INVITED REVIEW

Prostate Cancer

Circulating tumor cells and their role in prostate cancer

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Circulating tumor cells (CTC) have become an important biomarker in patients with advanced prostate cancer. CTC count has been demonstrated to be a prognostic factor for overall survival in patients with metastatic castration-resistant prostate cancer (mCRPC). In localized prostate cancer, a clear correlation between CTC counts and clinicopathological risk parameters and outcome has not been observed. Currently, the focus of research is shifting from CTC enumeration towards molecular characterization of CTC leading to the discovery of markers predicting treatment response. The role of androgen receptor splice variants expressed by CTC as markers of resistance to abiraterone and enzalutamide has been assessed by various studies. The identification of CTC markers predicting treatment response represents a key step to guide the selection of treatment (e.g., abiraterone/enzalutamide *vs* taxanes), particularly in patients with mCRPC. As an alternative to CTC, the analysis of circulating tumor DNA has been shown to enable a noninvasive disease characterization having high potential to promote precision oncology.

INTRODUCTION

Prostate cancer (PCa) is the most prevalent malignancy and the third leading cause of cancer death in men in the United States.[1] Morbidity and mortality by PCa are mainly caused by PCa metastases.[2] One important step in the sequence leading to the development of distant metastases is loss of adhesion and entry into the systemic vasculature with migration of tumor cells away from the primary tumor.[2] This step results in entry of tumor cells into the blood stream. Circulating tumor cells (CTC) are potentially detectable in the peripheral blood before the occurrence of clinically relevant metastases, and these cells are capable of forming new metastases.[3–7] The evidence of CTC in the peripheral blood may, therefore, be a hint for the progress of tumor although clear data from clinical studies are missing in this context.

In the past, most of the studies focused on quantification of CTC (CTC enumeration), which has been shown to correlate with disease outcome but does not exploit the full potential of circulating cells as biomarkers.[8,9] Molecular analysis of the CTC may provide a real-time overview of the tumor characteristics and its mutations.[10] This information might help predicting the tumor's response to different treatment options, particularly in cases of metastatic castration-resistant prostate cancer (mCRPC). Therefore, qualitative molecular analysis of CTC collected through liquid biopsies might be an important step for the implementation of personalized treatment strategies in the more and more complex therapeutic landscape of PCa.

Molecular analyses of CTC are useful because several studies have shown that the genomic information of CTC is comparable to the primary tumor tissue and/or metastases.[11] It is assumed that CTC are able to reflect all tumor characteristics including the intratumoral and intertumoral heterogeneity.[12–16] However, it is still not known whether CTC represent characteristics of all metastases or only the most invasive/aggressive clones. Because the half-life of CTC has been estimated to be in the range of hours, it is suggested that CTC provides a real-time representation of the tumor's characteristics, which offers the opportunity to take tumor snapshots at various time-points during the therapy.[17–19]

The diagnostic and therapeutic potential of CTC as promising biomarkers with prognostic and predictive value for potential clinical outcome and therapy response is a highly relevant topic for solid malignancies.[20,21] Concerning urologic oncology, there is a rising number of studies investigating the potential role of CTC, particularly in local and metastatic PCa.[10,22–24] The aim of this review is to give an overview on the current role of CTC in localized and metastatic PCa.

METHODS OF ISOLATION AND ENRICHMENT OF CTC

CTC are rare in the peripheral blood, estimated to account for one in a billion nucleated cells.[25] Therefore, one key step for a "liquid biopsy" aiming to detect CTC in the peripheral blood is the enrichment and isolation of these cells. There are different techniques for this purpose.

The CellSearch® system (Janssen Diagnostics, Raritan, NJ, USA) is the only Food and Drug Administration (FDA) approved CTC detection platform. After an immunomagnetic enrichment step, based on epithelial cell adhesion molecule (EpCAM), the second step is staining of the isolated cells with specific fluorescent antibody conjugates against cluster of differentiation 45 (CD45) and cytokeratin (CK) 8, 18, 19. In the next step, the sample is scanned on an analyser. In this system, CTC are defined as nucleated cells lacking the leukocyte marker CD45 and expressing cytokeratins. The size of a CTC

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has to be at least 4 μm × 4 μm and it has to show specific morphological features, judged by trained operators.[26,27] There is evidence that smaller and CK-negative CTC exist, which are even more aggressive. They seem to appear after epithelial-mesenchymal transition (EMT) or neuroendocrine differentiation.[28–30] These cells do not get isolated by the CellSearch® system.

