selection bias. Future studies with larger cohorts are required to validate the clinical implications of the genomic features identified by the current study, as well as to reveal additional features that are only attainable with higher statistical power. For the three patients with VTT who completed the ICI treatment, two of them exhibited a prominent reduction in the primary tumor mass, but not of the VTT, and we speculated that the VTTs' lack of response to ICI may be related to the genetic features of VTT. However, we were unable to show this directly, and instead, it was deduced from the genetic features identified in the VTT samples from the group that underwent surgery. The three patients were unfit for surgery, and therefore no tissue was available for genetic testing pre- and post-ICI treatment. Nevertheless, the differing responses to ICI highlight the

potential differences in the immune microenvironment between the primary tumor and VTT in ccRCC patients, and this merits further study on additional surgical cases that are also treated with ICI.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://bigd.big.ac.cn/gsa-human/, HRA000795.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of the First Medical Center of PLA General Hospital (S2017-100-01). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SN: data collection and drafting the article. KL: data analysis and manuscript revision. CP: data collection and analysis. YY: data collection. QH: data collection. SW: data collection. BC: data collection. XM: data collection and design of this work. XZ: design of this work. BW: design of this work and data analysis. All authors contributed to the article and approved the submitted version.

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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.2021.646338/full#supplementary-material

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Body Composition Variables as Radiographic Biomarkers of Clinical Outcomes in Metastatic Renal Cell Carcinoma Patients Receiving Immune Checkpoint Inhibitors

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Background: Immune checkpoint inhibitors (ICI) have revolutionized the treatment of metastatic renal cell carcinoma (mRCC). Biomarkers for mRCC patients treated with ICI are limited, and body composition is underutilized in mRCC. We investigated the association between body composition and clinical outcomes in ICI-treated mRCC patients.

Methods: We performed a retrospective analysis of 79 ICI-treated mRCC patients at Winship Cancer Institute from 2015-2020. Baseline CT images were collected at mid-L3 and segmented using SliceOMatic v5.0 (TomoVision). Density of skeletal muscle (SM), subcutaneous fat, inter-muscular fat, and visceral fat were measured and converted to indices by dividing by height(m)^2 (SMI, SFI, IFI, and VFI, respectively). Total fat index (TFI) was defined as the sum of SFI, IFI, and VFI. Patients were characterized as high *versus* low for each variable at gender-specific optimal cuts using overall survival (OS) as the primary outcome. A prognostic risk score was created based on the beta coefficient from the multivariable Cox model after best subset variable selection. Body composition risk score was calculated as IFI + 2*SM mean + SFI and patients were classified as poor (0-1), intermediate (2), or favorable risk (3-4). Kaplan-Meier method and Log-rank test were used to estimate OS and PFS and compare the risk groups. Concordance statistics (C-statistics) were used to measure the discriminatory magnitude of the model.

Results: Most patients were male (73%) and most received ICI as first (35%) or second-line (51%) therapy. The body composition poor-risk patients had significantly shorter OS (HR: 6.37, p<0.001), PFS (HR: 4.19, p<0.001), and lower chance of CB (OR: 0.23,

p=0.044) compared to favorable risk patients in multivariable analysis. Patients with low TFI had significantly shorter OS (HR: 2.72, p=0.002), PFS (HR: 1.91, p=0.025), and lower chance of CB (OR: 0.25, p=0.008) compared to high TFI patients in multivariable analysis. The C-statistics were higher for body composition risk groups and TFI (all C-statistics ≥ 0.598) compared to IMDC and BMI.

Conclusions: Risk stratification using the body composition variables IFI, SM mean, SFI, and TFI may be prognostic and predictive of clinical outcomes in mRCC patients treated with ICI. Larger, prospective studies are warranted to validate this hypothesis-generating data.

Keywords: body composition, mRCC, immune checkpoint inhibitors, sarcopenia, adiposity, prognostic model, biomarkers

BACKGROUND

Immune checkpoint inhibitors (ICI) have become an important option for the treatment of metastatic renal cell carcinoma (mRCC) over the past 5 years (1). Nivolumab, a programmed death protein-1 (PD-1) inhibitor, was the first ICI approved for mRCC in 2015 (2). Since that time, several ICI-based combination treatment regimens have been approved for treatment-naïve mRCC including nivolumab plus ipilimumab, pembrolizumab plus axitinib, avelumab plus axitinib, and nivolumab plus cabozantinib (2). Despite the increased use of ICI for mRCC, a subset of patients do not respond to treatment with ICI-based treatment regimens. Furthermore, biomarkers to help determine which patients are more likely to respond to treatment with ICIs are limited. Hence, the identification of robust clinical biomarkers of response to ICI in mRCC is an unmet need in the field of genitourinary oncology.

At this time, body composition is under-studied as a biomarker in mRCC patients. The investigation of markers of body composition as prognostic biomarkers in mRCC patients has primarily been focused on body mass index (BMI) (3). Additionally, increased BMI has been shown to be a favorable prognostic factor in patients with several malignancies treated with ICIs including non-small cell lung cancer (NSCLC), melanoma, and mRCC (4–6). There is a growing body of literature investigating sarcopenia, measured by skeletal muscle index (SMI), as a possible biomarker. Although the majority of these studies have been performed in the peri-operative setting for mRCC patients undergoing nephrectomy (7, 8), sarcopenia has also been shown to be a significant predictor of OS in mRCC (9). Additionally, subcutaneous fat index (SFI) was found to be an independent predictor of mortality in mRCC patients treated with sunitinib (10). Other markers of adiposity such as total fat index (TFI), visceral fat index (VFI), or inter-muscular fat index (IFI) have not been found to be associated with clinical outcomes in mRCC patients. Furthermore, there have been no studies investigating markers of adiposity in mRCC patients treated with ICIs.

In this study, we performed a comprehensive investigation of the association between radiographic markers of body composition and clinical outcomes in mRCC patients treated with ICI-based treatment regimens. We used markers of both sarcopenia and adiposity to create a novel risk scoring system in this cohort of patients. We also compared the predictive value of our model to the validated international mRCC database consortium (IMDC) criteria and BMI. Importantly, we also assessed the association between a composite marker of adiposity, total fat index (TFI), in this cohort of ICI-treated mRCC patients. We hypothesize that the findings from this study may provide evidence for body composition markers to be considered for inclusion in updated prognostic risk models for mRCC patients treated with ICIs. These results may be helpful for practicing oncologists in the academic or community setting given the increasing use of ICIs for several malignancies including mRCC.

METHODS

Patients and Data

We performed a retrospective analysis of 79 ICI-treated mRCC patients at Winship Cancer Institute of Emory University from 2015-2020. This study was approved by the Emory University Institutional Review Board. Inclusion criteria for this study were: (1) confirmed histologic diagnosis of RCC, (2) receipt of at least 1 dose of ICI and (3) availability of computed tomography (CT) scans within 2 months of ICI-initiation. Baseline CT images were collected at mid-L3 and segmented using SliceOMatic v5.0 (TomoVision) by one author (DJM). Adequate training was confirmed by an intra-observer variation < 1.3%. We collected the density of skeletal muscle (SM), subcutaneous fat, inter-muscular fat, and visceral fat using the following Hounsfield Unit (HU) references ranges (-29 to + 150 HU for skeletal muscle, -190 HU to -30 for subcutaneous and inter-muscular fat, -150 to -50 HU for visceral fat) (11, 12). Each density was converted to an index by dividing by height $(m)^2$ (SMI, SFI, IFI, and VFI, respectively). Total fat index (TFI) was defined as the sum of SFI, IFI, and VFI. Additional clinical data was collected including demographics, Eastern Cooperative Oncology Group Performance Status (ECOG PS), number and type of prior systemic therapies, sites of metastatic disease, and baseline BMI. IMDC criteria were used to characterize patients as favorable, intermediate, or poor-risk (13).

We used three different measures of clinical outcomes: overall survival (OS), progression-free survival (PFS), and clinical benefit (CB). OS and PFS were calculated as the number of months elapsed from the first dose of ICI to date of death or radiographic or clinical progression, respectively. CB was defined

as a best radiographic response of complete response (CR), partial response (PR), or stable disease (SD) for ≥ 6 months per response evaluation criteria in solid tumors version 1.1 (RECISTv1.1) (14). CB is a two-level variable, in which the responder is defined as having a best radiographic response of CR, PR, or SD ≥ 6 months and the non-responders were defined as PD or non-evaluable.

Statistical Analysis

Statistical analysis was performed using SAS Version 9.4, and SAS macros, which was developed by the Biostatistics Shared Resource at Winship Cancer Institute (15). The significance level was set at $p < 0.05$ and descriptive statistics for each variable were reported. The univariate association (UVA) of each covariate with OS and PFS was tested by proportional hazard model with a reported hazard ratio (HR) and its 95% confidence interval (CI) being reported. In the analysis for CB, we used logistic regression and modeled the probability of response to find the odds ratio (OR). Each fat index was characterized as high *versus* low for each variable at gender-specific optimal cuts using overall survival (OS) as the primary outcome through a bias-adjusted log-rank test searching algorithm (16). A prognostic risk score was created based on the beta coefficient from the multivariable Cox model (MVA) after best subset variable selection (17). Body composition risk score was calculated as IFI + 2*SM mean + SFI, and patients were classified as poor (0-1), intermediate (2), or favorable risk (3-4). The prediction performance by BMI, IMDC Risk Group, and our body composition risk score was measured and compared by Uno's concordance statistics (C-statistics) (18). The area under the curve (AUC) was reported for the discrimination for CB analysis. Kaplan-Meier method and Log-rank test were used to estimate OS and PFS and compare the risk groups.

RESULTS

Demographic Information and Baseline Disease Characteristics

Descriptive statistics for demographic information and baseline disease characteristics are presented in **Table 1**. Most patients were males (73.4%) and median age was 61.0 years old. The majority of patients were Caucasian (n=61, 77.2%) and more than one-fifth (n=17, 21.5%) were African Americans. Most patients had clear cell RCC (n=55, 74.3%) and patients were primarily intermediate (54%) or poor-risk (30%) per IMDC criteria. More than one-quarter of patients (n=19, 27) had three or more metastatic sites at baseline. The majority of patients received no prior systemic therapy (35%) or one (51%) prior line of systemic therapy prior to initiating ICI. Anti-PD-1 monotherapy was the most common treatment regimen (n=47, 59.5%), while 40.5% received an ICI combination regimen.

Risk Group Analysis

The MVA of the association between body composition and TFI with clinical outcomes is presented in **Table 2**. The body composition poor-risk patients had significantly shorter OS (HR: 6.37, CI: 2.40-16.92, p<0.001), PFS (HR: 4.19, CI: 1.87-9.42, p<0.001), and lower chance of CB (OR: 0.23, CI: 0.05-0.96, p=0.044) compared to favorable risk patients in MVA. Intermediate risk patients also showed a trend towards shorter PFS (HR: 2.05, CI: 0.98-4.29, p=0.057) compared to favorable risk patients. There was a step-wise decline in median OS and PFS from favorable risk to intermediate risk to poor risk patients per Kaplan Meier estimation (OS: 44.5 months *versus* 24.6 months *versus* 6.3 months; PFS: 12.4 months *vs.* 4.8 months *vs.* 2.5 months, **Table 2** and **Figures 1**, **2).** The C-statistics were higher for our body composition risk groups compared to IMDC and BMI for all three clinical outcomes (**Table 3** and **Supplemental Figure 1**).

TABLE 1 | Demographics and baseline characteristics.

Variable		n (%)
Gender	Male	58 (73.4)
	Female	21 (26.6)
Race	White	61 (77.2)
	Black	17 (21.5)
	Asian	1 (1.3)
ECOG PS	0-1	58 (75.3)
	2+	19 (24.7)
	Missing	2
ccRCC	Yes	55 (74.3)
	No	19 (25.7)
	Missing	5
Anti-PD-1 Monotherapy	Yes	47 (59.5)
	No	32 (40.5)
Prior Lines of Therapy	0	28 (35.4)
	1	40 (50.6)
	2	7 (8.9)
	3+	4 (5.1)
Number of distant metastatic sites	1	12 (15.2)
	2	27 (34.2)
	3+	40 (50.6)
IMDC Risk Groups	Favorable	12 (15.2)
	Intermediate	43 (54.4)
	Poor	24 (30.4)
Baseline BMI (Median: 26.2)	≤25	29 (37.2)
	>25	49 (62.8)
	Missing	1
Median (Optimal Cut-Off) Muscle and Adipose Variables	SMI	M: 44.0, F: 39.2
	Attenuated SM Mean	M: 35.1, F: 34.4
	SFI	M: 51.4, F: 69.8
	IFI	M: 4.4, F: 7.8
	VFI	M: 35.2, F: 37.4
	TFI	M: 98.7, F: 94.3
Median Age: 61.0 years		

ECOG PS, Eastern cooperative oncology group performance status; ccRCC, clear cell renal cell carcinoma; BMI, body mass index; SMI, skeletal muscle index; SM, skeletal muscle; SFI, subcutaneous fat index; IFI, inter-muscular fat index; VFI, visceral fat index.

Total Fat Index (TFI) Analysis

The categorical TFI analysis investigating the association with clinical outcomes is also presented in **Table 2**. Patients with low TFI had significantly shorter OS (HR: 2.72, CI: 1.43-5.17, p=0.002), PFS (HR: 1.91, CI: 1.09-3.35, p=0.025), and lower chance of CB (OR: 0.25, CI: 0.09-0.70, p=0.008) compared to high TFI patients in MVA. High TFI patients had significantly longer median OS (44.5 *vs.* 14.1 months, p=0.0012) and PFS (8.4 *vs.* 2.9 months, p=0.0015) compared to low TFI patients per

TABLE 2 | MVA* of association between body composition risk groups and TFI with clinical outcomes.

	OS		PFS		CB	
	HR (CI)	**p-value**	**HR (CI)**	**p-value**	**OR (CI)**	**p-value**
Body Composition Risk Group Analysis						
Poor Risk: Risk Score = 0-1 *n = 20*	6.37 (2.40-16.92)	**<0.001****	4.19 (1.87-9.42)	**<0.001****	0.23 (0.05-0.96)	**0.044****
	Median Survival: 6.3 months **24-Month Survival:** 29.2%		**Median Survival:** 2.5 months **12-Month Survival:** 15.0%			
Intermediate Risk: Risk Score = 2 *n* = 42	1.56 (0.61-3.95)	0.350	2.05 (0.98-4.29)	0.057	0.49 (0.15-1.59)	0.238
	Median Survival: 24.6 months **24-Month Survival:** 53.1%		**Median Survival:** 4.8 months **12-Month Survival:** 26.6%			
Favorable Risk: Risk Score = 3-4 *n* = 18	1		1		1	
	Median Survival: 44.5 months **24-Month Survival:** 82.1%		**Median Survival:** 12.4 months **12-Month Survival:** 54.3%			
Categorical Total Fat Index (TFI) Analysis* **						
Low n = 34	2.72 (1.43-5.17)	**0.002****	1.91 (1.09-3.35)	**0.025****	0.25 (0.09-0.70)	**0.008****
High n = 45	1		1		1	

** MVA controlled for race, gender, clear cell RCC, Baseline BMI, Age, anti-PD-1 monotherapy, IMDC risk groups and number of prior lines of therapy.*

***Statistically significant at the level of p < 0.05.*

****High vs low TFI determined by optimal cut analysis.*

Bold p-values represent statistically significant values.

Kaplan-Meier estimation (**Supplemental Figure 2**). Additionally, TFI had higher C-statistics for predicting OS, PFS, and CB compared to both IMDC and BMI (**Table 3**). Notably, TFI was significantly better at predicting clinical benefit compared to BMI (C-statistics: 0.646 *vs.* 0.522, p=0.012).

DISCUSSION

Overall, we found that increased adiposity and increased attenuated SM mean were associated with improved outcomes in this cohort of mRCC patients treated with ICI-based treatment regimens. We used two different methods to present the association of adiposity with clinical outcomes. First, we created a novel prognostic risk scoring system which included two measures of adiposity: SFI and VFI. In a secondary analysis, we showed that low TFI was significantly associated with worse outcomes. This is an important study in that it provides hypothesis-generating data regarding the prognostic risk associated with certain radiographic measurements of body composition. Although BMI has been associated with improved outcomes in mRCC patients, this is the first and most comprehensive study investigating risk associated with different components of adipose tissue and skeletal muscle on clinical outcomes in ICI-treated mRCC patients. Importantly, this study highlights the prognostic value of CT imaging in measuring adiposity in mRCC patients.

The hypothesis-generating data presented in this study has important clinical implications for mRCC patients initiating therapy with ICI-based treatment regimens. Namely, the significant association of body composition variables including SFI, IFI, attenuated SM mean, and TFI highlight the under-utilization of data collected by CT imaging in oncology patients treated with immunotherapy. These results add to a growing body of literature supporting the inclusion of body composition variables in updated prognostic and predictive models in mRCC patients treated with ICI-based treatment regimens. Radiographic body composition measures are particularly attractive as clinical biomarkers because baseline imaging is performed as standard of care for patients starting on a new line of systemic therapy. A recent study by Higgins et al. found that there was high correlation for adipose tissue and muscle measures between CT images and magnetic resonance imaging (MRI) (19). It should be noted, that there was a bias towards 10.34% lower measures of subcutaneous fat density on MRI which contributed to our decision to only including patients with baseline CT imaging in this study. Future studies are required to standardize the process of segmenting MRI images before the results from this study may be generalized to patients undergoing baseline MRI prior to ICI-initiation.

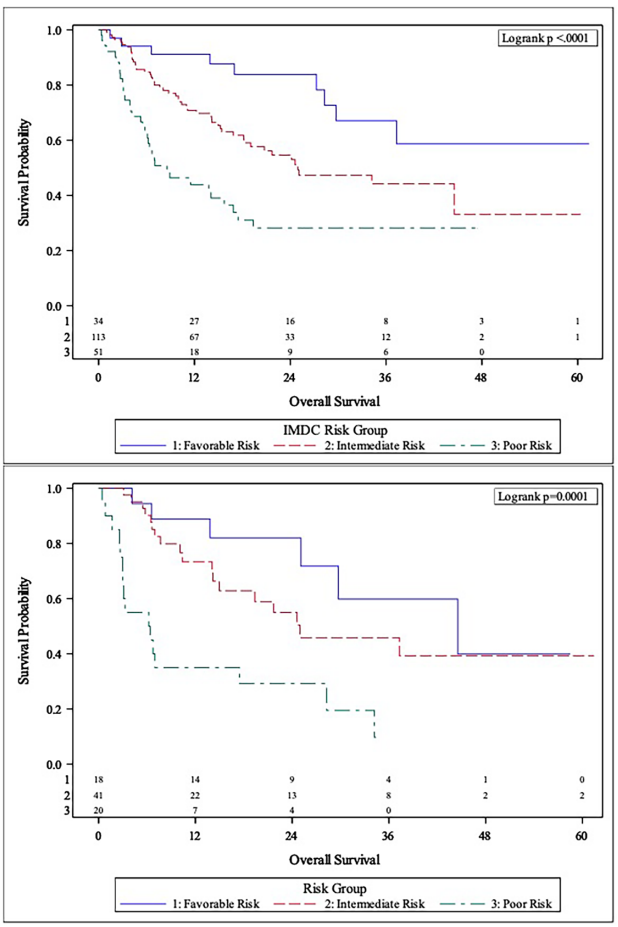

FIGURE 1 | Comparison of Kaplan-Meier curves between IMDC risk groups (Top panel) and body composition risk groups (Bottom panel) for overall survival (OS).

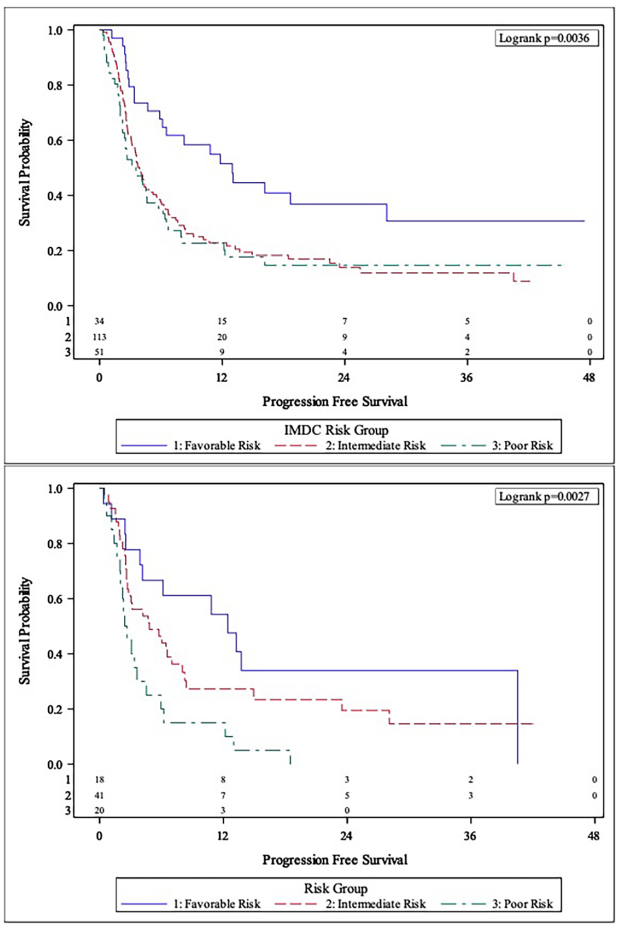

FIGURE 2 | Comparison of Kaplan-Meier curves between IMDC risk groups (Top Panel) and body composition risk groups (Bottom Panel) for progression-free survival (PFS).

Increased subcutaneous fat was associated with improved outcomes in this study, which is consistent with our group's previous findings of an association between increased SFI and longer OS and PFS in phase 1 clinical trial patients treated with immunotherapy (20). This is the first study, to our knowledge, to find an association between SFI and ICI-treated mRCC patients. This finding highlights the obesity paradox in cancer, in which obesity has been shown to contribute to carcinogenesis, yet obese patients may be more likely to have improved outcomes to treatment (21). Increased BMI has been associated with improved outcomes in ICI-treated NSCLC and melanoma patients (4, 5). This association was also described in patients with mRCC (6). A large retrospective study (n=736) found similar tumor mutational burden and genomic alterations between high and low BMI mRCC patients treated with ICIs (6). One possible explanation for this association is the fact that adipocyte PD-L1 expression increases during

TABLE 3 | Comparison of C-statistics between body composition risk groups, TFI, IMDC, and BMI.

	OS C-Statistic	p-value Comparison to IMDC	p-value Comparison to BMI	PFS C-Statistic	p-value (comparison to IMDC)	p-value (comparison to BMI)	CB C-statistic	p-value (comparison to IMDC)	p-value (comparison to BMI)
Risk Group	0.648	0.749	0.228	0.612	0.738	0.313	0.637	0.513	0.136
TFI	0.626	0.987	0.186	0.598	0.878	0.174	0.646	0.400	**0.012***
IMDC	0.613	Not Available		0.575	Not Available		0.584	Not Available	
BMI	0.562			0.544			0.522		

**Statistically significant at the level of $p < 0.05$.*
Bold p-values represent statistically significant values.

adipogenesis, suggesting that adiposity promotes tumor immune evasion which may be reversed by ICI-based treatment regimens *via* increased effector T-cells (22, 23). Surprisingly, we also found that increased IFI was associated with improved outcomes and was one of the variables chosen for inclusion in our risk group analysis. It is possible that increased IFI in this population is a reflection of total body adiposity, given that the Pearson correlation coefficients with SFI and TFI were 0.637 and 0.655, respectively (both $p<0.001$, **Supplemental Figure 3**). Taken together, we provide evidence that radiographic markers of adiposity such as SFI and IFI may be clinical biomarkers of improved outcomes in ICI-treated mRCC patients.

An important finding with significant clinical relevance presented in this study is that TFI was independently associated

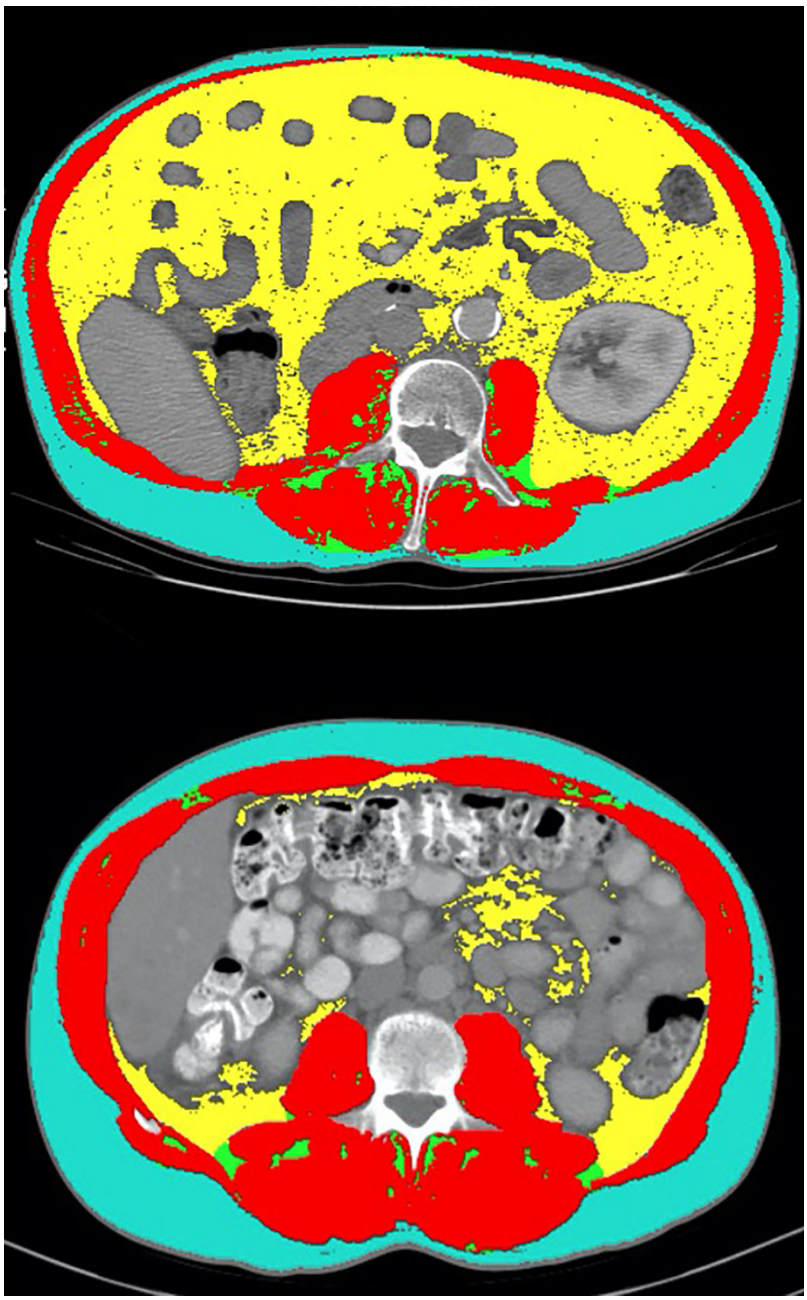

FIGURE 3 | Comparison of select CT segmentation results between two patients with similar BMI but disparate TFI and clinical outcomes. The first patient (top panel) had a BMI of 24.9 and a TFI of 119.97 (high). He had a best radiographic response of partial response on treatment with anti-PD-1 combination therapy. The second patient (bottom panel) had a BMI of 24.4, but a TFI of 44.88 (low). This patient had a best radiographic response of progressive disease on treatment with anti-PD-1 combination therapy.

with OS, PFS, and CB in MVA. Additionally, the C-statistics for predicting clinical outcomes were all higher for TFI than BMI, including a significantly higher C-statistics for predicting CB. This highlights the possible predictive and prognostic value of TFI which has not been previously described in the literature for mRCC patients. One possible explanation for this observation in this cohort is that adipose tissue is a secondary lymphoid organ and houses populations of T-cells (24). The value of TFI as a potential biomarker is highlighted in **Figure 3**, which compared two patients with similar BMI but disparate TFI and clinical outcomes on treatment with anti-PD-1 combination therapy. The first patient had a BMI of 24.9 and a high TFI of 119.97. He had a best radiographic response of PR on treatment. The second patient had a BMI of 24.4, but a low TFI of 44.88. This patient had a best radiographic response of progressive disease on anti-PD-1 combination treatment. This radiographic representation combined with the higher C-statistics of TFI compared to BMI suggest that TFI may be a better marker of total body adiposity than BMI in mRCC patients treated with ICI.

Interestingly, we found that attenuated SM mean was a better prognostic marker than SMI in our analysis. Decreased attenuated SM mean has been used as a marker of myosteatosis, given that it

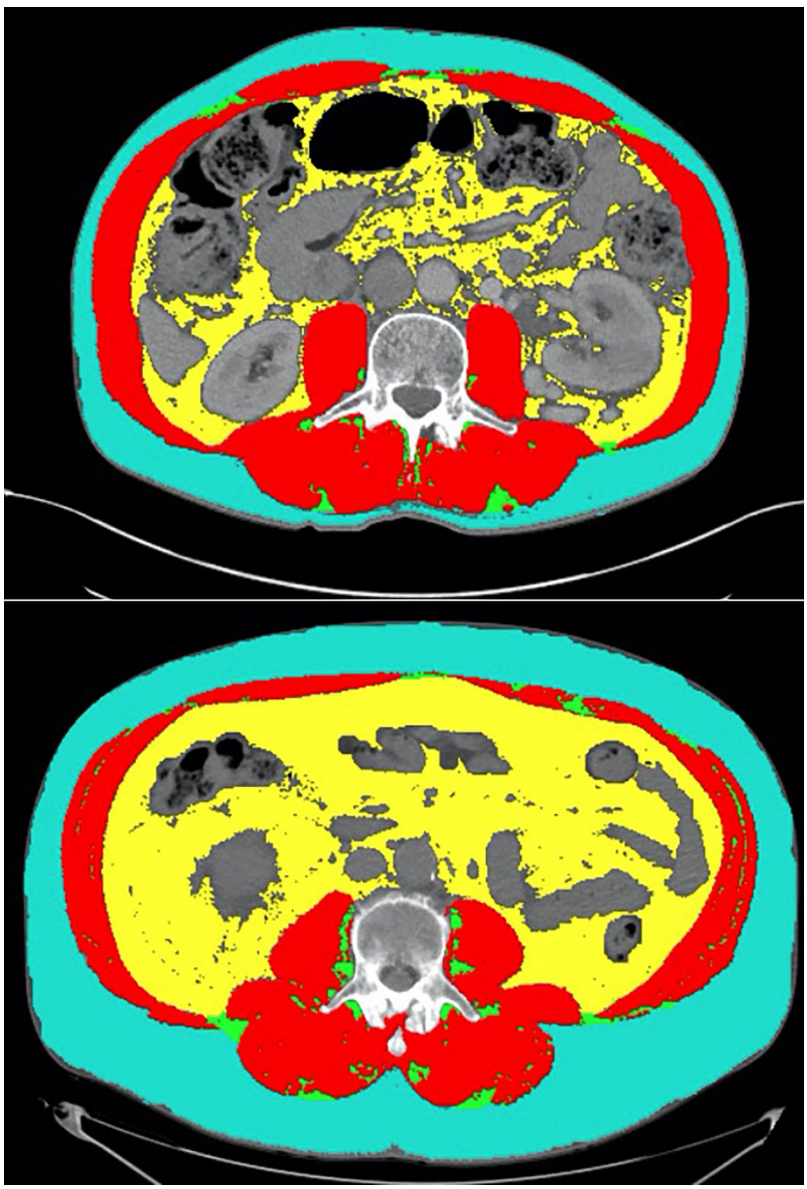

FIGURE 4 | Representative CT segmentation results from two patients with similar SMI but disparate attenuated SM mean. The first patient (top panel) had an SMI of 52.48 and an attenuated SM mean of 41.45 (high). He had a partial response on treatment with anti-PD-1 monotherapy and had remained progression free for over 9 months at the time of last follow-up. The second patient (bottom panel) had a SMI of 59.38 and an attenuated SM mean of 24.97 (low). This patient had progressive disease as his best radiographic response to anti-PD-1 monotherapy.

is inversely associated with intramuscular lipid deposition (25). Hence, we found that increased myosteatosis was associated with poor outcomes. This adds to data from a recent study which found that high skeletal muscle gauge (SMI x attenuated SM mean) was modestly associated with improved outcomes in melanoma patients treated with ICI combination therapy with nivolumab and ipilimumab (26). This is also consistent with our findings in a separate analysis that increased attenuated SM mean was significantly associated with improved outcomes in ICI-treated urothelial carcinoma patients (27). This provides further support for attenuated SM mean as an adjunctive biomarker to SMI in quantifying risk associated with sarcopenia and myosteatosis in ICI-treated patients with malignancies of the GU tract. The clinical utility of myosteatosis as a prognostic marker is highlighted by comparing two patients treated with anti-PD-1 monotherapy who had similar baseline SMI, but disparate attenuated SM mean and clinical outcomes (**Figure 4**). One of the patients (top panel) had an SMI of 52.48, but had an attenuated SM mean of 41.45, which is above the optimal cut in our analysis. He had a PR on treatment with anti-PD-1 monotherapy and had remained progression-free for over 9 months at the time of last follow-up. The second patient had a SMI of 59.38 and an attenuated SM mean of 24.97, which is below the optimal cut. This patient had progressive disease as his best radiographic response to anti-PD-1 monotherapy. This is an example of how attenuated SM mean can be used as an adjunct biomarker in analyzing the quality of skeletal muscle on CT imaging.

There are limitations to this study that should be noted. First, this is a heterogenous population of mRCC patients which included all patients who received at least one dose of ICI regardless of RCC subtype or line of therapy that ICI was received. We attempted to diminish the effect of these variables in our analyses by controlling for several baseline disease characteristics in MVA. Additionally, this is a relatively small sample size and the results presented in this study should be validated in a larger study. This is also a retrospective analysis which is vulnerable to selection bias, although we included all patients with available CT imaging and receipt of at least 1 dose of ICI with adequate clinical data availability. Future studies may explore the relationship between radiographic measures of body composition and metabolomic data in mRCC patients treated with ICI. Additionally, investigation of the predictive and prognostic value of our body composition risk score in patients treated with targeted therapy may provide insight into whether our system is specific for ICI-treated patients.

CONCLUSIONS

Risk stratification using the body composition variables IFI, SM mean, SFI, and TFI may be prognostic and predictive of clinical outcomes in mRCC patients treated with ICI. These variables may be considered in updated prognostic models for ICI-treated mRCC patients. Larger, prospective studies are warranted to validate this hypothesis-generating data.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Emory University IRB. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

DM, SG, YL, and MB were involved in the study design and concept, statistical analysis, and drafting the manuscript. DM, SC, and MB were involved in the identification and selection of patients. DM collected and analyzed all CT images. DM, AO, SE, and BM were involved in data collection and quality control. All other authors were involved in the care of the patients included in this study. All authors were involved in the review and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.707050/full#supplementary-material

Supplementary Table 1 | Univariable analysis of muscle and adipose variables and clinical outcomes. *Patients dichotomized as high *vs.* low at optimal cut. **Statistically significant at $p < 0.05$. SMI, skeletal muscle index; IFI, inter-muscular fat index; SM, skeletal muscle; SFI, subcutaneous fat index; VFI, visceral fat index; BMI, body mass index.

Supplementary Figure 1 | Receiver operating characteristic (ROC) curve comparison between body composition risk groups (Top panel) and TFI (Bottom panel) with IMDC risk group and BMI.

Supplementary Figure 2 | Kaplan-meier curves for high *vs.* low total fat index (TFI) for overall survival (OS, top panel) and progression-free survival (PFS, bottom panel).

Supplementary Figure 3 | Scatter plots of intermuscular fat (IFI) *versus* subcutaneous fat index (SFI, top panel) and total fat index (TFI, bottom panel).

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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.

frontiers in Oncology

CASE REPORT
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Metastatic Urachal Carcinoma Treated With Several Different Combined Regimens: A Case Report

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Urachal carcinoma is a rare bladder malignance. This study presents a case of an elderly patient with urachal carcinoma who was found to have pulmonary metastases 1 year after 5 recurrent resections. The patient was treated with up to 7 different chemotherapy regimens, including a VEGF monoclonal antibody and anti-PD-1 antibody. This is the first report of PD-1 antibody being used in patients with urachus, although the disease progressed after only four cycles of the application. The patient's disease was controlled by the FOLFIRI combined with the VEGF monoclonal antibody regimen. The most prominent issues at present are the difficulty of obtaining drugs for rare cancers and the lack of late-stage clinical trials to guide therapeutic decisions.

Keywords: urachal carcinoma, serum marker, immunity therapy, targeted therapy, combination chemotherapy

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INTRODUCTION

The first case of urachal carcinoma was reported by Begg in 1973 (1, 2), and subsequent reports are based on single case descriptions and institutional experiences. The urachus, a fibrous remnant of the allantois, usually regresses during fetal life, but its lumen stays in place in approximately one-third of adults. Urachal carcinoma accounts for 0.35%–0.7% of all bladder tumors and presents at an advanced stage and poor prognosis. For different types of pathology, there are different surgical approaches, among which, immunotherapy and targeted therapies for urachal carcinoma are still being explored.

CASE PRESENTATION

Patient: a 62-year-old male presented with a history of gross hematuria for 1 month. The patient immediately underwent a transurethral biopsy of the urachal tumor, which revealed a 3.0×2.0×3.0 cm adenocarcinoma and some mucinous adenocarcinoma (**Figure 1**). He requested to preserve the bladder to ensure the quality of life. When mucinous adenocarcinoma was again found in the bladder 3 months later, he began to receive systemic treatment with a standard bladder carcinoma regimen consisting of gemcitabine and cisplatin, which lasted for 4 cycles and followed by a 2 transurethral tumour electrosurgery within 3 years. He finally underwent a radical cystectomy 3 years after the onset of the disease. Pathology showed a 6.0×5.3 cm adenocarcinoma with positive immunohistochemistry for AE1/AE3, CK7, CK20, and CK19. Four weeks after surgery with paclitaxel and cisplatin, the patient began to receive chemotherapy, which was repeated every

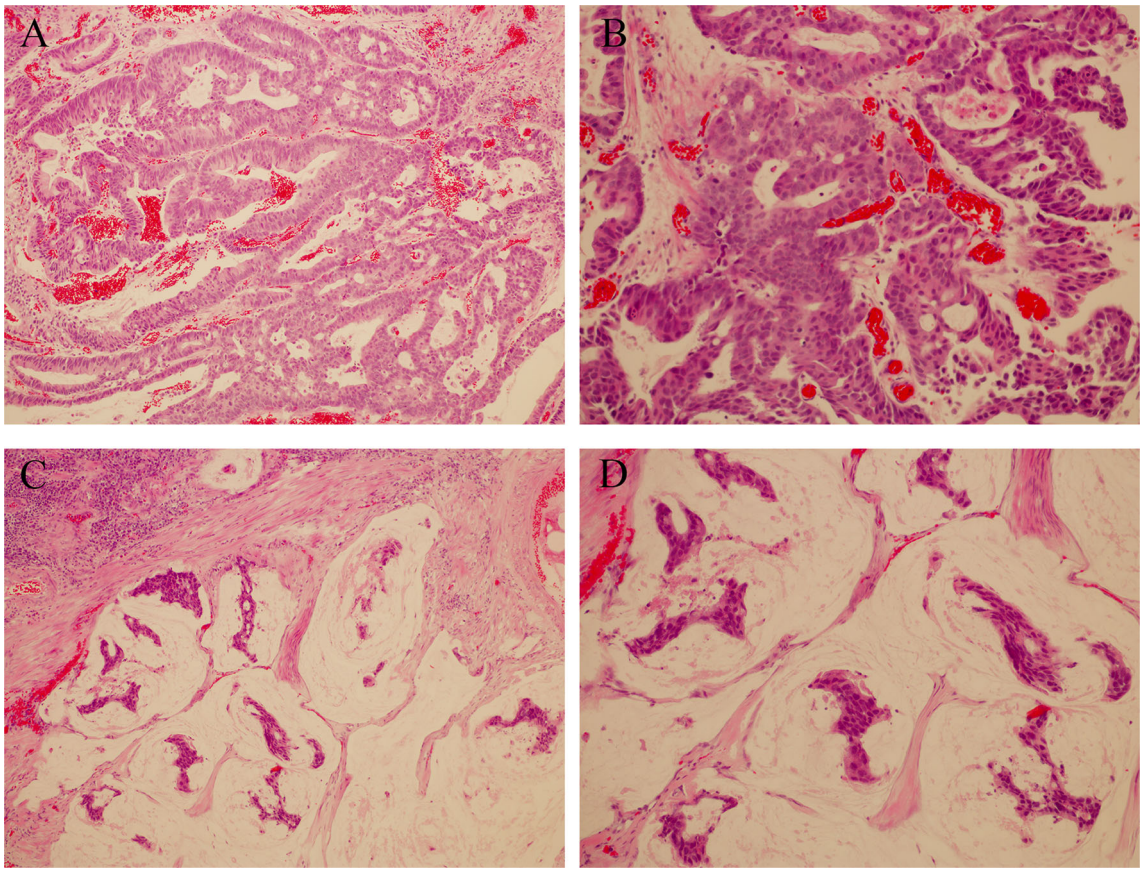

FIGURE 1 | **(A, B)** Hematoxylin and eosin staining; magnification ×100 and ×200, Duct cells are observed in the loose stroma. **(C, D)** Hematoxylin and eosin staining; magnification ×100 and ×200, Urachal mucinous adenocarcinoma, groups of tumour cells surrounded by extracellular mucin.

3 weeks for 4 cycles. CT scans that performed after a treatment of 8 months showed progressive pulmonary metastases. The patient started to receive treatment with gemcitabine and nedaplatin in combination with Endostar. A CT scan performed after a treatment of 3 cycles (3 months) showed no significant reduction in the lesion, so anti-PD-1 antibody was added. This regimen was well-tolerated, and CT scans done after 3 cycles showed stable disease. However, scans after 5 cycles showed significant clinical and radiological progression, after which, he was discontinued from the anti-PD-1 antibody and switched to bevacizumab, irinotecan, 5-fluorouracil (5-FU), and folinic acid, which are delivered weekly for 3 weeks (**Table 1**). After 6 cycles, a 50% reduction in the size of the metastatic lung lesions was observed by CT and the CEA decreased from 588.1 to 205.7 ng/ml (**Figure 2**).

TABLE 1 | Details about the treatments (schedule and dosage).

Time	Programme		CEA ng/ml
2015.11	DDP 40 mg, GEM 1.8 g	4 cycle	19.2
2019.03	DDP 30 mg, Taxol 400 mg	4 cycle	17.3
2020.04	NDP 120 mg, GEM 1800 mg, YH-16 30 mg	3 cycle	307.9
2020.07	NDP 120 mg, GEM 1800 mg, Tislelizumab 200 mg	3 cycle	588.1
2020/10	Irinotecan 280 mg, CF 750 mg, 5-FU 4.9 g Bevacizumab 500 mg	6 cycle	205.7

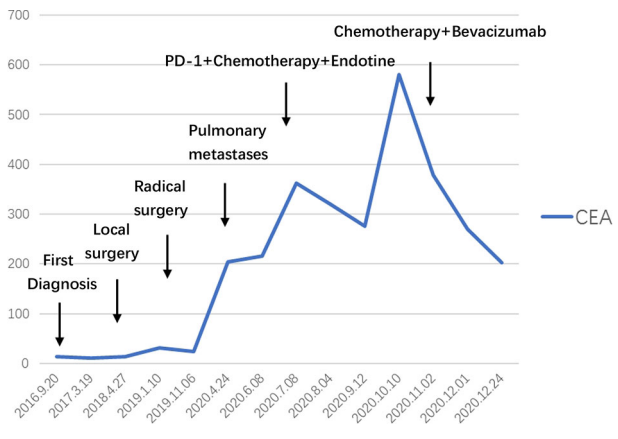

FIGURE 2 | Timeline of the Serum marker.

DISCUSSION

Urachal carcinoma shares certain histological and biological characteristics with intestinal tumour, and there are no clinical randomised trials available for its treatement due to its rarity. Early symptoms of both urachal and bladder carcinoma are gross hematuria visible to the naked eye, so they are often confused. However, patients with urachal carcinoma often have mucus-like substance in their urine, which is consistent with its type of pathology (3). In 2006, Asley et al. (4) proposed the Mayo staging, which is simpler and clearer than any previous staging. Initially, the overall prognosis for urachal carcinoma was low, with a 5-year survival rate of only 6% (5), but the rate has been improving with the use of new drugs. As with other intestinal malignancies, the persistent elevation of tumour markers predicts disease progression, with CEA, CA125, and CA19-9 markers being the most closely associated with the disease. Siefker-Radtke (6) found elevated serum CEA levels in 59% of patients with urachal carcinoma. Zong (7) noted that the elevated CEA and CA125 are suggestive of the recurrence of urachal carcinoma. In our case, we observed that serum CEA significantly increased after pulmonary metastases. After treatment, the decrease in marker levels may be correlated with the response to systemic therapy.

Surgery is the main form of treatment for urachal carcinoma. En bloc surgical removal of the umbilicus, urachal ligament, and partial cystectomy with pelvic lymphadenectomy are the preferred interventions (8). However, unlike other carcinomas, no standard

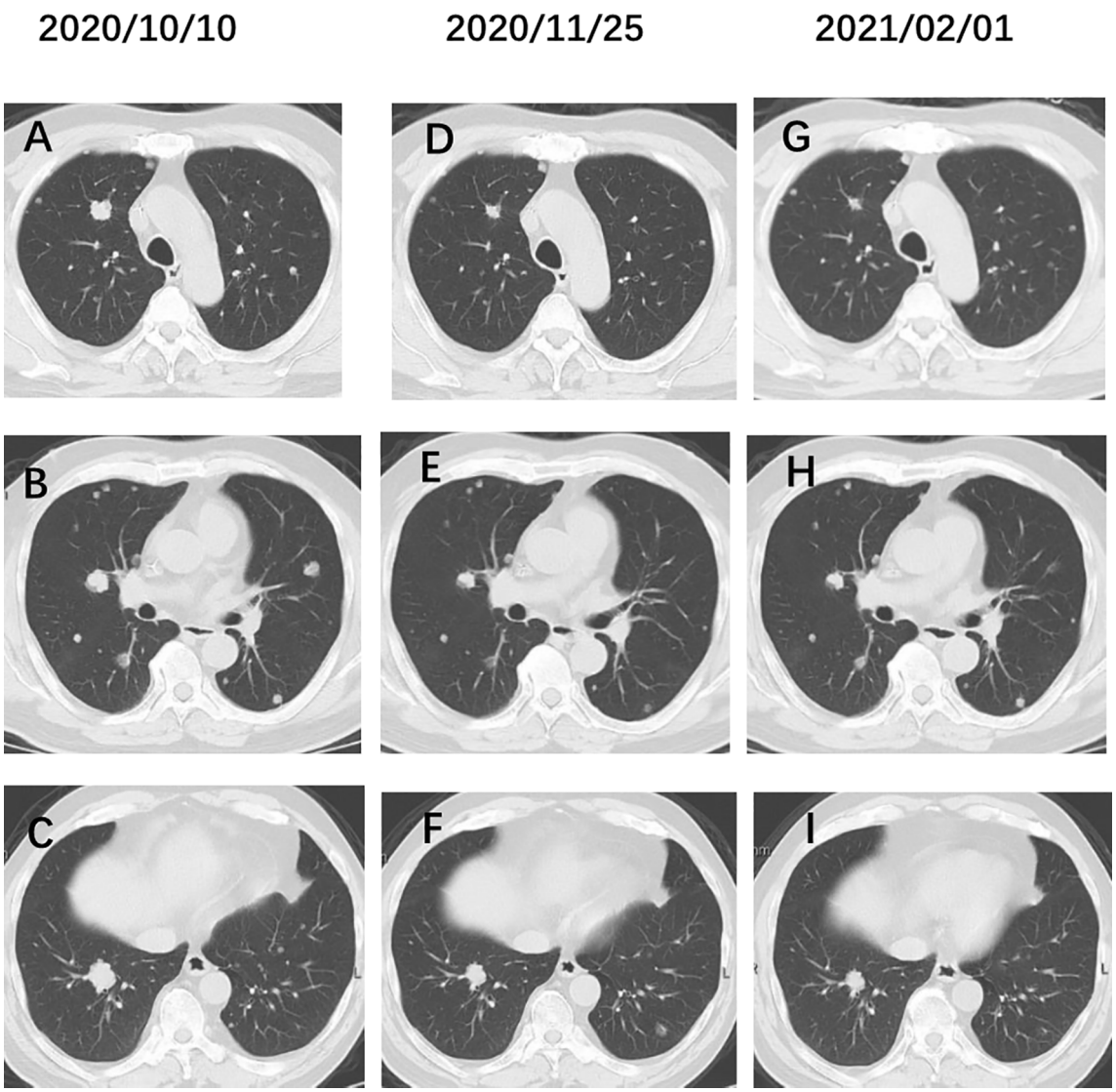

FIGURE 3 | **(A–C)** Chest CT before administration of Irinotecan. **(D–F)** Chest CT after 3 cycles Irinotecan and bevacizumab therapy. **(G–I)** After 6 cycles Irinotecan and bevacizumab therapy. The pulmonary metastatic lesions were reduced by 50%.

adjuvant chemotherapy regimen is yet available for treatment. In this reported case, the patient underwent five successive surgeries, and he was not immune to distant metastases despite the use of postoperative adjuvant chemotherapy. The choice of regimens was based largely on case reports and single institution experiences, and we have seen better response rates with 5-FU based regimens. Szarvas et al. (9) found that two therapies combined (cisplatin-based and 5-FU-based) had the best response rate (43%) than being applied alone. Tazi E and Mohile SG each described patients' response to chemotherapy with irinotecan, a commonly used regimen for colorectal carcinoma (10, 11). In our case, the patient's markers continued to decline and the metastatic lesions shrank after the use of the FOLFIRI regimen.

In addition to the use of drugs targeting cytotoxicity, we used a combination of VEGF monoclonal antibody (bevacizumab) based on genetic testing that identified KRAS mutations in the patient (p.G12V), and achieved a favorable result of 50% reduction after 6 cycles Irinotecan and Bevacizumab therapy in metastatic lesions (**Figure 3**). Lee et al. (12) identified somatic SnVs/indels and SCANs in 17 patients by using the WES and OncoScan platforms. KRAS, MYC, and growth factors appear to be involved in pathogenesis, suggesting potential roles in targeted treatment of urachal cancer. Our case also confirms this result. KRAS mutations and microsatellite instability appear to be common in urachal carcinoma, and the presence of KRAS mutations is associated with better overall survival (OS) (13, 14). In intestinal tumours, we observed that patients with microsatellite instability do not benefit from 5-FU therapy, and similarly, it should be used with caution in patients with urachal carcinoma. Testa et al. (15) reported tumour regression in a patient with metastatic urachal carcinoma, who was treated with a second-line multikinase inhibitor (sunitinib) after failure of platinum-containing combination chemotherapy. Therefore, we suggest that mutation analysis targeting members of the EGFR pathway in patients with urachal carcinoma may provide additional therapeutic information.

Jordan Kardos' study was the first overall transcriptome profiling of urachal carcinoma, demonstrating the similarity of this cancer to colorectal carcinoma (16). They reported tumors with mutations in DNA MMR proteins and POLE are rare, and described the successful treatment of a patient by using the anti-PD-L1 antibody atezolizumab. We treated the patient with an anti-PD-1 antibody, although it progressed after only 4 cycles, which is probably due to its low TMB, as mutational load correlates robustly with predicted neoantigen burden (17). The case reported by Sahu et al. was also treated with immunosuppressants, but the treatment was discontinued after one cycle due to immune pneumonia (18). By reviewing 76 articles (19), Claps concluded that personalized treatment could be the most suitable option for urachal carcinoma. Immunotherapy may have potential utility for urachal carcinoma, but clinical studies are still needed.

CONCLUSION

Urachal carcinoma is highly similar to colorectal carcinoma, and the FOLFOX and FOLFIRI regimens for colorectal carcinoma are the most effective chemotherapy regimens for its treatment. We recommend performing mutational analysis for members of the EGFR pathway, as EGFR inhibitors are of interest for the treatment of urachal carcinoma. We look forward to the development of prospective clinical studies to further improve the diagnosis and treatment of ureteral carcinoma.

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.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HZhe and WS performed the research, wrote the paper. XF, HZhe, and WS were performed therapy to a patient. HZha (Correspondence) contributed to supervision of this study and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Prostate Cancer Progression: as a Matter of Fats

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Advanced prostate cancer (PCa) represents the fifth cause of cancer death worldwide. Although survival has improved with second-generation androgen signaling and Parp inhibitors, the benefits are not long-lasting, and new therapeutic approaches are sorely needed. Lipids and their metabolism have recently reached the spotlight with accumulating evidence for their role as promoters of PCa development, progression, and metastasis. As a result, interest in targeting enzymes/transporters involved in lipid metabolism is rapidly growing. Moreover, the use of lipogenic signatures to predict prognosis and resistance to therapy has been recently explored with promising results. Despite the well-known association between obesity with PCa lethality, the underlying mechanistic role of diet/obesity-derived metabolites has only lately been unveiled. Furthermore, the role of lipids as energy source, building blocks, and signaling molecules in cancer cells has now been revisited and expanded in the context of the tumor microenvironment (TME), which is heavily influenced by the external environment and nutrient availability. Here, we describe how lipids, their enzymes, transporters, and modulators can promote PCa development and progression, and we emphasize the role of lipids in shaping TME. In a therapeutic perspective, we describe the ongoing efforts in targeting lipogenic hubs. Finally, we highlight studies supporting dietary modulation in the adjuvant setting with the purpose of achieving greater efficacy of the standard of care and of synthetic lethality. PCa progression is "a matter of fats", and the more we understand about the role of lipids as key players in this process, the better we can develop approaches to counteract their tumor promoter activity while preserving their beneficial properties.