In addition to the CellSearch® system, a variety of other approaches for CTC enrichment and detection exist. Polymerase chain reaction (PCR)-based approaches with or without previous enrichment steps have been assessed in various studies. CTC presence varies depending on the used mRNA marker to detect CTC and the method of enrichment.[31] The Adnatest platform (Adnatest®, Qiagen GmbH, Hilden, Germany) combines immunomagnetic enrichment of CTC with subsequent RNA isolation and reverse transcription PCR for PCa associated transcripts.[23,32] Recently, various groups have used mRNA analysis of whole blood-derived ribonucleic acid (RNA) to detect CTC-associated transcripts without prior enrichment of CTC.[33,34]

The EpicScience platform (Epic Science, San Diego, CA 92121, USA) uses high-throughput imaging, in which nucleated cells are plated on glass slides. It is based on the separation between nucleated cells (e.g., white blood cells, and CTC) and red blood cells. After lysis of red blood cells, it uses cytokeratin, leukocyte marker CD45, and nucleus staining with 4'6-diamidino-2-phenylindole (DAPI). Following this step, each slide is scanned by a fiber-optic array scanning technology (FAST), which records the precise coordinates of each cell and analyzes each cell for different parameters. This algorithm proposes potential CTC which have to be confirmed by a trained reader. It is therefore independent of EpCAM expression.[35]

Moreover, there are several label-free methods, based on cell size and morphology.[36] Separation of CTC from the peripheral blood is based on three-dimensional microfilters and bilayers. The success rate depends on several parameters, such as pore size, blood flow rate (high flow rates may lead to squeezing of CTC through pores, the slow flow rate can cause accumulation of leukocytes and clotting of the blood), and rigidity of the membrane.[36,37] In microfluidic devices, CTC seem to be detected in a higher proportion of patients and in higher counts.[17,38] Isolation of CTC by these devices offers the opportunity for detailed molecular analysis of tumor diseases. As the majority of these label-free techniques lead to the isolation of viable cells, these platforms have a significant potential for assays requiring viable cells (such as *in vitro* culture and xenografts).

The gold standard until today is the CellSearch® system. In the future, the focus will switch from quantification (like CTC enumeration) to molecular analyses. Because the CellSearch® system is mainly designed for quantification and not characterization, development and further improvements of other approaches, which enable extensive molecular analyses of CTC, are needed.

SIGNIFICANCE OF CTC IN LOCALIZED PCa

CTC have been most intensively investigated in metastatic PCa. In localized disease, the role of CTC has been addressed only by few studies, showing conflicting data on the added value of CTC analysis to the currently established risk stratifications, depending on the technique used for enrichment and isolation of CTC.

A significant amount of patients with localized PCa develop tumor recurrence despite curative intended surgical therapy.[39] Current risk stratification is mainly based on clinical parameters and evaluation of pathological tumor specifications after prostatectomy. Most of the published studies investigated the correlation between CTC in localized PCa with other clinical and pathological risk stratification

parameters (e.g., prostate-specific antigen [PSA] concentration, pathological tumor stage [pT-stage], lymph node stage [pN-stage], Gleason score) aiming to evaluate the value of CTC as an additional parameter.

In studies using the CellSearch® system to enrich and isolate CTC, no correlation was found between CTC count and other clinical-pathological parameters:

Thalgott *et al.*[22] analyzed CTC in a group of twenty patients with localized PCa before radical prostatectomy. They observed only one patient (5.0%) with one CTC per 7.5 ml blood. Consistent with the study from Davis *et al.*[40] (see below), there was no difference in the CTC-positive rate between patients with localized PCa and the healthy control group. In a second study from the same authors,[41] 20% of a cohort of 15 patients with localized PCa undergoing neoadjuvant docetaxel chemotherapy were CTC positive at baseline compared to 5% in the healthy control group. There was no difference in biochemical recurrence, independent from the patient status (CTC positive or not), before therapy. Moreover, they did not see any correlation between CTC and clinicopathological risk parameters.

Davis *et al.*[40] not only detected CTC in 21% of patients but also in a comparable proportion of the control group comprising patients without PCa (20%). In addition, the authors used more than the usually processed blood (22.5 ml instead of 7.5 ml) probably increasing the rate of CTC-positive patients compared to studies using only 7.5 ml. The authors did not observe any correlation with established pathological or clinical risk parameters.