Keywords: lipid metabolism, fatty acids, prostate cancer, castration resistance, obesity, microenvironment, lipidomics

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INTRODUCTION

Prostate cancer (PCa) is the second leading cause of cancer death in men in the US and the fifth cause worldwide (1). While primary PCa is successfully treated with surgery, about 30% of PCa cases recur. Androgen deprivation therapy (ADT) is the standard of care for androgen-sensitive metastatic PCas (mASPC). mASPC are initially responsive to ADT but will eventually develop

resistance, a disease stage known as metastatic castration-resistant PCa (mCRPC) (2). Management of mCRPC was primarily based on taxanes (i.e., Docetaxel, Cabazitaxel). However, in the last decade, second-generation androgen-receptor (AR) signaling inhibitors (i.e., enzalutamide) and intra-tumor androgen synthesis inhibitors (i.e., abiraterone) have been approved for the treatment of mCRPC with improved survival benefits. Unfortunately, efficacy is not long-lasting due to the occurrence of several mechanisms of resistance, including the overexpression of AR splicing constitutive active variants (i.e., AR-V7) (3). Therapeutic strategies based on the radioactive isotope radium-223 or cell-based immunotherapy (Sipuleucel-T) are also not resolutive (2, 4). Treatments with PARP inhibitors have been recently approved by the U.S. Food and Drug Administration (FDA) for the treatment of mCRPC patients harboring tumors with defects in DNA damage response (DDR), especially in the gene BRAC2, opening a new area for precision oncology in advanced PCa (5). This is also supported by the recent approval of two genetic tests, BRACAnalysis CDx and FoundationOne CDx to identifying mCRPC who have DDR genetic alterations and thus will most likely respond to PARP inhibitors (i.e., Olaparib and Rucaparib). (https://bit.ly/2z5Lu5C; https://bwnews.pr/2ZtfCSS). Clinical trials testing immune check-point inhibitors (ICI) are ongoing in mCRPC patients with disappointing results so far (6) Thus, strategies to boost responses to ICI are currently sought. Alterations of lipid metabolism in PCa cells were first observed long time ago using radiolabeling approaches and linked to AR signaling modulation (7, 8). However, the last decades have faced a change in the perspective of lipid role in cancer development and progression. In addition of being building blocks for membrane synthesis and energy fuel, lipids have emerged as key players in mediating oncogenic signaling, endoplasmic reticulum (ER) and oxidative stresses, non-apoptotic cell death (i.e., ferroptosis), and inflammatory stimuli (9). More recently, a lot of attention has been paid on the impact of lipids on the tumor microenvironment (TME), in particular on the immune TME (10). The plasticity of lipid metabolism rewiring allows PCa cells to thrive in hostile and nutrient-deprived environments and to spread to distant tissues. As a result, new mechanisms of therapy resistance and disease progression associated with lipid metabolism rewiring have recently emerged. This has also been supported by the recent advances in analytical techniques including high-resolution mass spectrometry (MS)-based lipidomics and MS-based imaging (MSI) that allow to measure hundreds of lipid species at once, Including rare lipid species, and to provide spatial resolution. These new technologies have uncovered the complexity and heterogeneity of lipid metabolism rewiring in a way that could not have been assessed before, opening new possibilities for both biomarkers and therapeutics discovery (9). Indeed, the combination of lipid metabolism modulators with standard of care is now strongly pursued.

In this review, we describe how lipid metabolism rewiring, including alterations in both *de novo* lipid synthesis, uptake, transport, storage, and utilization, contributes to PCa progression and therapy resistance and we discuss how these vulnerabilities can be exploited therapeutically. We emphasize the recently uncovered role of lipid metabolism in immune TME and the potential impact of *de novo* lipogenesis inhibitors as immunotherapy sensitizers. This review also briefly describes the advances in measuring and imaging lipids and how these more sophisticated analytical techniques contribute to improve biomarker discovery.

This review highlights that PCa progression is "a matter of fats" and lipids are rediscovered protagonists of oncogenic signaling, stress adaptation, and tumor-TME crosstalk. The more we understand about these aspects the better we can develop strategies to counteract their tumor supportive functions while enhancing their health-promoting roles.

LIPID METABOLISM REWIRING IN PCa DEVELOPMENT AND PROGRESSION

Since the discovery in the mid-1990s of OA-519, an oncogenic antigen encoding for Fatty acid (FA) Synthase (FASN) highly expressed in breast cancer, research on the role of lipid metabolism in cancer has proceeded quite slowly with respect to other fields (11). However, interest for the mechanisms through which lipids promote tumorigenesis and tumor progression has been regained in the last decade, paralleled by the rapid development of high-resolution analytical techniques to interrogate the lipidome in a comprehensive and unbiased manner. While the majority of attention has been focused on the *de novo* FA synthesis dysregulation as hallmark of PCa development and progression, the perspective has lately changed to include many aspects of lipid metabolism spanning from FA uptake and transport, FA oxidation, lipid storage, and remodeling.

Alterations in *De Novo* FA Synthesis

While non-transformed prostate cells obtain the majority of lipids for membrane synthesis and energy fuel from the diet and circulation, PCa cells show an increased in *de novo* FA synthesis from glucose or glutamine, despite circulating lipids [reviewed in (12)]. This results in increased production of phospholipids and sphingolipids to support new membrane synthesis in proliferating PCa cells but also in a net accumulation of intra-tumor lipids mostly as triglycerides stored lipid droplets (LD) (13) (**Figure 1**). LD accumulation, which is associated with a more aggressive disease, provides an excellent reservoir for building blocks and energy in conditions of nutrients deprivation such as those encountered during PCa progression and metastatic spread. Moreover, LDs prevent lipotoxicity due to excessive accumulation of free FAs (see below). This increased net lipid production, known as "lipogenic phenotype", is observed at early stages of PCa development and it is further enhanced in mCRPC. Consistently, enzymes or transcriptional factors (TFs) involved in *de novo* FA synthesis such as the TF sterol regulatory element-binding proteins (SREBPs), ATP citrate lyase (ACLY), Acetyl-CoA carboxylase (ACC), and FASN are overexpressed in

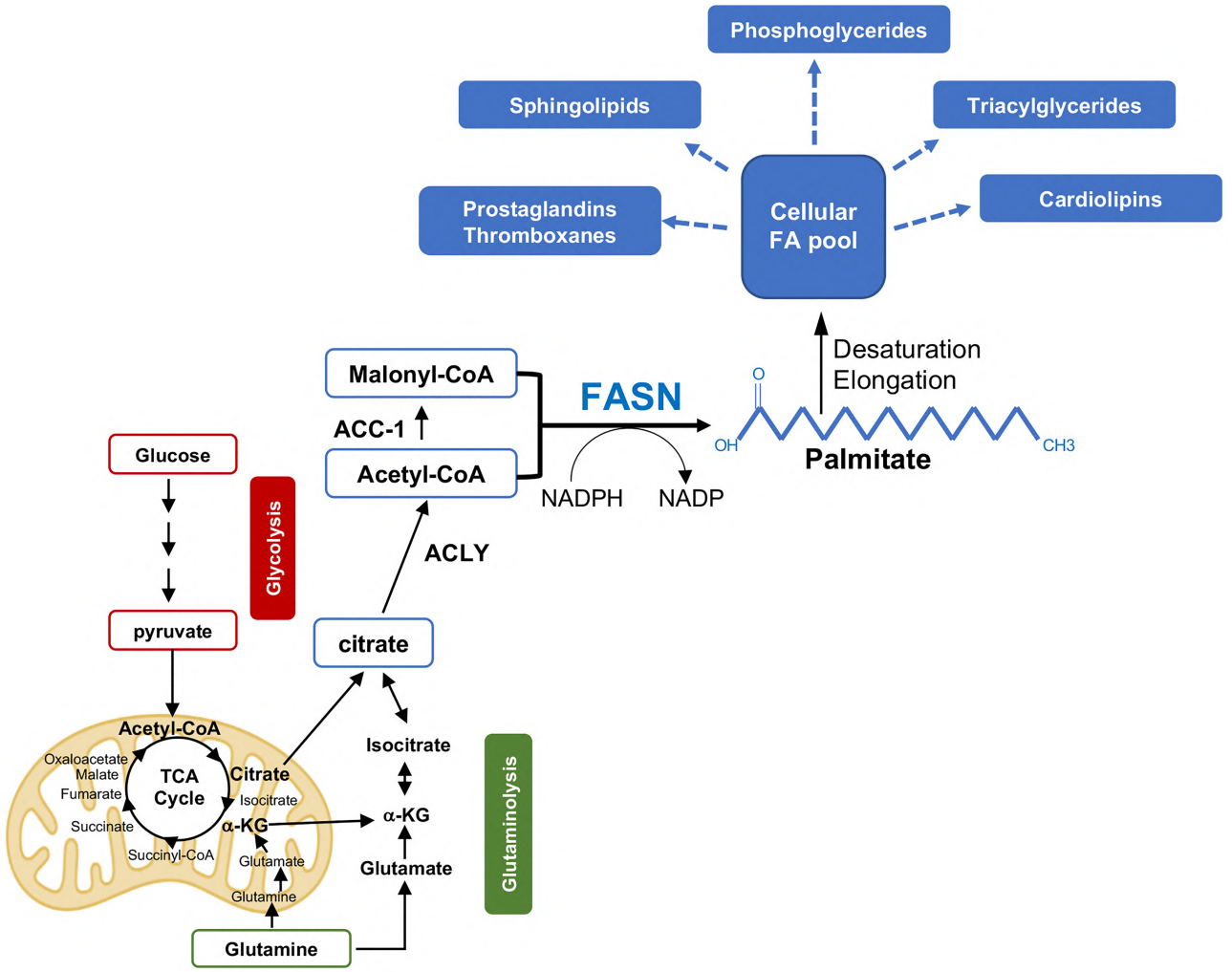

FIGURE 1 | *De novo* FA synthesis in PCa progression. Acetyl-CoA derived from glucose and glutamine metabolism is exported to the cytosol in the form of citrate and reconverted to acetyl-CoA by the enzyme ATP citrate lyase (ACLY). Acetyl-CoA is then converted to Malonyl-CoA by the Acetyl coenzyme A carboxylase a (or ACC-1). Fatty acid synthase (FASN) catalyzes the condensation of Acetyl-CoA and Malonyl-CoA (in the presence of the reducing equivalent icotinamide adenine dinucleotide phosphate (NADPH)) to generate the 16-carbon FA palmitate, a saturated FA that undergoes further modifications (i.e., elongation/desaturation) to form more complex lipids. The latter serve as energy source, building blocks, and inflammatory/immune modulators to sustain PCa progression and CR. αKG, alpha-ketoglutarate; NADPH, Nicotinamide adenine dinucleotide phosphate.

primary PCa and especially in mCRPC. Specifically, FASN, the key lipogenic enzyme responsible for the synthesis of the 16C saturated FA palmitate from acetyl-CoA and malonyl-CoA, was found among the top ten genes overexpressed in AR-V7-driven CRPC metastases (mets) (14–16). In line with this, the interrogation of the Cancer Genome Atlas and other publicly available datasets uncovered a positive association between FASN expression and worse clinico-pathological features, including Gleason grade, tumor stage, lymph node positivity, shorter time to recurrence, cancer-free survival, and overall survival [reviewed in (17)]. As a result, great efforts are directed to exploit the lipogenic phenotype in mCRPC (see below). The work of Swinnen and coworkers has been instrumental to demonstrate the tight control of *de novo* FA synthesis by androgens/AR signaling, the major driver of PCa development and progression to mCRPC (7, 8). A feedforward mechanism between SREBP and AR was initially described, whereby AR promotes SREBP activation and nuclear translocation while SREBP regulates AR promoter activity and expression (18, 19). Later on, Chan and coworkers identified AR-binding site in FASN gene promoter, suggesting AR-mediated direct regulation of FASN expression (20). This evidence has been supported by immunoprecipitation sequencing (ChIP-seq) experiments that revealed AR binding sites in several lipogenic enzymes in CRPC samples, besides FASN (21). Altogether these data suggest that both indirect and direct mechanisms of AR-mediated control of the lipogenic program exist. Cai and coworkers analyzed AR cistrome and demonstrated that

activation of lipid biosynthesis is a major function of AR signaling during PCa progression. Specifically, increased expression of AR-V7 turned out to be crucial for the reactivation of the lipid synthesis in CRPC, suggesting a key role of this splicing variant in regulating lipid metabolism in the CRPC setting. The authors also identified an AR-dependent lipogenic gene expression signature that predicts poor patient outcome (16). Our recent study has uncovered the existence of a reciprocal modulation between FASN and AR, in particular AR-V7, and it has proposed FASN inhibition as a non-canonical approach to indirectly antagonize AR-V7 and potentially overcome therapy resistance to enzalutamide and abiraterone (22). Overexpression of ACLY, ACC, and FASN has been consistently associated with increased PCa cell proliferation, tumor growth, migration and invasion, activation of oncogenic signaling, protection from chemotherapeutics-induced apoptosis, features that are reversed using genetic/pharmacological inhibition of enzyme activities [reviewed in (12)]. However, new roles for *de novo* FA synthesis in PCa progression have recently emerged. These involve post-translational modifications, DNA damage response, redox maintenance, ER and oxidative stress and resistance to ferroptosis, a lipid peroxidation-mediated non-apoptotic form of cell death [reviewed in (9)]. Palmitoylation of Wnt-1, RAS-related protein Rab-7a, alpha-tubulin, and eIF3L initiation factor are some of the post-translational modifications mediated by FASN and regulated by AR that activate oncogenic signaling in PCa (23–25). More recently, a palmitoyl-protein signature has been described in PCa derived extracellular vesicles (EVs), membrane-enclosed particles that play an important role in cancer progression as source of nutrients, signaling molecules, immune modulators, and circulating biomarkers, uncovering another potential mechanism of support to PCa progression (26).

In 2016, Wu and coworkers demonstrated the involvement of FASN in DNA repair and resistance to genotoxic insults. The authors found that FASN up-regulation regulates PARP-1 expression through NF-κB and SP1 modulation and increases Ku protein recruitment and DNA repair through activation of non-homologous end joining (27). Evidence for a direct interaction of FASN with MRN (MRE11-RAD50-NBS) complex has also been reported (28). By consuming NADPH, high rates of *de novo* FA synthesis also maintain redox balance and increased NADP/NADPH ratio, which is needed to support oxidative reactions such those in the pentose phosphate pathway for nucleotide synthesis (29). FASN expression/activity is also crucial in counteracting ER and oxidative stress in PCa by promoting saturated FA acids (SFA) synthesis and the remodeling of ER and mitochondrial membranes (22, 30–32). Furthermore, the increased production of SFAs and their acylation in phospholipids give rise to membranes characterized by a high ratio of SFA and polyunsaturated FAs (SFA/PUFA). These changes affect membrane fluidity, microdomains formation (i.e., lipid rafts), and lipid peroxidation (33, 34). SFA-enriched membranes affect the uptake of certain chemotherapeutics such as doxorubicin and promote the resistance to ionizing radiation (35, 36). Since SFAs are more resistant to lipid peroxidation, metastatic PCa cells with SFA-enriched membranes would be most likely less susceptible to oxidative stress-induced ferroptosis, an iron-dependent form of cell death induced by reactive oxygen species (ROS)-mediated lipid peroxidation (37). Targeting *de novo* lipogenesis and the Lands cycle has recently been shown to induce ferroptosis in KRAS-mutant lung cancer (38) and we anticipate similar results in mCRPC. These new findings have opened new possibilities for combinatorial treatments.

Alterations in FA Modelling

Once palmitate (16:0) and stearate (a 2C-elongated FA, 18:0), the most abundant SFAs, are synthesized or acquired from the diet (**Figures 1**, **2**) they usually undergo further modifications including desaturation and elongation. Desaturation of *de novo* synthesized SFAs involves the introduction of a cis-double bond to the acyl chain at the delta-9 (Δ9) position by stearoyl-CoA desaturases (SCDs) to generate the monounsaturated FAs (MUFA) palmitoleate and oleate (39). As humans lack delta-12 (Δ12) and delta-15 (Δ15) desaturases, PUFAs need to be acquired from the diet. Hence, α-linoleic acid (LA, an omega-6 PUFA) and α-linolenic acid (ALA, an omega-3 FA) are essential diet-derived PUFAs, which are required for the generation of further desaturated PUFAs (i.e., arachidonic acid), eicosanoids (i.e., prostaglandins and thromboxanes), and lipoxins, all of which play crucial roles as signaling molecules and mediators of PCa progression (40). LA and ALA desaturation is primarily catalyzed by the FA desaturases FADS1-3. Two human isoforms of SCD exist, SCD1 and SCD5 (41, 42). SCD1, the most abundant SCD in human cells, is highly expressed in human PCa with respect to normal tissues (43). Consistently, PCa cells upregulate *de novo* FA synthesis to generate SFA and MUFA-rich phospholipids that partition into detergent-resistant lipid rafts to markedly alter signal transduction, vesicular trafficking, and cell migration (44, 45). SCD1 pharmacological inhibition with BZ36 was shown to repress proliferation of AS LNCaP and CRPC C4-2 cells *in vitro* and *in vivo* through the abrogation of phosphatidylinositol generation and consequent inhibition of AKT pathway (46). Inhibition of SCD1 was also shown to activate 5' AMP-activated protein kinase (AMPK) and glycogen synthase kinase-3 (GSK3β), resulting in decreased β-catenin transcriptional activity (46).

SCD1 silencing also results in changes in the composition of cardiolipins, the major constituents of mitochondria membranes. As a result, alterations of mitochondria membrane favor the release of cytochrome c and the induction of apoptosis (43). Thus, overexpression of SCD-1 may represent a protective mechanism to apoptosis that PCa cells adopt, especially during stress. As an oxygen and NADPH-consuming process, desaturation occurrence is particularly challenging during cancer progression where hypoxic conditions are frequently observed. To overcome this, cancer cells tend to accumulate MUFAs in LDs, hydrolyze LDs, and assemble MUFA into PLs under hypoxic conditions. While the increase in MUFA incorporation in cellular membranes enhances their fluidity, it also reduces their PUFA/MUFA ratio, providing a

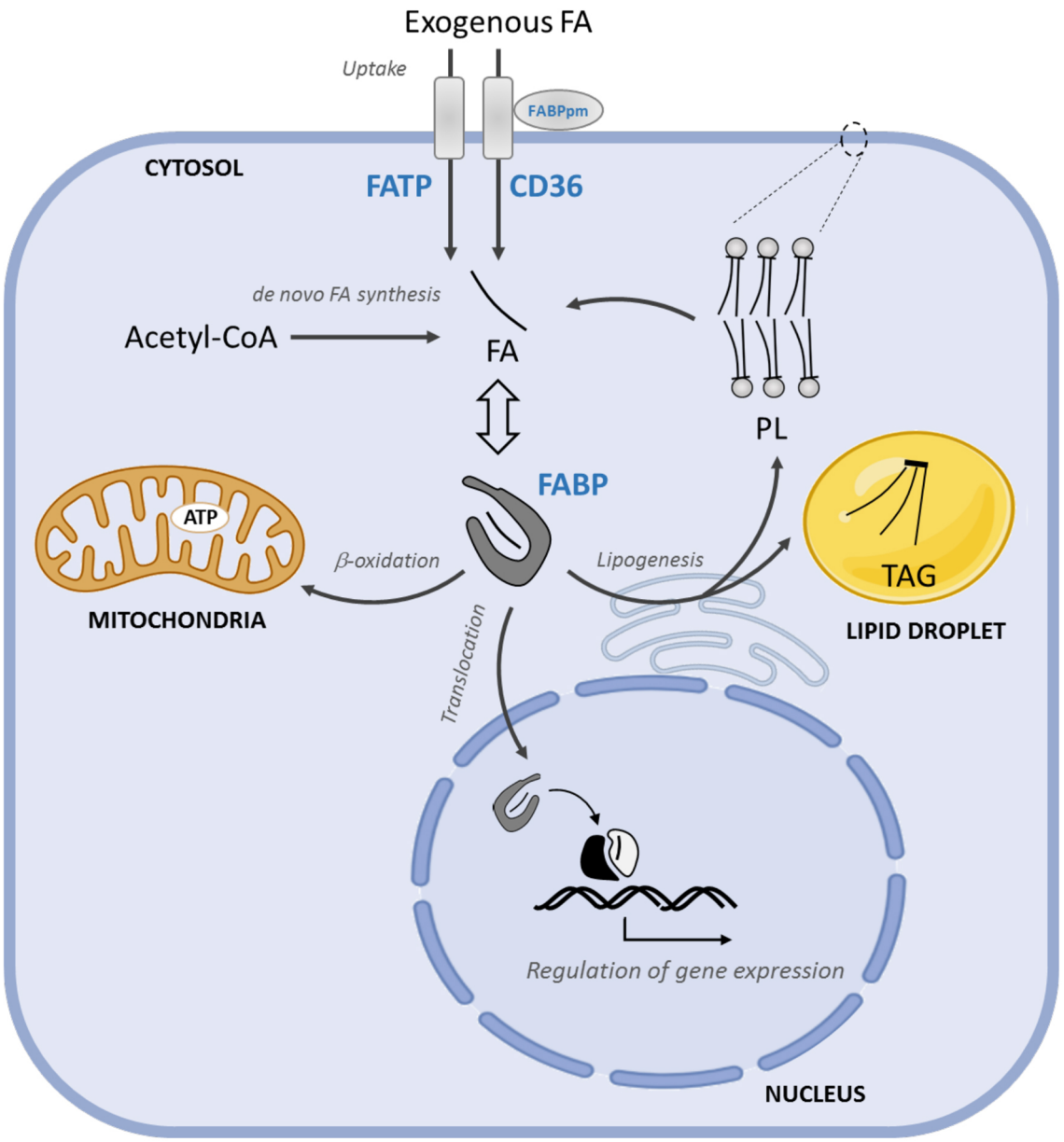

FIGURE 2 | FA uptake, intracellular transport, and FAO in PCa progression. The uptake of exogenous FA is mediated by membrane FA transporters, CD36, FATPs and FABPpm. FABPs solubilize cytosolic FAs and coordinate their intracellular transport towards FABPs solubilize cytosolic FAs towards storage (mainly TAG) or structural (principally PL). Moreover, FABPs coordinate their intracellular transport lipids to the mitochondria for energy supply and to the nucleus where FA regulate gene expression storage (mainly TAG) or structural (principally PL) lipids, to the mitochondria for energy supply and to the nucleus, where FA regulate gene expression. FATP, FA transport proteins; FABPs, FA binding proteins; TAG, triacylglycerides; PL, phospholipids.

robust protection from ferroptosis (47, 48). Thus, SCD-1 inhibitors are currently tested in the preclinical setting to induce ferroptosis (9, 48, 49).

Besides desaturation, FAs undergo elongation, a process that is catalyzed by a class of enzymes called elongases (ELOngation of Very Long fatty acids; ELOVLs), comprising seven members (ELOVL 1-7). ELOVLs add two carbon units to the carboxyl end of FA chains. While their precise functions are still not fully clarified, ELOVL-1, -3, and -6 predominantly elongate SFAs and MUFAs, ELOVL-2 and -4 elongate PUFAs, ELOVL-5 elongates MUFAs and PUFAs, and ELOVL-7 elongates SFAs and PUFAs (50). ELOVL-7 was the first elongase identified as overexpressed in human PCa tissues with respect to adjacent non-tumoral tissues (51). ELOVL-7 is induced by androgens and when overexpressed in LNCaP xenograft promotes tumor growth in mice fed high-fat diet (HFD). *In vitro* FA elongation assay and FA composition analysis showed that ELOVL-7 is preferentially involved in FA elongation of very-long-chain SFAs included in

phospholipids and neutral lipids (i.e., cholesterol ester) and when silenced it reduces androgens synthesis and CRPC tumor growth (51). Both ELOV-7 and ELOVL-5 are among the lipogenic genes overexpressed following AR reactivation and thus considered critical for the progression to CRPC (16). Consistently, Centenera and coworkers showed a significant increase in AR-regulated elongation of fatty acyl chain phospholipids, mediated by ELOVL-5, in both PCa cells and patient-derived explants. ELOVL-5 silencing markedly altered mitochondrial morphology and function, leading to enhanced ROS generation and suppression of PCa cell proliferation, 3D growth, *in vivo* tumor growth, and metastasis formation. These features were rescued by the supplementation of cis-vaccenic MUFA, a direct product of ELOVL-5 elongation. These data suggest that lipid elongation is a metastasis-promoter metabolic pathway, which is targetable *via* ELOVL-5 (52). Aside from membrane lipid elongation, ELOVL-5 has more recently been involved in the generation of eicosanoids, inflammatory lipids with potent pro-tumorigenic signaling effects (9).

Alterations in FA Uptake

The cellular uptake of free FAs, either derived from the hydrolysis of triacylglycerols (TAGs) in very low-density lipoproteins (VLDL), chylomicron or adipocytes, require their transport across the plasma membrane. The mechanisms and the identity of the proteins involved in this process are still not fully understood (53, 54). This is use, in part, to the use of bulky fluorescent, non-metabolizable FA analogs or indirect measurement of FA uptake. So far, the best characterized mediators of FA uptake are the scavenger receptor CD36, membrane-associated FA binding protein (FABPpm), and transmembrane FA transport proteins (FATPs) (**Figure 2**).

CD36 (also known as FAT, SCARB3, SR-B2, GP4 and others) is a ubiquitously expressed plasma membrane glycoprotein that binds diverse ligands, including FA, thrombospondin, oxidized low-density lipoproteins (LDL) and anionic phospholipids. CD36 is involved in FA uptake, clearance of apoptotic cells, and angiogenesis and it has been implicated in several diseases, including cancer (55–58). In skeletal muscles, CD36 has also been found in the outer mitochondrial membrane, where it might be involved in FA oxidation (FAO) under muscle contraction (i.e., exercise), although this aspect is still controversial (59, 60).

CD36 drives tumor progression in glioblastoma, melanoma, oral, and other carcinomas and it is required for stem cell self-renewal, tumor initiation, and metastatic potential in preclinical models (61, 62). CD36 is overexpressed more commonly in mets than in primary tumors and associated with poor prognosis (62–65). Furthermore, CD36 mRNA levels positively correlate with epithelial-mesenchymal transition (EMT) in several cancers, including PCa (63). In human PCa, CD36 protein was detected in both epithelial and stromal cells and equally expressed in tumor and adjacent normal regions, preventing its use as diagnostic biomarker (65). The discrepancy between the mRNA and protein findings may be ascribed to post-transcriptional mechanisms (66). Once in the cytosol, the fate of a FA largely depends on the cell metabolic status and ongoing signaling activation, resulting in FA incorporation in structural (mainly membrane PL) or storage lipids (in the form of TAG), in FA employment as second messenger or inflammatory molecule, and in FA use as fuel. The assessment of CD36 protein or mRNA levels has been used as FAO proxy in some studies. This *a priori* association is, however, often misleading, as in the case of PCa where enhanced FA uptake in human PCa and patient-derived xenografts (PDX) results in increased incorporation of FAs into complex lipids without FAO alteration. Consistently, CD36 ablation in PTEN knockout (KO) PCa mouse model failed to alter FAO (65). As expected, FA uptake impairment increases *de novo* FA synthesis as a compensatory mechanism, prompting the concomitant use of FA uptake and synthesis inhibitors. Accordingly, the combination of CD36 and FASN inhibitors significantly reduced PCa proliferation *in vivo* and in patient-derived PCa explants and it increased sensitivity to ionizing radiation, suggesting a potential synergistic effect in the clinical setting (65–68)

FABPpm is located in the outer plasma membrane leaflet and in mitochondria, displaying different function in each compartment (54). Despite the name, FABPpm is not related to the cytosolic FA binding proteins (see below). FABPpm expression is regulated by androgens in AS PCa cells, while its expression and function in CRPC cells is still largely unknown (69).

FATP1-6, also known as solute carrier family 27 (SLC27A1-6), are differentially expressed in a wide variety of tissues with different subcellular localizations. Their role as FA transporters and their function are still not fully clarified (54, 70). FATP1 is involved in FA metabolism and cancer progression (71–73). FATPs expression is highly heterogeneous in PCa tissues and cell lines and it varies across databases and detection methodologies (64, 74). The expression of FATP-6 is increased in enzalutamide-resistant LNCaP cells compared to the parental cells but no association with prognosis was observed in the clinical setting (75). Thus, further investigation on the role of FATP1-6 in PCa progression is required.

Besides those described above, other mechanisms for scavenging lipids from the extracellular *milieu* (76) may be involved. Recently, VLDL endocytosis has been described in breast cancer as a new mechanism to acquire exogenous FA, which may potentially occur also in advanced PCa (74, 77). The plasticity of cancer cells to obtain FA sources should be carefully taken into account during the planning and the design of therapeutic strategies. Dual targeting of FA uptake and synthesis holds promise for translation into the clinical setting.

Alterations in FA Transport

Once in the cytosol, free FAs bind to FA binding proteins (FABPs), which increase their solubility in the intracellular aqueous *milieu*. FABPs are small (~15kDa) proteins that bind medium and long chain FAs as well as other lipophilic molecules, including eicosanoids, bile salts, lysophospholipids, and retinoic acid [reviewed in (78, 79)]. So far, ten different FABPs have been described in humans (FABP1-9, and the less characterized FABP12) showing tissue specificity and both redundant and distinctive functions (80). Acting as lipid chaperones, FABPs

coordinate intracellular transport and lipid metabolism, and serve as sensors to signal FA supply to the nucleus (**Figure 2**).

FABP5 is the most characterized and highly expressed FABP in human PCas and cell lines, especially in CRPC cells (81–88). Different mechanisms account for FABP5 upregulation, including a positive feedback loop mediated by include proliferator-activated receptors (PPAR) PPARβ/δ (89), CpG island hypomethylation (85), and gene amplification (90). The latter is highly frequent in advanced CRPC (88). In human PCas, a positive correlation between FABP5 expression and androgen signaling responsive genes was observed. While FABP5 mRNA did not correlate with clinico-pathological features, FABP5 protein levels were significantly associated with high Gleason score and reduced patient survival (82, 84). Furthermore, FABP5 mRNA, protein, and serum levels were all increased in lymph node mets, suggesting FABP5 as a potential prognostic biomarker (91).

Consistent with a role in PCa progression, genetic or pharmacological inhibition of FABP5 decreased cell proliferation, colony-formation, invasive potential of PC3 and the more aggressive PC3-M cells. *In vivo*, tumor growth, mets formation, vascular endothelial growth factor (VEGF) expression, and microvessel density were also significantly reduced (81, 82, 92).

One of the main functions of FABPs is to escort both exogenous and *de novo* synthesized FAs towards nuclear receptors, such as the PPARs and modulate the expression of genes involved in cell survival, growth, migration, and invasion [reviewed in (93)]. A direct interaction between FABP5 and PPARβ/δ or PPARγ has been demonstrated using *in vitro* and cell-based assays (94, 95) and it accounts for some of the pro-tumoral effects of FABP5 in PCa (89, 89, 95–98). In 2016, Forootan and coworkers showed that FABP5 promotes VEGF expression and angiogenesis through FABP5-mediated FA transport to PPARγ. In CRPC, this mechanism overcomes the canonical AR-mediated regulation of VEGF/angiogenesis, suggesting FABP5/FA/PPARγ pathway as a potential therapeutic target (97). PPAR-independent FABP5-mediated regulation of gene expression has also been described in PCa (98, 99). Other FABP5-mediated oncogenic mechanisms include the activation of SREBP-1c and the hypoxia-inducible factor 1-alpha (HIF-1α), although their roles in PCa has not been explored yet (100, 101).

FABP5 is also secreted by adipocytes, and it may potentialy contribute to the tumor supportive role of periprostatic fat (102). Finally, FABP5 has been found in urinary extracellular vesicles, where it may serve as a prognostic PCa biomarker (103).

Both pro-tumorigenic and anti-tumorigenic roles have been ascribed to FABP4 according to tumor type and TME. While FABP4 acts as a tumor suppressor when ectopically expressed in DU145 PCa cells, recent studies suggest FABP4 involvement in adipose-PCa crosstalk. According to this model, FABP4 promotes FA release from adipocyte TAG to fuel mets formation while cancer cells induce changes in adipocyte metabolism to promote FA release (104, 105). Consistently, Herroon and collaborators showed that adipocyte-derived conditioned media increases FABP4 and CD36 expression in PCa and breast cancer cells, their proliferation, invasion, and LD accumulation. This was also associated with a significant increase in several cytokines, VEGF, and HIF-1α. Conversely, inhibition of FABP4 impaired adipocyte-derived conditioned media-induced invasion (106). Furthermore, HFD was shown to induce FABP4 expression in PC3 bone tumors but not in subcutaneous ones, indicating that bone marrow-derived adipocytes may promote specific metabolic alterations in PCa bone mets (106). Oncomine data and immunohistochemistry (IHC) confirmed increased expression of FABP4 mRNA and protein in bone mets, especially in areas enriched for infiltrating adipocytes (64, 106). Mechanistically, FABP4 expression is dependent on PPARγ, which in turn is activated by FA/FABP4, suggesting the existence of a feedforward mechanism that sustains high FABP4 levels in PCa cells.

Similar to FABP5, FABP4 is also secreted by adipocytes and it plays a role as adipokine. Circulating FABP4 levels correlates with obesity and some features of the metabolic syndrome in both mice and humans (107–109) and may impact PCa progression. Indeed, serum FABP4 levels were associated with high Gleason grade (110, 111). Several evidence suggest a link between FABP4 intracellular levels and PCa aggressiveness. Ectopic expression of FABP4 promotes DU145 PCa cell invasion *in vitro*, while *in vivo* FABP4 knockdown (KD) reduces tumor growth and lung mets formation (112). Haung and coworkers also uncovered FABP4-mediated tumor/TME crosstalk that sustains PCa invasive potential. According to this, not only PCa-secreted FABP4 increases PCa invasiveness by upregulating matrix metalloproteinases (MMP 2 and 9) but it also induces stromal cells to secrete interleukin-8 and -6, further promoting PCa invasiveness (110). Conversely, FABP4 inhibition was shown to decrease HFD-induced mets, adipocyte infiltration, reactive fibroblasts and serum IL-8. Altogether, these data support a critical role for FABP4 in shaping the TME and promoting PCa progression (110). Since both FABP 4 and 5 are also expressed in macrophages and endothelial cells, they may contribute to tumor-TME crosstalk through other mechanisms.

Although less characterized, other FABPs are involved in PCa onset and progression. FABP4, FABP5, FABP8, FABP9, and FABP12 loci were found in a commonly amplified region within the chromosome 8 (8q21.13), frequently observed in human PCas mets. In line with this, increased mRNA levels of FABP 4, 8, 9, and 12 were associated with increased Gleason score and PCa recurrence (90). In 2020, Liu and coworkers also demonstrated that FABP12 promotes EMT and PCa cell motility, at least in part, through a PPARγ-dependent pathway (113), while FABP9 suppression inhibits PC3 cell invasive potential in PPARγ-independent manner (86).

Altogether these data strongly support a role for FABPs in PCa progression and their potential use as therapeutic targets (9).

Alterations in FA oxidation

While the majority of reports describe AR-mediated regulation of *de novo* FA synthesis in PCa progression, evidence is

accumulating that both FA synthesis and FAO are regulated by AR signaling and contribute to castration resistance (CR) in a fine-tuned manner. For FAO to occur, FAs need to be converted to fatty acyl-CoAs by long chain Acyl-CoA Synthetases (ACSLs) and to cross the outer mitochondrial membrane. The latter is mediated by Carnitine palmitoyltransferase 1 (CPT-1), specifically the isoform CPT-1A. CPT-1A allows FA-CoAs across the mitochondrial membrane through the conversion to FA-carnitine, a rate-limiting step for FAO [reviewed in (12, 114)]. FAO is transcriptionally regulated by the PPAR family which mainly activate the expression of CPT-1 and other FAO enzymes in response to glucose deficiency, and post-translationally *via* the allosteric inhibition of CPT-1 by malonyl-CoA. The latter is mediated by the activation of AMPK, which phosphorylates and prevent ACC-2 (or ACCβ, the isoform expressed in the mitochondria) to synthesize malonyl-CoA [reviewed in (12, 114)]. Once in the mitochondria, FAs are oxidized to acetyl-CoA, which is used for energy production, generation of reducing equivalents to maintain redox homeostasis, or as substrate for new anabolic processes. During hypoxia or in response to drug treatment, cancer cells appear to favor FAO to rapidly generate ATP and NADH and promote survival. Indeed, targeting FAO with etomoxir was shown to reduce hypoxic areas in combination with radiation in metastatic PCa sphere (115).

The group led by Schlaepfer has been instrumental in uncovering the role of FAO in PCa progression to CRPC. In 2017, the authors showed that CPT1A isoform is abundant in high-grade PCa compared to benign tissues, and they demonstrated a synergistic effect in combining CPT-1A inhibitors with anti-androgen therapy. Mechanistically, the authors uncovered that CPT1A inhibition decreases AKT and inositol polyphosphate-5-phosphatase K (INPP5K) activation, resulting in increased AR activity and sensitivity to enzalutamide. Combination of FAO inhibitors (etomoxir, ranolazine, and perhexiline) with enzalutamide displayed a synergistic inhibitory effect, suggesting that co-targeting FAO and AR may have anti-cancer efficacy in mCRPC clinical setting (116). In 2019, the same group provided evidence for a new link between FAO and CR. The authors demonstrated that androgen withdrawal (which mimics the standard of care therapy for metastatic PCa) increases CPT-1A expression and FAO activity, which supports CRPC growth and antiandrogen resistance by supplying acetyl groups for histone acetylation (117). In a follow-up study, the authors showed that CPT-1A overexpression promotes antioxidant defenses, which foster PCa progression (118). Finally, last year, the group put forward the involvement of FAO in immunomodulation. Using the TRAMPC1 PCa model, the authors demonstrated that FAO inhibition with ranolazine decreases Tim3 content in CD8+ tumor-infiltrating T cells, increases macrophages, and decreases blood myeloid immunosuppressive monocytes, suggesting that targeting FAO stimulates anti-cancer immunity (118).

Besides CPT-1A, other FAO enzymes are involved in PCa progression. Combining proteomics and metabolomics, Biomme and coworkers identified the mitochondrial 2,4-dienoyl-CoA reductase (DECR1), an auxiliary FAO enzyme, as critical for CRPC. DECR1 participates in redox homeostasis by controlling the ratio between saturated and unsaturated phospholipids. As a result, DECR1 KO induced ER stress and sensitized CRPC cells to ferroptosis. Furthermore, DECR1 deletion impaired lipid metabolism and reduced CRPC tumor growth *in vivo* (119). Similar results were obtained by Nassar and coworkers using different models. The authors confirmed DECR1 KD-mediated cellular accumulation of PUFAs, enhanced mitochondrial oxidative stress, and lipid peroxidation. Specifically, DECR1 KD selectively inhibited PUFA oxidation, resulting in the suppression of proliferation, migration of PCa cells (including those resistant to enzalutamide), and metastasis formation in mouse xenograft models (120). These new findings implicate PUFA oxidation *via* DECR1 as an unexplored facet of FAO to promote PCa progression.

Yajun and coworkers also uncovered the involvement of the FAO regulator nuclear envelope protein Sun2 in PCa progression. The authors found a reduction of Sun2 expression in PCa tissues compared with adjacent normal tissues, which correlated with higher Gleason grade, postoperative T stage, lymph node invasion, and shorter PCa-free and overall survival. Sun2 silencing increased FAO activity, feature that was reversed by the use of etomoxir, suggesting a new role for Sun2 in promoting PCa progression through FAO modulation (121). Finally, Itkonen and coworkers identified enoyl-CoA-isomerase 2 (ECl2), a novel AR target involved in FAO. ECl2 was found overexpressed in PCa samples and associated with poor outcome, suggesting its possible involvement in PCa progression (122).

Alterations in Lipid Storage

Under excess of nutrients, *de novo* or acquired FAs are incorporated in TAGs and accumulate as LDs, organelles composed by deposits of TAGs and cholesterol esters, and surrounded by a monolayer of PLs. LDs represent a reservoir and source of lipids for cancer cells, particularly under stress conditions such as hypoxia (123). Increased abundance of LDs is a feature of many aggressive cancers, including PCa [reviewed in (9)]. The terminal step in TAG biosynthesis is catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT) enzymes, which transfer an acyl chain from fatty acyl CoA to diacyl glycerol (DAG). DGAT1 is overexpressed in PCa compared to normal epithelium and a recent study demonstrated that inhibition of DGAT1 reduces cell proliferation and migration *in vitro* and tumor growth *in vivo* by regulating intracellular lipids and non-centrosomal microtubule-organizing center (MTOC) protein GM130 (124). Similar results were also independently obtained by Mitra and coworkers (125). Using label-free Raman spectroscopy, Yue and coworkers demonstrated an aberrant accumulation of esterified cholesterol in LDs in high-grade PCa and mets due to the loss of the tumor suppressor PTEN, the activation of PI3K/AKT pathway, and the consequent activation of TF SREBP and LDL receptor (LDL-R). LD accumulation required the occurrence of cholesterol

esterification. As a result, pharmacological and genetic inhibition of cholesterol esterification using cholesterol acyltransferase (ACAT) significantly suppressed cancer proliferation, migration, invasion, and tumor growth *in vivo* (126). This finding suggests ACAT as a potential target in PTEN mutated/deleted CRPC, which account for around 70% of CRPCs.

TAGs in LDs are sequentially hydrolyzed by three different lipases, the adipose triglyceride lipase (ATGL), the HS lipase (HSL), and the monoacylglycerol lipase (MAGL) [reviewed in (9)]. In 2011, Nomura and coworkers showed that MAGL is increased in androgen-independent human PCa cell lines, and that pharmacological or genetic inhibition of MAGL impairs PCa aggressiveness. Furthermore, MAGL was found as part of an EMT and stem-like gene signature, suggesting MAGL as a potential therapeutic target in advanced PCa (127). These data highlight LDs are critical players in supporting PCa progression, especially under stress.

Alterations in Phospholipid Synthesis and Membrane Remodeling

FAs are essential building blocks for PLs. Early studies showed that a substantial fraction of the FAs acquired by PCa end up in PLs, which together with cholesterol and sphingolipids are the major constituents of membranes. In 2003, Swinnen and coworkers demonstrated that FASN plays a major role in the synthesis of PLs partitioning into detergent-resistant membrane microdomains, the latter being involved in key cellular processes including signal transduction, intracellular trafficking, cell polarization, and cell migration (45). PLs can be synthesized *de novo* but they can also be dynamically remodeled. For *de novo* PL synthesis, FAs are first incorporated in phosphatidic acid (PA) followed by phosphatidylcholine (PC), and phosphatidylethanolamines (PE) synthesis through the Kennedy pathway, although PE can also be generated from phosphatidylserines (PS) by headgroup exchange. PS is synthesized in the ER by headgroup exchange from PC and PE. Phosphatidylinositol (PI) is indirectly synthesized from PA, while cardiolipins (CL) are synthesized locally [reviewed in (9)]. PLs remodeling is catalyzed by phospholipases which can release acyl chains at different positions depending on the subclass of enzymes (PLA, PLC, PLD), while PL reacylation is catalyzed by a class of acyltransferases such as lysophosphatidylcholine acyl transferases (LPCAT). Our group demonstrated that *de novo* PC synthesis is required for cell cycle completion, upon cell division (128). Many enzymes involved in PL synthesis and remodeling are highly dysregulated in PCa. Lipin-1, a phosphatidic acid phosphatase (PAP) that regulates the rate-limiting step in PL synthesis is overexpressed in high-grade PCa and in PCa cells resistant to chemotherapy (i.e., Docetaxel). cBioPortal data also showed that patients with Lipin-1 amplification are characterized by decreased survival. Lipin-1 KD decreased both PCa cell proliferation and migration through RhoA activation, increased PA levels, and induced autophagy through the inhibition of PI3K/AKT/mTORC1 pathway., Lipin-1 depletion with propranolol sensitized cancer cells to rapamycin, suggesting new combination therapies (129, 130). Choline kinase alpha (ChoKa), the first enzyme of the Kennedy pathway, is also overexpressed in several cancer including PCa (131). Priolo and coworkers showed that the oncogene MYC increases ChoKa expression, as well as lipid synthesis (132). In line with this, positron emission tomography (PET) with PL-precursors ^{11}C-choline or ^{18}F-fluoro-choline has shown promising results in the detection of PCa recurrence and mets (133). Asim and coworkers demonstrated that ChoKa expression is regulated by androgens and it is positively associated with tumor stage. The authors also uncovered a role for ChoKa as a chaperone that binds to AR ligand-binding domain (LBD), enhancing AR stability. Consistently, ChoKa inhibition decreased AR protein levels and AR transcriptional program, and inhibited the growth of PCa cell lines, human PCa explants, and tumor xenografts (134), suggesting ChoKa as a marker of tumor progression and a potential therapeutic target. PLs remodeling is catalyzed by phospholipases, including PLA2, which is also involved in the generation of signaling FAs such as arachidonic acid (AA, see below) and lysophospholipids (LysoPLs). Phospholipase A2 Group IIA (PLA2G2A), especially the secretory form, is overexpressed in almost all human PCa specimens and correlate with high tumor grade. Blocking sPLA2-IIa function compromises CRPC cell growth, highlighting sPLA2 as a potential therapeutic target for CRPC. Serum sPLA2-IIa levels were increased in PCa patients and associated with high Gleason score and advanced disease stage, suggesting that serum sPLA2-IIa may serve as a PCa prognostic biomarker. A recent report also associated the expression of PLA2G2A with ferroptosis resistance through PUFA depletion in PCa membranes (135, 136). LysoPLs can stimulate PCa cell migration through several mechanisms, including the activation of the cationic channel T transient receptor potential vanilloid 2 (TRPV2), and the activation of lysophosphatidic acid (LPA)/LPA-R/mitogen-activated protein kinase (MAPK) pathway (137, 138). LysoPLs are also substrates for MAGL, whose expression is dysregulated in aggressive PCa (see above). LysoPLs can be reacylated by enzymes such as ysophosphatidylcholine acyl transferases (LPCATs). Grupp and coworkers demonstrated that the expression of lysophosphatidylcholine acyltransferase 1 (LPCAT1), a key enzyme in Lands' cycle remodeling pathway, correlates with PCa progression and resistance to chemotherapy (i.e., Paclitaxel) and might be used as prognostic biomarker of clinical outcomes and biochemical recurrence (139). LPCAT1 mediates CRPC growth *via* nuclear re-localization and Histone H4 palmitoylation in an androgen-dependent fashion, increasing mRNA synthesis rates. Silencing of LPCAT1 reduced the proliferation and CRPC cell invasive potential, suggesting this enzyme as a potential therapeutic candidate in CRPC (140).

Alterations in Cholesterol Metabolism

Cholesterol is a major constituent of cell membranes, LDs, and a precursor of androgens synthesis. It is evident that alterations in cholesterol synthesis and metabolism are associated with PCa pathogenesis and progression (141, 142). PCa cells can acquire cholesterol from exogenous sources, including circulating lipoproteins (i.e., VLDL, and LDL) and exosomes, from intra-

cellular storage (i.e., LDs), and from *de novo* cholesterol synthesis. All these processes are significantly altered in PCa, especially in aggressive PCa and CRPC. As mentioned above, Yue and coworkers demonstrated an aberrant accumulation of esterified cholesterol in LDs of high-grade PCa and mets due to PTEN loss-mediated activation of the PI3K/AKT pathway, and consequent increase of SREBP and LDL-R (126). However, low levels of LDL-R and high squalene monooxygenase (SQLE) expression were recently detected in high Gleason grade-human PCas and associated with lethal disease. According to these new results, PCas that progress to lethal disease rely on *de novo* cholesterol synthesis (via SQLE), rather than transcellular uptake (via LDL-R) or cholesterol esterification (via Sterol O-Acyltransferase 1, SOAT1) (142). The association of SQLE overexpression with lethal disease was validated in a second study from the same group looking at three different prospective cohorts (143). Absolute SQLE expression was associated with lethal cancer independently of Gleason grade and stage and with increased histologic markers of angiogenesis. SQLE expression at PCa diagnosis was found to be prognostic for lethal PCa both after prostatectomy and in a watchful waiting setting (143). Conversely, vitamin D-regulated catabolic enzyme sterol-27-hydroxylase (CYP27A1), which converts cholesterol to 27-hydroxycholesterol was detected at low levels in tumors characterized by high Gleason grade and high expression of cholesterol synthesis enzymes, including SQLE. Low expression of CYP27A1 was also associated with higher risk of lethal cancer, independent of SQLE (144). Altogether, these data support the notion that intra-tumor cholesterol accumulation (via increased synthesis or reduced catabolism) is a feature of lethal PCa. As expected, the key enzymes for cholesterol synthesis, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR, the first-rate limiting enzyme) and 3-hydroxy-3-methyl-glutaryl-coenzyme A synthetase (HMGCS) are regulated by androgens and upregulated in PCa, especially in CRPC and contribute to CR [reviewed in (12)]. HMGCS and HMGCR were found overexpressed in stromal cells when co-cultured with PCa cells to support PCa progression, suggesting that HMGCS and HMGCR in both PCa epithelium and stroma, might serve as theraputic targets (145).

THE ROLE OF LIPIDS AS SIGNALING MEDIATORS IN PCa

Besides their function as building blocks and energy suppliers, lipids can function as intra- and extracellular messengers and mediators of malignant behavior. Several classes of lipids are involved in signaling, including sphingolipids and eicosanoids. Tumor-promoting functions have been described for several sphingolipids, including sphingosine, spingosine-1-phosphate (S1P), ceramide, and ceramide-1-phosphate (C1P) (146). By using isotopic FA labeling strategy coupled with metabolomic profiling platforms to comprehensively map palmitic acid incorporation into complex lipids in cancer cells, Louie and coworkers elucidated that cancer cells, including PCa cells, and tumors robustly incorporate and remodel exogenous palmitate into structural and oncogenic glycerophospholipids, but mostly in sphingolipids and ether lipids. FA incorporation into oxidative pathways was reduced in aggressive PCa cells, and instead shunted into pathways for generating signaling lipids such as ceramide and sphingomyelin, suggesting a role for sphingolipids in PCa progression (147). In line with this, Increased levels of S1P were found in more aggressive PC. Pharmacological inhibition (with ABC294640) of sphingosine kinase 2 (SphK2), one of the two Sphk isoforms that catalyzes the synthesis of S1P from sphingosine, effectively reduced CRPC cell proliferation and xenograft tumor growth by targeting AR and the oncogene MYC (148). Classically, ceramide induces senescence and growth inhibition in cancer. However, recent studies suggested that ceramide effects are context dependent and rely on downstream effectors, which can both promote or inhibit tumor growth (149). Along the line, increased expression of acid ceramidase (AC) was observed in PCa. AC significantly altered the expression of ceramide species without affecting the total levels. In AC-overexpressing DU145 cells, low levels of C14-C20 ceramides (long chain ceramides) and elevated levels of C24, C24:1 ceramides (very long chain ceramides) were indeed detected. This was associated with increased proliferation, migration and augmented tumorigenicity *in vivo*, which were reversed by pharmacological or genetic AC inhibition (150, 151). Although AC-mediated oncogenic mechanisms are still unknown, it is likely that AC-induced very long chain ceramide species promote cell growth while long chain ceramides induce cell apoptosis [reviewed in (151)]. Consistently, LC/MS-based lipidomics in plasma from patients with primary PCa, mHSPC, and mCRPC, showed that elevated circulating ceramide levels are associated with poor outcomes across tumor stages progression from localized PCa, mHSPC, to mCRPC. Patients with elevated ceramide levels were more likely to have metastatic relapse, therapeutic failure (ADT/docetaxel), and shorter overall survival. The authors also validated a previously published prognostic 3-lipid signature with potential clinical traslation (152). Both ceramide and C1P are activators of PLA2, an enzyme that releases AA for subsequent conversion to prostaglandins, molecules involved in inflammation, immunity, and tumor growth modulation (see below). Increased levels of prostaglandins, like PGE2, are associated with enhanced PCa proliferation and invasion, which can be reversed by the use of cyclooxygenases (COX) inhibitors, suggesting the involvement of PGE2 in PCa progression. Contrasting results have been however obtained, highlighting the need for more validation studies (153, 154). Phosphoinositides represent another class of critical signaling molecules and central mediators of the PI3K/Akt/mTORC1 signaling axis. Activation of PI3K results in the rapid conversion of PI(4,5)P2 into PI(3,4,5)P3, leading to AKT activation. PI(4,5)P2 it-self can also play a major role in recruiting cytosolic proteins, facilitating processes like fusion, membrane budding, and the formation of signaling platforms [reviewed in (9)]. Finally, glycerolipid-derived mediators, such as DAG, LysoPA and LysoPC are involved in cancer progression. DAG, generated from the hydrolysis of PI(4,5)P2, functions as a

second messenger that triggers the oncogenic activation of protein kinase C (PKC). Sustained levels of DAG and activated PKC signaling were reported as a mechanism of resistance to FASN inhibitors, suggesting the assessment of DAG as predictive biomarker of FASN activity and the therapeutic combination of FASN and PKC inhibitors (155).

ONCOGENIC AND ENVIRONMENTAL REGULATION OF LIPID REWIRING IN PCa

Lipid metabolism rewiring is very dynamic. Cancer cells, including PCa cells, adapt their metabolism in response to changes in nutrients supply, hormonal status, growth factors stimuli as well as epi/genetic alterations in oncogenes (i.e., MYC, PI3K/AKT) or tumor suppressor genes (i.e., PTEN, p53, RB), commonly found in mCRPC, as comprehensively described in our recent review (31). Here, we focus on the impact of systemic metabolism and environmental factors, in particular diet, to PCa metabolism rewiring and disease progression.

Both obesity and sustained consumption of fat-enriched diets alter nutrient gradient in the TME, which may favor cancer cells/TME metabolic symbiosis, inflammation, cancer progression, and chemoresistance (10). SFA-enriched diet is sufficient to promote mCRPC in the nonmetastatic PTEN KO mouse model *via* an aberrant lipogenic program orchestrated by SREBP (156). In Hi-MYC mouse model, HFD-induced obesity (enriched for SFA) amplifies a c-MYC-mediated oncogenic transcriptional signature, which is associated with lethality in patients (156–158). Using the same Hi-MYC mouse model, Blando and coworkers also showed that HFD-induced obesity enhances, whereas 30% caloric restriction reduces growth factor (AKT/mTORC1 and STAT3) and inflammatory (NFκB and cytokines) signaling and PCa progression (159). Consistently, reduced dietary fat intake was shown to delay PCa progression to CRPC and to prolong survival in xenograft models, suggesting low-fat diet as a promising adjuvant intervention during ADT (160). Besides SFAs, the ratio between omega-3 (n-3)/omega-6 (n-6) PUFAs also affect PCa progression. Omega-3 but not omega-6 PUFAs slowed down the growth of CRPC in PTEN KO mouse model in part by accelerating proteasome-dependent degradation of AR protein (161). In line with this, an isocaloric 20% kcal fat diet consisting of n-6 and n-3 FAs in a ratio of 1:1 (n-3 diet) reduced tumor growth rates, tumor volumes, and serum PSA levels in LAPC-4 xenografts with respect to n-6 FAs-based diet (n-6 diet). n-3 diet-tumors were characterized by low proliferation, increased apoptosis, and reduced levels of COX-2, PGE-2, and VEGF. Furthermore, LAPC-4 cells proliferation in medium containing n-3 diet serum was reduced by 22% with respect to n-6 diet (162). Several clinical trials are ongoing to evaluate the effect of n-3 PUFA in patients with advanced PCas, as well as in active surveillance and PCa prevention (NCT00458549, NCT03753334, NCT03753334, NCT00253643, NCT02176902, NCT02333435). Results from these studies will be valuable to understand whether nutrition intervention should be implemented in the management of PCa patients prior to or along with ADT/AR signaling inhibitors.