Meyer *et al.*[42] analyzed preoperative CTC in a cohort of 152 patients with localized PCa. Eleven percent of this cohort had CTC preoperatively. Again, they could not observe a significant correlation of CTC presence with T-stage, Gleason score, or PSA level. Furthermore, there was no correlation between CTC positivity and biochemical recurrence (median follow-up: 48 months).

In a cohort of 59 patients with localized PCa, Tsumura *et al.*[43] evaluated the CTC count before and immediately after brachytherapy. They could show a change in CTC positivity after surgical manipulation (insertion of needles) in 11.8% of the patients. There was no correlation of CTC status with other variables such as PSA at diagnosis, previous use of neoadjuvant androgen deprivation therapy (ADT), type of brachytherapy, Gleason score, and biopsy positivity core rate.

Studies using a microfluidic device for CTC detection identified CTC in a higher proportion of patients and found considerably higher CTC counts than studies using CellSearch® system (Stott *et al.*[17]; 42% of patients with ≥14 CTC per ml, median count = 95 CTC per ml; Todenhofer *et al.*[39]; 50% of patients with ≥1 CTC per ml, median count = 4.5 CTC per ml). Stott *et al.*[17] could show a decline of CTC in six of eight patients within 24 h after prostatectomy. Todenhöfer *et al.*[38] could not find any correlation between the presence of CTC with age, serum PSA level, pT stage, pN stage, Gleason score, or risk category. Furthermore, the number of CTC in patients with CTC positivity was not associated with pT-stage, N-stage, or Gleason score. There was no surveillance for clearance of putative CTC from the blood after prostatectomy in this study.

In PCR-based approaches, associations with clinicopathological risk parameters depend on the cohort and the genes used for CTC detection. Joung *et al.*[31] could not find any association between prostate stem cell antigen (PSCA) mRNA levels with other clinicopathological risk parameters. Helo *et al.*[44] used PCR for kallikrein-related peptidase 2 (KLK2), PSA, and PSCA mRNA. They were not able to demonstrate an association between presence of these transcripts with unfavorable disease

features. Bianco *et al.*[45] could show an association of PSA mRNA and T-stage/biochemical recurrence (BCR) in a cohort of African Americans.

In localized PCa, the clinical value of CTC analyses remains to be determined. Compared with the prognostic value of CTC in metastatic PCa, where several studies showed that CTC detected by the CellSearch® system is associated with an unfavorable outcome, no clear association with outcome has been reported in localized disease. In studies analyzing prostate-cell associated transcripts as a surrogate parameter for CTC like in Joung *et al.*[31] or Bianco *et al.*,[45] a prognostic value for biochemical recurrence could be shown. The question whether this should result in more frequent follow-up examinations after surgery in CTC-positive patients remains to be elucidated in a clinical study. There is currently no clear rationale for a different treatment of CTC-positive patients with localized PCa compared to CTC-negative patients (e.g., receiving adjuvant chemotherapy).

Due to the low median number of CTC encounted using the CellSearch® system, a use of its CTC count as a continuous parameter is unlikely to add additional information in the localized setting. Microfluidic-based devices for CTC detection and enrichment lead to the detection of a higher number of CTC. Therefore, these techniques could provide a broader dynamic range, which prospectively may lead to a longitudinal assessment of CTC count as an additional continuous risk parameter regarding disease status and therapy response.

In addition to the quantitative analyses of the CTC count, qualitative analyses of molecular features of CTC may provide additional information about the tumor. In view of the fact that most of the localized PCa develop multifocally, CTC may represent the quintessence of molecular heterogeneity. Furthermore, CTC are discussed as originated cells from the most aggressive subpopulation inside of the prostate as they are cells which already left the prostate and infiltrated the blood circulating system.[38] However, the potential additional information that CTC may provide compared to analysis of the different tumor foci is unclear. Moreover, there is not sufficient evidence yet for a clear correlation of the molecular characteristics of CTC and multiple tumor foci.[11,46]

Table 1 summarizes results from studies assessing CTC in patients with localized PCa.

ROLE OF CTC IN METASTATIC PCa

In an advanced disease such as mCRPC, CTC have been demonstrated as a valuable biomarker. **Table 2** summarizes studies on CTC in metastatic PCa. In the past, most of the studies focused on the prognostic value of CTC enumeration and the role of CTC count as an early treatment response biomarker for patients with metastatic PCa. de Bono *et al.*[47] published a landmark study in 2008 contributing to the FDA approval of the CTC enumeration by the CellSearch® system for clinical use in patients with mCRPC. In this study, it was

Table 1: The summary of results from studies assessing circulating tumor cells (CTC) in patients with localized prostate cancer

Technique	Study	Patient (n)	CTC presence	Summarized observations
Microfluidic device, EpCAM antibody-coated microposts	Stott *et al.*[17]	PCa (cM0): n=19, before and after prostatectomy	42% CTC positive (defined as 14 CTC per ml); median CTC count: 95 CTC per ml; range: 38–222 CTC per ml	Six of eight patients with decline of CTC counts within 24 h after prostatectomy
Microfluidic device, ratchet mechanism	Todenhofer *et al.*[38]	PCa: n=50, before radical prostatectomy	50% CTC positive (≥1 CTC per 2 ml; median count: 4.5 CTC per ml; range: 0.5–208.5 CTC per ml)	No association with histopathologic parameters. AR expression in CK+ CTC
CellSearch®	Thalgott *et al.*[41]	PCa (cM0): n=15, before neoadjuvant chemohormonal therapy; HC: n=15	PCa: 20% CTC positive (≥1 CTC per 7.5 ml); HC: 5% CTC positive	No difference in BCR between patients with or without CTC; no association with clinicopathological risk parameters
CellSearch®	Thalgott *et al.*[22]	PCa (cM0): n=20, before radical prostatectomy; HC: n=20	PCa: 5% CTC positive (≥1 CTC per 7.5 ml); HC: 0% CTC positive	High risk population: median PSA=21 ng ml^{-1} and 95% patients ≥cT3a
CellSearch®	Davis *et al.*[40]	PCa: n=97, before prostatectomy; HC: n=20	PCa: 21% CTC positive (≥1 CTC per 22.5 ml); HC: 20% CTC positive	No association with histopathologic parameters; 18/20 patients negative after surgical procedure; <10% with >2 CTC per 22.5 ml
CellSearch®	Meyer *et al.*[42]	PCa (cM0): n=152, before radical prostatectomy	11% CTC positive (≥1 CTC per 7.5 ml)	No association with clinicopathologic risk parameters; no difference in BCR between patients with or without CTC
CellSearch®	Tsumura *et al.*[43]	PCa (cM0): n=59, before brachytherapy	Preoperative: 0% CTC positive; perioperative: 11.8% CTC positive (≥1 CTC per 7.5 ml)	Change in CTC positivity immediately after surgical manipulation; no association with clinicopathologic risk parameters, neoadjuvant ADT or type of brachytherapy
CellSearch®, CD133/E-cadherin CTC fragments	Pal *et al.*[73]	PCa: n=35, high risk before prostatectomy	49% CTC positive (≥1 CTC per 22.5 ml); Median count=3 CTC per 30 ml	No association of cell search with other parameters; CD133 and E-cadherin positive CTC fragments associated with BCR at 1 year
RT-PCR for PSCA mRNA	Joung *et al.*[31]	PCa: n=103, with high risk	16.5% CTC positive	No association with clinicopathological risk parameters; PSCA mRNA risk factor for early BCR
RT-PCR for KLK2, PSA and PSCA mRNA	Helo *et al.*[44]	PCa (cM0): n=37; healthy controls: n=19	PCa: 8% CTC positive (≥80 CTC mRNAs per ml); HC: 0% CTC positive	No association with unfavorable disease features
RT-PCR for PSA mRNA	Bianco *et al.*[45]	PCa: n=246, before prostatectomy	African Americans: 27% CTC positive; Caucasian Americans: 23% CTC positive	Association with T-stage and BCR in African Americans

ADT: androgen deprivation therapy; AR: androgen receptor; BCR: biochemical recurrence; CD: cluster of differentiation; CK: cytokeratin; CTC: circulating tumor cells; HC: healthy controls; KLK2: kallikrein-related peptidase 2; PCa: prostate cancer; PSCA: prostate stem cell antigen; RT-PCR: reverse transcription polymerase chain reaction; EpCAM: epithelial cell adhesion molecule; PSA: prostate-specific antigen