Obesity is also associated with increased fat storage in the adipose tissue. Interestingly, several tumors grow in anatomic proximity to adipose cells. This is the case of PCa, which grows adjacent to the peri-prostatic adipose tissue (PPAT) and develops mets in fatty bone marrow [reviewed in (163)]. Adipocytes can act as driving force to promote PCa cells migration to PPAT. Laurent et al. demonstrated that PPAT-derived adipocytes secrete the chemokine CCL7, which diffuses to the peripheral zone of the prostate, stimulating the migration of CCR3 expressing PCa cells. The latter is reversed by CCR3/CCL7 axis inhibition. In human PCas, CCR3 receptor expression is associated with higher occurrence of aggressive disease with extended local dissemination and biochemical recurrence (164). CCR3 is also potentially involved in the homing of PCa cells to the bone. Using *in vitro* migration assays, the same authors demonstrated that soluble factors released by human primary bone-marrow-derived adipocytes drive the directed migration of PCa cells in a CCR3-dependent manner. Furthermore, Oncomine microarray database uncovered increased levels of CCR3 mRNA in bone mets with respect to primary tumors, while IHC experiments demonstrated overexpression of CCR3 in bone *versus* visceral mets (165). Altogether, this evidence suggests the potential benefit of CCR3 antagonists in the treatment of advanced PCa. In a recent review, Nassar et al. not only describe the role of PPAT as a source of FAs and mitogens but also uncover the existence of a crosstalk between PCa and PPAT that sustains PCa pathogenesis and progression (166). In line with this, MRI-based PPAT measurements have provided new useful information in the prediction of PCa progression. Peri-prostatic fat area (PPFA) and PPFA to prostate area ratio (PPFA/PA) was reported as independent predictor of PCa, lymph node mets, Gleason score, tumor stage, and proliferation index (i.e., Ki-67) (167, 168). Thus, PPFA measurements along with transrectal ultrasound-guided biopsy may improve PCa detection and risk stratification. PPAT volume has been recently also associated with reduced progression-free survival in men with PCa on active surveillance and with poor response to ADT in patients with advanced PCa (169). These results highlight the crucial role of PPAT in PCa progression and the clinical value of MRI-based measurements of PPAT to predict prognosis and therapy response.

THE ROLE OF LIPIDS IN MEDIATING TUMOR-TME CROSSTALK

While the role of FAs in promoting inflammation and mediating inflammatory signaling has been largely characterized, more recent data suggest a key role for FAs in immune metabolism [reviewed in (10, 170)]. Both FA synthesis and oxidation are important regulators of immune responses. FA synthesis plays a role in antigen presentation and T cell activation, whereas FAO is a key feature of CD8 memory T cells (170). The source of lipids used for FAO in memory T cells is cell type specific. Central memory CD8 T cells cannot effectively take up lipids and rely on

lipolysis for FA supply, whereas tissue resident memory CD8 T cells require uptake of exogenous lipids for their survival and proliferation (171–173). In contrast to CD8 T cells, naïve and memory CD4 T cells require FA uptake and synthesis for full activation and proliferation (174). Regulatory T cells (Treg) and M2-like macrophages rely on lipid-dependent catabolism. Treg cells predominantly use FAO-fueled oxidative phosphorylation (OXPHOS) to generate energy and FAO inhibition with etomoxir suppresses Foxp3 expression in Treg cells without affecting T effector cells (Teff) cells. Thus, Treg cells display a survival advantage in low-glucose and lipid-rich environments over Teff cells and are well adapted to reside in fat tissue and lipid-rich TME, which is consistent with their increased frequency in the TME (175, 176). FAO is also required for the maturation and function of IL-4-induced anti-inflammatory M2 macrophages, which uptake FAs through CD36 and FATP1 to maintain their phenotype (177–180). FAO alterations and LD accumulation are also linked with dendritic cells dysfunction, highlighting the importance of lipids in antigen presentation (181–183).

Michelet and coworkers showed that HFD-induced obesity induces PPAR-driven lipid accumulation in Natural Killer (NK) cells, causing a complete 'paralysis' of their cellular metabolism and trafficking, resulting in blunted antitumor responses (184). Similarly, the integration of single-cell RNA sequencing, multiplexed immunofluorescence IHC, and mass-spectrometry approaches *in vivo*, uncovered that HFD-induced obesity impairs $CD8^+$ T cell function in TME due to a distinct metabolic adaptation to obesity by the tumor and T cells. While tumor cells increase fat uptake, tumor infiltrating $CD8^+$ T cells do not, leading to altered FA partitioning in HFD tumors, which impaired $CD8^+$T cell infiltration and function. Analysis of human cancers revealed similar transcriptional changes in $CD8^+$ T cell markers, suggesting the potential of lipid metabolism interventions to improve cancer immunotherapy (185). In contrast, obese cancer patients seem to respond to ICI, a phenomenon known as "Obesity paradox" (186). Thus, further studies are needed to clearly understand the impact of obesity and obesogenic HFD on immune therapy efficacy in patients.

Cancer cells not only suppress tumor immune surveillance, but they can also hijack the immune system to support their growth. For instance, ovarian cancer cells promote the efflux of cholesterol from macrophages which in turn drives a pro-tumoral M2 phenotype (187). Moreover, it has been reported that cancer cells can also promote tumor-associated myeloid-derived suppressor cells (MDSCs) to produce PGE2, an oxylipin with immune suppressive functions. This seems to occur through a cancer-dependent increase of Fatty acid transport protein 2 (FATP2) expression, which allow AA transport in MDSCs for PGE2 synthesis (188).

While the role of lipids in PCa immune TME has not been carefully investigated, early preliminary data showed increased expression of immune checkpoint PD-1, PD-L1, and PD-L2 in tumor tissues from PTEN KO mice fed HFD, suggesting an opportunity for ICI (189). Considering that the response to ICI has been so far disappointing, understanding whether obesity may boost response to ICI and "paradoxically" favor the use of immune therapy is crucial to identify a subset of mCRPC patients, who may potentially respond to immune therapies.

EXPLOITING LIPID METABOLISM REWIRING FOR THERAPEUTIC INTERVENTION

In light of the aforementioned changes in lipid metabolism during PCa progression, huge efforts have been directed on tackling enzymes and transporters involved in all the aspects of lipid metabolism (from FA uptake transport, *de novo* FA/cholesterol synthesis, sphingolipid and phospholipids synthesis, to lipid storage and lipolysis). Recently published reviews from our group and others have provided an exhaustive description of the small molecules/compounds targeting lipid metabolism tested so far in oncology (9, 31). Here, we emphasize those compounds that have already been approved for clinical use or are currently tested in clinical trials.

Inhibitors of *De Novo* FA Synthesis

The majority of therapeutic efforts have been focused on FASN, resulting in the development of several FASN inhibitors (i.e., Orlistat, C75, cerulenin, C93, Fasnall) with good results in the preclinical setting. Unfortunately, off-target effects, poor solubility and pharmacokinetics, and untoward side effects, including important weight loss, prevented their clinical translation [reviewed in (31)]. The development of TVB-2640, an orally available inhibitor of FASN β-ketoacyl-reductase domain has changed the perspective. A phase I clinical trial in cancer patients has been completed, showing the safety and efficacy of TVB-2640 in solid malignancies (NCT02223247). Combined with paclitaxel, TVB-2640 provided positive results in heavily pretreated breast cancer patients, while the non-orally available analog TVB-3166 was effective in mCRPC preclinical models (190–192). Phase II trials are now investigating TVB-2640 in several solid tumor types including HER-2 positive advanced breast cancer in combination with trastuzumab. (NCT03032484, NCT03179904, NCT02980029, NCT03808558). Our group also characterized a new oral-available small molecule irreversible FASN inhibitor (IPI-9119) with potential clinical translation. In the preclinical setting, we demonstrated that selective FASN inhibition antagonizes the growth of mCRPC, in part by inducing ER stress-mediated downregulation of AR-FL and AR-V7 protein levels and their transcriptional activity. As a result, IPI-9119 improved the response to enzalutamide in mCRPC cell lines and organoid models. Our data support FASN repression as a non-canonical approach to inhibit AR-V7, thus overcoming current resistance to standard of care for mCRPC. Multiplex immunofluorescence analysis combined with digital pathology of mCRPC tumor microarrays confirmed FASN/AR-V7 co-expression in about 80% of mCRCP patients resistant to enzalutamide and abiraterone, highlighting this patient subset as the ideal

candidate for the treatment with FASN inhibitors (22). Carefully designed clinical trials are still needed to adequately define the timing, combinations, and the suitable population to test.

Inhibitors of FA Oxidation

Targeting FAO in mCRPC has recently gained a lot of attention. Iglesias-Gato and coworkers have recently identified a subgroup of bone mets characterized by elevated expression of FAO enzymes, and thus potentially responsive to FAO inhibitors. These findings also underline the urgent need for adequate patient stratification when metabolic therapies are considered as therapeutic approaches (193). Combinations of FAO inhibitors (etomoxir, ranolazine, and perhexiline) and enzalutamide have been tested in mCRPC cell and xenograft models with positive results. Unfortunately, etomoxir use in the clinical setting has been terminated due to toxic side effects, mostly hepatotoxicity. In contrast, ranolazine and perhexiline are already approved for the treatment of heart diseases in Europe, US, and Australia (194), opening a potential safe avenue for the combinations of FAO and AR signaling inhibitors in mCRPC.

Inhibitors of Cholesterol Synthesis

Statins are commonly used to lower cholesterol levels and reduce cardiovascular risk. Statins use in the prevention of cancer risk has been evaluated with conflicting results. Their potential use in combination with the standard of care in the treatment of PCa has recently gained attention (31).

A clinical trial designed to test whether atorvastatin (an HMGCR inhibitor) delays the development of CR during ADT in metastatic or recurrent PCas is currently ongoing (NCT04026230). In the preclinical setting, HMGCR inhibition with simvastatin enhances the efficacy of enzalutamide and decreases AR/AR-Vs protein levels *via* inhibition of mTOR pathway (195).

A recent meta-analysis evaluated the effects of statins use on treatment outcomes (i.e., overall survival and cancer-specific survival) among patients with advanced PCa treated with ADT or AR signaling inhibitors. Statin use was associated with lower risk of all-cause mortality and cancer-specific mortality in advanced PCa patients treated with ADT, whereas inconsistent results were obtained with AR signaling inhibitors (196). Thus, future studies are still required to establish the efficacy of statins in combination with AR signaling inhibitors in mCRPC patients.

APPLICATION OF LIPIDOMICS AND MASS-SPECTROMETRY IMAGING IN PCa RESEARCH

Despite the crucial role of lipid metabolism in PCa progression and resistance to endocrine therapies, lipidomics studies have only recently reached the spotlight most likely due to the methodological challenge of analyzing simultaneously diverse lipid classes and molecular species and technical issues associated with these analytical techniques. An outstanding review has recently highlighted the current advances of lipidomics and mass-spectrometry imaging in cancer research and their critical role in precision medicine (9). Here, we briefly summarize studies using these technologies for the identification of new predictive/prognostic biomarkers in PCa.

Lin et al. performed LC/MS-based lipidomics in plasma samples from a discovery cohort of CRPC patients and identified forty-six lipids, predominantly sphingolipids, associated with poor prognosis. The authors derived a prognostic three-lipid signature (ceramide d18:1/24:1, sphingomyelin d18:2/16:0, phosphatidylcholine 16:0/16:0) as independent prognostic factor (197). More recently, the same group detected elevated circulating ceramide species in association with poorer clinical outcomes across the PCa progression and validated the three-lipid prognostic signature in an independent cohort (152). These studies not only identified an easily detectable prognostic biomarker but also highlighted the crucial role of sphingolipids in PCa progression. Similarly, Butler et al. profiled PCa cell lines, xenografts, and patient-derived explants under treatment with androgen and AR signaling inhibitors. Significant changes in lipid elongation for multiple phospholipid classes in response to androgen treatment were identified and reversed by enzalutamide, suggesting the utility of lipidomics to predict response to endocrine therapies (198). Lipidomics and transcriptomics integration in PCa and adjacent normal tissues also identified a strong accumulation of cholesteryl esters (CEs) most likely due to increased expression of scavenger receptor class B type I (SR-BI). CE accumulation was associated with disease progression and mets formation. In a discovery set, CE robustly differentiated PCa from normal tissue. In a validation set, CEs not only potently distinguished PCa from normal tissue, but it also discriminated PCa from benign prostatic hyperplasia (BPH) superior to PSA, suggesting CE, particularly, cholesteryl oleate, as a biomarker for PCa detection (199). Furthermore, targeted lipidomics in EVs derived from prostate and PCa cell lines uncovered differences in the molecular lipid species associated with PCa progression. These differences highlight the importance of characterizing the EV lipidome, which may lead to improved prognostic biomarkers (200). Despite the high resolution, sensitivity, and specificity of LC/MS-based lipidomics, these technologies fail to provide spatial information and to integrate the information of biomarker expression with tissue pathology and compartment distribution. The development of MSI has overcome this limitation. MSI thus represents an important step forward for the evaluation of metabolic reprogramming occurring in the TME. Matrix-assisted laser desorption ionization (MALDI)-MSI, where the sample is mixed with a UV-absorbing crystalline matrix material and ionized by the laser beam, is the most commonly used MSI method (201). Our group applied MALDI-MSI to investigate changes in lipid metabolism associated with gleason score. We detected increased levels of 31 lipids, including several phosphatidylcholines, PA, phosphatidylserines, phosphatidylinositols, and cardiolipins, in Gleason score 4 + 3 compared with Gleason score 3 + 4, suggesting these analytes as potential biomarkers of PCa

aggression worth further validation. Interestingly, we identified lipid changes in both regions of high tumor cell density, and in regions of tissue that appeared histologically "benign", implying the occurrence of precancerous lipid changes with prognostic significance (202). Using a similar approach, Andersen et al. identified increased levels of metabolites crucial for lipid metabolism in PCa, including metabolites involved in the carnitine shuttle as well as building blocks for *de novo* lipogenesis (203). The feasibility of spatial and rapid detection of metabolites associated with PCa onset and progression showcases MALDI-MSI as a promising and innovative diagnostic/prognosis tool in the clinical setting.

DISCUSSION

Lipid metabolism rewiring is highly dynamic throughout the course of PCa progression. Intracellular lipid changes due to either environmental cues or *de novo* FA synthesis/FAO increase PCa cells fitness and their capability to adapt to oxidative stress, hypoxia, ER stress, to maintain redox balance, and to counteract ferroptosis and genotoxic insults. Recent evidence also supports the role of lipids as key players in shaping TME metabolism, in particular immune metabolism. This is especially exacerbated by obesity or consumption of HFD diet, conditions in which cancer cells hijack lipids (with the support of tumor-surrounding adipose cells or cancer-associated fibroblasts) for their own benefit, impairing anti-tumor immunity. The rapid advance of lipidomics and MALDI-MSI has allowed to gain, a previously unforeseen, awareness of the dynamicity and adaptability of lipid rewiring during PCa progression, taking into account the influence of systemic metabolism and tumor-TME crosstalk. In the imminent future we anticipate the integration of MALDI-MSI, spatial transcriptomics, and digital pathology will further advance our current understanding of the biology of lipids in PCa progression and will offer opportunities for the identification of new druggable targets. Unfortunately, we still have a long road ahead to validate lipids as biomarkers and to translate the lipid-metabolism targeting drugs available so far in the clinical setting. The journey has started long time ago, but we are now fully equipped with the adequate models (i.e., patient-derived organoids, explants, xenografts, co-culture systems, immune-competent mouse models, etc.), technologies, and bioinformatics support to rapidly move forward.

PCa is "a matter of fats". The big challenge is to carefully identify and target those lipid and pathways that are tumor-friends while preserving those that protect our health and longevity.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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The Interplay Between Prostate Cancer Genomics, Metabolism, and the Epigenome: Perspectives and Future Prospects

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Prostate cancer is a high-incidence cancer, often detected late in life. The prostate gland is an accessory gland that secretes citrate; an impaired citrate secretion reflects imbalances in the activity of enzymes in the TCA Cycle in mitochondria. Profiling studies on prostate tumours have identified significant metabolite, proteomic, and transcriptional modulations with an increased mitochondrial metabolic activity associated with localised prostate cancer. Here, we focus on the androgen receptor, c-Myc, phosphatase and tensin Homolog deleted on chromosome 10 (PTEN), and p53 as amongst the best-characterised genomic drivers of prostate cancer implicated in metabolic dysregulation and prostate cancer progression. We outline their impact on metabolic function before discussing how this may affect metabolite pools and in turn chromatin structure and the epigenome. We reflect on some recent literature indicating that mitochondrial mutations and OGlcNAcylation may also contribute to this crosstalk. Finally, we discuss the technological challenges of assessing crosstalk given the significant differences in the spatial sensitivity and throughput of genomic and metabolomic profiling approaches.

Keywords: prostate cancer, metabolism, epigenetics, mitochondria, TCA cycle

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INTRODUCTION

Prostate cancer (PCa) is the most common cancer affecting men in the developed world. The incidence is the second highest after lung cancer in men worldwide (1, 2).

Deciphering the functional impact of prostate cancer genomics on disease progression has been a challenge in comparison to other cancer types for many reasons including sample accessibility and the limited availability of model systems. Multi-focal sampling of prostate cancer in patient samples with downstream DNA and RNA sequencing have revealed both inter- and intra-patient heterogeneity in primary tumours and metastatic samples (3, 4). Despite this heterogeneity, it has been possible to sub-type prostate cancers based not only on gene fusion status but also on the abundance of mutations associated with biological drivers of the disease, and in particular mutations affecting androgen receptor (AR) signalling), PI 3-Kinase/Akt, and DNA repair pathways (3).

GENOMIC FEATURES OF PROSTATE CANCER

In considering the crosstalk between genetic changes in prostate cancer and metabolic dysregulation, it is helpful to focus on some of the principal oncogenic drivers—AR activity, c-Myc amplification and overexpression and mutations in phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and in TP53.

AR

Over the decades, a focus on targeting the androgen receptor (AR) signalling axis to block AR *via* androgen deprivation therapy and AR antagonists has been a conventional therapy in PCa. Aberration in AR ranges from point mutations such as W741R, V757A, R846G, H874Y, and T877A in ligand binding region of the AR imparting insensitivity towards AR antagonists (1). In addition, deletions in the region of G589-A628 has been identified in patients with CRPC, disrupting second zinc-finger domain of AR, developing resistance to AR antagonists or AR targeted therapies (1). By using ChIP-seq and transcriptomic profiling networks of AR, target genes have been identified in cell lines and in tumour samples (2). These datasets have provided insight into AR crosstalks with other transcription factors and regulated biological processes. Recent transcriptomic and cistromic studies have revealed AR as a modulator of autophagy and DNA repair (3–7). Massie et al. employed a combination of transcript profiling and ChIP-seq to identify androgen receptor target genes and pathways in prostate cancer cell lines (8). Through a meta-analysis of clinical transcriptomic data and subsequent validation using immunohistochemistry, the authors identified calcium/calmodulin-dependent kinase kinase 2 (CAMKK2) as a clinically relevant regulator of metabolism. They went on to knockdown CAMKK2 and also inhibit this kinase with a small molecule inhibitor, which impaired tumorigenesis in a prostate cancer xenograft model. Pairing these interventions with ^{13}C-glucose metabolic flux analysis using mass spectroscopy, they showed that targeting CAMKK2 inhibited the incorporation of hydrocarbons into TCA cycle metabolites and amino acids. This work illustrates the use of genomics and metabolomics to identify metabolic regulators that are affected by AR activity. Other studies have shown that AR-associated gene targets include key components/enzymes of glucose homeostasis, mitochondrial respiration, and fatty acid oxidation (9–14).

Pathway enrichment analysis on AR-regulated gene networks has unearthed enrichments for metabolic processes amongst which the most prominent are lipid synthesis and degradation pathways (15). Importantly, the vast majority of AR-regulated metabolic enzymes are cytosolic or associated with organelles other than mitochondria; however, high rates of metabolic activity arising from AR-regulated pathways feed metabolites into mitochondria. Important examples of lipid-metabolising enzymes that are AR-dependent include FASN, ELOVL5, and ACACA (acetyl-CoA carboxylase alpha) (16). The precise functional effects of aberrant lipid metabolism remain to be determined but include changes in membrane fluidity and the generation of acetyl CoA to support the post-translational modification of proteins (acetylation and glycosylation), prominent amongst which are histones that are of relevance to the crosstalk between metabolism and the epigenome (see below) (17). In addition, a number of other important oncogenic drivers of prostate cancer also sustain aberrant lipid metabolism (see below); as such, this biology is arguably a convergence point for prostate cancer tumorigenesis and, consequently, may offer opportunities for the development of new treatments and repurposing of existing drugs (8, 18).

C-MYC

Myc is copy number amplified and overexpressed in poor-prognosis prostate cancer and exerts an impact on tumour metabolism. It has been shown to affect expression levels of enzymes of oxidative/glycolytic pathway including hexokinase 2, phosphofructokinase, enolase 1, and lactate dehydrogenase A and also GLUT1 levels (19–21). Interestingly, many of these effects are synergistic with the hypoxia-inducible factor 1 (HIF1) function (22). c-Myc also regulates glutamine transporter and mitochondrial glutaminase GLS1 expression through miRNA23a/b and subsequently enhances glutamine metabolism (23, 24). c-Myc may play a significant role in global metabolic reprogramming, such as fuelling citric acid cycle intermediates into anabolic pathways on similar line with AR (25). In addition, c-Myc expression is inversely correlated to AR activity, emphasizing the precise balance and regulation of oncogenic transcription factors thresholds playing a significant role in PCa cells (26). Interestingly, an integrative analysis of metabolomics based on mass spectroscopy revealed differential expression of metabolites; association of AKT1 and MYC activation correlated with accumulation of metabolites of aerobic glycolysis and dysregulated lipid metabolism in human tumours, mouse models, and also in cultured cells (RWPE-1 cells), establishing the oncogene-associated metabolic signatures in PCa (27). In a recent *in vivo* study, a high-fat diet (HFD) led to both metabolic dysregulation and upregulated the MYC transcriptional cascade. These changes favoured H4K20 histone hypomethylation at the promoter regions of MYC-regulated genes, supporting enhanced cell proliferation and tumour growth. This study exemplifies the link between the activity of oncogenic transcription factors and feedback effects on the epigenetic landscape of cancer genomes, a theme we explore further in this review (28).

PTEN

PTEN is a well-established tumour suppressor exhibiting both protein and lipid phosphatase activities. Loss of PTEN function is common in various cancers including bladder, brain, and prostate cancers, often through the deletion of a single gene copy of PTEN at chromosomal location 10q23 (29, 30). It is a negative regulator of oncogenic PI3K/AKT signalling network and plays a

vital role in both lipid and glucose metabolism including mitochondrial functions (31, 32). *In vivo* studies with transgenic models overexpressing PTEN showed an overall change in the metabolic profile with increase in mitochondrial oxidative phosphorylation and coupled with reduction in glucose and glutamine uptake (33).

PTEN has been used as the basis for the transgenic modelling of prostate cancers, and this has revealed that deletion of this tumour suppressor leads to the activation of SREBP1, a transcription factor that regulates lipogenic genes (34). This transcriptional program is enhanced by co-deletion of PTEN with other factors (for example PML1), and tumorigenesis in these models, analogous to c-Myc, is enhanced through a high-fat diet. In a separate study using a prostate-specific conditional PTEN-null (PTEN−/−) transgenic mouse model of cancer, increased pyruvate dehydrogenase activity was shown to be required for tumorigenesis. Genetic ablation of pyruvate dehydrogenase A1 (Pdha1) in PTEN −/− tumors inhibited tumour growth, and this was associated with the reduced expression of lipogenic genes, which were components of a gene network regulated by sterol regulatory element-binding transcription factor (SREBF). Importantly, nuclear Pdha1 was found to sustain this transcriptional activity by supporting histone H3K9 acetylation at sites bound by SREBF1, putatively supporting its transcriptional activity. Interestingly, whereas the knockdown of Pdha1 in PTEN −/− prostate cancer cells reduced acetylation of histone H3 Lys9 (H3K9ac) at these sites, it had not impact at E2F1 binding sites associated with cell cycle progression genes (35). This specificity, and in fact the molecular basis of the crosstalk between metabolite pools and site-specific, as opposed to global, changes in chromatin modifications or DNA methylation remain largely undefined in this and other published studies identifying similar interplays. One example is the nuclear contribution of ATP-citrate lyase to the provision of acetyl-CoA for histone acetylation in lung cancer (36), and others will be highlighted in the course of this article. Overall, these examples of crosstalk suggest that metabolic reprogramming may sustain and be sustained by transcription factors reinforced by metabolically dependent chromatin modifications.

p53

p53 mutations are amongst the most common features of various cancer types including treatment-resistant prostate cancer (37–39). It is often marked by a loss of one allele and inactivity of the second allele resulting in p53 inactivity resulting in cell cycle deregulation and genomic stability (40, 41). Gain-of-function mutations in p53 can also confer oncogenic properties and resistance towards therapeutics (42). p53 regulates metabolism by inhibiting the expression of genes of pentose phosphate shunt pathway and counteracting Myc- and HIF-induced glycolytic flux (43). p53 can also impair nuclear factor kappa B-dependent glucose uptake and glycolysis by repressing the expression of glucose transporters, GLUT1/4 and GLUT3 (44).

p53 also regulates glutamine metabolism through activation of phosphate-activated mitochondrial glutaminase (GLS2) and mitochondrial glutaminase promoting ATP generation *via* oxidative phosphorylation (45). In addition, p53 inhibits AR activity; also, a loss of p53 function enhances Myc activity in PCa (46, 47). This may be partly explained by the enhanced amino acid metabolism and mitochondrial activity arising from p53 deletion. For example, p53 deletion results in mitochondrial biogenesis and in mitochondrial dysfunction mediated by PGC-1α mitochondrial in PC3 prostate cancer cells (48). p53 loss also results in enhanced serine/glycine biosynthesis and changes in one-carbon metabolism that support DNA methylation and nucleotide production (49).