Table 2: Role of circulating tumor cell (CTC) in metastatic prostate cancer

Sample	Study	Technique	Parameter	Summarized observations
CTC	Vogelzang *et al*.[51]	CellSearch®	CTC count	In mCRPC patients treated with chemotherapy unfavorable CTC counts (≥5 cells per 7.5 ml) associated with lower OS. Changes from favorable to unfavorable with shorter OS, best prognosis if change from unfavorable to favorable during therapy
CTC	Lorente *et al*.[49]	CellSearch®	CTC count	Thirty percent decline in CTC count after treatment with abiraterone/chemotherapy associated with OS in patients with mCRPC
CTC	Chang *et al*.[52]	CellSearch®, RT-PCR	CTC count, stem-cell/EMT related genes	Unfavorable CTC counts (≥5 cells per 7.5 ml) prognostic for shorter OS. Positive stem-cell expression associated with poor prognosis. EMT without prognostic value
CTC	Thalgott *et al*.[74]	CellSearch®	CTC count	Categorical CTC-count status independent predictor for TR, PFS and OS, 3 weeks following treatment initiation with docetaxel. Continuous CTC-inconsistent surrogate marker in mCRPC patients
CTC	Bitting *et al*.[53]	CellSearch®	CTC count	CTC enumeration prognostic in men with mCRPC
CTC	Miyamoto *et al*.[55]	CTC chip, immunofluorescence staining	AR phenotype	"AR-off" phenotype in the majority of CTC. Increase of "AR-on"/"AR-mixed" CTC during abiraterone therapy associated with decreased OS
CTC	Steinestel *et al*.[24]	Adnatest, PCR	AR-V7 expression	AR-V7 expression in 49% of the patients. AR mutations in 5%. Presence of AR-V7 predictive for resistance to subsequent anti-AR or chemotherapy. AR-V7 presence positively correlated with the number of prior treatment lines
CTC	Antonarakis *et al*.[23]	Adnatest, PCR	AR-V7 expression	AR-V7 expression in 29% of the patients. Presence of AR-V7 predictive for abiraterone and enzalutamide resistance and prognostic for PFS and OS
CTC	Antonarakis *et al*.[57]	Adnatest, PCR	AR-V7 expression	AR-V7 presence in 46% of the patients. Presence of AR-V7 not prognostic for resistance to taxanes and not prognostic for PFS and OS. Longer PFS in AR-V7 positive patients treated with taxanes compared to anti-AR treatment. No difference in AR-V7 negative patients between the treatments
CTC	Nakazawa *et al*.[75]	Adnatest, PCR	AR-V7 expression	Frequent AR-V7 status conversions, reversions only during taxane therapy. Clinical association of these changes was not reported
CTC	Onstenk *et al*.[60]	CellSearch®, PCR	AR-V7 expression	AR-V7 detected in 55% of the patients. No association between AR-V7 in baseline CTC with resistance to cabitaxel. No prognostic value for PFS and OS
CTC/PBMC	Todenhofer *et al*.[34]	Paxgene RNA extraction kit	AR-V7 expression	Patients treated with abiraterone: 0% PSA-RR in AR-V7 positive patients, worse OS in AR-V7 positive patients
CTC	Scher *et al*.[61]	EpicScience platform, AR-V7, immunocytochemistry	AR-V7 expression	AR-V7 positive patients treated with AR-signaling inhibitors: 0% PSA-RR, worse rPFS and OS; in AR-V7 positive patients treated with taxanes: poor survival, not predictive for PSA-RR
CTC/cfDNA	de Laere *et al*.[56]	CellSearch®, PCR, Illumina sequencing on RNA	AR-variants	In 30 patients with CRPC, 25 patients with any AR perturbation, 15 patients with intra-AR structural variations, and 14 with multiple AR-Vs. Most expressed AR-V3, presence of any ARV associated with progression-free survival after second-line endocrine treatment. Six of 17 poor responders were AR-V7 negative
CTC	Danila *et al*.[33]	CellSearch® profiling kit, PCR for *TMPRSS2-ERG*	*TMPRSS2-ERG* expression	No association of *TMPRSS2-ERG* and PSA decline/other clinical outcomes in patients treated with abiraterone
CTC	Reig *et al*.[65]	PBMC isolation, PCR for *TMPRSS2-ERG*	*TMPRSS2-ERG* expression	In patients treated with taxanes: worse OS in *TMPRSS2-ERG* positive patients
CTC	Attard *et al*.[64]	CellSearch®/FISH for *AR/PTEN/ERG*	*ERG* rearrangements	In patients treated with abiraterone: poor PSA response in patients with *ERG* rearrangements (CTC, tumor and metastatic tissue)
RNA	Heck *et al*.[54]	PAXgene blood RNA Kit	*TMPRSS2*, *KLK2*	Poor OS in patients with unfavorable 2GP (≥1 marker positive). Better performance of 2GP in OS than PSA decline. Correlation between conversion to favorable 2GP during treatment with improved OS, PSA-PFS, clinical PFS

EMT: epithelial-mesenchymal transition; AR: androgen receptor; AR-Vs: androgen receptor variants; PSA: prostate-specific antigen; PFS: progression free survival; OS: overall survival; 2GP: 2-gene panel; *ERG*: erythroblastosis virus E26 oncogene homolog gene; *TMPRSS2*: transmembrane protease, serine 2; RNA: ribonucleic acid; RT-PCR: reverse transcription polymerase chain reaction; *KLK2*: kallikrein-related peptidase 2; mCRPC: metastatic castration resistant prostate cancer; PBMC: peripheral blood mononuclear cell; CTC: circulating tumor cells

demonstrated that a favorable CTC count (<5 cells per 7.5 ml blood) predicts a significantly improved progression-free survival (PFS) and overall survival (OS) compared to an unfavorable CTC count (≥5 cells per 7.5 ml blood) in abiraterone-treated patients. CTC were defined as nucleated (DAPI^{pos}), CK^{pos}, and CD45^{neg} cells with a diameter ≥4 µm. Moreover, the authors could show that conversions of the CTC count from favorable to unfavorable and vice versa were associated with an improvement or deterioration of the prognosis. This change could already be shown 2–5 weeks after the treatment's start, which means that the CTC count as early response marker outperforms a 30%–50% decline in PSA (significant for the prognosis after 6–8 weeks).

The prognostic value of CTC count and the value as early therapy response biomarkers have been confirmed in other studies. Danila *et al*.[9]

showed that baseline CTC count was associated with OS. Olmos *et al*.[48] demonstrated that CTC count changes predict a change in prognosis in patients treated with any chemotherapy. Scher *et al*.[8] observed that changes in CTC count were strongly associated with OS at various time points during therapy with docetaxel monotherapy (or combination therapy). Furthermore, they demonstrated again that CTC count can outperform PSA as an early treatment response marker. A recently published study by Lorente *et al*.[49] showed that a 30% CTC decline after treatment from an initial unfavorable CTC count (≥5 cells per 7.5 ml blood) is independently associated with OS after abiraterone/chemotherapy in patients with CRPC. These results were confirmed in several other studies.[22,26,50–53]

Heck *et al*.[54] recently developed a prognostic model derived from PCa-enhanced transcripts in whole blood of CRPC patients and

explored its applicability as a surrogate of treatment response. They showed that an unfavorable 2-gene panel (2GP) (meaning: ≥1 marker positive) correlates with poor OS. A conversion to favorable 2GP during treatment correlated with improved OS, PSA-PFS, and clinical PFS.

The characterization of CTC significantly broadens the options for their use in patients with PC as a sole enumeration provides only limited information. By limiting CTC analysis on their quantification, molecular features of these cells with potential predictive value for the selection of the most promising treatment for individual patients are ignored. This led to the shift of the research focus from pure CTC quantification toward qualitative analyses. In the dense therapeutic landscape of the mCRPC, molecular analyses of CTC may be a key step for the implementation of personalized treatment strategies.

In the context of CTC characterization in mCRPC, the most intensively evaluated target is the androgen receptor (AR):

Miyamoto *et al.*[55] studied the transition of AR phenotypes in CTC under different therapies and in different disease stages. They showed that the AR phenotype of CTC was highly heterogeneous with a majority being of the "AR off" phenotype (PSA[neg], PSMA[pos]). In hormone-naïve patients, "AR-on" cells (immunofluorescence: PSA[pos], PSMA[neg]) are dominant. Most of the "AR-on" CTC changed into "AR-off" after androgen deprivation therapy. After the onset of CRPC, an increasing quantity of "AR-mixed" (PSA[pos], PSMA[pos]) and "AR-on" cells could be observed. The increase of these cells during abiraterone therapy was associated with a decreased OS.

de Laere *et al.*[56] demonstrated that in most of CRPC patients, AR pertubations can be detected.[56] In 50% of the patients, intra-AR structural variations were present, including AR variants (AR-V) in most of the patients. AR-V positive patients express multiple AR-V, and in most of the cases, AR-V3 could be found. The presence of any AR-V was associated with PFS after the second-line endocrine treatment.