Many of the transcriptional effects of p53 on metabolic gene expression are likely to arise from changes in an impact on the activity of chromatin regulators and hence histone methylation and acetylation. p53 gain of mutants modulates chromatin regulatory genes, including the methyltransferases mixed-lineage leukaemia family of histone methyltransferases 1 and 2 (MLL1 and MLL2) and acetyltransferase monocytic leukaemia zinc finger protein (MOZ) resulting in genome-wide upregulation of histone methylation and acetylation (50). p53 negatively modulates H2Bub1 expression independently of the role of p53 as a transcription factor, establishing it as a significant epigenetic modulator (51).

As highlighted, these oncogenic drivers have a significant impact on the balance between glycolytic and TCA cycle activity in cancer cells. It is worth reflecting on the fact that in normal prostate cells, both the TCA cycle and OXPHOS are impeded, and there is a net secretion of citrate. By contrast, in PCa, OXPHOS activity is increased in cancer cells in localised disease and a new dynamic exists between cancer cells and the tumour microenvironment. This dynamic entails increased production and turnover of citrate, a reduction in citrate secretion, and lactate exchange between tumour and stromal cells (52). Lactate exchange sustains both catabolism and anabolism and supports OXPHOS activity in cancer cells. As prostate cancer progresses to a treatment-resistant, metastatic state, TCA cycle activity is once again impaired, and cancers develop a Warburg-like metabolism otherwise termed aerobic glycolysis. So far, no single study has evaluated these metabolic states alongside the genomic landscape of tumour cells and other cell types within the tissue (**Figure 1**).

THE CROSSTALK BETWEEN EPIGENOMES AND METABOLISM

We have previously outlined the contribution of a number of transcription factors, including p53, c-Myc, and hypoxia-inducing factor (HIF), to prostate cancer progression acting in part by regulating the expression of metabolic enzymes. Metabolism can, in turn, alter the accessibility of chromatin to these factors supplying or restricting hydrocarbon adducts required for epigenetic alterations, principally consisting of histone modifications and DNA methylation (53) (**Figure 2**). This dynamic relationship may allow cells to rapidly adjust their transcriptional programs in response to treatment or environmental stress and provide the basis for plasticity and the emergence of new cell lineage characteristics in resistant cells (54).

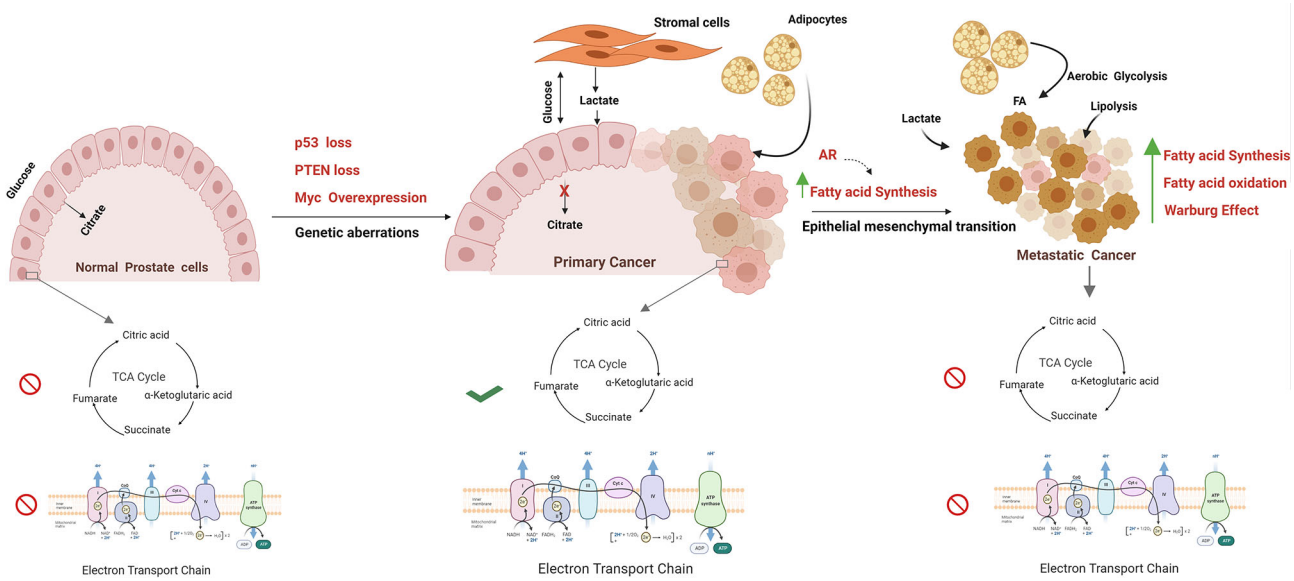

FIGURE 1 | Metabolism alterations in prostate cancer cell. The figure represents the metabolic alterations during prostate cancer metastasis. The normal prostate epithelial cells are characterised with increased release of citrate in seminal fluid, abbreviated Krebs cycle, and reduced oxidative phosphorylation rate. Normal prostate cells undergo transformation due to genetic aberrations; for example, MYC overexpression, PTEN loss, p53 loss, mutations, and other tumor suppressors result in activation of TCA cycle and oxidize citrate and generate Acetyl CoA for lipid biosynthesis. Besides, these fatty acids are fuelled into TCA cycle through lipolysis of adipocytes. Increased Warburg effect is a common feature in metastatic PCa with high lactate secretion, provided by cancer-associated fibroblasts. Progression towards mCRPC is marked by epithelial to mesenchymal transition. In metastatic stage, energy demand is met by both fatty acid oxidation and fatty acid synthesis. The figure has been drawn using Biorender software.

DNA AND HISTONE METHYLATION

DNA methylation involves the addition of a methyl group to the 5′-carbon of cytosine in CpG dinucleotide sequences catalysed by a family of DNA methyltransferases (DNMTs). CpG islands are CpG-rich regions, located proximal to promoter region of genes with high expression. The DNA methylation/demethylation can result in inhibition/activation of transcription of genes, and in prostate cancer, malignant transformation is a common feature as a result of DNA methylation (55). A study in a castration-resistant prostate metastasis exhibited a novel epigenomic subtype associated with hypermethylation and somatic mutations in TET2, DNMT3B, IDH1, and BRAF and also identified differential methylation associated with transcriptional expression of AR, ERG, and Myc oncogenic drivers (56).

In assessing early-stage drivers of prostate cancer based on their incidence, epigenetic alterations, and particularly DNA methylation change, and gene fusions are far more prevalent than somatic point mutations or indeed copy number alterations in most genomic loci (57). For example, GSTP1 promoter hypermethylation is a feature of >60% of localised prostate cancers, and by contrast, TP53 point mutations/copy number deletions are associated with approximately 10% of localised prostate cancers (58–60). Epigenetic changes are also known to be affected by perturbations in metabolic pools and, in the case of methylation, by changes in TCA cycle metabolites and metabolites associated with serine, glycine, and polyamine biosynthesis and the one-carbon cycle (61). In the TCGA prostate cancer dataset, the prostate tumours with the highest genome-wide levels of DNA hypermethylation carry IDH1 point mutations, and this suggests that perturbations in alpha-ketoglutarate and succinate levels associated with these mutations disrupting methylation status by inhibiting TET enzyme activity and the conversion of methyl- to 5-hydroxymethylcytosine marks (62). Ten–eleven translocation (TET) proteins are dioxygenases involved in the regulation of demethylation by oxidizing 5-methylcytosine to 5-hydroxymethylcytosine. Both expression and activity of TET proteins are deregulated in various ranges of cancers including prostate cancer. Mutations in TET2 and reduced TET have been associated with poor prognosis in in prostate cancer (63). The activity of the TET enzyme is regulated by metabolites from TCA cycle and oxygen pool. Dioxygenases utilise a metabolite, 2-oxoglutarate (2-OG), as an essential cofactor that is generated by isocitrate dehydrogenases (IDH).

Isocitrate dehydrogenase, an $NADP^+$-dependent enzyme, which decarboxylates isocitrate to α-ketoglutarate in the TCA cycle, has been found to carry heterozygous mutations in the prostate including other cancers such as acute myeloid leukaemia (AML) (64). Another study by Ghiam et al., using mutational and array comparative genomic hybridization analyses, has identified *IDH1* mutations (R132, R172, or R140 mutations) in localized prostate cancer (PCa) (65). IDH mutations can impair dioxygenase activity by restricting the availability of this cofactor and in turn enhancing the steady-state levels of DNA methylation genome-wide. This DNA hypermethylation

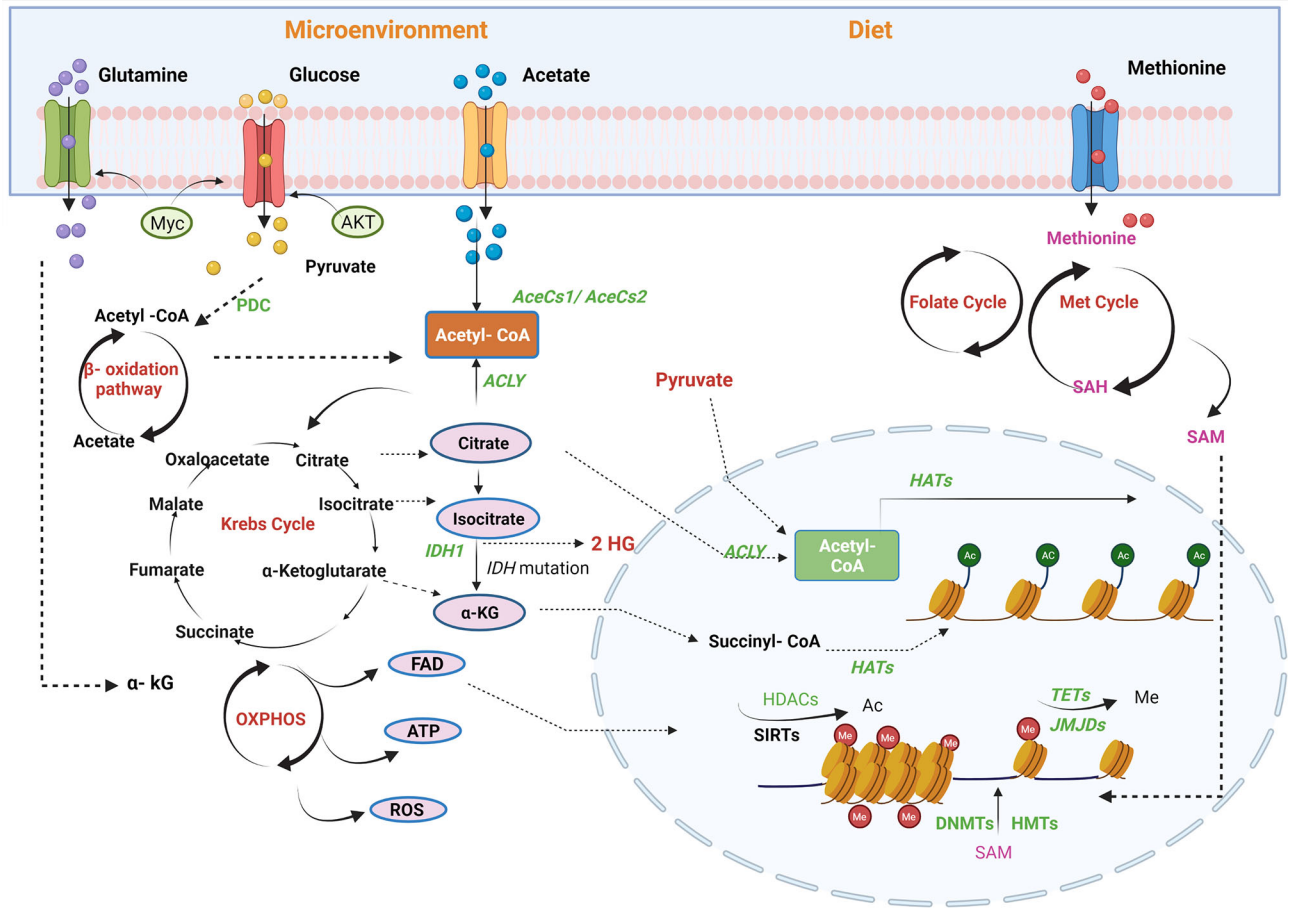

FIGURE 2 | Regulation of PCa epigenome by metabolic pathways. Metabolites are the mediators of epigenetic regulation through mitochondrial and nucleus crosstalk. Different factors contribute to epigenetic regulation, for example, microenvironment and availability of nutrients through diet. Acetyl-CoA, a connecting link between different metabolic pathways, provides acetyl groups in the cell, catalysed by histone acetyltransferases (HATs) for histone modifications. In mitochondria, acetyl-CoA is generated *via* fatty acid oxidation, catalysis of pyruvate-by-pyruvate dehydrogenases complexes (PDC), and by utilising acetate by acyl-CoA synthetase short-chain family member 1/2 (AceSS1/AceSS1). Furthermore, TCA cycle metabolites, pyruvate, citrate, and acetate translocates into nucleus to generate acetyl-Co A pool by pyruvate dehydrogenase complex, ACLY and AceSS2, respectively. Another TCA cycle metabolite, α-ketoglutarate (KG), also translocates into the nucleus and is utilised by histone demethylases [Jumonji C domain-containing (JMJD)] and DNA demethylase [ten–eleven translocation (TET)]. In addition, isocitrate also diffuses into the cytosol and is converted to α-KG by isocitrate dehydrogenase enzyme. FADH2, a by-product of β-oxidation, is oxidised by electron transport chain into flavin adenine dinucleotide (FAD), which translocates to the cytosol or nucleus. The amino acid cycles, namely, methionine and folate, generate an S-adenosyl methionine (SAM), a methyl group donor utilised by DNA methyltransferase (DNMT) and histone methyltransferases (HMTs). Acetyl-CoA, acetyl-coenzyme; ACLY, ATP-citrate lyase; SIRTs, sirtuins; TETs, ten–eleven translocation family; α-KG, alpha-ketoglutarate; HDAC, histone lysine deacetylase; DNMT, DNA methyltransferase; ATP, adenosine triphosphate; FAD, flavin adenine dinucleotide; TCA, tricarboxylic acid; DNMT, DNA methyltransferase; JMJD, Jumonji C domain-containing histone demethylase. The figure has been created with Biorender.com.

phenotype, relative to the cohort as whole, has been observed in the small percentage of prostate cancer TCGA cases carrying IDH mutations (66, 67). A second impact of IDH mutations on DNA methylation has been deciphered through the use of pre-clinical models, which show that these mutations lead to the accumulation of an oncometabolite, R (–)-2-hydroxyglutarate (2HG) (68, 69). *In vitro*, ectopic expression of IDH1 mutants generate high levels of an oncometabolite, (R)-2HG, which perturbs DNA and histone methylation by inhibiting α-ketoglutarate-dependent enzymes including TET dioxygenases and histone demethylases Jumonji 2 (JMJD2) and JMJ C domain-containing histone demethylase-1 (JHDM1) (70–72). In addition, the perturbation in TCA cycle metabolites arising from these mutations also leads to the stabilization of HIF-1α under normoxic conditions and enhanced glycolytic activity (73).

DNA methyltransferase 1 (DNMT1), the methyltransferase enzyme that modulates gene expression by methylating cytosine residues within CpG dinucleotides, regulates DNA methylation and is found to be overexpressed in higher in localized, metastatic, and hormone-resistant PCa compared with benign prostate hyperplasia (BPH) (74–77). In PCa, high DNMT1 expression has been associated with high grade/stage cancers (78).

Overall, although methylation changes are high-incidence events in localised prostate cancer, yet there is limited evidence

to suggest that genome-wide increases in methylation are prognostics. By contrast, chromatin relaxation and increased enhancer activity, associated with histone acetylation, are a feature of castrate-resistant prostate cancer (79).

HISTONE ACETYLATION

Histone acetylation occurs on lysine residues and reflects the balance of activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (80, 81). HATs utilise acetyl-CoA derived from a number of metabolic processes, and consequently, nutrient availability and utilisation can, in principle, affect the steady-state levels of histone acetylation in tumours (82–84). For example, in PTEN-null prostate cancers, nuclear pyruvate dehydrogenase A1 (PDHA1) is a source of acetyl-CoA for histone H3 K27 acetylation and, as consequence, sustains SREBP1 transcriptional activity (85). Pyruvate dehydrogenase complex (PDH) is another example of an enzyme that links glycolysis and the TCA cycle. It converts pyruvate, a glycolytic metabolite to acetyl CoA in the mitochondria (86). The pyruvate dehydrogenase complex (PDC), however, has also been found in the nucleus in prostate cancer (87). Mitochondrial PDH regulates the availability of citrate in mitochondria for lipid biosynthesis, whereas nuclear regulates expression of sterol regulatory element-binding transcription factor (SREBF)-target genes by mediating histone acetylation. In addition, an amplified expression of PDHA1, both at protein and gene level, have been reported in prostate tumours (87). *PDHA1 gene* knockout in prostate cancer cells developed alterations in tumor cell metabolism with an increase in expression of glutaminase1 (GLS1) and glutamate dehydrogenase1 (GLUD1), leading to an increase in glutamine-dependent cell survival (88). All these outcomes indicate that PDH supports prostate tumorigenesis not only by regulating lipid biosynthesis but also by utilising alternate metabolic pathways for cell survival.

Altered acetyl-CoA levels significantly affect the substrate specificity of CBP and p300 acetyltransferases. For example, at a low concentration of acetyl CoA, p300 has the highest specificity for histone H4K16, for which specificity is 10^{18}-fold higher than CBP (89). The acetyl-CoA-producing enzyme ATP-citrate lyase (ACLY) regulates histone acetylation levels in a nutrient-dependent manner in cells (36, 89). The location of ACLY in both nucleus and cytosol further suggests that it plays a role in both histone acetylation and lipid biosynthesis (90). In a limited nutrient environment (low glucose levels), cancer cells can still modulate and increase acetyl CoA pool by AKT (S473)-mediated ACLY phosphorylation and upregulates histone acetylation marks in prostate tumors (91).

Whilst a number of recent pre-clinical molecular studies have highlighted important crosstalk between acetyl-CoA production and histone acetylation, contributing tumorigenesis, nothing similar has yet been possible in the study of clinical disease. This in part is due to the dynamic changes that occur in the metabolic states of tumours, which makes it technically very challenging to generate robust high-throughput metabolomic data on a similar scale and resolution to genomic data (refer to *Future Perspectives* for more discussion). Given the significant progress that has been made in developing epigenetic drugs as cancer therapeutics, it is of course vital to learn more about this interplay because greater functional and clinical understanding could support ultimately the use of metabolic drugs as sensitising agents. Prostate cancers are also susceptible to inhibitors of fatty acid oxidation, a prominent source of acetyl CoA, and examples include etomoxir and perhexiline (92). These are examples of co-dependencies between metabolic and epigenetic activities that maybe amenable to combinatorial treatments if patient stratification is possible. If we consider mitochondrial activity as a primary determinant of the availability of acetyl-CoA within cancer cells, then what is the evidence that mitochondrial mutations exist within tumour cells and also have an impact on the epigenome?

CROSSTALK BETWEEN MITOCHONDRIAL ACTIVITY AND THE EPIGENOME

Unlike many cancer types, OXPHOS activity is enhanced in the transition from benign/untransformed tissue to cancer in the prostate gland—as discussed above. Given the prominent role that mitochondria play in the turnover of acetyl-CoA, this change might be expected to correlate with increased acetylation. Thus far, no translational studies have attempted to assess the relationship between mitochondrial activity and histone acetylation or indeed chromatin relaxation. A number of studies have, however, shown that mitochondrial mutations accumulate during prostate cancer progression.

The human mitochondrial DNA encodes 13 polypeptides crucial for oxidative phosphorylation, 22 transfer RNA molecules, and 2 ribosomal RNA molecules essential for mitochondrial translational machinery, and the rest is encoded by nuclear genome (93–95). This coordinated expression of subunits of mitochondrial proteins and replication machinery through mitochondrial and nuclear genes is regulated by a bidirectional flow of intermediates (metabolites) and polypeptides including enzymes and is the key of co-regulated biologies of nuclear and mitochondrial processes (96). In addition to somatic mutations in nuclear genome, the mitochondrial genome shows a 55-fold higher incidence of mutation rate in comparison to nuclear genome in PCa (97). A sequencing study identified mutational hotspots in the mitochondrial genomes of 384 prostate cancer and went on to associated mitochondrial mutational burden with Myc amplification and disease recurrence in a subgroup of poor-prognosis patients (98). The functional basis for this relationship remains undefined; however, preclinically researchers have been able to deplete prostate cancer cells of mitochondrial DNA using sub-toxic doses of DNA-damaging agents, creating so-called rho-null derivatives. These depleted cell lines have reduced levels of histone acetylation (principally histone H3K9, H3K18, and H3K27), which suggests that mitochondrial content could affect the epigenetic landscape of tumours (99). In other studies, focussing on the impact of mitochondrial mutations on the tumorigenic potential of prostate cancer cells, it has been possible to use rho-null

derivatives as acceptor lines in cell fusion experiments to re-complement the cells with mutated mitochondrial genomes. Since the acceptor and wild-type lines remain isogenic in their autosomal genomes, the enhanced metastatic potential of the resultant cybrids has been attributed to the mutations present in the mitochondrial genome. Using this principle, Petros et al. generated cybrids in PC3 cells with mitochondrial DNA (mtDNA) ATP6 T8993G mutations and engrafted them into immune-compromised mice, resulting in enhanced tumorigenesis compared to wild-type cells and increased production of reactive oxygene species (100). Whilst these distinct studies highlight the impact of mitochondrial activity on the epigenome and of mitochondrial mutations on tumorigenesis, no signal study has related these mechanistically using the models available.

OGLCNACYLATION: A RHEOSTAT CONNECTING METABOLIC DYSREGULATION TO TRANSCRIPTION

Histone acetylation and the OGlcNAcylation of chromatin are significant features of enhancers in the prostate cancer genome (101). Histone acetylation has been associated with increased lipid turnover under the influence of a number of the genomic drivers of prostate cancer that were discussed earlier, and particularly with PTEN-loss and Myc overexpression (28). OGlcNAcylation at enhancer sites occupied by c-Myc suggests that these metabolite-dependent modifications may sustain oncogenic activity in a feed-forward manner—transcriptionally driven metabolic dysregulation supporting oncogenic transcriptional activity (**Figure 3**). OGlcNAcylation, as a post-translational modification sustained by a metabolic adduct (UDP-GlcNAc), is an abundant feature of cancer cells and is, unlike other post-translational modifications, catalysed by a single enzyme, OGlcNAc transferase (OGT) (102, 103). UDP-GlcNAc is synthesised by the hexosamine biosynthesis pathway and utilised both by OGT and by enzymes in the endoplasmic reticulum for the N-linked glycosylation and proper folding of newly synthesised proteins. Consequently, whilst enzymes in the hexosamine biosynthesis pathway are AR dependent, and many are overexpressed in prostate cancer, there is not necessarily a direct relationship between their activity and the OGlcNAcylation status of OGT substrates (104). Indeed, recent studies indicate that hexosamine biosynthesis itself may restrain the emergence of castrate-resistant prostate cancer (105). OGT activity itself, however, can support cancer progression, and the challenge is in determining the nature of the substrates, and biological processes are the primary mediators of this crosstalk. There are a number of biologically compelling candidates including c-Myc, FOXM1, and HIF1a, all of which are known to be OGlcNAcylated and more active due to OGT function (106). There are also broader impacts of OGT activity; at the chromatin level, it is known to modify histones, and transcriptionally, it is known to regulate RNA polymerase II activation and processivity working in concert with cyclin-dependent kinases such as CDK7 and CDK9 (107). By implication, OGT activity reflects a nutrient-replete/"fed" state sufficient to permit glucose, glutamine, UTP, and acetyl-CoA to be used to provide adequate UDP-GlcNAc as a substrate for OGT (108). However, that alone is unlikely to provide an understanding of crosstalk; we need to establish more clearly how OGT selects protein substrates, and we also need to account for a second enzymatic activity ascribed to OGT, its proteolytic function. A recent study has indicated that in some cell types, OGT can sustain cell proliferation through a non-catalytic function that needs to be fully characterised (109). This in turn also needs to be put into a spatial context, since OGT can function as different isoforms in distinct organelles within the cell, mitochondria, and nuclei being principal examples. That final aspect is of course reminiscent of TCA cycle enzymes such as PDHA1 and ATP-citrate lyase as previously described in the context of acetylation.

Given that a limited number of metabolic pathways may be the mediators of feedback effects of oncogenes and tumour suppressors on chromatin/the epigenome, how do we use this knowledge to benefit patients? To achieve this, we arguably need to fill a knowledge gap in our ability to identify metabolites and understand how changes in metabolic activity occur spatially within tumours.

FUTURE PERSPECTIVES

As outlined in this article, there are significant examples of crosstalk between metabolic pathways and other cancer drivers; it remains challenging to prove that this crosstalk plays a causative role in prostate cancer progression. This is because changes in metabolic activity will inevitably impact on the redox state of the cell and the availability of metabolites for anabolic metabolism and sustain transcription and DNA replication. In addition, most metabolic processes are also contributors to the functions of untransformed cells in immune system and tumour micro-environment and are affected by the availability of nutrients and by crosstalk between ranges of cell types. In the big picture, how do we achieve cancer selectivity in modelling this interplay and in targeting this crosstalk?

First, an important factor to address is our lack of knowledge of the metabolome. As it stands, a metabolomics study can only identify maximally approximately 10% of the metabolite signals that are measurable using mass spectroscopy or other methods. This means that our understanding of the activity of mitochondria and metabolic pathways in cancer cells is constrained by our capacity to identify novel metabolites ("oncometabolites") in a sensitive and unbiased manner. This missing information is by contrast increasingly an alien concept in the field of cancer genomics due to the capacity to sequence at high scale and decode genomic data. This has led, for example, to the discovery of highly cancer-specific non-coding transcripts and somatic DNA mutations; we have no equivalent signatures thus far in the cancer metabolome. Addressing this point is predominantly a technical challenge.

Second, clinical disease is by definition molecular heterogeneous. We know that this is true when we assess bulk sequencing data and look for high incidence mutations in

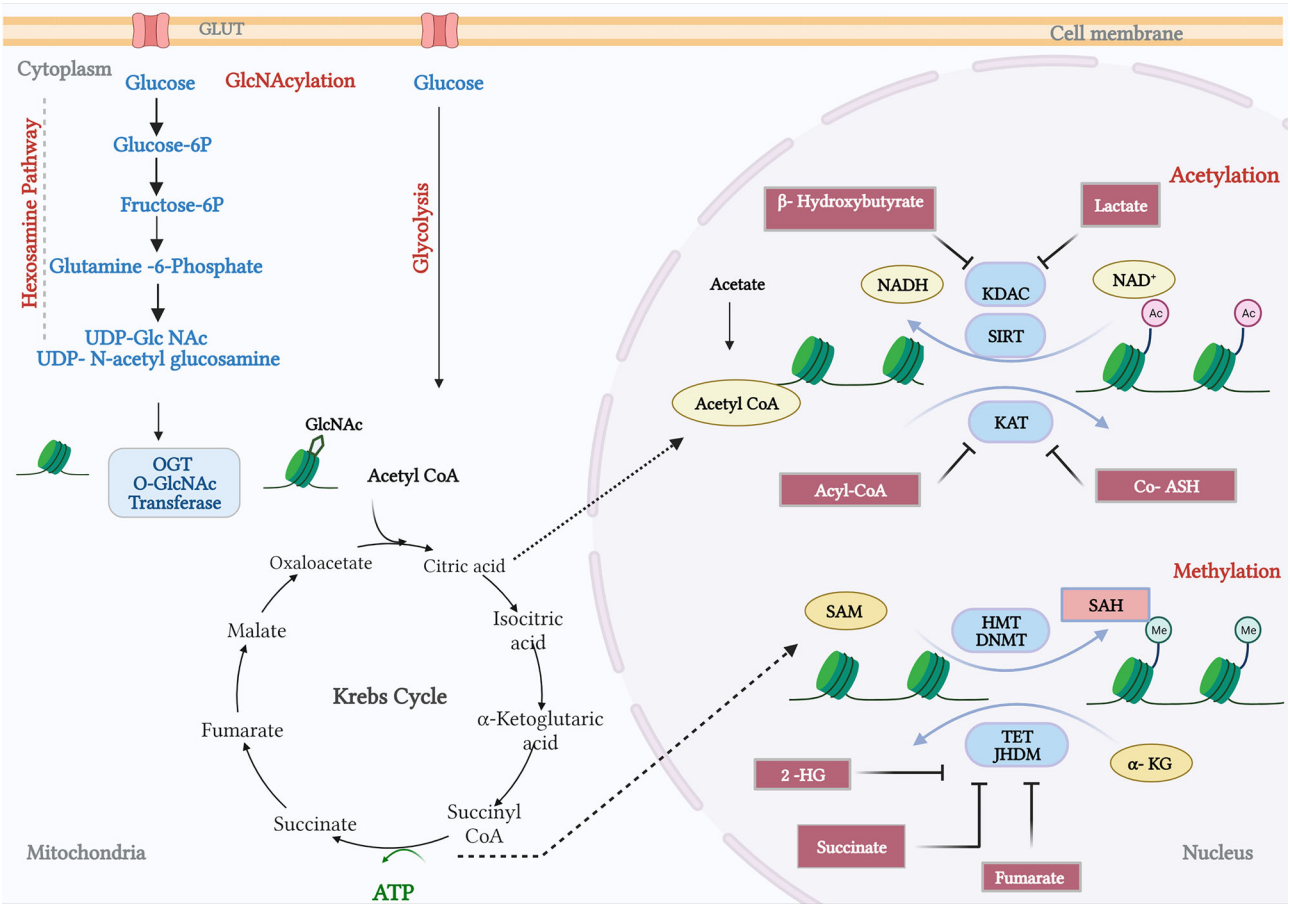

FIGURE 3 | Crosstalk between metabolism and prostate cancer genomics. The hexosamine pathway catalyses the conversion of glucose and glutamine to provide a metabolite UDP-glucosamine (UDP-GlcNAc). The O-GlcNAc transferase (OGT) facilitates the N-acetylglucosamination (GlcNAcylation) or Glycation process. In addition, the Krebs cycle fuels metabolites private and citrate, which gets converted by acyl-COA synthetase and pyruvate dehydrogenase. Products from fatty acid oxidation, fatty Acyl CoA, inhibit the activity of lysine acetyltransferases (KAT). KAT reactions also release CoA-SH, which acts as an inhibitor. B-Hydroxybutyrate, a ketone from FA, and lactate, a by-product of glycolysis, have been shown to inhibit lysine deacetylase (KDAC). Amino acid metabolism and TCA cycle fuels in methionine and ATP, respectively, and synthesizes S-adenosylmethionine (SAM). Histone methyl transferases and DNA methyl transferases catalyse SAM to S-adenosylhomocysteine (SAH), which in turn can inhibit DNMTs and HMTs. TCA cycle intermediates, succinate, fumarate, α-ketoglutarate (α-KG), and 2-hydroxyglutarate (2-HG) acts as inhibitors of TET demethylases and Jumonji-C (JMJC) domain-containing histone demethylases (JHDMs). The figure has been adapted and modified from the review (101).

multi-focal tumour samples from a single patient. However, recently, it has been possible to combine spatial information including pathology and genomic data using new platforms that permit RNA extraction, library preparation, and sequencing on a solid-phase surface/glass slide. This spatial dimension permits molecular information to be mapped onto distinct cell sub-populations and interpreted more readily in the context of associations between cell types at tumour–stromal interface and elsewhere. As a consequence, spatially resolved transcriptomics was declared to be the Method of the Year for 2020 by Nature Methods (110). Equivalent spatial resolution of those metabolic signals that we can attribute to known metabolites would provide great insights into cell–cell crosstalk in prostate cancers. This is important to test hypotheses, for example, around the compartmentalisation of metabolism in cancers, such as the idea of a lactate shuttle between cancer and stromal cells and compartmentalisation of glycolysis and oxidative phosphorylation between distinct cell types that communicate with each other in tumours (111). Significant progress is being made in developing single-cell spatial and *in situ* methods for the sensitive detection of well-known metabolites, and mass spectrometry imaging is showing promise in defining more complex and accurate metabolic classifiers of disease (112). However, there is a need for new devices/biomedical engineering to capture metabolic information in real time in an operating theatre, as the signals can be significantly affected by environmental factors. As matter of concern, the metabolomic signals are dynamic/unstable, and sequencing data (DNA) is stable, and technology needs to address those differences (113–117). With the vast increase in

prostate cancer genomic data and other data types (clinical, pathological, imaging, metabolomics, and proteomics), there is a significant challenge in assimilating, refining, and deciphering biologically informative signals that reflect crosstalk. Machine-learning algorithms and artificial intelligence promise to alleviate this issue, since they are, in many cases, data-type agnostic. Their impact is exemplified in the sphere of medical imaging. Digital pathology, aided by artificial Intelligence (AI), has decoded large datasets to improve the reliability of diagnostic pathology and improve the prediction of treatment outcome and patient survival (118). Additionally, multiparametric MRI has significantly improved sampling of clinical significant prostate cancers at biopsy, and improvements in both the scanners and analytical approaches employed on the resultant data will further enhance and standardise this work across clinical centres (119). We can anticipate a future in which spatial genomic and metabolomic data are aligned to radiomic features and imaging to risk stratify patients and simultaneously inform treatment selection (119).

AUTHOR CONTRIBUTIONS

RS wrote the manuscript and made the figures. IM discussed the themes and provided advice during the drafting process. All authors contributed to the article and approved the submitted version.

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REVIEW
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Personalized Medicine for Prostate Cancer: Is Targeting Metabolism a Reality?

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Prostate cancer invokes major shifts in gene transcription and metabolic signaling to mediate alterations in nutrient acquisition and metabolic substrate selection when compared to normal tissues. Exploiting such metabolic reprogramming is proposed to enable the development of targeted therapies for prostate cancer, yet there are several challenges to overcome before this becomes a reality. Herein, we outline the role of several nutrients known to contribute to prostate tumorigenesis, including fatty acids, glucose, lactate and glutamine, and discuss the major factors contributing to variability in prostate cancer metabolism, including cellular heterogeneity, genetic drivers and mutations, as well as complexity in the tumor microenvironment. The review draws from original studies employing immortalized prostate cancer cells, as well as more complex experimental models, including animals and humans, that more accurately reflect the complexity of the *in vivo* tumor microenvironment. In synthesizing this information, we consider the feasibility and potential limitations of implementing metabolic therapies for prostate cancer management.

Keywords: prostate neoplasia, lipid metabolism, obesity, metabolism, patient-derived xenograft, metabolic targeting, metabolic heterogeneity

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INTRODUCTION

Urological cancers accounted for 13.1% of 19.3 million new cancer incidence worldwide in 2020 (1). Prostate cancer is the most commonly diagnosed urologic cancer, followed by bladder, kidney, testis, and penile cancers (1) and frequently occurs in men over 65 years of age (2). More than 80% of men are diagnosed with localized disease, and the majority of these patients will have indolent tumors that are slow to progress, with low risk of experiencing prostate cancer-specific death (3). For these men, active surveillance, curative intent surgery or radiotherapy, are mostly effective with 10-year disease-specific survival rate of >90% (4). However, approximately one third of patients will experience disease progression and develop metastases, most commonly to bone, but also to other soft tissues such as liver and lung (5, 6). For these men, androgen deprivation therapy (ADT) is standard of care and while initially effective at reducing tumor burden, residual cancer cells adapt to low systemic androgen levels and therapy resistant metastatic castrate-resistant prostate cancer (mCRPC) develops, where

tumorigenesis is driven by adaptive androgen receptor (AR) changes and intra-tumoral steroid biosynthesis (7). There are limited therapeutic options in managing this advanced stage disease, necessitating the development of novel targeted therapies and/or neo-adjuvant therapies that either prevent progression or treat mCPRC (**Figure 1**).

The hallmarks of cancer, proposed by Hanahan and Weinberg (8), comprise a series of biological capabilities acquired during the multistep development of human tumors, of which 'deregulated cellular energetics' is one. Cancer invokes an increase in energy production to sustain proliferation, and metabolic 'rewiring' is often invoked to maintain this requirement. Alterations in metabolic reprogramming include adaptation in nutrient acquisition, preferential utilization of substrates, and transcriptional changes that alter intracellular metabolic signaling pathways. Exploiting such metabolic reprogramming is proposed to enable the development of targeted therapies in cancers (9), leading to an explosion of interest in the field of cancer metabolism.

Metabolic inhibitors have been used for cancer therapies for many years, including the anti-metabolite class of chemotherapy (10, 11), and other agents have been developed for the treatment of advanced breast cancer, colorectal cancer, and hematological malignancies (12). This firmly establishes the principle that metabolic vulnerabilities can be effectively targeted for cancer treatment. However, to date, there are no metabolic inhibitors approved for use in prostate cancer, which we posit is due to a knowledge gap in understanding the molecular and cellular reprogramming and associated changes in substrate utilization in human tumors, and the marked heterogeneity of this disease.

Herein, we will discuss how metabolism is reprogrammed in prostate cancer, in both localized and mCRPC, which likely have different metabolic needs. We will focus on literature employing studies in immortalized prostate cancer cells and expand to more complex environments, including animal models and human studies. We will then outline the factors contributing to variability in prostate cancer metabolism, including genetic drivers and alterations in the tumor microenvironment (TME), and lastly discuss the feasibility of metabolic targeting in patients and potential limitations in prostate cancer management.

PROSTATE CANCER METABOLISM

The prostate gland secretes large amounts of citrate (~1000-fold than blood plasma) as the major constituent of prostatic fluid (13). The accumulation of zinc within the prostate gland by ZIP1 (SLC39A1) competitively inhibits mitochondrial aconitase (ACO2) activity, which hinders citrate oxidation and Tricarboxylic Acid (TCA) cycle flux (14–16). Hence, unlike other well-differentiated tissues, which rely on oxidative

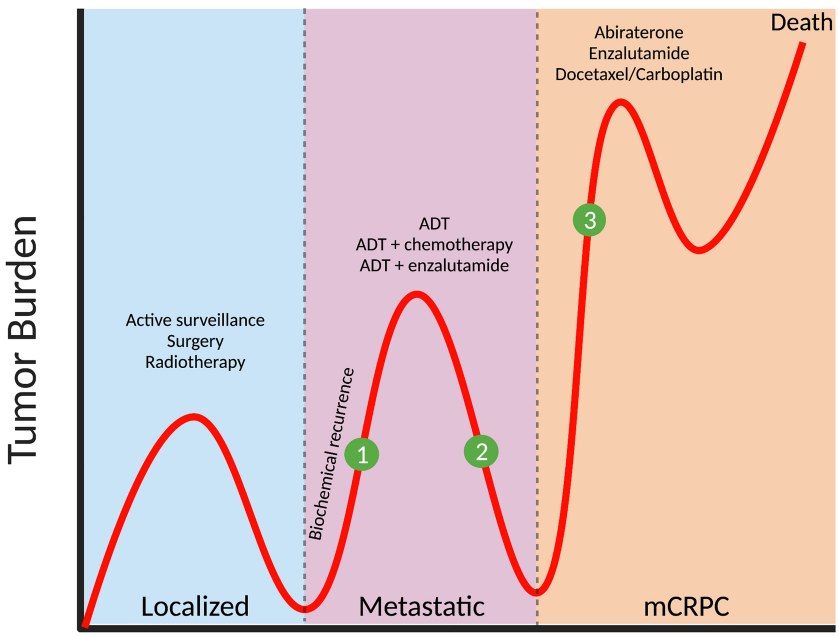

FIGURE 1 | Prostate cancer progression and potential stages for intervention with metabolic therapies. The majority of patients (>80%) are diagnosed with localized prostate cancer, with treatment including active surveillance (for low-risk tumors), or surgery/radiotherapy (for intermediate- and high-risk tumors). In one third of patients, biochemical recurrence (defined as a rise in prostate specific antigen, PSA, and indicative of active tumor growth) occurs and metastases develop at distant organs, and androgen-deprivation therapy (ADT) is administered. While initially effective, tumors eventually progress to metastatic castrate-resistant prostate cancer (mCRPC) and treatments include abiraterone, enzalutamide and chemotherapy such as docetaxel or carboplatin. Clinical intervention with relevant metabolic inhibitors, that are designed to slow tumor growth, could be applied (1) at the time of biochemical recurrence, thereby delaying the need for ADT, (2) in combination with ADT to target metabolic vulnerabilities induced by androgen withdrawal or (3) to treat mCRPC in combination with, or after existing therapies.

phosphorylation to produce ATP, normal prostate epithelium depends on aerobic glycolysis with glucose and aspartate as the primary carbon donors (17). In malignant prostate tissues, ZIP1 expression and citrate production are decreased, while ACO2 expression is increased, converting prostate cells from citrate-producing to a citrate-oxidizing phenotype (18–22). These changes enhance the capacity for energy production to support proliferation and metastasis, and provide evidence that metabolic adaptation occurs in prostate cancer (**Figure 2**).

Glucose

Glucose is a primary substrate for most cells. Glucose is transported into cells and undergoes glycolysis, resulting in the production of pyruvate. A proportion of pyruvate undergoes reduction to lactate, but the majority enters the mitochondria for further processing in the TCA cycle for eventual oxidative phosphorylation, which enables energy production. The glycolytic intermediates generated in the breakdown of glucose are also used for nucleotide, amino acid, and lipid biosynthesis (23). In contrast, most cancer cells utilize glucose differently, producing lactate at high rates despite the presence of oxygen in a phenomenon termed Warburg metabolism or aerobic glycolysis (24).

Glucose utilization in increased in prostate cancer compared to normal tissues, and actively contributes to the growth of prostate cancer cells. Treatment of AR-positive LNCaP prostate cancer cells with the synthetic steroid R1881 induced transcriptional upregulation of glucose transporters (GLUT1, GLUT12) and glycolytic enzymes (HK1/2, and PFKB2), increased glucose uptake, glucose entry into glycolysis, and glucose storage into lipids (*de novo lipogenesis*) (25–27). Meanwhile, studies conducted in AR-negative cells (PC3 and DU145) reported higher glucose uptake and increased lactate production compared to AR-positive cells (LNCaP and 22Rv1) (28–30). These observations indicate that AR signaling promotes the entry of glucose-derived pyruvate into the TCA cycle for eventual complete oxidation, while aerobic glycolysis is increased in the absence of AR signaling in immortalized prostate cancer cells.

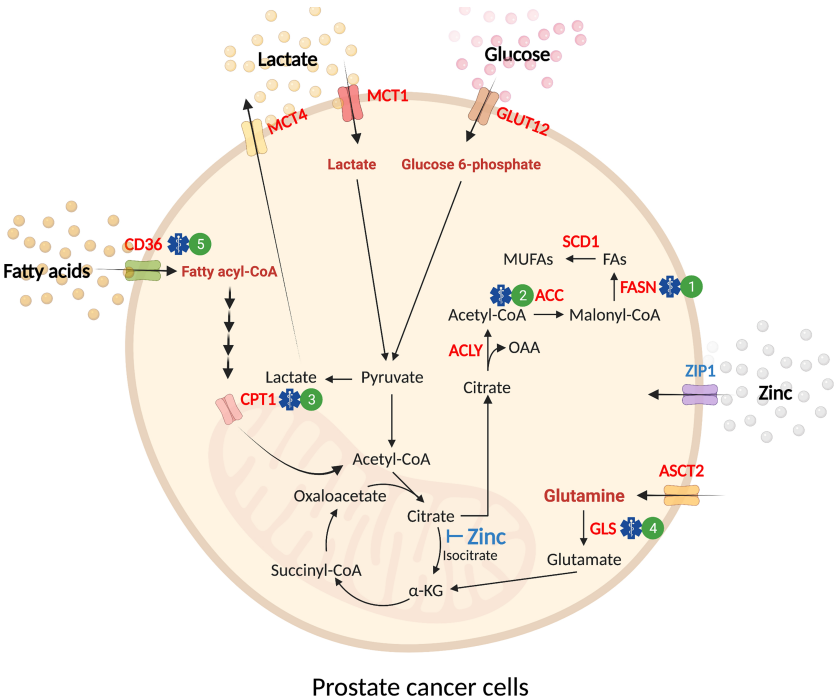

FIGURE 2 | Substrate utilization in prostate cancer. Normal prostate epithelial cells exhibit a glycolytic phenotype due to the inhibitory effect of mitochondrial zinc accumulation in the TCA cycle (*blue text*). Malignant transformation of prostate epithelial cells leads to an increase in the uptake of exogenous nutrients (glucose, glutamine, fatty acids, and lactate) and *de novo* synthesis of lipids (*red text*). These substrates are utilized for energy production in the mitochondria to accommodate increasing energy demands in malignancy. In prostate cancer, glucose uptake is mediated by GLUT12 before it is catabolized into pyruvate. While a proportion of glucose-derived pyruvate enters the TCA cycle for oxidation, a fraction of pyruvate is reduced to lactate and transported out of the cell by MCT4. The influx of extracellular lactate is mediated by MCT1. In mitochondria, the outflow of citrate to cytosol provides substrate for *de novo* synthesis of fatty acids (i.e. lipogenesis). ASCT2 supplies exogenous glutamine as a fuel source through deamination by glutaminase (GLS) before further conversion into α-KG to feed the TCA cycle. Fatty acid uptake is mediated by fatty acid translocase (FAT)/CD36 before transport into the mitochondria by CPT1. In mitochondria, fatty acids undergo β-oxidation, producing acetyl-CoA that feeds into the TCA cycle. Pre-clinical treatments for prostate cancer are denoted by a blue symbol and corresponding number. 1) FASN (*e.g.* TVB-2640, IPI-9119); 2) ACC (*e.g.* Firsocostat, PF-05175157); 3) CPT1 (*e.g.* Perhexiline); 4) GLS (*e.g.* CB-839); 5) CD36 (*e.g.* agents in development). α-KG, α-ketoglutarate; CPT1, carnitine palmitoyltransferase 1; FAs, fatty acids; GLS, glutaminase; MCT1, monocarboxylate transporter 1; MCT4, monocarboxylate transporter 4; MUFAs, monounsaturated fatty acids; OAA, oxaloacetate.

Enhanced aerobic glycolysis in response to androgen withdrawal is also observed in *in vivo* models. A metabolomic screen of an orthotopic xenograft model of TRAMP-C1 prostate cancer demonstrated increased glycolysis in tumors following androgen deprivation (31) while, *in vivo* and *ex vivo* metabolic imaging using hyperpolarized 1-[^{13}C]pyruvate in TRAMP tumors also points towards elevated glycolysis and higher lactate dehydrogenase (LDH) activity in the castrate setting (30).

Evidence from human studies similarly show different glucose utilization across the spectrum of prostate cancer, which is best illustrated by ^{18}F-fluorodeoxyglucose (^{18}F-FDG) cancer diagnostic imaging in patients. ^{18}F-FDG is taken up by tissues and 'trapped', and its accumulation is reflective of the tissues glycolytic activity (32). Notably, the diagnostic utility of ^{18}F-FDG imaging is limited to localized high-risk tumors and metastatic disease, indicating increased glucose uptake in rapidly growing malignant tissues and not indolent localized disease [*as reviewed in* (33)]. In addition, proteins that regulate glucose metabolism were increased in both localized and metastatic lesions of prostate cancer, including HIF-1α, GLUT1, HK2, PFKFB3, PFKFB4, PKM2, PDK1 (29, 34–37). Functional analysis of glucose metabolism showed increased *de novo* lipogenesis in localized prostate cancer tissues compared to patient-matched benign tissues (38); however, this was not accompanied by increased glucose oxidation, indicating that much of the additional citrate produced in the TCA cycle is exported into the cytosol for lipogenesis (38).

Lactate is produced *via* the reduction of pyruvate and is classically viewed as the by-product of excess glycolysis; however, it is becoming increasingly recognized as an important mediator of tumorigenesis in some cancers. Lactate is used to fuel the TCA cycle in some malignancies (*e.g.*, non-small cell lung cancer) (39) and inhibiting lactate influx into cells through the monocarboxylate transporter 1 (MCT1) reduces the metastatic potential of melanoma (40). Serum lactate dehydrogenase (LDH) is often increased in patients with high-grade prostate cancer and is associated with increased risk of mortality and disease progression in patients with metastatic prostate cancer (41, 42). Consistent with these observations, clinical studies utilizing hyperpolarized ^{13}C-pyruvate imaging reported a positive correlation between prostate cancer Gleason grade and the conversion of pyruvate to lactate (43). Interestingly, monocarboxylate transporter 4 (MCT4), the protein responsible for lactate efflux from cells, is increased in localized and metastatic tumors (29) and RNAi-mediated silencing of MCT1/4 in prostate cancer cells decreased cell growth (44), suggesting lactate production and its intracellular utilization are important for tumorigenesis. A more comprehensive investigation of lactate metabolism in prostate cancer is clearly warranted. Finally, the pentose phosphate pathway (PPP) is a glucose catabolic pathway that appears to be important in prostate tumor growth in AR/SREBP/6PGD-dependent manner (45). However, whether PPP plays a significant role in prostate cancer by generating nucleotide precursor or sustaining the NADPH pool for lipogenesis and redox homeostasis is yet to be elucidated.

Glutamine

Glutamine is a nonessential amino acid and the most abundant amino acid in the circulation (~500 μM). Glutamine functions as a carbon donor for lipogenesis *via* reductive carboxylation, a nitrogen donor for non-essential amino acid production and nucleotide biosynthesis (46), and as a fuel source (47–51). Glutamine anaplerosis starts with glutamine conversion into glutamate by glutaminase (GLS) then further conversion into α-ketoglutarate (AKG) to feed the TCA cycle by the actions of glutamate dehydrogenase (GLUD) and several transaminases, including glutamate–oxaloacetate transaminase (GOT), glutamate–pyruvate transaminase (GPT), and phosphoserine transaminase (PSAT) (46). While fourteen proteins are known to transport extracellular glutamine into cells, SLC1A5/ASCT2 is thought to be the major transporter, and its expression is upregulated in various cancers (52, 53). Glutamine can also donate its alpha nitrogen to serine, glycine, alanine, or aspartate following deamidation to glutamate (54). Serine feeds into one-carbon metabolism, which centrally integrates many pathways that are dysregulated within prostate cancer, strengthening the argument for targeting glutamine metabolism (55, 56). Additionally, enhanced aspartate metabolism has been implicated with epithelial to mesenchymal transition while increased levels of alanine has been identified within prostate cancer biopsies (57, 58).

Several lines of evidence demonstrate an important role for glutamine in prostate cancer growth and progression. ASCT2 is expressed in prostate cancer cells (*e.g.*, LNCaP, VCaP, PC3, and DU145) (53, 59, 60) and approaches that reduce ASCT2 expression/function suppress glutamine uptake and hamper cell proliferation (53, 59). In a similar manner, GLS expression is higher in prostate cancer cells (e.g., LNCaP, 22Rv1, DU145, and PC-3) as compared with non-malignant prostate epithelial cells (e.g., RWPE-1) (60–62), and selective inhibition of GLS reduced proliferation and survival (60–63).

Key findings in cultured cells have been recapitulated in mouse models. ASCT2 mRNA expression is decreased upon castration and increased in CRPC (59) and knockdown of ASCT2 suppresses growth and metastatic burden in PC3 xenografts in mice (59), although rates of glutamine uptake and downstream metabolism were not assessed in this study. GLS expression is increased post-castration in LNCaP and LAPC4 xenografts (61), and pharmacological inhibition of GLS1 reduces the tumor burden in PC3, but not LNCaP xenografts (61), highlighting the dependency on glutamine metabolism in AR-negative, hormone-insensitive prostate cancer (61). Consistent with this notion, analysis of TRAMP tumors utilizing [U-^{13}C] glutamine metabolic tracing reported upregulation of glutaminolysis to replenish TCA cycle intermediates and upregulation of GLS1 activity in castrate-resistant compared to androgen-dependent tumors (30).

The importance of glutamine metabolism in human prostate cancer is unknown. ASCT2 and GLS1 mRNA expression is high in human prostate cancer (59, 63, 64) and ASCT2 expression is significantly associated with shorter time to biochemical recurrence in recurrent prostate cancer (64). Temporal ASCT2 expression is also observed in human tumors, with decreased expression upon ADT treatment (1-6 months and 7-12 months)

and increased expression in recurrent tumors (59). In addition, expression of the GLS1 enzyme undergoes a shift in isoform from kidney-type glutaminase (KGA) to the more active isoform, glutaminase C (GAC). This shift occurs progressively from localized to mCPRC and neuroendocrine prostate cancer (NEPC) (61). While these observations signal an important role for glutamine metabolism in advanced stages of prostate cancer (*i.e.*, mCRPC and NEPC), studies evaluating glutamine uptake, glutaminolysis and ATP production in human prostate cancer are clearly needed.

Fatty Acids

Fatty acids are essential for the generation of structural cell membranes, energy production, and cellular signaling. Fatty acids are derived from adipose tissue lipolysis or from triglycerides stored in chylomicrons and very-low density lipoproteins, where they are transported from the circulation into cells. Several cell types, most notably hepatocytes and adipocytes, are capable of synthesizing fatty acids using other substrates, such as glucose and acetate, through a process called *de novo lipogenesis*. Fatty acids are the dominant metabolic substrate in most tissues where they undergo mitochondrial β-oxidation to generate acetyl-CoA, which feeds into the TCA cycle and oxidative metabolism.

Emerging evidence demonstrates an important role for fatty acid metabolism in prostate cancer. Fatty acid uptake is increased in immortalized prostate cancer cells (38, 65), which is often accompanied with increased energy production from fatty acid oxidation (65, 66). Treatment of prostate cancer cells with etomoxir, an inhibitor of fatty acid oxidation, reduces cell viability and proliferation, reinforcing the importance of this metabolic substrate for cancer progression (65, 67). Aside from the direct energy-generating mitochondrial fatty acid oxidation, peroxisomal fatty acid oxidation also supports prostate cancer growth (68, 69).

As mentioned above, prostate cancer is exceedingly lipogenic, highlighted by accelerated *de novo* synthesis of fatty acids driven by enhanced activity of sterol regulatory element-binding protein (SREBP) (70, 71), which induces the transcription of many genes involved in lipid metabolism, including ACLY, ACACA, FASN, SCD1 and LDLR (72). Studies employing pharmacological and genetic manipulation of key regulatory enzymes of lipid metabolism in immortalized cell lines and xenografts have demonstrated the importance of several lipid metabolism pathways in prostate cancer progression including increased *de novo* lipogenesis (i.e., *via* ACLY, ACC and FASN inhibition) (73–76), triacylglycerol storage (DGAT1) (77), cholesterol metabolism (SOAT1, HMGCS1, HMGCR, and SCARB1) (78–80), lipolysis (MAGL) (81), and fatty acid elongation (ELOVL5 and ELOVL7) (82, 83). Similarly, 2,4-dienoyl-CoA reductase (DECR1) and enoyl-CoA delta isomerase 2 (ECI2), auxiliary enzymes responsible for the degradation of unsaturated fatty acids, are also essential for prostate cancer growth and therapy resistance (84–86). Finally, studies using tandem mass spectrometry lipidomics have reported marked alterations in the prostate lipidome with cancer (38, 82, 87, 88), indicating the likelihood that other nodes of lipid metabolism are regulated in prostate cancer development and metastasis.