AR splice variant 7 (*AR-V7*) is coding for a truncated and constitutively active AR and shows a higher transactivating activity than a full-length AR. Various studies have evaluated the potential predictive value of CTC by investigating the association between the presence of AR-V7 and treatment outcome. The presence of AR-V7 in CTC was highly predictive for resistance to anti-AR treatments.[23,24] While Antonarakis *et al.*[23] could prove a predictive value of AR-V7 concerning an abiraterone/enzalutamide resistance, the same group could demonstrate that an AR-V7 expression is not predictive for resistance to taxanes.[57] In the latter cohort, the PFS of AR-V7[pos] patients was comparable to AR-V7[neg]. AR-V7[pos] patients treated with taxanes showed a longer PFS compared to patients treated with abiraterone or enzalutamide. Because the enzalutamide/abiraterone group was taken from the first study, this kind of comparison between two different cohorts has to be interpreted carefully. In a recent update from the Baltimore group the correlation of the presence of AR-V7 positivity with response to abiraterone or enzalutamide could be confirmed. However, in this larger cohort, a considerable proportion of AR-V7 positive patients (13.9%) showed a PSA response.[58] In these studies, the Adnatest system was used for CTC- and AR-V7-detection. Recently, the analytical validation of AR-V7 analysis in a Clinical Laboratory Improvement Amendments (CLIA) setting has been published showing the stable performance of the assay.[59]

Onstenk *et al.*[60] measured the AR-V7 expression in patients after they have been treated with at least docetaxel and starting with cabazitaxel. Their hypothesis was that treatment with AR-independent mechanisms (such as cabazitaxel) remains effective, due to the lack of any dependency to the missing ligand-binding domain in AR-V7. AR-V7 presence was detected in 55% of the patients and was more frequent in patients who had received abiraterone before (100% *vs* 35%). In terms of CTC- response rate (RR) or PSA-RR no association could be found between AR-V7 presence in baseline CTC and response to cabazitaxel. OS was not impacted by the presence of AR-V7.

Todenhofer *et al.*[34] used whole blood mRNA to detect AR-V7 mRNA in patients treated with abiraterone. Detection of AR-V7 transcripts was associated with inferior outcomes. AR-V7[pos] patients had a PSA RR of 0%. These results confirm the potential usefulness of AR-V7 as a prognostic and predictive biomarker for mCRPC using an alternative approach.

A predictive ability of AR-V7 as biomarker in CTC has been also observed by Scher *et al.*[61] They showed that CTC nuclear expression of the AR-V7 protein in men with mCRPC as a treatment-specific biomarker is associated with superior survival on taxane therapy over ARS-directed therapy using the EpicScience platform.[61] In summary, recent studies indicate that AR-V7 has an impact on the response to enzalutamide and abiraterone but does not impact the effect of chemotherapy and can be detected in CTC.

Gene fusion between transmembrane protease, serine 2 (*TMPRSS2*), and erythoblastosis virus E26 oncogene homolog gene (*ERG*) seems to represent a prostate-specific alteration in patients with advanced PCa.[62] This androgen-driven expression of the *ERG* oncogene after fusion with *TMPRSS2* occurs in 30%–70% of therapy-naïve prostate cancers.[63] The results concerning the prognostic role of this fusion remain inconsistent: in 41 patients treated with abiraterone after docetaxel failure, Danila *et al.*[33] analyzed a *TMPRSS2-ERG* status. They could not show that *TMPRSS2-ERG* status was able to predict PSA response or other clinical outcome parameters. Attard *et al.*[64] could show an association of *ERG* rearrangements with the magnitude of PSA response. However, this analysis correlates *ERG* status not only from CTC, but also from primary tumor tissue and metastatic lesions.

Reig *et al.*[65] analyzed *TMPRSS2-ERG* expression in a peripheral blood mononuclear cell (PBMC) of 72 patients treated with taxanes. They observed a significantly worse PSA-PFS in patients positive for *TMPRSS2-ERG*. Because this study did not include an enrichment of CTC, it is not comparable to the previous studies, which means that until now it is not yet possible to draw a conclusion of the predictive value of *ERG* rearrangements in CTC.

As with *ERG*, the value of tensin homolog (*PTEN*) loss in CTC as a potential marker of therapy resistance remains unclear. Loss of tumor-suppressor phosphatase and *PTEN* is frequently associated with *ERG* rearrangements and frequently occurs in CRPC progression.[63] To date, there are only limited data on the clinical value of *PTEN* analyses in CTC. A correlation between *PTEN* status and survival was found by Punnoose *et al.*[11]

In contrast to localized PCa, several studies have already demonstrated an added clinical value of CTC analysis in metastatic PCa. It could be shown that a favorable CTC count is a prognostic marker for improved PFS and OS in metastatic PCa, whereas no clear association between outcome and CTC count could be found in localized disease.