While studies in cells and mice provide a reasonably compelling narrative that distinguishes lipid metabolism as a hallmark of prostate cancer, studies in primary human tissue are limited. Our team recently performed functional metabolic analysis in freshly procured human prostate tissue. Fatty acid uptake, fatty acid storage into complex lipids and cellular membranes, and *de novo* lipogenesis were upregulated in malignant compared to benign prostate tissues (38). Further studies identified fatty acid translocase (FAT/CD36) as a key fatty acid transport protein in prostate cancer while inhibition of FAT/CD36 with a monoclonal antibody attenuated tumor growth in a prostate patient-derived xenograft (PDX) and PDX-derived organoids. While this study identified a role for altered lipid metabolism in localized disease, further studies are required to ascertain whether these, and other changes in lipid metabolism, occur in metastatic disease. Additionally, whether there are further alterations in fatty acid utilization in the setting of mCRPC, where AR activity is amplified, is yet to be determined. In this context, a recent study employing transcriptomics and proteomics in prostate cancer cell lines and patient samples identified several lipid-mediated transporters and increased rates of fatty acid, cholesterol, and low-density lipoprotein uptake with androgen stimulation (89). Hence, any potential therapeutic benefit is likely to require cotargeting of lipid supply.

Efforts to elucidate the metabolic landscape of prostate cancer have highlighted the importance of glucose, glutamine, and fatty acid in prostate cancer growth and progression, and it is evident that there is a '*metabolic switch*' from normal prostate epithelium to prostate cancer (90). However, the differences in metabolic regulation between localized and mCRPC tumors are less well defined. This highlights the need for comprehensive studies evaluating multiple substrates in a more complex system that reflect clinical tumors. The current advancement in patient-derived organoids (PDO) generation protocols (91, 92) and the creation of several PDX collections (93–96) will enable complex studies in identifying targetable metabolic vulnerabilities in different disease stages. However, a limitation of all *in vitro* studies is that metabolite concentrations in the TME are unknown. A widely held view is that commonly used cell culture medium (*e.g.*, RPMI, MEM, DMEM) contain significantly higher concentrations of glucose and amino acids than what is physiologically available, and often do not contain free fatty acids. Acknowledgement of this limitation and a better understanding of the constituents of the TME in different disease stages is required to move the field forward (see **Table 1**).

FACTORS INFLUENCING PROSTATE CANCER METABOLISM

Prostate cancer displays marked heterogeneity from a molecular, morphological and clinical perspective and consideration of the factors that influence metabolic selection is essential to better understand the metabolic requirements of human prostate tumors in their native environment (**Figure 3**).

TABLE 1 | Methodological considerations.

Limitations of experimental models used to assess metabolism in prostate cancer
• Most studies assessing metabolic regulation have been conducted in immortalized prostate cancer cells, including PC3, DU145 and LNCaP, which facilitate simple physiological and/or genetic manipulation and high throughput analysis, but bare limited resemblance to the complexity or heterogeneity of human tumors.
• Exposure of cells *in vitro* to supraphysiological nutrient levels in the culture medium unlikely recapitulate the condition in tumors, although it is noted that the concentration of metabolic substrates in the tumor microenvironment (TME) are currently unknown.
• *In vivo* studies using genetically engineered mouse models of prostate cancer overcome some of these issues, however the mutations do not replicate the genomic and phenotypic heterogeneity observed clinically.
• The use of human tissues or clinical studies are often impracticable due to limited access to patients under carefully controlled conditions and the technical difficulty in assessing tissue-specific metabolism *in vivo*.
• To overcome this limitation, prostate cancer patient-derived xenografts (PDXs) capture the heterogenous nature of tumor of origin. However, despite its perceived superiority over other approaches, PDXs lack stroma and immunological contribution.
• Combined approaches that integrate these complementary models are required to understand the metabolic landscape of prostate cancer and identify promising therapeutic strategies.

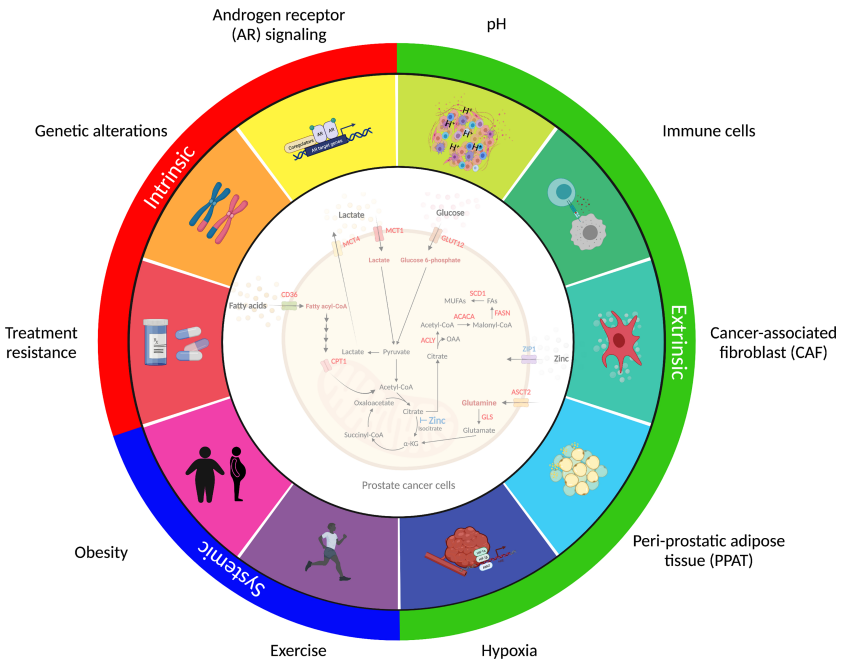

FIGURE 3 | Factors shaping prostate cancer metabolism. Prostate cancer metabolism is influenced by factors inside the cancer cell (*intrinsic*), immediately adjacent to the cancer cell (*extrinsic*), and derived from multi-system perturbations (*systemic*). The intrinsic factors (*red*) represent intracellular changes regulated by androgen receptor (AR) signaling activity, genetic alterations, and therapy resistance. The extrinsic factors (*green*) are extracellular signals derived from nearby cells that influence cancer cell metabolism and adaptation. These include dysregulated vascularization, acidosis, hypoxia and cancer-associated fibroblasts (CAF), immune cells and peri-prostatic adipose tissue (PPAT), which collectively contribute to the tumor microenvironment (TME). The systemic factors (*blue*) that regulate tumor metabolism include alterations in the body's metabolic and hormonal milieu, induced by short-term perturbations or long-term changes in metabolic state, including exercise and obesity, respectively. These complexities should be considered when assessing metabolic changes in prostate cancer and highlight the multiple challenges in implementing metabolic therapies into clinical practice.

Genetic Drivers of Metabolism

Specific oncogenic mutations can promote metabolic phenotypes in some cancers [*as reviewed in* (97)]. This is unequivocally the case in melanoma, where $BRAF^{V600E}$ mutations that account for ~80% of melanomas drive a metabolic program with a preference towards Warburg metabolism (98). Inhibition of oncogenic *BRAF* using drugs such as vemurafenib, dabrafenib or encorafenib cause profound reductions in glucose uptake and improve patient outcomes (99). While the use of oncogene-driven mouse models has been helpful in linking specific genomic alterations with aberrant metabolic phenotypes in some tumor types (100–103), the profound number of molecular aberrations and heterogeneity observed in human tumors make it challenging to identify single DNA or gene alterations that dictate metabolic regulation.

Acquisition of genomic alterations underpins prostate tumorigenesis. Comprehensive genomic characterization of prostate cancer has identified recurrent alterations in genes involved in androgen signalling, DNA repair, and PI3K signalling, such as *TP53*, *SPOP*, *PTEN*, *AR*, *FOXA1*, *MYC*, *ATM* and *APC*. However, the incidence of significantly mutated genes follows a long-tail distribution, where the frequent alterations are only detected in ~5-10% of cases, and many other genes are mutated in <3% of cases (104). This underpins a complex genetic landscape in prostate cancer and heterogeneous nature of the disease. There is limited evidence showing induction of metabolic remodelling by individual oncogenes, such as *MYC* amplification, which promotes fatty acid synthesis and accelerates prostate cancer progression (105, 106). However, the absence of a dominant and frequent genetic mutation in prostate cancer indicates that a 'common' oncogenic-driven metabolic phenotype is unlikely to exist, although this remains to be fully explored.

Neuroendocrine Prostate Cancer

The prominent pathology in prostate cancer is adenocarcinoma; however, in rare cases (<1%), NEPC tumors occur and present with an AR-null phenotype. While these are uncommon at diagnosis, there is increasing prevalence of therapy-induced NEPC that develops as an aggressive form of mCRPC. Treatment of NEPC presents an unmet clinical challenge in managing advanced prostate cancer. Emergence of an AR-null, NEPC phenotype is characterized by the expression of neuroendocrine markers such as synaptophysin, CD56, and chromogranin, with the absence of AR and AR-regulated gene expression (107). The genomic loss of tumor suppressors, dysregulation of specific transcription factors, and epigenetic modifications have been linked to the gain of neuroendocrine-like properties [*as reviewed in* (108)].

The metabolic regulation in NEPC disease states requires independent investigation. A previous study identified the requirement of increased serine biosynthesis following the loss of PKCλ/l to fuel the methionine salvage pathway, which in turn augmented NEPC differentiation through DNA methylation (56). This highlights the role of metabolism in epithelial cell differentiation, beyond energy production. NEPC is characterized by increased glucose uptake and glucokinase expression compared to adenocarcinoma, despite the suppression of GLUT12 (109). Transcriptomic analysis of NEPC PDX and patient specimens identified elevated glycolysis and lactate production as the metabolic feature of NEPC (110); however, these and other metabolic processes in NEPC are yet to be quantified using appropriate tracer methodologies.

Prostate Metabolism and the Tumor Microenvironment

Prostate tumor cells reside in close proximity to neighboring cells within the tumor microenvironment (TME). The major components of the TME include cancer-associated fibroblasts (CAFs), endothelial cells, mesenchymal cells, as well as immune cells such as mast cells, T cells, macrophages and monocytes. Each of these cell types secrete metabolites, hormones, extracellular vesicles and cytokines that could impact local metabolism. Several characteristic changes in the TME can impact metabolism, including dysregulated vascularization that involves disorganized and leaky blood vessels with low pericytes coverage, which in turn creates a hypoxic and acidic environment. Hypoxia has been implicated in the metabolic reprogramming of cancer cells, and upregulation of HIF-1α plays an important role in the regulation of glycolysis (111). Additionally, CAFs themselves undergo a significant shift from oxidative phosphorylation to aerobic glycolysis, altering substrate availability for nearby cancer cells (112). There are also remarkable alterations in immune responses and the inflammatory environment in the TME, creating an immunosuppressive milieu. Interestingly, this relationship is likely to be bidirectional, with evidence that the low pH induced by excess lactate production in cancer cells reduces T cell infiltration. In addition, the microbiome is an important modulator of various host processes such as metabolism and immunity, and microbiome dysbiosis is associated with tumor development, disease progression, and treatment response and resistance in prostate cancer (113). The complexity of intra-tumoral paracrine signaling is exacerbated by remarkable heterogeneity in the cellular composition between individual human tumors, as demonstrated by single-cell transcriptomic analysis (114). These findings highlight the need to develop methods to sample and define the components of the prostate TME, with a view to understanding the factors controlling tumor metabolism and, perhaps, determining targetable metabolic vulnerabilities.

Prostate Cancer Metabolism and Obesity

Obesity is a global epidemic affecting 281 million men (115) and more than 40% of men aged between 45-74 are obese (116), an age when the majority of prostate cancer diagnoses occur (117). While there is limited evidence that obesity is an initiator of prostate cancer (118, 119), epidemiologic evidence indicates that obese patients develop aggressive tumors with poor clinical outcomes (120, 121), although there is some conjecture with respect to mCRPC (122). Studies in rodents mostly confirm progression towards an aggressive phenotype in obesity [*as reviewed in* (123)] and many plausible mechanisms have been proposed to explain the link between obesity and aggressive prostate cancer (123), including increased free fatty acid supply, hyperinsulinemia, hypertriglyceridemia, altered endocrine signaling and low-grade inflammation (123). Notably, definitive evidence supporting these putative obesity-related drivers of prostate cancer progression is lacking. While not directly related to obesity, higher dietary saturated fat intake is associated with prostate cancer lethality (105) and raises the possibility that dietary interventions that reduce saturated fat intake and/or interventions to desaturate fatty acids might be efficacious in managing prostate cancer.

Periprostatic adipose tissue (PPAT) covers the prostate anteriorly and patients with more PPAT have worse cancer

prognosis (124), leading to the view that PPAT secreted factors stimulate tumorigenesis, particularly in obesity (123). Studies employing co-culture of prostate cancer cell lines and adipocytes (125), or the addition of PPAT secreted factors to prostate cancer cells (126, 127) support this possibility; however, co-grafting of patient-matched PPAT and localized prostate cancer PDX did not enhance prostate cancer tumorigenesis in mice (126). Nevertheless, changes in fatty acid delivery or adipose-secreted proteins (*i.e.*, adipokines) from PPAT are factors that may impact prostate cancer metabolism.

Exercise and Prostate Cancer

Observational studies indicate that exercise and physical activity are associated with decreased risk of prostate cancer incidence, and lower overall prostate cancer mortality. Notably, vigorous activity is associated with a reduced risk of advanced, high Gleason grade group, or fatal prostate cancer in men over 65 years of age (128). While the mechanisms underlying potential anti-tumorigenic effects of exercise remain elusive, several have been proposed and include reduced circulating insulin, insulin-like growth factor 1 and proinflammatory cytokines, reduced tumor vascularization, AR adaptations, reduced cholesterol, production of unknown 'exercise circulating factors' contained in exosomes and reprogramming of metabolic and immunological dysregulation (129, 130). Overall, local, systemic and external influences play a significant role in metabolic regulation and prostate tumorigenesis, although there remains much to be learnt in this space.

SYSTEMIC THERAPIES FOR PROSTATE CANCER

Hormone therapy is standard of care for patients with advanced prostate cancer, involving the use of gonadotropin-releasing hormone (GnRH) agonists or antagonists to suppress testicular testosterone synthesis. The use of androgen-targeted agents, such as enzalutamide (AR antagonist) and abiraterone (inhibitor of cytochrome P450 (CYP) C17 to block androgen synthesis), are used clinically to treat mCRPC (131). Meanwhile, Rucaparib (132) and Olaparib (133) (PARP inhibitor) have been recently approved for men with mCRPC harboring deleterious mutation of homologous recombination repair genes. While these discoveries improve current management of prostate cancer, the need for new therapeutics or adjuvant therapies continues as mCRPC remains lethal, and NEPC tumors are refractory to hormone therapy.

Metabolic changes, including insulin resistance, dyslipidemia, diabetes, and cardiovascular morbidity have been associated with ADT (134). Recent studies examining metabolomic profiles of men receiving ADT reported a reduction in acyl-carnitines and ketone bodies, indicating ADT-induced systemic changes in fatty acid metabolism (135, 136). Meanwhile, low-carbohydrate diets reversed this alteration in fatty acid metabolism while slightly increasing androgen suppression (137). This emphasizes the importance of diet in maximizing ADT therapeutic activity while minimizing its effects on altering metabolism. In addition, patients are often prescribed with exercise, anti-hypertensive, anti-hyperlipidemic and anti-hyperglycemic medications to attenuate the effects of ADT, and it is possible that these interventions induce metabolic changes that improve cancer outcomes, although the evidence for this is limited (discussed below).

Aside from the impact of ADT on systemic metabolism, it has been postulated that ADT induces metabolic vulnerabilities in the tumor itself that can be therapeutically targeted using combination approaches. The use of metabolic inhibitor(s) as an adjuvant therapy have improved the efficacy of existing therapies and prevented the development of resistance in several tumors (138). In prostate cancer, metabolic adaptations occur in prostate cancer cells following ADT, as well as androgen-targeted therapies, including enzalutamide or abiraterone, suggesting the possibility of co-treatment strategies (139, 140) (**Figure 1**). This was exemplified in prostate cancer PDXs where a synergistic effect was demonstrated following treatment of ADT (through castration) plus metformin (141). Thus, the possibility of metabolic targeting in combination with ADT should be further explored.

PUTATIVE METABOLIC TARGETING IN PROSTATE CANCER

Effective targeting of cancer metabolism relies on suppressing or modulating metabolic pathways identified as cancer 'dependent' and the use of metabolic agents is thereby limited by the defined therapeutic window of efficacy and toxicity in cancerous and non-cancerous cells. While there are no metabolic inhibitors approved for clinical use in patients with prostate cancer, several agents targeting *de novo* lipogenesis, fatty acid oxidation and glutamine oxidation are in pre-clinical or early phase clinical trials.

De Novo Lipogenesis Inhibitors

The lipogenic phenotype of prostate cancer raises the possibility of targeting *de novo* lipogenesis. In this context, fatty acid synthase (FASN) is a rate-limiting enzyme in this process, and several FASN inhibitors, including TVB-3166 and TVB-2640, suppressed tumor growth by 15% in 22Rv1 xenografts (142), and notably, induced up to 97% tumor growth inhibition in combination with paclitaxel (142). Another FASN inhibitor, IPI-9119, showed anti-tumorigenic activity in human mCRPC organoids and 22Rv1 and LNCaP-95 xenograft models (143). Phase I studies of TVB-2640, the first FASN inhibitor to enter clinical trials for prostate cancer, indicated a favorable tolerability profile as either monotherapy or in combination with taxane in four heavily pre-treated prostate cancer patients (144). Clinical studies are warranted to evaluate the clinical utility of FASN inhibitors in mCRPC.

Moreover, several drugs that target other enzymes in the *de novo* lipogenesis pathway are in clinical trials for other diseases, such as the ACC inhibitors Firsocostat (Gilead) and PF-

05175157 (Pfizer) for non-alcoholic fatty liver disease (145), and derivatives of these compounds could conceivably be adopted for treatment of prostate cancer. Indeed, PF-05175157 showed promising results in reducing proliferation and inducing apoptosis in localized prostate cancer patient-derived explants (88).

Fatty Acid Oxidation Inhibitors

Etomoxir is an irreversible inhibitor of carnitine palmitoyl transferase 1, which is the protein that transports fatty acids into the mitochondria for eventual oxidation. Treating mice with etomoxir reduced tumor growth in VCaP xenografts, without changing body weight or inducing systemic toxicity (67); however, etomoxir caused hepatotoxicity in patients with heart failure leading to the premature termination of a phase II clinical trial (146). While etomoxir is unlikely to progress to clinical trials for prostate cancer, two angina medications, ranolazine and perhexiline, may prove to be efficacious. Ranolazine is an FDA-approved partial inhibitor of fatty acid oxidation (147), while perhexiline is an TGA-approved competitive inhibitor of CPT1 (148). While neither drug reduces tumor growth alone, combining either compound with enzalutamide significantly decreased tumor growth *in vitro* and *in vivo* (149). Moreover, perhexiline alone showed no anti-tumorigenic activity in patient-derived explants, while cotreatment of perhexiline with the HSP90 inhibitor, AUY922, significantly reduced proliferation and increased apoptosis (150). These observations indicate that inhibitors of fatty acid oxidation may sensitize prostate cancer to other therapies, albeit through unknown mechanisms, and could be rapidly translated to the clinic.

Glutaminolysis Inhibitors

CB-839, an oral glutaminase inhibitor, showed encouraging safety and tolerability results in a phase 1 study conducted in patients with advanced and/or treatment-refractory solid tumors, including breast cancer, lung cancer, renal cell carcinoma and mesothelioma (151). Preclinical studies in DU145 cells and xenografts indicated a synergistic effect of CB-839 in combination with talazoparib (PARP inhibitor) (152), leading to an upcoming phase II open label study of CB-839 and talazoparib in patients with mCRPC (NCT04824937).

HMG-CoA Reductase Inhibitors

Statins are a class of drugs that inhibit the activity of HMG-CoA reductase and are widely used to treat patients with hypercholesterolemia. While observational studies demonstrate that statin use is associated with reduced cancer-specific mortality in patients with mCRPC receiving ADT (153), the results from one randomized trial indicates that short-term statin use does not impact tumor proliferation or serum prostate-specific antigen (PSA) compared to placebo (154). Similarly, statins alone did not reduce tumor burden in LNCaP xenograft and PDX trials; however, combination therapy with a re-purposed SREBP2 inhibitor, dipyridamole, significantly reduced tumor growth (155). Future studies exploring the safety and efficacy of this, and other combinations, in clinical studies are yet to be seen.

Metformin

Metformin is the current first-line treatment of type 2 diabetes. While the exact mechanisms of action of metformin are still incompletely resolved, the anticancer potential of metformin is indicated through the capacity to activate AMPK and inhibit the cell cycle and epithelial-mesenchymal transition [*as reviewed in* (156)]. However, epidemiology studies showed no effects in reducing prostate cancer incidence and minimal improvement in overall survival (157). Multiple clinical trials are currently underway to assess the therapeutic utility of metformin as a monotherapy, or in combination with androgen targeted agents (enzalutamide and abiraterone) in managing CRPC.

PROSPECTS AND CHALLENGES FOR IMPLEMENTING METABOLIC THERAPIES IN PROSTATE CANCER

Prostate cancer is slow growing by nature, providing sufficient time to implement therapies to delay progression or manage aggressive disease. For patients with intermediate risk disease, the median time to biochemical recurrence is ~4.25 years (158), necessitating the need for initiation of ADT, and in some patients, radiotherapy. Current clinical practice is to combine ADT with androgen-targeted therapy or chemotherapy, as this approach has been shown to increase overall survival (159). While effective in the short term, CPRC inevitably develops in ~5-8 years (160), which is then associated with a median survival ranging from 13-30 months (161–163). Overall, the time from diagnosis to end-stage disease for most patients is ~10-15 years, providing ample time for therapeutic intervention (**Figure 1**). This makes prostate cancer distinct to other more rapidly progressing cancers.

Of course, the overarching challenge in developing and utilizing 'metabolic therapies' for prostate cancer is to determine the appropriate strategy for the appropriate patient at the appropriate time, which as outlined above will vary between localized, metastatic and CRPC (see *Prostate Cancer Metabolism* section). We are, however, some way off implementing precise, actionable therapies as the focus of current research in cancer metabolism is predominantly pre-clinical and there is an urgent need for clinically based metabolic research. One emerging methodology, not yet applied to prostate cancer, is the use of intraoperative ^{13}C metabolic tracer infusions in human cancer patients, which overcomes limitations of *ex vivo* studies and by integrating systemic, TME and spatial parameters that shape metabolic phenotypes (164).

The clinical trajectory described above is generalized for patients with intermediate-risk prostate cancer, although in reality, each patient has individual prognostic features that dictate disease progression. Risk-stratification for prostate cancer is critical to guide appropriate treatment decision-

making. Towards this, it is worth considering whether there are subsets of patients who might benefit from metabolic therapies, either based on the reliance of an essential metabolic substrate, or specific tumor subtypes with common genomic aberrations or pathology. However, this has not been demonstrated, likely because of the remarkable heterogeneity of prostate cancer, diversity in metabolic substrate fluxes described in human tumors, and lack of appropriate biomarkers. In this context, mass spectrometry metabolic imaging is being refined to detect 'metabolic signatures' of prostate cancer, with evidence indicating that such imaging may aid in understanding biological processes and to help cancer diagnosis, prognosis and monitor response to therapies (165, 166).

A major challenge for the field is to define when metabolic therapies could be clinically applied. One option is during early-stage disease, following curative intent surgery or radiation when PSA levels are beginning to slowly rise, indicative of residual disease that is progressing. It is envisaged that metabolic therapies designed to reduce nutrient supply and/or ATP production could slow growth and delay the need for ADT. Alternatively, there is interest in the potential for metabolic therapies to be used to treat CRPC, because significant energy is required for the growth of highly aggressive therapy resistant tumors (**Figure 1**). More generally, it has been suggested that a better understanding of the association between metabolism and prostate cancer may lead to cancer prevention, although such strategies are opaque.

Overall, there is very little evidence from preclinical models or clinical studies that targeting a single metabolic pathway will be sufficient to slow prostate tumor progression. Firstly, this requires modulation of a single substrate, enzyme or metabolic pathway to limit tumor growth or increase tumor susceptibility to an adjunct therapy. In this context, metabolic inhibition, commonly leads to compensatory upregulation of other fuel utilization pathways to maintain pro-tumorigenic energy demands. For example, our work showed that this was the case with fatty acid transport inhibition, whereby blocking FAT/CD36 induced an increase in *de novo* lipogenesis in localized disease (38). Similarly, others showed that inhibition of FASN led to the upregulation of genes involved in steroid biosynthesis and increased intracellular cholesterol (143, 167). Thus, we posit that targeting dual processes will most likely be required for effective metabolic intervention in prostate cancer. Further to this, most tissues in the body readily utilize each of the substrates commonly used in prostate cancer, with evidence of dependencies in some tissues (*e.g.* glucose for red blood cells and brain). Hence, approaches that direct metabolic therapies to the tumor will be essential to minimize the likelihood of off-target effects. Such approaches are feasible as evidenced by the implementation of radioligand-therapy targeted to prostate-specific antigens in the clinic.

CONCLUSIONS

Prostate cancer invokes major shifts in gene transcription and metabolic signaling to mediate alterations in nutrient acquisition and metabolic substrate selection when compared to normal tissues. Exploiting such metabolic reprogramming is proposed to enable the development of targeted therapies for prostate cancer, yet there are several challenges to be overcome before this becomes a reality. Firstly, several metabolic substrates have been identified in prostate cancer, including (but not limited to) fatty acids, glucose, lactate and glutamine, all of which are 'required' substrates in prostate cancer. Thus, identifying the most appropriate substrate to be targeted, and in which type of prostate cancer, remains unclear. Somewhat related, there is a gap in our knowledge of metabolism in human tumors. The majority of studies that have defined metabolic regulation of prostate cancer have been limited to cell culture or genetically modified mouse models, which does not accurately reflect the complexity of the *in vivo* tumor microenvironment and the impact that this induces on prostate metabolism (**Table 1**). Thirdly, prostate cancer is notoriously heterogeneous and there is currently insufficient evidence to indicate that subgroups of patients or tumor subtypes, based on genomic aberrations or pathology, share common metabolic vulnerabilities. Hence, there is an urgent need for these gaps to be addressed before metabolic therapies can be designed and incorporated into clinical practice.

AUTHOR CONTRIBUTIONS

All authors (GF, MW, and RT) conceived the idea for the review, searched the literature, drafted the manuscript and provided critical revision of the manuscript for intellectual content. GF generated figures. MW and RT obtained funding and provided supervision. All authors contributed to the article and approved the submitted version.

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