At present, the research focus is shifting away from quantitative analysis toward molecular characterization to evaluate if molecular information of CTC can be used as a predictive marker for different therapy strategies in the complex therapeutic landscape of mCRPC.

FUTURE PERSPECTIVES

The recent progress in the development of CTC isolation and characterization techniques makes it likely that the information obtained by analyzing CTC will increase in the future. Beside the

enumeration and molecular analysis of CTC on cellular level, one promising approach for the future might be *in vitro* cultivation of CTC. *In vitro* grown cells, as molecular copy of the tumor and metastases *in vivo* might be a key step for the diagnostic and therapeutic future of PCa, although it is known that *in vitro* conditions differ from the microenvironment in the blood. Nevertheless, drug sensitivity of tumor-specific tissue might be tested *ex vivo* as a step toward individualized treatment.

Gao *et al.*[66] described the successful long-term culture of CTC from patients with advanced PCa; however, future improved methods for isolation and enrichment of CTC are required to guarantee viable, for cultivation feasible, CTC. Xenograft models using CTC for further *in vivo* drug testing may then provide a valuable tool for investigating the individual responsiveness of a tumor.

As the sequencing of single cells (e.g., CTC) is challenging, circulating DNA fragments from tumor cells (so-called circulating tumor DNA [ctDNA]) has been discussed as an alternative approach to obtain molecular information on tumor-associated DNA alterations in metastatic patients.[67] ctDNA is composed of small fragments of the nucleic acid of both nuclear and mitochondrial DNA and is not associated with cells or cell fragments.[68] This DNA contains coding and noncoding DNA-sequences and can be used to analyze microsatellite instability, mutations, methylation, polymorphisms and loss of heterozygosity and DNA integrity.[68,69] In recent studies, analysis of ctDNA has been used to assess AR alterations in patients with CRPC. Azad *et al.*[70] studied patients progressing after abiraterone, enzalutamide, or other agents. They could show that in those switching to enzalutamide, a higher copy number or mutation in *AR* exon 8 meant lower PSA-RR.

The results of Romanel *et al.*[71] are in accordance to this, showing that the presence of specific mutations of the *AR* and an increased number of *AR* copies were associated with abiraterone resistance in terms of PSA response.

Wyatt *et al.*[72] used cfDNA to detect copy number variations and mutations in *AR* and other PCa associated genes. They could show, that detection of PCa associated genes such as MYC proto-oncogene (*MYC*) (MYC gain), retinoblastoma protein 1 (*RB1*) (RB1 loss), tyrosine-protein kinase Met (*MET*) (MET gain) was associated with poor PFS in 65 patients receiving enzalutamide. Analyses and monitoring of cfDNA, therefore, might be an attractive alternative to CTC, although, to date, most of the available studies address CTC and not cfDNA.

CONCLUSIONS

Although the number of studies including CTC analysis has been constantly increased during the last decade, the optimal use of this biomarker in patients with PCa is still unclear. Although correlations of CTC numbers with outcome have been reported, CTC numbers and their changes are still not recommended to serve as parameters for clinical decision making in patients with PCa. The recent developments in the molecular analysis of CTC have significantly broadened the options for the use of CTC as biomarkers. The recent discovery of AR-V7 as a predictor of outcome response for treatment with abiraterone/enzalutamide led to a significantly increased interest in the discovery of predictive biomarkers. Beside CTC, circulating tumor DNA enables the noninvasive characterization of CTC and first studies indicate a potential role as markers used for precision oncology.

Although the CellSearch® system has been approved by the FDA, the use of analysis of CTC is limited in patients with PCa and has not been recommended yet in current guidelines for daily clinical practice. To promote the use of CTC analysis in standard practice, a clear influence of CTC test results on clinical decision making should be demonstrated. The identification of AR-V7 as a potential marker of resistance for treatment with abiraterone and enzalutamide has significantly promoted the interest in CTC as a tool for promoting personalized treatment in PCa patients. However, further data on the clinical relevance of AR-V7 positive CTC and the optimal technique for determination is required before being implemented in daily clinical practice.

AUTHOR CONTRIBUTIONS

MM was mainly responsible for data acquisition, drafting the manuscript and setup of tables. TT contributed to the planning of the manuscript, data acquisition, and writing of the manuscript. MH, SR, and JB contributed to data acquisition and reviewing the manuscript. AS was responsible for the planning of the review, selection of subtopics, and final review of the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

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